Serotonin Transporter Function and Emotional Behaviour

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Abstract

The serotonin transporter (5-HTT) is a key regulator of synaptic serotonin (5-HT), a monoamine that is significantly involved in the regulation of fear and anxiety processes. Importantly, low or null 5-HTT expression levels have also been associated with anxious and depressive phenotypes in several species. This remains controversial, however, with different research groups reporting conflicting results with most of the methodologies used so far to investigate whether such an association exists. An emerging 'neuroplasticity theory of 5-HT function' argues that low 5-HTT-expressing individuals, who are thought to have increased 5-HT availability, are not more vulnerable to anxious and depressive phenotypes per se but more sensitive to aversive and non-aversive life events. Subsequently, this increased sensitivity could lead to facilitated emotional learning in a 'for-better-or-for-worse' manner depending on their experiences in a given environment.

This thesis aimed to investigate this further by testing knockout mice for the 5-HTT (5-HTTKO mice) in approach–avoidance conflict tests, social assays and discrimination learning tasks with negative and positive emotional stimuli. Additionally, in a subset of mice, altered basolateral amygdala (BLA) function during an aversive learning task was also investigated via recordings of neuronal and haemodynamic responses to emotional cues.

The experiments reported here have shown that 5-HTTKO mice exhibit altered sensitivity towards emotionally-relevant negative and positive events and towards social influences. Moreover, 5-HTTKO mice exhibited superior aversive learning via augmented BLA function. However, the nature of the stimuli used in the experiments was crucial for the observation of such facilitated learning, given that learning about reward-predicting cues was not facilitated in 5-HTTKO mice.

Overall, the results obtained in this thesis provide evidence for the neuroplasticity theory of 5-HT function at the behavioural and neurophysiological level. However, further studies are warranted to clarify the boundaries of this theory.

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List of abbreviations

5-HT, 5-hydroxytryptamine (serotonin)
5-HTP, 5-hydroxy-L-tryptophan
5-HTT, serotonin transporter
5-HTTLPR, 5-HTT gene-linked polymorphic region
AB, attentional bias(es)
ABM, attention bias modification
ANOVA, analysis of variance
ATD, acute tryptophan depletion
BA, basal amygdala
BDFN, brain-derived neurotrophic factor
BLA, basolateral amygdala
BMA, basomedial amygdala
BNST, bed nucleus of the stria terminalis
BOLD, blood oxygen level-dependent
CBF, cerebral blood flow
CeA, central amygdala
$CMRO_2$, cerebral metabolic rate of O2 consumption
CPA, constant potential amperometry
CPE, carbon paste electrode
CR, conditioned response
CS, conditioned stimulus
CS+, conditioned excitor
CS−, conditioned inhibitor
CSa, ambiguous CS
dB, decibel(s)
DNA, deoxyribonucleic acid
DR, discrimination ratio
DRc, caudal subnucleus
DRN, dorsal raphe nucleus
EFP, extracellular field potentials
ENS, enteric nervous system
EPM, elevated plus maze
EPQ, Eysenck Personality Questionnaire
fMRI, functional magnetic resonance imaging
GAD, generalised anxiety disorder
GWAS, genome-wide association studies
HPA, hypothalamo-pituitary-adrenocortical
HPC, hippocampus
HPN, hyponeophagia
Hz, hertz
IN, interneuron
IP, intraperitoneal
IRQ, inter-quartile range
KO, knockout
LA, lateral amygdala
LFPs, local field potentials
MAO-A, monoamine oxidase-A
mPFC, medial prefrontal cortex
mRNA, messenger ribonucleic acid
MRN, median raphe nucleus
NOR, novel object recognition test
NSS, adrenomedullary neuroendocrine serotonin system
O_2, oxygen
PnO, oral pontine nucleus
PAG, periaqueductal grey
PFC, prefrontal cortex
PET, positron emission tomography
PN, principal neurons
PSS, peripheral serotonin system
PTSD, post-traumatic stress disorder
PV INs, parvalbumin parvalbumin-expressing interneurons
SC, subcutaneous
SEM, standard error of the mean
SSRI, selective serotonin reuptake inhibitor
UR, unconditioned response
US, unconditioned stimulus
TO_2, tissue oxygen
VEP, visual evoked potentials
vHPC, ventral hippocampus
WT, wild-type
VMAT2, vesicular monoamine transporter type-2

Chapter 1. General introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter/ neuromodulator in the central and peripheral nervous system. It has been associated with numerous physiological and behavioural functions, including cardiovascular regulation, sleep-wake cycle, sensorimotor integration and reactivity, reward-related processing and learning (Lucki, 1998; Berger et al., 2009; Jacobs & Fornal, 2010; Cassel, 2010; Hayes & Greenshaw, 2011). Considerable evidence also indicates that 5-HT is significantly involved in the regulation of fear and anxiety processes (Deakin & Graeff, 1991; Lowry et al., 2005). Additionally, changes in 5-HT signalling have been associated with both the pathophysiology and the treatment of depression and anxiety disorders (Ressler & Nemeroff, 2000; Sharp & Cowen, 2011; Hale et al., 2012). Concerning these latter processes, a fruitful line of research comes from studying the effects of alterations in the expression or function of a key structure of the serotonergic system: the serotonin transporter (5-HTT).

The 5-HTT regulates the magnitude and duration of 5-HT action following neuronal stimulation and thus has an active role in the serotonergic system (Murphy et al., 2004). Different approaches have been used to clarify the role of the 5-HTT and many have focused on studying the effects of gene variants in the gene coding for the 5-HTT that have an impact on its expression levels (Murphy et al., 2008). One outcome thought to result from different 5-HTT expression levels is a differential susceptibility to clinical anxiety and depression, with low 5-HTT-expressing individuals having a greater risk of developing these disorders, particularly after experiencing stressful adversity during their lifetime (Caspi et al., 2010). Another, more specific effect suggested is that different 5-HTT expression levels can affect the function of the amygdala (Hariri et al., 2002, 2005), a brain structure that is known to be involved in emotional processing. However, both claims have proved controversial, with

different research groups reporting conflicting results with most of the methodologies used so far (e.g. Risch et al., 2009; Murphy et al., 2013b).

An alternative theory, however, suggests that low 5-HTT-expressing individuals are not more vulnerable but more sensitive both to aversive and non-aversive life events (Belsky et al., 2009; Branchi, 2011; Homberg & van den Hove, 2012). In other words, they are not simply more susceptible to adversity but more responsive to experiences instead. Although some studies support this proposal (hereafter "the neuroplasticity theory of 5-HT function") empirical evidence is still lacking. In this thesis, it was hypothesised that if an individual is more sensitive to the environment, then he or she should encode aversive and non-aversive experiences more strongly compared to an individual with higher 5-HTT expressions levels. Subsequently, this could lead to facilitated emotional learning.

To help to clarify the role of the 5-HTT, this thesis aimed to test the neuroplasticity theory of 5-HT function in mice with genetic deletion of the (murine) 5-HTT gene. Given that these mice do not express the 5-HTT, they can be considered as an extreme model of an individual with low 5-HTT expression levels. This model was used to investigate if an extreme reduction of the 5-HTT could alter social anxiety and aversive and non-aversive learning. Additionally, in a subset of mice, altered amygdala function during an aversive learning task was also investigated.

This Introduction starts by providing an overview of the serotonergic system and how it is related to depression and anxiety disorders. This is followed by a description of the different types of genetic association studies conducted to investigate the effects of 5-HTT expression variation on emotionality in various human subpopulations, and a summary of the reported results. Afterwards, research focused on altered amygdala function associated with different 5-HTT expression levels is reviewed. Advantages of using mouse models are then explained, together with a description of translational tools used to investigate aspects

of altered brain function that are not possible (practically, ethically, or both) to investigate with humans. Finally, the neuroplasticity theory of 5-HT function is explained, and the aims of the thesis are stated.

1.1. The serotonergic system

1.1.1. Neuroanatomy of the serotonergic system

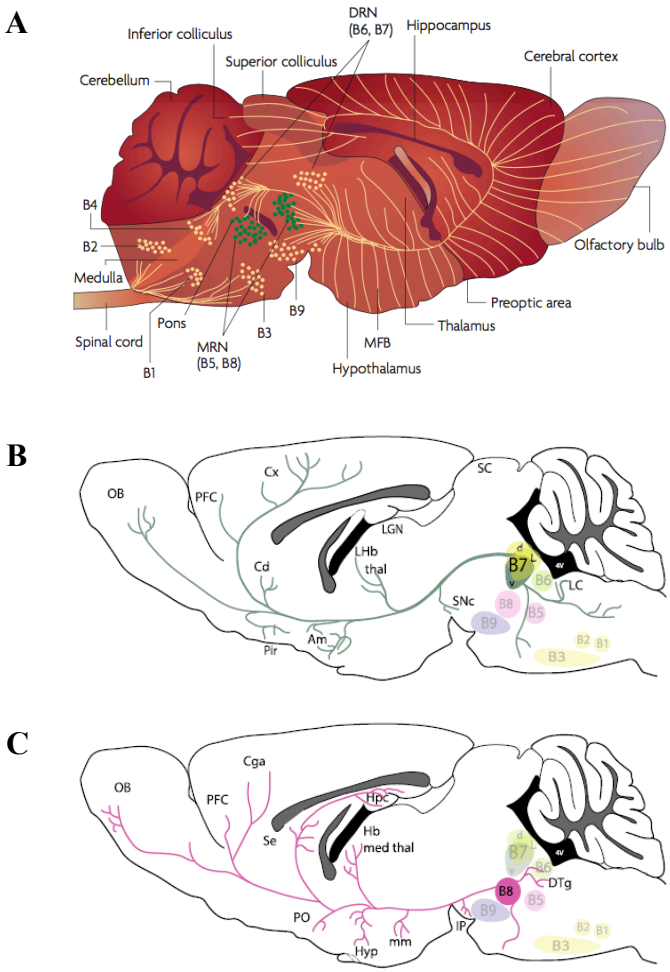


Figure 1.1. A) Central serotonergic system in the mouse brain. It includes nine raphe nuclei (B1-B9), including the dorsal raphe nucleus (DRN; B6 and B7) and the median raphe nucleus (MRN, B5 and B8). Source: Murphy and Lesch (2008). Reprinted by permission from Springer Nature Terms and Conditions for RightsLink Permissions Springer Customer Service Centre GmbH: Springer Nature, (Nature Reviews Neuroscience, Targeting the murine serotonin transporter: insights into human neurobiology, Dennis L. Murphy, Klaus Peter Lesch), Copyright 2008; **B, C)** Illustration of the principal targets of the DRN (B7) and the MRN (B8) in the mouse brain, obtained via the use of a selective anterograde labelling method. Note the projection of the DRN (B7) to the amygdala. Source: Muzerelle et al. (2016). Reprinted under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0). Licensed Material: Muzerelle, A., Scotto-Lomassese, S., Bernard, J.F. et al. Brain Struct Funct (2016) 221: 535. https://doi.org/10.1007/s00429-014-0924-4.

The serotonergic system consists of nine groups of neurons containing 5-HT cell bodies that are in the brainstem and can innervate virtually the entire central nervous system using their axonal projections. Most serotonergic neurons are in the raphe nuclei, with some also located in the reticular formation (Törk, 1990; Hornung, 2003). The identification and labelling of these groups (caudally to rostrally) as B1 to B9 was first done by Dahlström and Fuxe in rats (1964). However, a generally similar organisation of these groups is found across several mammalian species, including mice, rats, monkeys and humans (Azmitia & Segal, 1978; Felten & Sladek, 1983; Törk, 1990; Jacobs & Azmitia, 1992; VanderHorst & Ulfhake, 2006; Muzerelle et al., 2016). In mammalian species, the serotonergic system can be divided into two divisions/groups. First, a rostral group, in the caudal mesencephalon and the rostral pons, which projects mainly to the forebrain. Second, a caudal group, extending from the caudal pons to the caudal portion of the medulla oblongata, which projects mainly to the caudal brainstem and to the spinal cord (Törk, 1990; Hornung, 2003). Each group includes several nuclei that vary in their (afferent and efferent) connections with different structures, but also in cell densities and morphology.

Relevant to this thesis is the rostral group, given that it is especially involved in anxiety, fear and mood (Paul & Lowry, 2013). It is within this group of nuclei that the largest collection of serotonergic neurons in the brain is found (Törk, 1990; Hornung, 2003): the dorsal raphe nucleus (DRN; B7 and B6). In most species, the DRN is comprised of five subnuclei. The dorsal, ventral, ventrolateral, and interfascicular subnuclei are in a more rostral region of the DRN, whereas the caudal subnucleus (DRc) can be found more caudally in the DRN. Among the several regions targeted by the DRN are the cerebral cortex, neostriatum and amygdala, which receive projections from the rostral part of the DRN; and the hippocampus, entorhinal cortex and the locus coeruleus, targeted by the caudal part of the DRN (Hornung, 2010). Notably, there seems to be a topographic organisation in the

DRN innervation of the forebrain, with each forebrain region (e.g. different amygdala nuclei) having its own unique representation within the DRN groups (Lowry et al., 2005; Asan et al., 2013).

In addition to the DRN, the rostral group also includes another important cluster of serotonergic neurons: the median raphe nucleus (MRN; B5 and B8). This nucleus can be divided into a median region (MRN proper) and a paramedian region, although many cells extend into adjacent regions, such as the reticular formation, to form other clusters (e.g. oral pontine nucleus, PnO; Törk, 1990; Hornung, 2003). The MRN projects mainly to the basal forebrain, the septal region, the ventral tegmental area and hippocampus (Hornung, 2010).

The efferent projections of the MRN and DRN ascend to the forebrain in two pathways, the ventral pathway and the dorsal pathway. The ventral pathway comprises the MRN and DRc projections, whereas the dorsal pathway collects the DRN and PnO projections (Hornung, 2003). The MRN and the DRN also share some afferent projections, including those originating from limbic system structures (Hornung, 2003).

1.1.2. 5-HT signalling

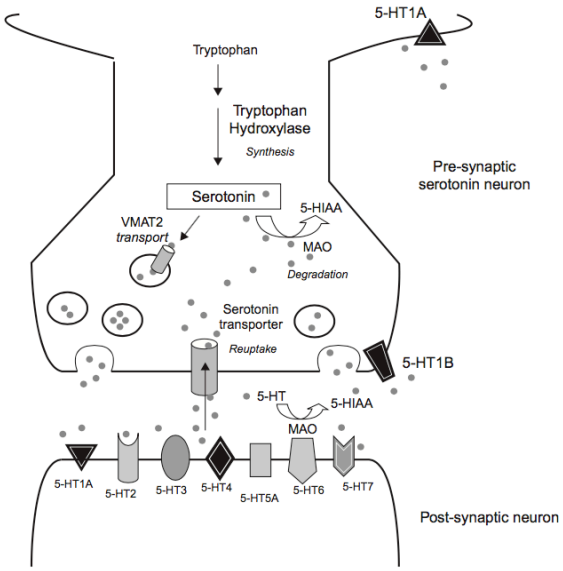


Figure 1.2. Scheme of a serotonergic synapse. It includes the serotonin transporter (5-HTT, the autoreceptors 5-HT_{1A} and 5-HT_{1B}, and 14 distinct receptors classified into seven families (5-HT_{1-7}). (MAO, monoamine oxidase; 5-HIAA, 5-hydroxyindole acetic acid; VMAT2, vesicular monoamine transporter 2). Source: O'Leary & Cryan, 2010. Reprinted from Handbook of Behavioral Neuroscience, vol. 21 / First Edition, Olivia F. O'Leary, John F. Cryan, The Behavioral Genetics of Serotonin: Relevance to Anxiety and Depression, pp. 749-790 (41 pages), Copyright (2010), with permission from Elsevier.

In neurons, 5-HT is synthesised from the amino acid L-tryptophan through two steps: hydroxylation of L-tryptophan, which forms 5-hydroxy-L-tryptophan (5-HTP); and decarboxylation of 5-HTP, which produces 5-hydroxytryptamine (i.e. 5-HT; Hasegawa & Nakamura, 2010). Afterwards, the vesicular monoamine transporter type-2 (VMAT2) stores 5-HT into vesicles, where it is protected from metabolism. Upon the firing of the serotonergic neuron and release into the synaptic cleft, 5-HT can exert its actions on postsynaptic serotonergic receptors. This mode of serotonergic transmission is known as wiring (synaptic) transmission. However, 5-HT can also act in a volume transmission mode. This "makes communication possible between all the cells of the brain via diffusion (…) [of 5-HT] in the extracellular fluid and cerebrospinal fluid", thus "enabling information

handling and trophic communication between all cells, including neuron–glia and glia–glia interactions" (Fuxe et al., 2007, p.19).

In addition to interacting with postsynaptic receptors located in non-5-HT neurons, 5-HT can also interact with presynaptic autoreceptors (e.g. 5-HT_{1A}, 5-HT_{1B}) of the serotonergic neuron(s). The autoreceptors' function is to work as an autoregulatory mechanism that senses abnormal levels of 5-HT in the extracellular space, and then inhibit cell firing (5-HT_{1A}) and further 5-HT release (5-HT_{1B}), in combination with other mechanisms (e.g. postsynaptic 5-HT feedback mechanisms) (Sharp et al., 2007). 5-HT can then be taken up by surrounding glial cells (e.g. via organic cation transporters [OCT] or plasma membrane monoamine transporters [PMAT], among other transporters; Busch et al., 1996; Engel et al. , 2004; Daws, 2009), which may terminate 5-HT activity by degrading it to its metabolite 5-hydroxyindole acetic acid (5-HIAA) via the enzyme monoamine oxidase-A (MAO-A) (Hirst et al., 1998; Inazu et al., 2001; Youdim et al., 2006). The primary mechanism terminating 5-HT action, however, is the rapid removal of 5-HT from the synaptic cleft and transport into the releasing neuron by the neuronal serotonin transporter (SERT or 5-HTT) (Blakely et al., 1994; Murphy et al., 2004). This protein, located in the perisynaptic membranes of nerve terminals (but also in somatodendritic membranes), is decisive in the "homeostatic regulation of the magnitude, duration, and spatial distribution of 5-HT signals reaching serotonin receptors" (Murphy et al., 2004, p.111). When 5-HT is returned to the nerve terminal, it can either be degraded/metabolised mainly by MAO-A (Bortolato et al., 2010), or recycled as 5-HT back into new synaptic vesicles.

The multiple effects of serotonin likely result from an interaction between various factors, including cellular specificity (e.g. serotonergic neurons can vary regarding axonal morphology and synaptic connections) and differential distribution of distinct receptors subtypes in the brain (Iversen et al., 2009; Hornung, 2010). The complexity of 5-HT

signalling is further increased as at least 14 distinct 5-HT receptor subtypes mediate this signalling (Barnes & Sharp, 1999; Hannon & Hoyer, 2008).

In addition to its modulatory effects in the brain, 5-HT also acts as a modulator of cardiovascular and gastrointestinal activity and can mediate hormonal responses to stress. Indeed, 5-HT and 5-HTT can be found in i) the enteric nervous system (ENS; e.g. within the gut, which contains the largest amount of 5-HT in the body), ii) the hypothalamo-pituitary-adrenocortical (HPA) system, and iii) the adrenomedullary neuroendocrine serotonin system (NSS) (Lucki, 1998; Murphy et al., 2008; Gershon, 2013). Furthermore, tissues that also contain functional 5-HTT, such as the heart, lungs, and blood vessels, are considered a peripheral serotonin system (PSS) given that they take up, store and release 5-HT (Murphy & Lesch, 2008).

1.1.3. 5-HT in depression and anxiety

Several lines of research have provided evidence that 5-HT plays a major role in emotional processing. For instance, neuroanatomical studies indicate that there is a strong serotonergic innervation of limbic structures directly involved in emotional processing, such as the amygdala and frontal cortex (Hensler, 2006; Asan et al., 2013). Additionally, pharmacological manipulations that increase or decrease 5-HT neurotransmission have been found to affect attentional processing, learning and memory of emotional/affective stimuli in several species (Harmer, 2008; Homberg, 2012; Bauer, 2015; further discussed in Chapter 6). However, the first and primary source of evidence has stemmed from research on the pathophysiology and pharmacological treatment of depression and anxiety disorders.

5-HT and depression

Depression is a mood disorder whose main features include a severe, pervasive and prolonged experience of an unpleasant (dysphoric) mood, often paralleled by mental anguish, anhedonia and loss of interest in the world, typically felt on a regular basis (e.g. most of the days) (Hyman & Cohen, 2013). Regarding the involvement of 5-HT in this disorder, it has often been observed, for instance, that depressed patients exhibit abnormalities in the serotonergic system, such as decreased 5-HT_{1A} and 5-HTT binding density (Drevets et al., 2007; Gryglewski et al. , 2014). Strong evidence of a 5-HT role in depression also comes from the fact that the first-line pharmacological treatment approach in depression consists of using selective serotonin reuptake inhibitors (SSRIs). SSRIs act firstly by inhibiting the functioning of the 5-HTT and raising extracellular 5-HT levels (Bel & Artigas, 1992; Nutt, 2002; Meyer, 2004). Improvements in mood, however, are only seen after a few weeks of treatment (i.e. after chronic administration, as opposed to an acute administration). One hypothesis for this situation is that continued SSRI administration over a few weeks leads to, among other processes, a desensitisation of 5-HT autoreceptors (e.g. 5-HT_{1A}, 5-HT_{1B}), which stops their inhibition of both neuronal firing and 5-HT release (Nutt, 2002; Sharp & Cowen, 2011). Desensitisation of 5-HT autoreceptors thus results in a further increase of extracellular 5-HT levels. Increasing 5-HT levels via SSRI administration is assumed to lead to enhanced neuronal plasticity (Castrén, 2005; Krishnan & Nestler, 2008; Branchi, 2011) and changes in emotional processing (Harmer et al., 2009), which are thought to be the main factors contributing to changes in mood, particularly if combined with psychotherapy (Cuijpers et al., 2009).

Another line of research investigating whether altered 5-HT signalling is associated with depressive symptoms involves inducing a temporary lowering of brain 5-HT activity through an acute “tryptophan depletion” (ATD) technique (Carpenter et al., 1998). Despite

inconsistencies between studies using this technique, both with healthy participants and recovered depressed patients, it is possible to lower mood with ATD in subpopulations of those groups (Ruhé et al., 2007; Feenstra & van der Plasse, 2010). This is the case, for instance, in healthy participants with vulnerability factors for the development of depression (such as a family history of depression, a genetic susceptibility factor for depression, or both; e.g. Neumeister et al., 2002; van der Veen et al., 2007). Although there is no consistent effect of ATD on mood of healthy participants who are not at risk for depression, it is possible to induce changes in emotional processing in these participants like those seen in people with depression (Harmer, 2008). Accordingly, a reduction of the processing of positive relative to negative affective emotional stimuli can be achieved in several tasks in healthy participants subjected to ATD, whereas generally an opposite effect can be induced by an acute SSRI administration (Harmer, 2008).

Despite the above-mentioned observations, it is unlikely that 5-HT influences mood directly. A good example supporting this assumption is that not all depressed patients show a significant response to SSRI treatment (e.g. Trivedi et al., 2006). This might be related to their genetic makeup, including gene variants affecting the serotonergic system (Serretti et al., 2006; Uher et al., 2009; Kato & Serretti, 2010). Additionally, when a SSRI is administered to healthy participants, they do not exhibit mood elation (Cowen, 2008). In fact, it has been shown that healthy participants subjected to an acute SSRI administration can exhibit an increase in threat processing, and not only the previously referred increased positive processing (Harmer, 2008; see also Chapter 6). This is consistent with clinical reports of increased anxiety symptoms in the early stages of SSRI treatment (Baldwin, 2005).

5-HT and anxiety

Anxiety can be defined as anticipation of future or potential threat and is associated with heightened arousal, vigilance, and increased avoidance behaviour in situations involving uncertainty, unpredictability, or uncontrollability (Lowry et al., 2005). It can result from a conflict between concurrently primed goals or response choices (e.g. an approach-avoidance conflict) and typically leads to heightened attention (e.g. to change the salience of stimuli), inhibition of the goals with the strongest negative associations and suppression of ongoing motor programs (Gray & McNaughton, 2000; Bannerman et al., 2014). It differs from fear, which i) is an emotional response to a real (or perceived) imminent and unambiguous threat, ii) is more associated with autonomic arousal related to fight or flight responses (as opposed to risk assessment), and iii) relies on different neuronal circuits (despite considerable overlap) (Cryan & Holmes, 2005; Tovote, 2015; American Psychiatric Association, 2013).

In normal subjects, anxiety is an adaptive response, particularly in response to potentially threatening situations. However, when it leads to an exaggerated and persistent response to a possible threat, causing distress or disability that interferes with function, then it might fulfil the criteria for an anxiety disorder (Hyman & Cohen, 2013). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, American Psychiatric Association, 2013), anxiety disorders include the generalised anxiety disorder, panic disorder, specific phobia, and social anxiety disorder (social phobia), among others. The various disorders can be distinguished by the nature of the eliciting stimulus (or stimuli) and the nature and intensity of the symptoms (Hyman & Cohen, 2013). For example, in the case of social anxiety disorder, there is an out of proportion fear or anxiety of situations where the individual feels that he or she is likely to be scrutinized by others. The individual fears that he or she will be negatively evaluated, anticipating a humiliating/embarrassing

situation, which could lead to offending others and/or being rejected by them (DSM-5, American Psychiatric Association, 2013). Despite the existence of several anxiety disorders, comorbidity between different disorders is common.

Both anxiety and depression involve negative emotional states, and the high levels of comorbidity between the two disorders suggest overlapping genetic and neuronal mechanisms (Gorman, 1996; Ressler & Nemeroff, 2000; Hyman & Cohen, 2013). Indeed, anxiety disorders commonly precede depression (Wittchen et al., 2000; Hettema et al., 2003). Additionally, abnormalities in the serotonergic system, such as altered 5-HT_{1A} and 5-HTT binding potential, have also often been observed in patients with anxiety disorders (Iny et al., 1994; Akimova et al., 2009; Spies et al., 2015). Moreover, as with depression, SSRIs are also the first-line pharmacological treatment for anxiety disorders (Hoffman & Mathew, 2008; Bandelow et al., 2012). Furthermore, tryptophan depletion has been found to reverse the therapeutic effects of SSRI treatment in patients with anxiety disorders (Bell et al., 2002; Argyropoulos et al., 2004). Therefore, the involvement of the serotonergic system in both disorders seems to be unquestionable, even if its exact role is still unclear.

The Deakin/Graeff hypothesis

One attractive hypothesis to explain the serotonergic mechanisms underlying the different symptoms of depression and the various anxiety disorders is the Deakin/Graeff hypothesis of anxiety and affective disorders (Deakin & Graeff, 1991; Graeff et al., 1996). According to the hypothesis, different types of aversive stimuli might activate different serotonergic pathways and receptor subtypes (i.e. different “5-HT systems”), and dysfunction in each one of them leads to a distinct set of symptoms (i.e. disorders). In short, 5-HT mediates different disorders by playing distinct roles in different 5-HT pathways.

According to the Deakin/Graeff hypothesis, the DRN-periventricular pathway, which innervates the periventricular and periaqueductal grey (PAG), would be involved in restraining fight-or-flight behaviour in response to acute unconditioned (i.e. innate) aversive stimuli. The DRN-forebrain pathway, which innervates the amygdala, hippocampus and prefrontal cortex (PFC), would be involved in risk assessment, fear and avoidance behaviour in response to acute conditioned aversive stimuli (i.e. stimuli predicting aversive events). Finally, the MRN-hippocampus pathway, projecting to the septo-hippocampal system, would be activated by chronic exposure to unconditioned and conditioned aversive stimuli, which would then promote resistance to chronic stress. When working under normal conditions, these systems would coordinate adaptive responses, such as suppression of flight in the PAG simultaneously to an increase in anticipatory anxiety in the amygdala (Deakin & Graeff, 1991; Graeff et al., 1996). However, dysfunction in the DRN-forebrain pathway might result in some anxiety disorders (e.g. GAD), whereas abnormalities in the MRN-hippocampus pathway might lead to depression. Dysfunction in the DRN-periventricular pathway might be related to panic disorder (see also Paul et al., 2014).

Despite the strong support that this hypothesis has received (Graeff et al., 1996; Hale et al., 2012; Paul & Lowry, 2013), it is worth mentioning that there are still some contradictory data to be explained by the Deakin/Graeff hypothesis (Guimarães et al., 2010). Furthermore, other factors besides the nature of the eliciting stimuli can affect which 5-HT pathway is activated, such as proximity of the threat and escapability from the stressor (Paul & Lowry, 2013). Additionally, more recent models have expanded the Deakin/Graeff hypothesis, either by including additional serotonergic pathways (e.g. Cools et al., 2008) or by specifying how 5-HT regulates aversive learning (e.g. Daw et al., 2002; Dayan & Huys, 2009).

In addition to the Deakin/Graeff hypothesis other theories have also implicated a role for 5-HT in anxiety. These theories emphasise that 5-HT either i) plays a primary role in motivation via general behavioural suppression or inhibition and/or ii) allows that more specific inputs related to, for instance, punishment, have greater control over behaviour than what they would normally be able to exert (Iversen, 1984; Soubrié, 1986; Gray & McNaughton, 2000)

1.2. 5-HTT expression variation and emotionality

Another line of research that aimed to clarify how alterations in 5-HT signalling affect the brain and behavioural processes, particularly those related to depression and anxiety disorders, has focused instead on investigating the genes that regulate the serotonergic system. Specifically, researchers have tried to understand the contribution of variation in the expression of genes regulating the proteins involved in the synthesis, storage, release, uptake and degradation of 5-HT (see a review by O'Leary & Cryan, 2010). One of the most significant genes under investigation by this approach is the 5-HTT gene (SLC6A4 in humans; Slc6a4 in mice; also known as SERT or 5-HTT gene; Murphy et al., 2008).

Polymorphisms in the 5-HTT gene

The human SLC6A4 gene is in chromosome 17q11.2, and it includes, among several constituents, a promoter region, 14 exons, and non-coding and intronic areas (Lesch et al., 1994). Variation in different parts of the 5-HTT gene can affect its expression, function and/or regulation (Nakamura et al., 2000; Murphy et al., 2004; Murphy et al., 2008; Ehli et al., 2012). Another source of 5-HTT variation is epigenetic regulation of the 5-HTT gene (e.g. DNA methylation occurring at the promoter region), which can affect, for instance, its

transcriptional function (Olsson et al., 2010; Booij et al., 2013; Provenzi et al., 2016). However, the most well-known and investigated loci are those concerning the variants modulating its transcriptional activity. In humans, 5-HTT levels can vary up to sevenfold between individuals (Lundberg et al., 2006; Lundberg et al., 2007), with considerable evidence suggesting that this is mostly driven by variation in the 5-HTT gene-linked polymorphic region (5-HTTLPR) of the 5-HTT gene promoter. The most studied of those variations in this region affects its length by changing the size of a repetitive sequence comprising 26 repeat elements with a deletion of repeat unit 6-8 (Heils et al., 1996; Lesch et al., 1996). The insertion or deletion of the repeat unit 6-8 leads to, among other low-frequency alleles of variable sizes, either a "short" 14-repeat allele (s allele) or a "long" 16-repeat allele (*l* allele) (Heils et al., 1996; Ehli et al., 2012; Nakamura et al., 2000).

Considering the modulation of transcriptional activity, several studies using different (*in vitro* or *in vivo*) techniques have reported that the *s* allele is associated with lower transcriptional potential compared to the *l* allele (Heils et al., 1996; Lesch et al., 1996; Greenberg et al., 1999; Heinz et al., 2000). However, other researchers have failed to find replicable differences in 5-HTT expression dependent upon genotype (Mann et al., 2000; Preuss et al., 2000; Shioe et al., 2003; Patkar et al., 2004; Murthy et al., 2010). One possibility underlying these different results could be the fact that single nucleotide polymorphisms in the 5-HTTLPR region can also affect the transcriptional activity of the 5-HTT gene. Notably, the rs25531 variant can change the *l* allele (by substituting a single base A for G) to make it functionally equivalent to the *s* allele (the L_G allele) (Hu et al., 2006; Wendland et al., 2006). In this case, the gain of function is only ensured with a L_AL_A genotype, which means that the 5-HTTLPR polymorphism should be functionally triallelic (*s*, L_G, L_AL_A). The existence of a triallelic 5-HTTLPR also means that up to 15% of

individuals considered *l* carriers in studies using a biallelic 5-HTTLPR could be, technically, *s* carriers for the study (Murphy et al., 2013a).

Nonetheless, even when the triallelic 5-HTTLPR is considered, there are conflicting findings. Such conflict can be seen, for instance, in positron emission tomography (PET) imaging studies of the 5-HTT using the selective ligand [^{11}C]DASB. Some authors have found evidence of increased binding of this ligand (reflecting increased 5-HTT expression levels) in carriers of the L_AL_A genotype compared to non- L_AL_A carriers (including carriers of at least one *s* allele; Praschak-Rieder et al., 2007; Reimold et al., 2007) whereas others have not (Murthy et al., 2010). Importantly, other factors might also affect these results (e.g. ethnicity of the participants, time of the year; Willeit & Praschak-Rieder, 2010).

Despite these conflicting results, several studies working on the assumption that a biallelic 5-HTTLPR polymorphism can influence 5-HTT expression levels, have found it to be associated with depression and anxiety disorders. These studies are discussed below. Unless otherwise specified, in this thesis 5-HTTLPR refers to the "biallelic" 5-HTTLPR" (with the originals *s* or *l* variants), which is the most often used promoter region variant in the referred studies and still the most commonly used, albeit controversially (Murphy et al., 2013a).

1.2.1. *Candidate gene association studies*

Gene association studies

Twenty years ago, Lesch and colleagues (1996) conducted a study that indicated an association between the 5-HTTLPR and the personality trait neuroticism (also known as negative affectivity), which is considered in some models to be a vulnerability factor for the development of anxiety and depression (Clark et al., 1994). Specifically, carriers of the *s*

allele scored higher in neuroticism than individuals homozygous for the *l* variant of the 5-HTTLPR. That is, healthy volunteers who had putatively lower 5-HTT levels, and thus greater 5-HT extracellular levels had a greater susceptibility to exhibit an anxiety-related trait, characterised by a negative bias in attention, interpretation and recall of information (Ormel et al., 2013). This result was a highly influential finding, even if it seemed paradoxical considering that the first line of treatment for patients with depression and anxiety disorders involves elevating 5-HT levels via SSRI administration. However, Lesch and colleagues (1996) pointed out that they had obtained their findings with a sample of the general population (i.e. healthy participants), whereas the effects of SSRIs were usually reported in patients. It was possible that elevating 5-HT levels in patients ameliorated a possible pre-existing dysfunction of the 5-HT system stemming from early brain development.

Subjects	Type of study	Is there any evidence of a significant association?	
		Yes	**No**
Clinical populations	▪ **Genetic association studies** 5-HTTLPR → symptoms of anxiety/depression disorders	Anxiety: Bengel et al., 1999; Lee et al., 2005; •Lin, 2007; Depression: Hauser et al., 2003; Hoefgen et al., 2005	Anxiety: •Blaya et al., 2007; Strug et al., 2010; Depression: Caspi et al., 2003; •Munafò et al., 2009a; •Risch et al., 2009; Willis-Owen et al., 2005; •Culverhouse et al., 2017;
	▪ **Gene × Environment interaction studies** 5-HTTLPR + adverse environment → symptoms of anxiety/depression disorders	Depression: Caspi et al., 2003; •Karg et al., 2011; Eley et al., 2004; Kaufman et al., 2004; Taylor et al., 2006;	Depression: •Munafò et al., 2009a; •Risch et al., 2009; •Culverhouse et al., 2017;
Healthy participants	▪ **Genetic association studies for complex traits** 5-HTTLPR → psychological/cognitive intermediate phenotypes	Lesch et al., 1996; •Schinka et al., 2004; •Sen et al., 2004; •Munafò et al., 2003 [partial support in some analyses]	•Munafò et al., 2003; Willis-Owen et al., 2005; Munafò et al., 2009b
	▪ **Genetic association studies for neuronal intermediate phenotypes** 5-HTTLPR → altered brain mechanisms	Hariri et al., 2002; Heinz et al., 2005; Pezawas et al., 2005; •Munafò et al., 2008; •Murphy et al., 2013b;	•Murphy et al., 2013b [marginally significant in some analyses; criticisms]; •Bastiaansen et al., 2014; [included a large-scale study]
	▪ **Gene × Environment interaction studies for intermediate phenotypes** ▪ 5-HTTLPR + adverse environment → psychological/cognitive intermediate phenotypes	Kuepper et al., 2012; Pluess et al., 2010; Stein et al., 2008;	Middeldorp et al., 2010; Araya et al., 2009;

Table 1.1. Selection of different types of genetic association studies investigating the effects of 5-HTT expression variation in various human subpopulations. •, meta-analysis.

This study led to hundreds of investigations in the following years aimed at elucidating the exact role of this polymorphism in the SLC6A4 gene, which was now a candidate gene for increased risk for anxiety and depression. Despite the variety of approaches and populations used by genetic association studies investigating the 5-HTTLPR, the effects of this polymorphism on individual differences in personality and on mental disorder susceptibility have remained highly controversial (see Table 1.1).

A traditional genetic association study typically involves comparing the genotypes of a clinical population with those of unaffected controls to verify if the frequency of the gene variant differs between the two groups (Flint et al., 2010). This approach initially provided support for a direct effect of the 5-HTTLPR on the risk for anxiety and depression (e.g. Lee et al., 2005; Hoefgen et al., 2005). However, many studies using this method have not found a significant association between the 5-HTTLPR and these disorders (e.g. Caspi et al., 2003; Strug et al., 2010). Indeed, results provided by a few meta-analyses have pointed towards a negligible effect of the polymorphism on the risk of developing anxiety and depression (e.g. Blaya et al., 2007; Munafò et al., 2009a; Risch et al., 2009).

Gene × Environment association studies

One possibility underlying the discrepancy across studies could be that carrying a genetic predisposition is not enough on its own to develop anxiety/depression, but an adverse environment would have to act as a stressor for its effects to be observed. That is, the 5-HTTLPR would not influence the risk for mental disorders by itself, but could moderate the serotonergic system response to stress and thus affect the individual's response to an adverse environment. Given that most genetic association studies do not consider the influence of the individuals' stress history, this could help explain the conflicting results obtained by different studies. This hypothesis was advanced by Caspi and collaborators (2003), who

showed a gene-by-environment (G×E) interaction between the 5-HTTLPR and stressful life events. In this study, carriers of the *s* allele exhibited more depression-related symptoms, diagnoses and suicidality at the age of 26 years old than individuals carrying a *l*/*l* genotype after experiencing stressful life events between ages 21 and 26 years (e.g. a disabling injury, being involved in a physically violent relationship, long-term unemployment). Additionally, it was shown that childhood maltreatment (3-11 years; e.g. exposure to child physical abuse, unwanted sexual contact, harsh discipline) could predict adult depression (18-26 years) in *s* allele carriers but not in homozygotes for the *l* allele. Evidence for a dose-response relationship was also found, with individuals with the *s*/*s* genotype showing higher risk of depression in relation to stressful life events compared with those carrying the *s*/*l* genotype.

Several studies have tried to replicate this G×E interaction finding (i.e. development of depressive disorder in response to childhood maltreatment or other stressful life events), but it was soon evident that this approach would also be surrounded by controversy. Although one meta-analysis supported the G×E interaction (Karg et al., 2011), others reported that no evidence existed to support this hypothesis (Munafò et al., 2009a; Risch et al., 2009). It was initially suggested that differences in methodological aspects of the studies could explain this discrepancy (e.g. biallelic versus triallelic 5-HTTLPR; sample age and gender composition; method of assessment of environmental adversity; Uher & McGuffin, 2008). However, criticisms were also made of the meta-analysis supporting the interaction between stress and 5-HTTLPR genotype on the risk for depression (Taylor & Munafò, 2016), and a more recent report of a collaborative meta-analysis on 31 data sets showed no evidence for such a G×E interaction (Culverhouse et al., 2017). According to Culverhouse and colleagues (2017), if such G×E interaction does exist, then it likely has a modest effect size and it is only observed in limited situations.

Genetic association studies for intermediate phenotypes

In addition to the previous two types of studies, more investigations along the lines of that conducted by Lesch and collaborators (1996) were also conducted, particularly by looking for an association between the 5-HTTLPR and psychological/cognitive intermediate phenotypes (see Table 1.1). The definition of intermediate phenotypes (sometimes called endophenotypes) varies depending on whether researchers see it as a risk indicator of the disorder or a mediating variable in the causal pathway from gene to disorder (Kendler & Neale, 2010). Considering the purpose of this thesis, they can be broadly defined as quantitative traits located between genetic variation and disorder (Flint et al., 2014), which are components of a more complex behaviour. They can vary in their form (e.g. anatomical, neurophysiological, psychological/cognitive) depending on how biologically close they are to the genetic influence (Flint & Munafò, 2007). However, in general, they are an internal process, stable over time, and crucially more objectively measured than the disorder itself. Importantly, they can work as a more genetically tractable target than the disorder according to some authors (Gottesman & Gould, 2003; Meyer-Lindenberg & Weinberger, 2006). Specifically, these authors believe that "the more behavioural the phenotype, the less directly it will be predicted by the genotype" (Meyer-Lindenberg & Weinberger, 2006, p. 818).

It is also believed that intermediate phenotypes should have a simpler genetic basis and be less influenced by multiple pathway effects than the disorders, although the strength of this claim has been disputed (Flint et al., 2014). However, if it is true, choosing a simpler phenotype closer to the candidate gene allows the detection of more direct and larger effects of gene variation. Using an approach focused on intermediate phenotypes could give genetic association studies a better chance to detect the effects of a polymorphism such as the 5-HTTLPR, with the additional benefits of the possibility of using healthy participants and smaller sample sizes than the above-mentioned studies. Although this reasoning is more

suitable to biological traits, personality traits such as neuroticism can also be used as an intermediate phenotype that mediates a possible association between the 5-HTTLPR and a mental disorder (Munafò et al., 2006).

Accordingly, several genetic association studies for complex traits have initially provided evidence for an influence of the 5-HTTLPR on anxiety-related traits, even though this effect was usually moderated by the measurement scale or inventory used (Schinka et al., 2004; Sen et al., 2004; Munafò et al., 2005). A later meta-analysis provided support for an effect of 5-HTTLPR on neuroticism measured with the NEO personality inventory but failed to find evidence for two other anxiety-related personality traits: harm avoidance and neuroticism measured with the Eysenck Personality Questionnaire (EPQ) (Munafò et al., 2009b). A large-scale and well-powered study conducted by Willis-Owen and colleagues (2005) measuring neuroticism with the EPQ questionnaire also failed to provide support for an effect of the 5-HTTLPR on neuroticism. In summary, genetic association studies for complex traits have also produced conflicting results.

1.2.2. Imaging genetics

The advent of neuroimaging techniques, such as functional magnetic resonance imaging (fMRI) and PET, allowed biological characterisation of the effects of candidate genes. These techniques can measure brain structure or function in a rapid and non-invasive way and can help discover neural mechanisms positioned between candidate genes and complex emergent phenotypic associations, such as psychiatric disorders or behaviour traits (Meyer-Lindenberg & Weinberger, 2006). In other words, they can be used to infer biological phenotypes that are closer to gene function than the psychological/cognitive ones mentioned above.

The use of imaging techniques for complementing association studies by identifying the effects of candidate genes at the level of neuronal systems and circuitry became known as "imaging genetics." This approach was particularly productive for the *in vivo* study of 5-HTTLPR effects on stable and reliable patterns of brain activation, which can be considered as trait-like phenomena (Hariri & Weinberger, 2003; Hariri et al., 2006; Hariri, 2009). This approach involved the use of relatively small samples of healthy participants (compared to sample sizes for genetic association studies) and procedures capable of activating brain systems considered to be involved in the disorders (e.g. anxiety) or related intermediate endophenotypes (e.g. neuroticism) by other lines of research. In the study of the 5-HTTLPR, the imaging technique most often used has been fMRI and the brain structure most investigated has been the amygdala.

The BOLD fMRI technique

Blood oxygen level-dependent (BOLD) fMRI is a technique that measures *in vivo* brain activity from a participant during a resting state but also while he or she is performing a task. Its primary use in neuroscience is to localise brain regions engaged in specific sensory, motor and cognitive processes in healthy subjects (Logothetis, 2008). However, it can also be used to study physiological properties of different brain regions if a researcher investigates, for instance, the duration and amplitude of the BOLD signal during the presentation of a stimulus by means of an event-related design (Passingham & Rowe, 2016; see also Appendix 1).

BOLD fMRI works by tracking the haemodynamic changes in blood vessels of the brain coupled with an increase in the local neuronal activity (Raichle, 1998; Lauritzern, 2005). Changes in energy metabolism of neurons resulting from increased activity prompt an increase in glucose uptake and oxygen (O_2) consumption in the brain tissue, which leads

to a local increase in cerebral blood flow (CBF) and cerebral blood volume (Small & Heeger, 2013). Given that the flow of oxygenated blood in the microcirculation of the active brain area is always greater than the changes in tissue O_2 consumption (i.e. the cerebral metabolic rate of O_2 consumption; $CMRO_2$) (Fox et al., 1988), this leads to a temporary situation of a higher proportion of oxyhaemoglobin to deoxyhaemoglobin (i.e. an oxygen-depleted haemoglobin) in that local area. The function of the MRI scanner is to detect changes in the relative amount in deoxyhaemoglobin within each voxel (a tissue volume unit of brain space). For example, an increase in the ratio of oxygenated to de-oxygenated blood per unit volume of brain tissue would lead to an increase in the BOLD signal (Passingham & Rowe, 2016). Logically, the deoxyhemoglobin content is influenced by the CBF, blood volume, $CMRO_2$ and the basal state of the region of interest, which makes the interpretation of the BOLD signal complex (Bartlett et al., 2008; Small & Heeger, 2013). Nevertheless, an increase in this signal is interpreted as resulting from the flow of oxygenated blood, which is closely related to increased cellular metabolism. Therefore, by measuring changes in O_2 availability, the BOLD fMRI signal provides an indirect measure of changes in neuronal activity.

Concurrent electrophysiology and fMRI experiments conducted in the visual system of monkeys have demonstrated that an increase in the BOLD response elicited by a visual stimulus in the primary visual cortex does indeed reflect an increase in neuronal activity (Logothetis et al., 2001; Goense & Logothetis, 2008). Using event-related designs (see Appendix 1) and analysing time courses, Logothetis and colleagues showed that the BOLD activation during the presentation of the visual stimulus mainly reflects neuronal input (i.e. perisynaptic activity) and intracortical processing within a cortical area, instead of neuronal output (spiking activity). Indeed, although spiking activity was frequently correlated with the BOLD response, it was possible to observe that whereas the spiking responses adapted a

couple of seconds after stimulus onset, the BOLD response could remain undiminished for the entire duration of stimulation. That is, there was a dissociation between spikes and cerebral blood flow. The best and most reliable predictor of the BOLD response was the local field potentials (LFPs), which reflect perisynaptic activity of the local neuronal population (Logothetis et al., 2001). The LFPs stayed elevated for the duration of the stimulus, thus they correlated with the BOLD response.

The amygdala

The amygdala is a brain structure important for the emotional processing of fearful, anxiogenic and rewarding stimuli (Klüver & Bucy, 1939; Weiskrantz, 1956; Everitt et al., 2003; Murray, 2007; Davis et al., 2010; Grupe & Nitschke, 2013; Duvarci & Pare, 2014; Tovote et al., 2015). Particularly, it is involved in the associative learning processes of stimuli that predict aversive (Davis, 1992; LeDoux, 2000) or rewarding outcomes (Balleine & Killcross, 2006; Tye et al., 2008). It also modulates anxiolytic or anxiogenic effects on behaviour (Tye et al., 2011), and contributes to attentional processes, such as those mediating vigilance or surprise, which play a crucial role in associative learning processes (Holland & Gallagher, 1999; Davis & Whalen, 2001; Roesch et al., 2012; Grupe & Nitschke, 2013). Additionally, it is a brain structure whose dysfunction (e.g. exaggerated reactivity) has been associated not only with depression (Sheline et al., 2001; Victor et al., 2010) but particularly with anxiety disorders (Etkin & Wager, 2007), which often result from altered aversive associative learning processes (Lissek et al., 2009; Graham & Milad, 2011). Some researchers also believe that amygdala reactivity can be used as a neural biomarker of psychological vulnerability to life stress (Swartz et al., 2015).

Despite some differences, the amygdala is a brain structure whose circuitry and function is well conserved across mammalian species (McDonald, 1998; Janak & Tye,

2015). It consists of a collection of several nuclei, with some of them usually divided into two or more subnuclei. Relevant for this thesis, however, are the two main sub-areas of the amygdala: the basolateral amygdala (BLA), which includes the lateral (LA), the basal (BA) and the basomedial (BMA) nuclei; and the central amygdala (CeA). These two sub-areas differ in their cell types, functional organisation, connections and behavioural function (Duvarci & Paré, 2014; Tovote et al., 2015). Together, these nuclei are crucial for the acquisition, expression and extinction of conditioned (i.e. acquired) fear memories, and are part of a wider network of structures involved in anxiety-related behaviours and innate fear (Davis et al., 2010; Tovote et al., 2015). Additionally, they are also involved in the acquisition of cue-directed, reward-seeking behaviour (Balleine & Killcross, 2006; Tye et al., 2008; Janak & Tye, 2015).

Altered amygdala function in individuals with varying levels of 5-HTT expression

In a seminal study in 2002, Hariri and collaborators showed that amygdala reactivity to emotional stimuli could be affected by 5-HTTLPR variation. This was achieved by measuring the fMRI BOLD signal during the administration of an emotional-face-matching task in 28 healthy participants previously genotyped for the S and L variants of the 5-HTTLPR. The stimuli presented during the emotional blocks, angry/threatening or fearful faces, were known to evoke amygdala activity (Breiter et al., 1996; Morris et al., 1996; Hariri et al., 2000). The perceptual task required the participants to match the affect of two presented faces with a simultaneously presented target face. This "emotional task" was interleaved with a "sensorimotor control task", where geometrical shapes were used instead of faces. The effect of 5-HTT genotype on amygdala processing resulted in greater reactivity specifically to the aversive stimuli in carriers of the *s* allele compared to those homozygous for the *l* allele.

The 5-HTT allele-dependent effect on amygdala reactivity was further replicated by the same research group with a larger independent sample (92 participants; Hariri et al., 2005), and by many other research groups testing healthy participants (and sometimes also patients; Munafò et al., 2008). Additionally, other imaging studies extended this finding by reporting additional effects of the 5-HTTLPR polymorphism on the neural circuits dealing with emotional processing, particularly those involved in amygdala regulation. This was the case with two studies showing that during the processing of aversive stimuli, *s* allele carriers exhibit (i) a decreased functional coupling between the amygdala and the anterior cingulate cortex (which could lead to a dysregulated amygdala response); and (ii) an increased coupling between the amygdala and medial PFC (mPFC) (which could reflect compensatory activity to regulate the amygdala) (Heinz et al., 2005; Pezawas et al., 2005). These results supported the hypothesis advanced by Hariri and collaborators (2002), and all the collected evidence strengthened the "imaging genetics" approach (Hariri et al. , 2006).

In addition, the first meta-analysis supported the hypothesis of an increased amygdala activation underlying the association between the 5-HTTLPR *s* allele and increased risk for anxiety-related traits (Munafò et al., 2008). It was based on data from 14 study samples (12 subjected to fMRI testing), and suggested that the polymorphism could explain up to 10% of phenotypic variance. A recent meta-analysis still found a statistically significant result with studies published between 2002 and 2011 (29 study samples, with 21 subjected to fMRI; Murphy et al., 2013b), though with a smaller effect size than previously advanced (~1%, with 4.6% as the best possible value within the estimate range). However, when 4 unpublished data sets (from fMRI studies) were added to the analysis, only a marginally significant result was obtained ($p = .050$). Furthermore, in an update to this recent meta-analysis which included the largest study conducted so far looking at this association (120 participants), only a trend towards significance was obtained ($p = .06$; 35 study samples,

22 subjected to fMRI; Bastiaansen et al., 2014). The large imaging study itself failed to find an association.

These recent meta-analyses led to several observations and warnings. Among these were a considerable between-study heterogeneity not attributable to study design and sample characteristics, and possible problems with genotyping quality (due to the Hardy-Weinberg equilibrium not being always met or reported; Munafò et al., 2008; Murphy et al., 2013b; Bastiaansen et al., 2014). However, the main criticism was that the studies conducted were underpowered, with Murphy and collaborators (2013b) suggesting that a sample size of 236 participants would be needed to meet the effect size advanced by their meta-analysis. No research group investigating the 5-HTTLPR *s* allele-driven amygdala heightened reactivity to emotional stimuli has ever conducted such a large-scale study. It is worth noting, however, that these meta-analyses were not specific only to the kind of studies proposed by Hariri and collaborators (2002). For instance, they also included studies examining just the resting state amygdala activity (via arterial spin-labelling perfusion magnetic resonance imaging) instead of amygdala activity in response to affective stimuli, on the basis that the method did not significantly moderate the effect size provided.

Limitations of candidate genes association studies and use of intermediate endophenotypes

In addition to the limitations mentioned so far, two other important caveats can be mentioned regarding not only genetic association studies but also imaging genetics studies. First, according to Murphy and colleagues (2013a), it was and still is common practice in many studies to genotype participants only for the biallelic 5-HTTLPR, thus ignoring other variants near the 5-HTTLPR known to also have a considerable impact on the 5-HTT gene transcriptional activity. This is the case for the already mentioned rs25531, which can indeed

produce either false positives or false negatives. Although large samples can protect against these two false results, the second caveat considers precisely the number of participants used. It is clear that a large number of genetic association studies and imaging genetics studies are in general underpowered (Flint & Munafò, 2004; Munafò et al., 2009a; Button et al., 2013), and those investigating the 5-HTTLPR are no exception (e.g. Munafò et al., 2009a; Murphy et al., 2013b). Underpowered studies have a lower probability of finding a true effect, and can only detect the largest of effects (i.e. those that pass the threshold for discovery), a situation that leads to an inflated estimation of the effect size (Button et al., 2013). An initial inflated estimation of the effect size (the "winner's curse") was what might have happened in the study conducted by Hariri and colleagues in 2002 (i.e. the initial claim that the 5-HTTLPR accounted for 40% of the variance in amygdala activation has been reduced to ~1% in the latest meta-analysis).

Overall, the results obtained with genes such as SLC6A4, but also with other types of genetic studies (e.g. linkage studies), seem to indicate that it is unlikely that a single gene variant can have a decisive risk for anxiety and depression. Risks for these disorders likely stem from gene-gene and gene-environment interactions, and thus the 5-HTTLPR can only contribute to a minimal extent to the variation in the measures used (Flint et al., 2010; Bogdan et al., 2013). Additionally, the strategy of investigating intermediate phenotypes to detect larger effects of single genetic polymorphisms seems to be less powerful than previously thought, particularly when compared to gene association studies. Accordingly, for some authors, investigation of intermediate phenotypes does not appear to guarantee larger genetic effects than those associated with the mental disorders or complex traits, nor provide more genetically tractable targets (Flint & Munafò, 2007; Flint et al., 2014). They argue, however, that intermediate phenotypes are better used to help to understand

mechanistic pathways; if not for candidate genes, then for genes identified through large-scale genome-wide association studies (GWAS).

1.2.3. Mouse models of genetically altered 5-HTT expression

One way of understanding mechanistic pathways with intermediate phenotypes is by modelling and studying them in mice (Tarantino & Bucan, 2000). Indeed, although it is not possible to completely model a psychiatric disorder in this species, it is possible to model intermediate phenotypes of that disorder that can be congruent with the human biology and genetics (Gould & Gottesman, 2006). One strategy consists of investigating candidate genes in mice to elucidate their role in molecular pathways known to be involved in the disorder, and their contribution to variation in behaviour, cognition and emotionality (Seong et al., 2002; Cryan & Holmes, 2005).

The mouse (*Mus musculus*) and human genome share considerable similarities. For instance, according to Waterston and colleagues (2002): the proportion of mouse genes without any homologue in the human genome is likely to be less than 1%; and, additionally, conserved synteny (i.e. regions where local gene order remains largely intact) can be found in corresponding regions of more than 90% of the mouse and human genomes. Importantly, mice are currently the most genetically tractable mammalian species (Humby & Wilkinson, 2006) given how much more amenable they are to genetic manipulations (e.g. gene modification in the germ line) compared to other species (e.g. rat; Seong et al., 2002; Hofker, 2011; Wahlsten, 2011). Additionally, the mouse is a model very comparable to the human in several aspects, including organ systems, tissues and physiological systems and behavioural traits (Hofker, 2011).

The extensive knowledge of the mouse genome and the level of conservation between human and mouse genes thus strengthens research investigating the relationships between genes, proteins and neural circuits underlying human behaviour in the mouse (Bargman & Gilliam, 2013). Relevant for the investigation of psychiatric intermediate phenotypes is also the degree of conservation between mouse and man regarding subcortical structures involved in the processing of emotion, such as the hippocampus and the amygdala, and in various relevant physiological and behavioural responses (e.g. fear, aggression, behavioural responses to drugs; Tecott, 2003; Cryan & Holmes, 2005).

One way to investigate the role of a candidate gene is by using a reverse genetics approach, where the consequences of the alteration of a candidate gene are studied by examining the phenotype of a genetically modified mouse carrying an altered form of that gene. This can be achieved by gene targeting, where a precise alteration is made in a predetermined location of the mouse genome (i.e. where the mouse version (orthologue) of the human candidate gene is located). Alternatively, transgenic technology can be used, in which a foreign (e.g. human) gene is introduced (somewhat imprecisely) into the mouse genome. Changes in the phenotype of a mouse carrying the altered gene detected by comparison with its wild-type (WT) mouse version can give an indication of the function of that gene.

For instance, researchers can remove or "knock out" a specific gene from an animal's genome via gene targeting to investigate the absence of function of that gene. Such a procedure is thought to exaggerate the effects obtained with more subtle point mutation manipulations (that alter but not eliminate the gene function; Seong et al., 2002). An advantage of using this technique is that it can be done in mice generated by inbreeding, which makes them ~99% homozygous. Keeping practically every gene identical between the experimental and littermate mice, apart from the altered form of a particular gene in one

of them, increases the probability that any differences found between the two lines will arise from the induced alteration. The effect attributable to a change in a single locus is thus inflated in inbred strains, which can help to better understand the function of a candidate gene by reducing the confounds associated with the genetic heterogeneity in a human population (Phillips et al., 2002; Flint et al., 2010). The reduction of confounds is further complemented by using littermate controls and exposing them to the same environmental conditions provided to the experimental mice, which helps to control for G×E interactions (Barkus, 2013). Epistatic (gene-gene interaction) effects can also be investigated if the mutation is studied in different genetic backgrounds (e.g. Holmes et al., 2003a).

For several years the study of intermediate phenotypes has been used in mouse models to investigate anxiety and depression (Holmes, 2001; Cryan & Mombereau, 2004; Cryan & Holmes, 2005). Although there is no orthologue of the 5-HTTLPR in the mouse (Heils et al., 1998), one of the most relevant mouse models in the study of anxiety and depressive disorders targets the murine 5-HTT gene: the knockout mouse for the 5- HTT gene (5-HTTKO mouse).

Examples of phenotypic changes in the 5-HTTKO mice		Effect	Authors/year
Biochemical features	▪ Extracellular 5-HT levels	↑	▪ Mathews et al., 2004; Jennings et al., 2010
	▪ 5-HT clearance and uptake	↓	▪ Montañez et al., 2003; Pan et al., 2001; Zhou et al., 2002;
	▪ 5-HT content in brain and periphery	↓	▪ Bengel et al., 1998; Kim et al., 2005
	▪ 5-HT synthesis	↑	▪ Kim et al., 2005
Anatomical changes	▪ Pyramidal neuron spine density	↑	▪ Wellman et al., 2007
	▪ Barrel-like patterns in somatosensory cortex	absent	▪ Persico et al., 2001
	▪ Cell density in neocortex	↑	▪ Altamura et al., 2007
Alterations in the expression and function of 5-HT receptors	▪ Expression and function of 5-HT_{1A} receptors in the DRN and amygdala	↓	▪ Fabre et al., 2000; Holmes et al., 2003b; Li et al., 2000
	▪ 5-HT_{1B} receptors density in the substantia nigra	↓	▪ Fabre et al., 2000
	▪ Density of 5-HT_{2C} receptors in the amygdala	↑	▪ Li et al., 2003
	▪ 5-HT_4 receptor binding site density in the amygdala	↓	▪ Jennings et al., 2012
Physiological features	▪ DRN 5-HT neurons firing activity	↓	▪ Gobbi et al., 2001
	▪ Neuroendocrine and adrenomedullary responses to acute and chronic mild stress	↑	▪ Lanfumey et al., 2000; ▪ Li et al., 1999;Tjurmina et al., 2002)
	▪ Body weight (after 3 months)	↑	▪ Holmes et al., 2002c; Murphy & Lesch, 2008
Behavioural features	▪ Homecage activity	↓	▪ Holmes et al., 2002a
	▪ Locomotor activity (motor function)	−	▪ Bengel et al., 1998; Sora et al., 2001; Holmes et al., 2003b
	▪ Exploration in novelty-based tests		
	o horizontal and vertical activities	↓	▪ Holmes et al., 2003b;
	o thigmotaxis	↑	▪ Kalueff et al., 2007a
	▪ Anxiety-like behaviours	↑	▪ Holmes et al., 2003b; Line et al., 2011
	▪ Depression-like behaviour	↑	▪ Holmes et al., 2002b; Lira et al., 2003;
	▪ Fear extinction recall	↓	▪ Wellman et al., 2007

Table 1.2. Selection of phenotypic changes in the 5-HTTKO mice. ↑, increased; ↓, reduced; −, no significant genotypic/group differences.

5-HTT knockout mice

There is more than one method to genetically modify a mouse so as not to express the 5-HTT but the commonly used and most well-known one was introduced by Bengel and collaborators (1998), who made a constitutive 5-HTTKO mouse by disruption of the murine 5-HTT gene via deletion of exon 2 near its 5' end. This leads to a complete absence of 5-HTT expression throughout both development and adulthood, resulting in not only several alterations of the serotonergic system in this mouse model, but also in many other biochemical, anatomical and physiological changes (Bengel et al., 1998; Kim et al., 2005; Murphy et al., 2008). Particularly relevant to this thesis are the following changes: the large (up to 10-fold) increase in basal extracellular 5-HT levels in several brain regions (Fabre et al., 2000; Mathews et al., 2004; Shen et al., 2004; Jennings et al., 2010; see also Table 1.2); a prolonged 5-HT clearance that is indistinguishable from diffusion alone (Montañez et al., 2003); and, finally, a greatly reduced 5-HT reuptake (e.g. a residual uptake via the dopamine transporters; Pan et al., 2001; Zhou et al., 2002). These changes amplify and prolong the duration of serotonin signalling at serotonin receptors (Murphy & Lesch, 2008).

5-HTTKO mice, therefore, provide an extreme model of the 5-HTTLPR and have been used to investigate some of the claims relating to the effects of that polymorphism advanced by genetic association studies conducted with human populations. Accordingly, across several tests, these mice exhibit increased anxiety-like behaviours (Holmes et al., 2003b; Lira et al., 2003; Line et al., 2011) and depressogenic behaviour (during exposure to stressful situations), though there is less consistency across tests in the latter case (Holmes et al.2002b; Lira et al., 2003). Additionally, 5-HTTKO mice also exhibit exaggerated adrenomedullary responses to stress (e.g. increase in plasma epinephrine to immobilization), increased anxiety-like responses after being exposed to predator odour, and increased

depression-like behaviour following repeated stress (Tjurmina et al., 2002; Adamec et al., 2006; Carroll et al., 2007; Wellman et al., 2007; see also Table 1.2).

Despite the possible influence of development alterations playing a role in the results mentioned above (see some examples in Table 1.2l; see also Chapter 6, section 6.1, and Appendix 1), it is important to mention that there has been consistency in these behavioural findings in other ways. Notably, 5-HTTKO rats have also been generated (Smits et al., 2006; Homberg et al., 2007a) and found to exhibit similar altered anxiety- and depression-like behaviours to the 5-HTTKO mice (reviewed by Kalueff et al., 2010). Additionally, consistency regarding the important role of 5-HTT expression in the modulation of those behaviours has also been found by using a genetically modified mouse model which is opposite to the 5-HTTKO mice: a mouse model with an overexpression of 5-HTT levels compared to its WT controls (Jennings et al., 2006).

5-HTT-overexpressing mice

Transgenic technology has been used to insert and express the human 5-HTT gene in mice to create another gain-of-function mice of the 5-HTT (Jennings et al., 2006). Compared with the gene targeting technique, transgenic technology has little control over where the foreign DNA construct is incorporated into the genome, and how many copies are integrated at random locations (Wolfensohn & Lloyd, 2003; Carter & Shieh, 2010). This sort of DNA incorporation can lead to an overexpression of the gene product which is useful to study the consequences of an enhanced function of a given candidate gene (Tecott, 2003). Indeed, the mice generated by Jennings and collaborators (2006) were 5-HTT-overexpressing mice (5-HTTOE mice), exhibiting two- to threefold greater 5-HTT expression levels than their WT controls. Notably, this is a similar variation to that reported by some authors for the human population (Lundberg et al., 2007).

Compared to their WT controls, 5-HTTOE mice have increased levels of 5-HTT expression in the raphe nuclei and several regions of the forebrain, including the amygdala (Jennings et al., 2006; Barkus et al., 2014). Moreover, opposite to the 5-HTTKO mice, they exhibit a reduction in basal and potassium-evoked extracellular 5-HT levels during *in vivo* microdialysis studies, and a decrease in electrically evoked fluctuations in extracellular 5-HT concentrations measured by fast-scan voltammetry in brain slices (Jennings et al., 2006; Jennings et al., 2010).

A significant finding obtained with 5-HTTOE mice was that they exhibit reduced anxiety-like behaviours in the same tasks in which 5-HTTKO mice show exaggerated anxiety-like behaviour (Jennings et al., 2006; Line et al., 2011). The results obtained with 5-HTTOE mice strengthened those obtained with 5-HTTKO mice, given that the opposite direction of changes in anxiety-like behaviours was observed, resulting from opposing alterations of the 5-HTT gene (enhancement and deletion). Importantly, administration of a SSRI in 5-HTTOE mice not only increases extracellular 5-HT levels towards WT levels but, if acutely administered (30min) before an anxiety test such as the elevated plus maze (EPM), it can reverse the low-anxiety phenotype typically exhibited by these mice in the EPM (Jennings et al., 2006).

These observations suggest the opposite behavioural changes observed with the 5-HTTKO mice and 5-HTTOE mice in anxiety tests might result from opposite alterations in extracellular 5-HT levels. Contrary to this hypothesis, there are another 5-HTT gain-of-function mice that do not typically differ from their WT controls in tests measuring anxiety-like behaviours. This is the case of the 5-HTT Gly56Ala knock-in mouse (Veenstra-VanderWeele et al., 2009), which does not significantly differ from the WT controls in the EPM, elevated zero maze and open field, for instance (Veenstra-VanderWeele et al., 2012; Kerr et al., 2013). However, importantly, the gain-of-function in these mice (e.g. increased

brain 5-HT clearance) occurs despite no changes in protein levels. Taken together, these results and those obtained with 5-HTTKO mice suggest that levels of 5-HTT expression can affect anxiety-like behaviours in mice and that this can be associated with changes in 5-HT transmission.

1.2.4. Invasive recordings from the basolateral amygdala in mouse models

Researchers have considered using imaging techniques to study more quantitative intermediate phenotypes in mouse models since these started to be used to study aspects of psychiatric disorders (e.g. Seong et al., 2002; Gould & Gottesman, 2006). Although it is possible to combine small-animal fMRI systems with electrophysiology, this is problematic for several technical reasons, which are difficult or costly to counteract. Particularly, the electrodes implanted in the region of interest can cause artefacts in imaging data and the electromagnetic field of the fMRI can interfere with the neuronal recordings (Martin, 2014). Additionally, an animal subject would not be able to move around freely and thus imaging would be incompatible with many of the tasks already validated to study emotional learning and behaviour. Furthermore, the problem of different spatiotemporal scales between imaging and neuronal activity signals would remain.

However, another important reason to use animal models is the possibility of using invasive techniques, either alone or combined with other techniques in behaving subjects that would be either unethical or impractical to conduct in human studies. Accordingly, an alternative, invasive technique to fMRI has been developed that can provide a signal surrogate for the BOLD fMRI response in freely, behaving mice, which can be combined with other electrophysiological recordings. This technique is known as tissue O_2 (hereafter, TO_2) amperometry.

Tissue oxygen amperometry

The BOLD fMRI signal is based on TO_2 availability, even though it is not obtained by direct measurement of changes in brain TO_2 but instead by changes in the state of blood oxygenation in the local microcirculation (Raichle, 1998). TO_2 is associated to deoxyhemoglobin through i) the local oxygen concentration gradient in tissue and ii) the oxygen-haemoglobin dissociation curve (Thompson et al., 2003). This means that O_2 readily dissociates from the haemoglobin and is released for use in tissues using O_2 at a high rate, which is the case for brain tissue (Offenhauser et al., 2005). Importantly, changes in TO_2 are similarly influenced by changes in CBF, blood volume and O_2 consumption (Offenhauser et al., 2005; Logothetis, 2007; Viswanathan & Freeman, 2007). Considering this, it has been argued that the direct measurement of changes in TO_2 concentration could provide a signal reflecting the balance of local O_2 delivery and consumption analogous to the BOLD signal (Zheng et al., 2002; Thompson et al., 2003; Viswanathan & Freeman, 2007; Lowry et al., 2010). Moreover, measuring these changes in TO_2 directly might provide a more accurate signal, with a better spatio-temporal resolution than the BOLD signal itself (Logothetis, 2007).

Tissue O_2 changes can be directly measured by either TO_2 polarography or TO_2 voltammetry, which are invasive techniques that involve implanting a working electrode (sensor) in the brain region of interest. Both allow a direct and continuous measurement of the time course of TO_2 availability *in vivo*, either by measuring it as partial pressure (polarography) or by electrochemical reduction of dissolved O_2 (amperometry) in the extracellular fluid.

TO_2 polarography involves the use of a noble metal electrode to measure changes in the partial pressure of TO_2, which is linearly related to TO_2 within the measured area (Logothetis, 2007; Bartlett et al., 2008). The working electrode reduces the O_2 at its surface,

which induces changes in the measurable current flow that are proportional to the local O_2 concentration. This technique has been used, for instance, by Viswanathan and Freeman (2007), who supported the claim made by Logothetis and colleagues (2001) that the best predictor of the BOLD signal are LFPs. They created an experimental situation where LFPs could be elicited in the primary visual cortex without concomitant spike discharge and then performed colocalised TO_2 amperometric polarography. They observed that changes in TO_2 were more closely coupled with LFPs compared to neuronal spiking and that spiking activity was not necessary to induce changes in TO_2 responses (and in the BOLD signal, by extrapolation).

An alternative but similar method to the TO_2 polarography is a type of voltammetry known as TO_2 constant potential amperometry (CPA). CPA involves having a working carbon paste electrode (CPE) held at a specific, constant voltage (-650mV, relative to a reference electrode) to reduce O_2 at its surface (Lowry et al., 1997; Bolger et al., 2011). The acceptance of electrons by the O_2 produces a measurable change in current, with the magnitude of change being proportional to the local concentration of O_2 available (Hitchman, 1978). TO_2 monitored using CPA at a CPE (hereafter, TO_2 amperometry) has a high temporal resolution (which allows real-time, submillisecond recordings), high sensitivity for its target analyte (O_2) and provides interference-free signals (Bolger et al., 2011). Compared with TO_2 polarography with noble metal electrodes, it has a higher temporal resolution, its sensors (CPEs) are more stable, less fragile and are less prone to surface poisoning (Bolger et al., 2011; Martin, 2014).

Importantly, in addition to providing a reliable index of CBF under conditions of physiological stimulation (Lowry et al., 1997), it has been shown in an *in vivo* experiment using fMRI concurrently with TO_2 amperometry that the BOLD and TO_2 signals were correlated in the rat cerebral cortex (Lowry et al., 2010). Additionally, other experiments

combining TO_2 voltammetry with LFPs measurements demonstrated that changes in the TO_2 signal occur rapidly following neuronal activity (Li et al., 2011), and that these techniques can be used simultaneously in freely behaving rodents during a discriminative fear conditioning procedure (McHugh et al., 2013, 2014).

In conclusion, TO_2 amperometry is an appropriate technique to investigate the amygdala haemodynamic responses from behaving mice, particularly because it can be simultaneously combined with other invasive techniques such as LFPs recordings.

Local field potentials

When a recording electrode is placed into the brain, it is possible to measure the potential difference (in Volts), relative to a reference electrode, with sub-millisecond time resolution. The change in voltage recorded over time reflects changes in the extracellular medium caused by all the aggregated ionic exchanges between the intracellular cytosol and the extracellular fluid occurring in multiple neurons of the brain tissue surrounding the recording electrode (Reed et al., 2015). That is, any transmembrane current leads to an intracellular and an extracellular voltage deflection, and extracellular voltage deflections are the source of local field potentials (LFPs) (Buzsáki et al., 2012).

For instance, following an excitatory synaptic input, positive charge enters the neuron (inward current) leading to a local extracellular (current) sink. To balance this, another current is prompted (along the neuron) in a distant site of the same neuron through an opposing ionic flux from the intracellular to the extracellular space (Buzsáki et al., 2012; see their Fig 2.A). This distant site thus becomes an extracellular (current) source for the sink region, and when the (outward) current flows back to the sink site, it alters the extracellular medium (causing a voltage deflection). The superposition of sinks and sources generated by multiple neurons at a given time in a delimited space is what causes

extracellular field potentials (EFP). The weighted spatiotemporal sum of these potentials (i.e. the mean EFP) is what the recording electrode detects (Logothetis, 2008; Berens et al., 2010; Logothetis & Panzeri, 2015). According to Logothetis and colleagues, this signal comprises several types of neural activity, including action potentials, spiking of a small neural population, and perisynaptic activity of a neural population. The latter type of activity within a volume of brain tissue is what a LFP essentially measures, which includes excitatory and inhibitory postsynaptic potentials but many other processes capable of generating extracellular currents (e.g. intrinsic currents and resonances; Berens et al., 2010; Buzsáki et al., 2012)

To analyse LFPs, it is usual to first filter the recorded mean EFP voltage trace and focus on its low-frequency components, thus avoiding the effects of action potentials. These components are thought to mostly reflect cooperative activity in neural populations (Logothetis, 2008, p. 10 of the supplementary information). Then, one way of dealing with the ambiguity associated with the multitude of processes generating a LFP and the changes in its frequency composition over time is to decompose the signal into a set of frequency bands (Logothetis & Panzeri, 2015). These band-limited, oscillatory components are thought to be generated by different processes and are typically associated with different brain states, though their consequences vary according to the function of the brain region where they are generated (Buzsáki & Draguhn, 2004). Their limits are largely arbitrary and vary between studies and species.

Of importance for this thesis are oscillations in the theta frequency band (θ, 4-12Hz). Oscillations in this frequency band have been associated with the processing of aversive stimuli, particularly because several structures involved in fear learning seem to process information and communicate with one another via theta-related activity synchronisation. Synchrony of LFPs indicates that the activity of two simultaneously recorded distant

networks is correlated and this likely underlies an interaction between two brain structures, possibly leading to facilitated synaptic transmission (Frederick et al., 2015).

Moving from human imaging studies to research with genetically modified mice

An important function of mouse models is that they can be used to investigate experimental hypotheses advanced by human imaging studies examining the influence of genetic variation on the function of neural circuits underlying certain emotional behaviours (Cryan & Holmes, 2005). This was the case with 5-HTTOE mice in a study conducted by Barkus and colleagues (2014), who recorded changes in TO_2 and LFPs from the BLA of these mice and their WT controls while they were fear conditioned. It was shown that, compared to WT controls, 5-HTTOE mice exhibit significantly reduced amygdala TO_2 responses to an aversive cue predicting footshock delivery, and that these were accompanied by reduced theta oscillations. Importantly, this study also reported that there was a significant correlation between theta oscillatory power and TO_2 responses evoked by the aversive cue in WT mice but not in 5-HTTOE mice.

This approach can thus work as an important alternative to imaging genetics studies to investigate the same fundamental questions in a more controlled and thorough way. Questions such as whether variation in 5-HTT expression levels affects the processing of aversive cues, and what are the underlying neuronal mechanisms, can be investigated in a more direct manner than by measuring brain activity in an indirect way (via the BOLD signal) in participants with only a putative variation in 5-HTT expression levels. Using TO_2 amperometry in a mouse model in which 5-HTT expressions levels had been directly manipulated and measured, allowed Barkus and colleagues (2014) to confirm that variation in 5-HTT expression can influence TO_2 responses (and by extrapolation, the BOLD signal) in the amygdala during the processing of aversive stimuli. Additionally, the parallel

recording of LFPs made it possible to probe into a potential neuronal mechanism underlying such altered amygdala activity (see also Bocchio et al., 2016). This study not only complemented imaging genetics studies on the study of 5-HTTLPR effects on amygdala reactivity, but it also advanced new lines of enquiry for the function of the 5-HTT.

1.3. "Plasticity genes" and a neuroplasticity theory of 5-HT function

"Vulnerability genes" versus "plasticity genes"

A common premise of most gene-environment interaction studies investigating the 5-HTTLPR is that *s* allele carriers (particularly with a *s*/*s* genotype or equivalent) have a latent increased risk to certain psychiatric disorders that is activated when they are exposed to environmental adversity/stress (Caspi et al., 2010). The theoretical framework underlying those studies is known as the diathesis-stress model of psychopathology and postulates that some individuals have a genetic make-up that makes them more vulnerable to negative environmental conditions whereas others are not so affected (i.e. they are resilient to those conditions). However, the persistence of a genetic variant throughout evolution associated only with disadvantage leads to a paradox when considered in the light of the principles of natural selection, which would predict their elimination from the genetic pool if that was the case (Uher, 2009; Homberg & Lesch, 2011).

Adopting an evolutionary perspective, other authors proposed the hypothesis that some gene variants make some individuals not merely more "vulnerable" to adverse situations but instead more responsive (or susceptible) to both negative and positive environmental conditions. This is the case, for instance, for the genetic differential-susceptibility perspective of Belsky and colleagues, who suggested that individuals with certain gene variants vary in their plasticity to environmental influences (Belsky et al., 2007,

2009). That is, they are more malleable to different environments. Such malleability (or plasticity) leads them to be at a greater disadvantage in adverse environments (i.e. the "vulnerability" considered by diathesis-stress models), but also to greater advantages when exposed to environmental support and enrichment, or just absence of adversity (e.g. "vantage sensitivity"; Pluess & Belsky, 2013). Belsky et al. (Belsky et al., 2009) therefore suggested that some of the so-called "vulnerability genes" or "risk alleles" could be better conceptualized as "plasticity genes", given that they seem to make individuals more susceptible or plastic to environmental influences. Individuals without such "plasticity genes" are predicted to be less responsive to the environment, suffering less in stressful environments but benefiting less in more supportive ones. This new "for-better-and-for-worse" perspective of the role of the *s* allele could help explain the inconsistencies found in genetic association studies, which usually do not consider positive life events.

Research on the 5-HTTLPR has provided support for this "for-better-and-for-worse" hypothesis, either with G×E interaction studies in clinical populations (Eley et al., 2004; Kaufman et al., 2004; Taylor et al., 2006), or G×E interaction studies for intermediate phenotypes, such as neuroticism or anxiety sensitivity (Stein et al., 2008; Pluess et al., 2010; Kuepper et al., 2012; see Table 1.1). For instance, in a study conducted by Eley and colleagues (2004), adolescents with the *s*/*s* genotype exposed to high-stress family environments were associated with a higher level of depressive symptoms compared to *l*/*l* carriers. However, in low-stress family environments the *s*/*s* genotype was associated with reduced depressive symptoms compared to the *l*/*l* genotype. In another example, it has been shown that more negative lifetime events are associated with greater neuroticism in healthy participants with the *s*/*s* genotype compared to *l*-allele homozygotes. However, more positive lifetime events can be associated with less neuroticism and increased life satisfaction among carriers of the *s*/*s* genotype but not *l*-allele homozygotes (Pluess et al.,

2010; Kuepper et al., 2012). Additionally, compared to *l*-allele carriers, children homozygous for the *s* allele are more responsive to both supportive and unsupportive parenting, exhibiting higher and lower positive affect, respectively (Hankin et al., 2011).

Some studies on the 5-HTTLPR have not supported the genetic differential-susceptibility perspective, including studies for intermediate phenotypes (e.g. Araya et al., 2009; Middeldorp et al., 2010). However, the first meta-analysis on G×E interaction with 5-HTTLPR studies in children under 18 years old provided some support for the hypothesis, at least in Caucasian samples (van IJzendoorn et al., 2012). Additionally, experimental research with human participants and animal models with varying 5-HTT expression levels has also been used to provide support for the "for-better-and-for-worse" effect associated with low 5-HTT-expression levels (Homberg & Lesch, 2011; Homberg & van den Hove, 2012; Kiser et al., 2012). For instance, Carola and colleagues (2008) investigated susceptibility to the long-term behavioural effects of altered maternal care in heterozygous mice for the 5-HTT (5-HTT +/– mice), which also exhibit decreased 5-HTT expression, decreased 5-HT clearance and elevated extracellular 5-HT levels compared to WT controls (Bengel et al., 1998; Montañez et al., 2003; Mathews et al., 2004; Jennings et al., 2010). 5-HTT +/– mice subjected to low maternal care (until day 19) exhibited increased anxiety and depression-related behaviour when tested at an adult age (8-10 weeks) compared to 5-HTT +/– mice subjected to high maternal care. Importantly, no effect of maternal care was seen in WT controls. Additionally, other studies with human *s* allele carriers, rhesus monkeys carrying an orthologue of the *s* allele, and 5-HTTKO rodents have all shown that subjects with low or null 5-HTT expression levels can exhibit cognitive improvements in several tasks (e.g. reversal learning; Homberg & Lesch, 2011; see chapter 7). Therefore, evidence has been collected across species supporting "for-better-and-for-worse" effects on subjects carrying genetic variants leading to low or null 5-HTT expression levels.

A neuroplasticity theory of 5-HT function

It has been hypothesised that an increased responsivity to the environment could depend upon the availability of 5-HT. This hypothesis was advanced by Branchi (2011), who proposed that increased 5-HT levels can affect sensitivity to the environment via enhanced neuronal plasticity, thus working as a permissive/catalyser factor that can lead to change without determining the direction of such change. Such direction of change can be determined by several factors acting at different levels of the individual (e.g. molecular, behavioural), and the environment is a crucial element of those factors.

There is evidence that antidepressants can facilitate plasticity processes (Pittenger & Duman, 2008; Castrén & Hen, 2013), including "neurogenesis and selective neural elimination, arborisation and retraction of axons and dendrites, and synaptic formation and pruning" (Castrén, 2005, p. 244). For instance, Maya-Vetencourt and colleagues (2008) have shown that chronic SSRI administration can restore neuronal plasticity in the adult visual system of an adult rat that has been subjected to an impairment of visual ability early in its development. This was seen in the form of a reinstatement of a juvenile-like form of plasticity in adulthood, and increased brain-derived neurotrophic factor (BDNF) protein expression in the visual cortex of fluoxetine-treated rats. Furthermore, other animal studies have shown that SSRI administration can induce developmental-like plasticity (e.g. reverse the established state of maturation of neurons) in the dentate gyrus (Kobayashi et al., 2010) and amygdala (Karpova et al., 2011). Additionally, Karpova and colleagues (2011) showed that increased plasticity in the amygdala due to SSRI treatment (e.g. increased BDNF mRNA levels in the BLA) can also lead to an enduring loss of a fear conditioned memory after extinction training. According to these researchers, their findings suggest a putative biological mechanism underlying the enhanced effect of combining antidepressant drugs and psychotherapy (Karpova et al., 2011). Indeed, when considering clinical data, there are

reports that combining antidepressant treatment with psychotherapy works better than either treatment alone (Pampallona et al., 2004; Cuijpers et al., 2009).

The above-mentioned result of Maya-Vetencourt and colleagues (2008) can be linked to a study that showed altered neuronal plasticity in the visual pathways of healthy participants subjected to chronic administration of an SSRI. Using visual evoked potentials (VEP) as a marker for synaptic activity, Normann and colleagues (2007) showed that healthy participants exhibited augmented stimulus-induced VEP plasticity to a prolonged presentation of visual stimuli after chronic SSRI administration, compared to a pre-treatment period. Importantly, they also showed that changes in some components of the VEPs in depressed patients occurred in the opposite direction to those observed with SSRI-treated healthy controls, suggesting an impairment of synaptic plasticity in the patients. This is consistent with research indicating that biological markers of neuronal plasticity, such as BDNF expression levels, are reduced in depressed patients, but are either at normal (Karege, et al., 2005) or even increased levels (Chen et al., 2001a) in those receiving antidepressants treatment. Similarly, antidepressant-treated depressed patients have been shown to exhibit increased levels of BDNF in serum compared to antidepressant-free patients (Shimizu et al., 2003).

Besides considering SSRI treatment as a factor triggering an increase in neural plasticity, Branchi (2011) considers that the *s* allele leads to a higher susceptibility to change, either for the worse (e.g. increased vulnerability to psychopathology when facing an adverse environment) or for the better (e.g. increased capacity to recover with psychotherapy). Considering the second situation, it has been shown that children homozygous for the *s* allele are more responsive to cognitive behaviour therapy when suffering from anxiety disorders (Eley et al., 2012). Additionally, they exhibit reduced risk for depression after a history of maltreatment if positive social supports are available to them on a regular basis (Kaufman et

al., 2004). Adult *s* allele carriers have also been found to be more responsive to some therapies than adults homozygous for the *l* allele, as is the case, for instance, with the exposure-based behavioural therapy program for agoraphobia (Knuts et al., 2014).

Underlying these observations seen in the *s* allele carriers is the assumption that these individuals should have higher levels of neuronal plasticity compared to homozygotes for the *l* allele (Branchi, 2011). Consequently, low levels of neuronal plasticity make the latter less vulnerable to psychopathology. At first glance, this seems to conflict with the above-mentioned research indicating low levels of neuronal plasticity measured with BDNF levels in people with depression. However, it should first be noted that *l*/*l* carriers are not immune to depression or psychopathology. Moreover, when they do suffer from a mental disorder, they show a worse capacity to recover, probably resulting from a lower impact of a supportive environment (e.g. psychological therapy; Branchi, 2011). Additionally, it has been shown that acute and chronic stress can reduce hippocampal BDNF mRNA levels (e.g. Smith et al., 1995; Rasmusson et al., 2002), and that elevated early-life psychosocial adversity can decrease plasma BDNF levels in *l*/*l* carriers (Buchmann et al., 2013). Moreover, other factors can affect BDNF expression. For instance, the Met allele of the BDNF gene val66met polymorphism is associated with decreased processing and release of BDNF. Notably, at least two well-powered studies have shown that *l*/*l* carriers homozygous for the BDNF Met allele who had experienced early-life adversity (e.g. sexual abuse experience) showed increased vulnerability to depression, which was comparable to carriers of the SS/SL+Val/Val (Grabe et al., 2002; Comasco et al., 2013). Therefore, it is possible that the samples from depressive patients used in the above-mentioned studies could include both *s* allele carriers and homozygotes for the *l*/*l* allele.

Considering the ideas articulated by Belsky's and Branchi's and also by other researchers (e.g. Homberg & van den Hove, 2012), it is possible to describe them in this

thesis as a "neuroplasticity theory of 5-HT function", where 5-HTT expression levels and associated changes in 5-HT availability do not affect emotionality *per se* but influence sensitivity to the environment instead. That is, low 5-HTT expression levels and high 5-HT availability are neither advantageous or disadvantageous by themselves; they promote neuronal plasticity and the environmental conditions will determine whether the consequences will be beneficial or detrimental. Importantly, Branchi et al. (2013) have already provided initial empirical evidence for this theory with a pre-clinical study in mice. In this study, mice chronically treated with an SSRI in an enriched environment after a ~3-week period of stress (a daily exposure to a stressful task, e.g. forced swim), showed less anhedonia and an increase in brain BDNF levels compared to saline-treated controls. By contrast, chronic SSRI administration during a ~3-week period of exposure to a stressful-condition resulted in a faster and more pronounced worsening of the depression-like profile (e.g. more anhedonia and lower brain BDNF levels). Limited effects of SSRI administration were observed in constant environmental conditions. Therefore, the effect of SSRIs can be driven by the quality of the environment (Branchi et al., 2013).

1.4. Aims of this thesis

It has been suggested that hypervigilance, deeper processing of emotional stimuli and selective cognitive processing biases could underlie the enhanced emotional responsivity to different environments associated with low or null 5-HTT expression, and increased 5-HT availability (Homberg & Lesch, 2011; Homberg et al., 2016; Fox & Beevers, 2016). However, direct evidence for the neuroplasticity theory of 5-HT function at the behavioural and neurophysiological levels is still relatively scarce. Therefore, the main goal of this thesis was to investigate the neuroplasticity theory of 5-HT function by testing a mouse model

known to have increased extracellular 5-HT levels, the 5-HTTKO mouse, in several behavioural tasks, using a variety of negative and positive emotional stimuli. Particularly relevant was the investigation of whether altered sensitivity could facilitate aversive learning, as some human studies with participants (putatively) varying in their 5-HTT expression levels seem to suggest (e.g. Garpenstrand et al., 2001; Lonsdorf et al., 2009). However, other forms of altered emotionality and learning were also investigated.

The first set of experiments (Chapter 3) was intended to build upon previous research on the anxiety phenotype of 5-HTTKO mice by extending it to the investigation of social anxiety. Two non-social conflict-based anxiety tests (elevated plus maze and hyponeophagia) were first used to replicate previous findings of increased anxiety in 5-HTTKO mice relative to WT controls (Holmes et al., 2003b; Line et al., 2011). Afterwards, two assays were used to investigate if 5-HTTKO mice also exhibited altered social anxiety: the reciprocal social interaction test and the three-chamber social approach test. In addition to this, the latter test included a measure for social novelty preference to investigate social recognition memory, thought to reflect sensitivity to social stimuli in the environment.

After this comprehensive assessment of their anxiety profile, two discriminative (Pavlovian) fear conditioning experiments were administered to 5-HTTKO mice to investigate whether the absence of the 5-HTT affects aversive learning via enhanced sensitivity to emotionally-relevant cues (Chapter 4). The first experiment required subjects to learn to discriminate between a fully predictive aversive cue (P(shock|cue#A = 1) and a fully predictive non-aversive cue (P(shock|cue#B = 0). The second experiment, conducted on a different batch of mice, consisted of the same task but with the fully predictive aversive cue now replaced with a cue that was only partially reinforced (P(shock|cue#A = 0.2), thus becoming an ambiguous aversive cue. This second experiment was conducted because a previous study in the laboratory had shown that aversive conditioning to the ambiguous cue

was more sensitive to variation in 5-HTT expression than the conditioning to the fully predictive aversive cue. Extinction learning and contextual fear conditioning were also investigated with the 5-HTTKO mice.

Subsequently, in Chapter 5, an experiment was conducted to probe into the neuronal substrates of the behavioural phenotype detected when testing 5-HTTKO mice in the discriminative fear conditioning task using fully predictive aversive cues (Experiment 1 of Chapter 4). TO_2 amperometry and LFPs were simultaneously recorded from the BLA of 5-HTTKO mice and WT controls during behavioural testing in a slightly extended version of the same conditioning task. This procedure not only allowed the investigation of the neuronal substrates of the behavioural phenotype, but also allowed comparisons with the results previously obtained with 5-HTTOE mice tested in the same task (Barkus et al., 2014).

Afterwards, it was crucial to address the issue of whether the behavioural phenotypes observed in the discriminative fear conditioning tasks (Chapter 4) were due to elevated 5-HT levels at the time of conditioning, or due to neurodevelopmental changes in the neuronal circuits underlying fear learning in the 5-HTTKO mice. Therefore, non-transgenic mice were subjected to the same conditioning tasks but with increases in 5-HT levels following SSRI (citalopram) administration (Chapter 6). If similar behavioural alterations to those obtained with the 5-HTTKO mice were observed, then increased support for the neuroplasticity theory of 5-HT would be gathered.

Finally, emotional learning in a positive (i.e. appetitive) situation was also investigated with 5-HTTKO mice (Chapter 7). 5-HT is involved in the regulation of reward-related behaviour (Hayes & Greenshaw, 2011) and if the neuroplasticity theory of 5-HT is correct, then these mice should exhibit increased sensitivity not only to aversive events but also to non-aversive events. Accordingly, 5-HTTKO mice and their WT controls were subjected to a Pavlovian conditioning procedure similar to the discriminative aversive

conditioning procedure used previously, but using a food reward cue (i.e. P(reward|cue#A) = 1; P(reward|cue#B) = 0). Additionally, this discriminative appetitive learning was followed by reversal learning, to investigate cognitive flexibility and extinction learning, for comparison with the data obtained with the fear extinction learning (Chapter 4).

Chapter 2. General methods

This chapter provides complementary information for the experiments reported in this thesis (Chapters 3-7). It includes information on (i) the generation of 5-HTTKO mice, (ii) animal welfare and logistics, (iii) apparatus and behavioural tests, (iv) surgical and histological techniques, and (v) data analyses used. Further details relative to each experiment can be found in the relevant chapters.

2.1. Experimental subjects

Every experiment reported in this thesis used mice as subjects. All experiments used 5-HTTKO mice and their WT littermates, apart from those described in Chapter 6, which used C57BL/6J mice. The 5-HTTKO mice and WT controls were generated as previously described (Bengel et al. 1998), that is, they were generated on a 129P1 (129P1/ReJ) × C57BL/6J hybrid background, before being repeatedly backcrossed onto a C57BL/6J background for more than eight generations (see below for more details). They were originally provided to the University of Oxford by the laboratory of Klaus P. Lesch (University of Wuerzburg, Germany), which means that all subjects used in this thesis are the progeny of the Bengel et al. model. However, all the KOs and WTs used in this thesis were bred at the University of Oxford.

Animal procedures followed the United Kingdom Animals Scientific Procedures Act (ASPA, 1986) under project licences 30/2561 and 30/3068, and personal licenses 30/9559 (Chapters 3-7), 30/5488 (Chapter 5) and 30/6220 (Chapter 6). Experiments were approved by a local ethical review for the University of Oxford (UO), and codes of practice drawn up by the Department of Experimental Psychology (DEP, UO), were followed throughout. Mice

were housed at Biomedical Sciences Building (BSB, UO) and experiments were conducted at the Jeffrey Gray Behavioural Neuroscience Unit (BNU), located in the same building.

2.1.1. Generating 5-HTTKO mice

5-HTTKO mice were created by replacing exon 2 of the murine 5-HTT gene with a drug-resistant gene (PGK neomycin-polyA expression cassette) in 129 R1 ES cells (129P1/ReJ background), which were then microinjected in C57BL/6J blastocysts (Bengel et al., 1998). The resulting chimeric male mice were mated with female C57BL/6J mice, and thereafter backcrossed onto that parental strain for at least eight generations. The C57BL/6J strain is recommended as a background strain for behavioural studies (Crawley, 2008; Wahlsten, 2011), and is preferable to the 129 background strain for investigating emotional phenotypes because of high levels of anxiety in the 129 (Võikar et al., 2001).

The breeding strategy was the heterozygote mating of mice not closely related (i.e. not brothers or sisters). Mating 5-HTTKO +/– mice is usually a preferred breeding strategy given that it controls for differences in maternal behaviour related to genotype (Holmes & Cryan, 2006).

The KOs and WTs used in the experiments were age-matched littermates originated from as many different breeding pairs as possible. WT littermates are the recommended controls for the KO_S because they control for potentially confounding differences in the genetic background (see Appendix 1) and in early life experiences (e.g. differential maternal care; Holmes & Cryan, 2006; Crusio et al., 2009; Carter & Shieh, 2010).

2.1.2. Genotyping

5-HTTKO mice were identified by PCR genotyping on tissue obtained by ear biopsy during the marking for individual identification of subjects in each cage by ear punching. 5-HTTKO primers for exon2, intron2 and neo-new (Eurofins MWG Operon, Ebersberg,

Germany) were used for genotyping. This was initially done personally by me, with the help of Greg Daubney (DEP, UO) and Amarjit Bhomra (Welcome Trust Centre for Human Genetics, UO), and later by Harlan Laboratories (Envigo RMS, Loughborough, UK).

2.2. Logistics

Mice were housed with their littermates in small groups (2-6) of the same sex to maintain the social groups established before they were weaned off parental care (Bailey et al., 2006). In situations where this was not possible, a pup was housed with others weaned at the same time to avoid single housing, which can stress mice and also lead to detrimental effects in several behaviours (Garattini & Valzelli, 1981; Martin & Brown, 2010).

Mice were housed in cages containing sawdust bedding, sizzle nest and cardboard enrichment (e.g. a roll); and held in a colony room under constant temperature conditions (21 ± 1 °C) on a 12h light/dark cycle (lights on between 07:00 and 19:00h), with water and food always available. Subjects were usually provided several weeks of acclimatisation to the new housing conditions before being tested. However, when this was not possible, care was taken to provide at least one week of acclimatisation to the colony room (and two cage changes) before the beginning of an experiment. Testing was coordinated with cage changing to control for the increase in general activity usually seen in mice immediately after the change (Bailey et al., 2006; Wahlsten, 2011).

During testing periods, subjects were transported to a holding (staging) room located within the BNU, where they would wait for at least 30-45 minutes before being tested. Apart from providing consistency of experiences before testing, this is thought to also reduce stress levels by temporally providing a regular acclimatisation environment, and by reducing the transportation time to the experimental room (Bailey et al., 2006; Bailey & Crawley, 2009).

Coordination of testing with the light cycle was done by having mice tested at fixed times of the day and outwith the light-dark transitional periods of the colony room (i.e. subjects were tested between 09:00 and 18:00h). Testing in the morning and early afternoon was always preferred to the testing in the later afternoon. Acclimatising periods to the holding room and, in the majority of the experiments reported here, to the experimental room just before testing, also help keeping light levels constant (Wahlsten, 2011). Each mouse was individually transported and acclimatised to the experimental room in a clean cage (Bailey & Crawley, 2009), although adjustments and exceptions were made in some experiments.

Mice are sensitive to pheromones, which mediate their communication, particularly in situations where they work as sex attractants or alarm odours (e.g. when a stressed mouse causes dispersal of other mice; Ferguson et al., 2001; Wolfensohn & Lloyd, 2003). In some tests, such as the elevated plus maze, they can also leave odour trails. Therefore, pieces of apparatus were cleaned immediately after testing each mouse, usually with disinfectant ('Trigene'/'Anistel', Tristel, Snailwell, UK) but sometimes with a 10% alcohol solution, or simply water (e.g. in the three-chamber apparatus), and allowed to dry. Male mice were always tested before female mice, and non-experimental subjects were exposed to the apparatus first in some experiments. Counterbalancing of the test order for genotype and home cage within a batch of animals tested on the same day also addressed the problem of having lingering odours or pheromones biasing the results.

Mice were usually experimentally naïve before testing, but when this was not the case, they were tested from the least invasive to the most invasive test, as suggested by others in the field (McIlwain et al., 2001; Crawley, 2008). Experimental subjects and littermates in the same home cage were provided substantial handling before each experiment. This involved being first grabbed by the tail and then individually exposed to the hands and forearms of experimenter for up to 1.5-2 mins per day during the 3 days prior to the start of

the start of the experiment. If mice were exposed to consecutive days of testing (e.g. Chapter 3), they were usually handled only before the first test, and different experimental rooms were used for each test.

Male and female mice were used throughout. Experimental and control groups were matched on age, sex and breeding parents wherever possible. To control for time-of-day effects, experimental and control mice were tested together in matched pairs, where possible. When this was not possible, balancing of the conditions (e.g. genotype; A=WT, B=KO) was always ensured, usually in an ABBA/BAAB/BAAB/ABBA sequence.

2.3. Behavioural tests

This section describes several of the behavioural tests and conditioning tasks used in this thesis, although the description of some more specific tests is left to the relevant chapters (e.g. the footshock reactivity test, in Chapter 4). Tests to measure anxiety-like behaviours, social assays and a discriminative appetitive learning task were used only once in this thesis. Therefore, this section describes the purpose of these tests but further details about the apparatus and procedures can be found in Chapters 3 and 7. However, given that several chapters in this thesis used discriminative aversive learning tasks, this section explains not only the purpose of those tasks but also provides details about the apparatus and procedures utilised to avoid repetition throughout the thesis. Diagrams of the structure of a fear conditioning session can be found in the relevant chapters.

2.3.1. Approach-avoidance conflict tests for anxiety-like behaviour

Anxiety-related behaviours are most often measured in ethologically-based tests, which take advantage of what it is known of the natural, unconditioned behaviours of mice (Rodgers et al., 1997). A common strategy is to induce a state of anxiety in the mouse by

making it temporarily experience a conflict when forced to follow only one of two of its natural tendencies. For instance, mice are a natural exploratory and foraging species, which also exhibit an aversion to open, brightly lit, or elevated spaces (Bailey & Crawley, 2009; Andrew Holmes & Cryan, 2006). A situation of conflict can then be created by subjecting the mouse to a novel but open arena under very bright lighting (i.e. offering no protection from potential predators). This makes its natural tendency to explore a novel environment inhibited by a conflict between (i) entering a potentially dangerous but also potentially rewarding situation, and (ii) withholding entrance. Whereas with fear responses a mouse clearly wants to leave a dangerous situation (active avoidance), in the test of anxiety-like behaviour there is a conflict between cautiously approaching the novelty (approach behaviour) and not doing so (passive avoidance) (Gray & McNaughton, 2000; McNaughton & Corr, 2004). A behavioural test following these principles is called an approach-avoidance conflict test, and this thesis includes two such tests (Chapter 3).

Elevated plus maze

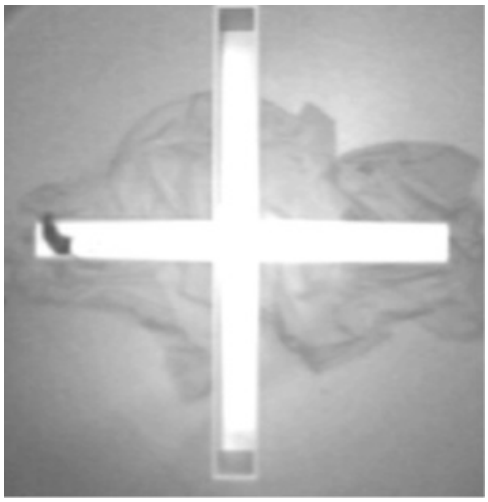


Figure 2.1. Elevated plus maze used in Chapter 3. It consisted of two sets of opposed arms (35×6 cm) perpendicularly united in a plus sign formation, and was elevated 70 cm above the floor. The 'closed arms' were enclosed by 20 cm high walls. Laboratory coats were placed on the ground under the open arms to prevent injury to a mouse that might fall.

The elevated plus maze (EPM) owes its development to observations of Montgomery (1955), who reported that when rats are placed in mazes consisting of open and closed alleys, they consistently prefer to explore the enclosed ones. Handley and Mithani (1984) adapted these findings into an animal model for the study of anxiety with the use of their 'X-maze' and the demonstration that rats injected with anti-anxiety drugs explore open areas of the maze more. Further work with rats (e.g. Pellow et al., 1985) and mice (Lister, 1987) helped establish this task that would become the most popular rodent model of anxiety-like behaviour (Litvin et al., 2008).

The EPM consists of two sets of opposed arms perpendicularly united, which makes it a maze with four arms arranged in a plus sign formation (see Figure 2.1). Two opposing arms are enclosed by walls whereas the other two are not, hence the labelling of 'closed arms' and 'open arms', respectively. The entire apparatus is elevated above the floor. The lack of walls in open arms, rather than the height of the maze, is the main anxiogenic stimulus in

this test, which is thought to be related to greater fear of exposure to predation (Treit et al., 1993; McNaughton & Zangrossi, 2008). This can also be observed in thigmotaxis, a rodent preference for staying near vertical surfaces as opposed to open areas.

It is generally accepted that when a rodent is placed in this apparatus, it will experience an approach-avoidance conflict while choosing between exploring the open arms and staying in the closed arms. An open arm is potentially rewarding for animals with a natural tendency to explore novel environments, but its aversive properties also make it potentially dangerous, especially by contrast with the safe, enclosed arms (Pellow et al., 1985; Bannerman et al., 2014).

The measurements in the EPM are usually spatiotemporal in nature; for example, time spent on the open arms expressed as a percentage of total time spent in all arms, with the number of closed arm entries (instead of total entries) used as an index of motor activity (Rodgers & Dalvi, 1997; Holmes & Cryan, 2006). Time spent in the centre platform, although related to risk assessment, is ambiguous and thus usually not used for data analysis (but see Rodgers & Dalvi, 1997).

Hyponeophagia

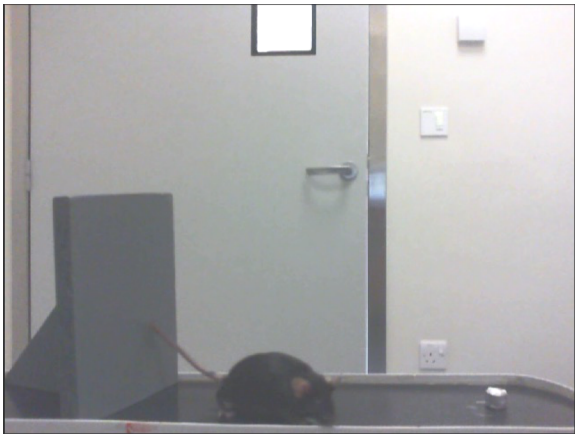



Figure 2.2. Left, Apparatus used for the first hyponeophagia test of Chapter 3 ('HPN-1 test'). It consisted of a translucent plastic jug (12.5 cm high, 18 cm long, 13 cm at its biggest width) placed upside down on a white plastic base. The food well was topped up with sweetened condensed milk. **Right,** Apparatus used for the second hyponeophagia test of Chapter 3, which was conducted in a more anxiogenic environment ('HPN-2 test'). Subjects were tested on the arm of an elevated Y-maze, which was raised 79.5 cm above the floor. The food well contained sucrose pellets.

When rodents encounter food in a novel environment, they usually consume very small amounts of it and only after considerable investigation, particularly if it is also novel food (Bailey & Crawley, 2009; Deacon, 2011). This unconditioned inhibition of feeding when exposed to a novel environment is termed hyponeophagia, which is also called novelty-suppressed feeding. The seminal observation by Hall (1934) of a negative correlation between feeding and defecation in rats exposed to a novel environment provided the first indication that hyponeophagia can be used as a behavioural measure of anxiety in rodents. Novelty as an anxiogenic factor in this situation is further strengthened with the observation that both behaviours attenuate with repeated exposures to the environment (Samuels & Hen, 2011).

The typical hyponeophagia test involves presenting a desirable food to a rodent in a novel and potentially dangerous environment, thus leading it to experience a conflict between avoiding and approaching and consuming the food (Dulawa & Hen, 2005). The

measures commonly recorded are latency to the first contact with food and latency to continuous feeding (~3s), with longer latencies indicating greater anxiety (Trullas & Skolnick, 1993; Bailey & Crawley, 2009; Samuels & Hen, 2011).

The apparatus should be structurally different from the subject's home cage and brightly lit (Carr & Lucki, 2010; see two examples in Figure 2.2). Lighting intensity is a key anxiogenic component in this test, with greater intensity generating a more anxiogenic environment. To force consumption in the new environment, the food used in the test is made desirable by food-depriving the subject overnight before testing.

This conflict-based anxiety test is a good alternative to exploration-based tasks, such as the EPM, for situations where locomotion is thought to be a confounding variable when anxiety-related behaviours are being measured (Andrew Holmes & Cryan, 2006). The downside of this task is that genetic manipulations can affect appetite and food preference and latency scores often do not exhibit normal distributions (Bailey & Crawley, 2009; Deacon, 2011). The latter problem can be tackled by applying cut-offs for latency as well as the use of transformations or nonparametric statistics.

2.3.2. Social assays

Reciprocal social interaction test



Figure 2.3. Apparatus used for the reciprocal social interaction test of Chapter 3. It consisted of a (20×30 cm) Plexiglas arena with 15 cm high walls.

The social interaction test of anxiety was initially developed for male rats with the aim of measuring changes in anxiety alone without using appetitive or aversive strategies to motivate animals (File & Hyde, 1978; File, 1980; File & Seth, 2003). Unfamiliar male rats naturally engage in social interaction (e.g. sniffing each other) when placed in a situation in which neither has established territory yet. A conflict-based test using this natural tendency was created by letting two unfamiliar subjects freely interact in a delimited arena for 10 mins while modulating social interaction via manipulation of two ethologically relevant sources of anxiety: the novelty of the test arena and bright lighting. The time spent in active social interaction by a pair of subjects is inversely related to the familiarity with the arena and to the intensity of the lightning (Mathews, 2001) and, therefore, it has been used by others as an (inverse) measure of anxiety. However, this is only the case when no concomitant

increases in the exploration of the arena are observed, and decreases in social interaction are correlated with increases in other emotional manifestations, such as freezing and defection (File, 1980; File & Seth, 2003). Care is also taken in matching pairs of rats by weight and, sometimes, by singly housing the subjects before the test (e.g. 5 days), to increase social interaction.

Given that mice also have a natural tendency to investigate novel conspecifics (Ferguson et al., 2003), the social interaction test was also used with this species. However, it proved less reliable with mice due to high levels of locomotor activity, low levels of social interaction, more aggressive behaviours between male mice compared with male rats, and less reliable effects of novelty of the test arena, among other problems (de Angelis & File, 1979; Lister & Hilakivi, 1988; Lister, 1990). This led to the development of mouse versions of the task (de Angelis & File, 1979; Bailey & Crawley, 2009; Barkus et al., 2012; Sato et al., 2013; see Figure 2.3). In these versions, the arena is typically made familiar by means of individual exposures for several (~10) minutes on each of the 2 to 5 consecutive days before the administration of a 5- or 10-minute test session. The test is conducted under one light condition only, chosen accordingly with the research aims. Unless experimentally necessary for research purposes, prior social isolation is avoided with mice because it makes them more aggressive (Valzelli, 1973). Mice are usually matched by weight, sex and strain or genotype.

Three-chamber social approach test

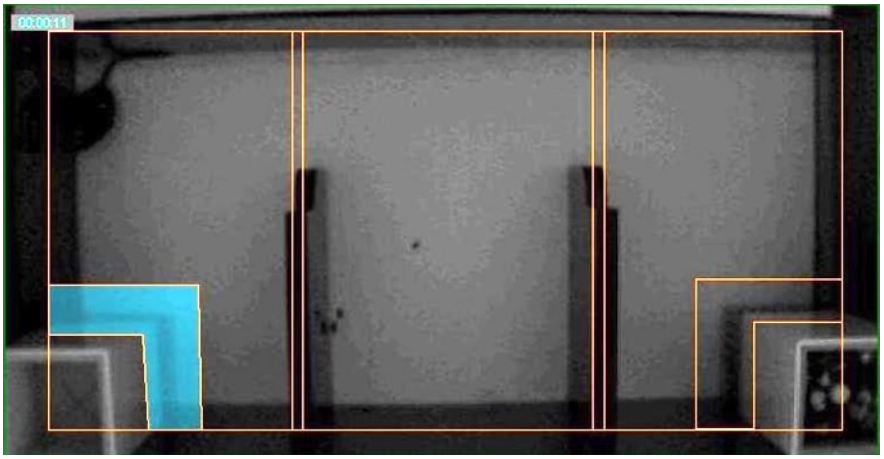


Figure 2.4. Apparatus used for the three-chamber social approach test in Chapter 3. It was a rectangular, three-chamber box made of Plexiglas, with two (15 cm high) walls dividing three (20×30 cm) adjacent compartments. The dividing walls included manually retractable doors which could give access to the side chambers. Steel wire cage enclosures (8.1×8.1×21.8 cm), placed vertically inside the apparatus, were used to hold the stimulus mice during the test. The area highlighted in blue delineates a 'proximity area'.

Mice are social animals that establish group territories and engage in high levels of reciprocal social interactions, particularly those regarding parenting behaviours (Crawley, 2007; Silverman et al., 2010). Crucial to the establishment of social relationships in any species is the ability to remember conspecifics by means of social recognition and social memory (Ferguson et al., 2001). Accordingly, rodents are not only capable of forming short-term memories of recently encountered conspecifics (i.e. social recognition), making them able to discriminate these from novel individuals, but they are also capable of long-term recognition of familiar animals (i.e. social memory) (Engelmann et al., 1995; Silverman et al., 2010; Thor & Holloway, 1982; Winslow, 2003).

Combining these defining characteristics with the aforementioned tendency to investigate novel individuals, social assays have been developed to measure social recognition. For example, if a novel conspecific is introduced to a subject, removed after a certain period of time and then reintroduced to the same subject, the latter will show social

recognition if it spends less time investigating the former on the second exposure compared with the first one (Thor & Holloway, 1982). This change in the duration of investigation is used as a memory index. Alternatively, researchers can use a social discrimination procedure, where on the second trial the subject is simultaneously exposed to the previously encountered, familiar conspecific and to a novel conspecific (Engelmann et al., 1995; Winslow, 2003). The subject will exhibit social recognition if the time spent investigating the familiar conspecific is reduced, particularly because it shows a preference for investigating the novel conspecific. This preference for social novelty as an indication of social recognition is measured by comparing the difference in time spent investigating each conspecific.

In addition to these assays, other approaches have been developed using choice tests, making use of differential and spontaneous behavioural responses by the test animal, which chooses to interact with one or both conspecifics (Gheusi et al., 1994; Crawley, 2007). One of those approaches, aiming to minimize the contributions of stimulus mice to the social interactions, uses a three-chamber apparatus where two stimuli mice (familiar and novel) are placed in opposite chambers and enclosed in wire cages (Moy et al., 2004; Nadler et al., 2004; see Figure 2.4) In the 'three-chamber social approach test' the subject is placed in a central, adjacent chamber at an equidistant position from both stimulus mice. The subject is then left to freely explore the entire apparatus (i.e. either the central chamber or any of the side chambers) and to choose for how long to interact with either the familiar or the novel stimulus mice, or both. Given that this approach was developed to study disorders associated with social approach abnormalities, particularly autism, the trial where a conspecific is introduced for the first the time was also designed to monitor for the lack of initiation of social interaction by the subject (Crawley, 2007). The task gives the subject a choice of exploring three chambers: a 'neutral' central chamber; a side chamber containing an unfamiliar mouse enclosed in a wire cage enclosure; or a side chamber containing an empty

wire cage enclosure. The task thus comprises a habituation phase to the apparatus, a social approach or sociability phase and, finally, a preference for social novelty phase.

The apparatus used in this thesis for the three-chamber social approach test (see Figure 2.4) differs in a few aspects from the original one developed by Crawley and colleagues (Moy et al., 2004; Nadler et al., 2004). For instance, the entrance to the side chambers is not located at the middle of the central walls, the cages for social stimuli are not placed in the middle of the side chambers, and retractable doors were added to the apparatus (see further details in Chapter 3, section 3.6.2). The apparatus used in this thesis was designed and validated by Tomasz Schneider (a former researcher at the DEP; see Ufartes et al., 2013) to allow combining social tests with voltammetry in the future (so the cables/wires could move around the cage without obstacles). The red-tinted colour was chosen for easy observation from side and recording from every angle, and the retractable doors allowed for easier manipulation of the apparatus without exposing the experimenter to the animals from above.

Although the three-chamber social approach task was particularly developed to model autism-related behaviours, such as a qualitative impairment in social interactions (Chadman et al., 2008), it is important to notice that social phobias and social anxiety also include avoidance of social interactions, and that a comorbid feature of autism is anxiety (Muris et al., 1998). Therefore, models such as these might not only be useful to evaluate genetically modified mice relevant to autism disorders, but also those ones relevant to understand anxiety disorders (Kalueff et al., 2007d).

2.3.3. Discriminative appetitive conditioning

An adaptively advantageous capacity for animals' survival and reproduction is to be able to anticipate, and thus better prepare for, upcoming biologically important events such as prey animals, predators, mates and offspring (Bouton, 2007). This can be achieved by (i)

detecting, among other cues, specific sounds or odours, places or times of occurrence that are consistently correlated with those events (Machado & Silva, 2002), and (ii) subsequently learning the relations between those events. In the laboratory, Pavlovian conditioning is used to investigate the learning about the cueing function of stimuli in animals (Machado & Silva, 2002), and has been a particularly suitable procedure for research on the neural basis of learning and memory (Rescorla, 1988).

Pavlovian (or classical) conditioning is a form of associative learning through which animals learn to associate stimuli in their environment. A stimulus can be defined as "any physical event, combination of events, or relation among events (…) [that] has been shown to exert control over behaviour" (Catania, 1991, p.G38). In a classic Pavlovian conditioning task, a subject's behaviour changes when it is exposed to a paired presentation of two stimuli: (i) a stimulus with no biological significance for the animal (i.e. a motivationally neutral event), such as presentation of a tone; and (ii) a motivationally salient stimulus, such as delivery of a desired food, capable of automatically and reliably eliciting a specific response from the animal when presented alone (e.g. salivation). Importantly, although the initial presentation of the former stimulus (i) might elicit some response (e.g. an orienting response), it does not produce the automatic response elicited by the latter stimulus (ii) (Roberts, 1998). However, after several pairings of the two stimuli, where the neutral stimulus (i) usually precedes the motivationally salient one (ii) by a short interval, the neutral stimulus begins to elicit a new response. This response can be similar to the one naturally elicited by the motivationally salient stimulus but it does not have to be (Hall, 2001).

The acquired new function of the neutral stimulus in a Pavlovian conditioning task is conditional upon the exposure to the paired presentations. Therefore, the response elicited by this stimulus is named the conditioned response (CR) and the process leading to its development is called conditioning (Hall, 2001; see also Dinsmoor, 2004). Accordingly, the initial neutral stimulus becomes a conditioned stimulus (CS), given that it depends on

conditioning to acquire control over the CR. Since the automatic response to the motivationally salient stimulus does not depend on the conditioning procedure (i.e. it is innate), these two elements of the conditioning process are called the unconditioned response (UR) and unconditioned stimulus (CS), respectively (Bouton, 2007). In addition, the animal learns about many different stimuli besides the CS and US. This is the case with the features of the situation where they are presented (i.e. the context), which can be associated with either the CS or the US (Rescorla, 1988).

In this thesis, appetitive conditioning was studied by using the delivery of sucrose solution as the US to a food-deprived subject, which elicits approach behaviour and consumption. The procedure used was discriminative conditioning (also known as Pavlovian differentiation, or differential conditioning), where two stimuli are presented on separate trials, one (CS+) always paired with the US, and the other (CS–) never paired with the US (Catania, 1991; Zielinski, 1979). By reinforcing (i.e. pairing with the US) only one of the stimuli, two different CS-US contingencies are learned, thus generating a discriminative emotional value (i.e. valence) for the two stimuli (Likhtik & Paz, 2015).

2.3.4. Discriminative fear conditioning

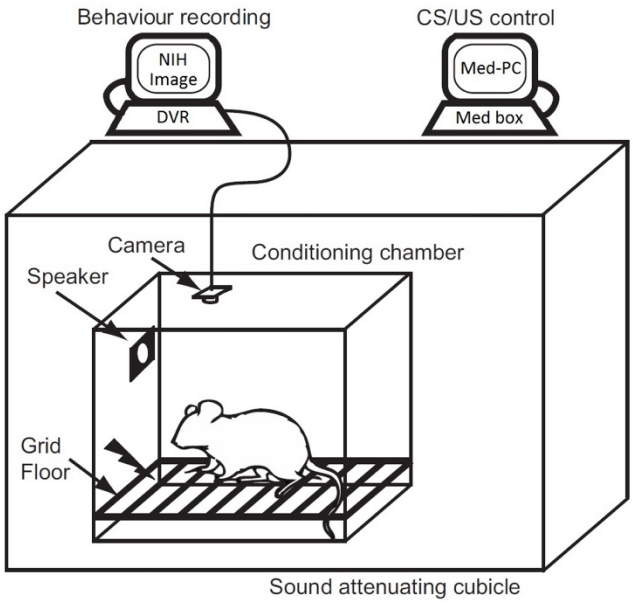


Figure 2.5. Diagram of the apparatus used for the discriminative aversive learning experiments of Chapters 4 and 6. Adapted from McHugh et al. (2014). Reprinted under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014.

Fear responses are evoked by a specific external threat, typically an explicit and imminent stimulus that is usually short-lived, leading to either an intense escape or avoidance response (Cryan & Holmes, 2005; Wahlsten, 2011). These responses can be conditioned to an initially motivationally neutral stimulus if this is associated with an aversive event (e.g. a footshock) by means of Pavlovian conditioning. Several chapters in this thesis include this form of associative learning (Chapters 4-6).

Pavlovian fear conditioning is a type of conditioning where the CR resulting from the paired presentations of an innocuous stimulus with an aversive or noxious US consists of elicited fear. Pavlovian fear conditioning has been particularly useful for investigating learning and memory processes because it produces a "rapid, robust, and enduring learning" (Maren, 2008, p. 1661), amenable to invasive, *in vivo* brain recordings (Maren & Quirk,

2004). However, it has also been used for studying emotional processes with animal models, particularly those believed to be involved in the aetiology of anxiety disorders (LeDoux, 2000; Cryan & Holmes, 2005; Lissek et al., 2005; Fanselow & Ponnusamy, 2008).

In a typical experiment with rodents placed in a chamber from which they cannot escape, the aversive US is a mild electrical footshock, which elicits an activity burst (e.g. running or hopping). The dominant behavioural CR is conditioned freezing, that is, immobility except for breathing-related movements (Bouton, 2007; Fanselow & Ponnusamy, 2008). Clearly, the UR and CR are not similar in this situation. Freezing is an adaptive response that, for instance, enables rodents to have a higher chance of survival when facing a predator which has not yet detected or attacked them (Fanselow & Ponnusamy, 2008). However, in the laboratory situation just described, freezing is a learned response towards stimuli previously associated with an aversive situation (i.e. it is only exhibited after the association is made). These stimuli can be contextual, namely the spatial, temporal and interoceptive cues present at the time of US presentation (Myers & Davis, 2008), or a discrete CS presented just before an US delivery, such as a tone or a light.

Importantly, even when a discrete CS is paired with an US, the animal still associates the learned CS-US contingency with the background context where that learning occurred. This has led to the distinction of two forms of Pavlovian fear conditioning: 'cued fear conditioning', where a discrete CS is used; and 'context fear conditioning', where the context is the CS (e.g. Phillips & LeDoux, 1992). To investigate cued fear conditioning, subjects are usually fear conditioned in one context and then tested under extinction session in a novel, distinct context. This helps to dissociate the effects of the discrete CS from those of the context CS which, according to different theories, can work either as an occasion setter, or simply make up a compound CS with the discrete CS (Bouton, 2004). When contextual fear conditioning is investigated, it is necessary to provide the subject with time to explore the context before delivering footshocks because a representation of the context (i.e. context

encoding) is necessary for conditioning to occur (Fanselow & Ponnusamy, 2008; Maren et al., 2013).

Two conditioning chambers (ENV-307A, Med Associates Inc., Lafayette, IN, USA) inside sound-attenuating cubicles were used for fear conditioning in this thesis. They had a houselight, a floor of steel rods connected to a scrambled-shock generator (ENV-414S, Med Associates) and a loudspeaker connected to an audio stimulus generator. To provide two different conditioning contexts, each sound-attenuating cubicle was associated with a specific scent (almond or lavender) and a distinctive wall layout (plain white or black and white stripes). The delivery of stimuli in the conditioning chambers was controlled by a script written in Med-PC IV software programme (Med Associates). A miniature video camera with a wide-angle lens was placed above the chambers during each experimental session and its output was stored on a DVR digital video recorder (see Figure 2.5).

A discriminative fear conditioning procedure was used, in which two auditory cues were presented, one of them co-terminating with a 0.5s mild (0.3 mA) footshock (CS+), but the other one not followed by footshock (CS–). The cues were two 30s-long, 72 dB auditory cues: a 2900 Hz continuous tone and a white noise. The allocation of each type of auditory cue to the CSs was fully counterbalanced across subjects. The CS presentations were interspersed with a mean inter-cue interval of 80s (range: 60-100s). Every session started with a 300s lead-in period prior to the first cue presentation, and ended with a 60s-lead-out period after the last cue presentation (total duration: 1380s). Contexts were counterbalanced across mice.

Mice underwent a cued fear conditioning procedure comprising two phases: a 2-day conditioning phase (conditioning sessions 1 and 2, or C1 and C2, respectively), followed by another phase consisting of three extinction sessions administered in a different context. Given that during the first extinction session subjects only experienced five opportunities to learn that the CS+ was no longer followed by the US, that session is comparable to a test of

fear memory recall. Accordingly, in this thesis, the first extinction session is called fear memory recall testing session (FMR), whereas the following two are called extinction sessions (extinction sessions 1 and 2, or E1 and E2, respectively).

The structure of all sessions was virtually identical, consisting of the presentation of five cues of each type per day, in a pseudo-random order. Order and carry-over effects of the sequence of CS type presentations was controlled by combining intrasubject and intergroup counterbalancing of the order of cue presentations (Christensen, 2007). Briefly, two fixed sequences of CS presentations were created for each session following two rules: the first two presentations of each session had to be a CS+ and CS–, and no more than two presentations of each CS type in a row were allowed. The difference between the sequences was that one sequence (e.g. ABBA) had the opposite order of the other one (e.g. BAAB). Half of the mice in each group would receive one sequence, while the other half would have the other version.

The behavioural measure used to assess acquired fear was conditioned freezing. There are several advantages of measuring this CR, such as it being non-invasive and sensitive to the manipulations of parameters such as the number of CS-US pairings administered, which affect the level of learning and fear (Maren, 2008; Fanselow & Ponnusamy, 2008). Measuring freezing in this thesis was achieved by processing the video output with a script in the NIH Image software environment, also known as ImageJ (Schneider et al., 2012), which measured freezing behaviour by comparing two video frames sampled from two consecutive seconds, and computed the number of altered pixels from the first to the second frame. For each second of the session analysed, an instance of freezing corresponded to a percentage of changed pixels (from the previous second) that was less than or equal to a threshold previously calibrated for immobility apart from movements of the flanks caused by breathing.

The freezing behaviour relevant to the fear conditioning analyses was calculated by selecting the relevant period for analysis (e.g. the presentation of a given 30-second long CS) and dividing the number of seconds where freezing occurred (e.g. 23) by the total duration of that relevant period, times 100 (e.g. (23/30) × 100 = 76.7%). After calculating the percentage of freezing in the 30s before CS onset and in the 30s-long CS period, CS-evoked changes in freezing behaviour (Δfreezing) were calculated by subtracting the former percentage from the latter (e.g. CS+ evoked Δfreezing = '% freezing during the CS+ period' minus the '% freezing during the pre-CS+ period'). This Δfreezing measure controls for possible background group differences in freezing behaviour or activity levels. This process was done for every cue administered in an experimental session before averages were calculated for each type of CS used. Additionally, a discrimination leaning index was also calculated by subtracting the CS– evoked Δfreezing score from the CS+ evoked Δfreezing score (Lissek et al., 2005).

2.4. Surgery

Surgery was performed for the implantation of intracranial electrodes. Mice were given pre-operative analgesia through subcutaneous (S.C.) injections of the opioid Vetergesic (i.e. buprenorphine; 0.08 mg/kg) and the non-steroidal anti-inflammatory Metacam (i.e. meloxicam; 5 mg/kg). The local anaesthetic Marcain (i.e. bupivacaine; 2 mg/kg, S.C.) was injected into the scalp. Anaesthesia was achieved with the inhalable ether Isoflurane, first in an induction chamber (4% in 4 L/min) and then via a nose mask (1.5-2% in 1 L/min) during surgery (David Kopf Instruments, model 942; Tujunga, CA, USA). A subcutaneous injection of saline (0.5 ml) was given before and after the surgery to maintain the fluid balance of the mice during the intervention. Body temperature was monitored via a

rectal thermometer and maintained at 37° using a homeothermic blanket during the entire (~2 h long) procedure. Mice received another dose of Vetergesic several hours after surgery, and another dose of Metacam the following day. They recovered for at least seven days after surgery before any testing began.

To place the electrodes in the discrete region of the BLA, a stereotaxic surgery was performed. After fixing the mouse's head in the stereotaxic frame, a scalp incision was made in order to expose two anatomical skull landmarks: bregma, the junction of the sagittal and coronal (bregmatic) sutures of the brain; and lambda, defined as the "intersection between the lines of best fit through the sagittal and lambdoid sutures" (Carter & Shieh, 2010, p. 74). A micromanipulator carrying an electrode holder was then used to make bregma a zero-reference point, after assuring that the mouse's head was positioned in the flat (or level) skull position (i.e. that the dorsal-ventral measurement at bregma and lambda were within 100 μm of each other). Using the coordinates empirically adjusted over the years in the laboratory from those suggested by the stereotaxic atlas (Paxinos & Franklin, 2004), it was possible to place the tip of an electrode into the BLA by adding or subtracting them from the zero reference point coordinates by means of linear scales assembled to the micromanipulator (David Kopf Instruments, model 940-B). The coordinates for the implantation of CPE and LFP electrodes were -1.35mm anterior-posterior, ± 3.1mm medial-lateral and -5mm dorsal-ventral relative to bregma.

Electrodes were lowered into the brain through small holes drilled in the skull. Small screws were also inserted into the skull to provide anchoring for the dental cement. After the positioning of the electrodes, each one of them was inserted into a pedestal plug (MS363, Plastics One). Dental cement ('Simplex Rapid', Associated Dental Products, Wilts, UK) was used to secure the electrodes and pedestal to the skull.

2.5. Histological methods

To verify electrode placement, implanted mice had their brains fixed, sectioned and stained at the end of testing. After injecting mice with a terminal dose of an anaesthetic (sodium pentobarbitone, 200 mg⁄kg via a 0.3 ml intraperitoneal injection), a transcardial perfusion was performed. This was achieved by rinsing the blood out of veins and arteries with physiological saline (0.9% NaCl) and delivering the cross-linking fixative paraformaldehyde (4%), which stops autolysis, fixes proteins into to place and kills microorganisms that can degrade the brain tissue (Carter & Shieh, 2010; Carlson, 2011). Afterwards, each brain was placed in a pot containing the same fixative used during perfusion for 3 days, and then immersed in a 30% sucrose–formalin solution for 24 h before being frozen. This cryoprotection in a sucrose solution minimises artefacts caused by freezing. At a later stage, 40 μm coronal slices were sectioned using a microtome with a steel knife, mounted on glass microscope slides and stained with the Nissl stain cresyl violet (5% solution, Thermo Scientific RA Lamb Dry Dyes, Paisley, UK) to enhance the visualisation of brain structures for light microscopy.

2.6. Data analyses

Software

Data were processed in Microsoft Excel 2013 for Windows 7 and in Microsoft Excel for Mac (version 15), whereas statistical analyses were carried out using IBM SPSS Statistics (version 22, IBM Corporation). Graphs were created in Prism (version 7, GraphPad Software, Inc.). They usually show the mean value ± 1 SEM (standard error of the mean), apart from a few clearly identified exceptions. Processing of the tissue oxygen data and local field

potentials data (see Chapter 5) was achieved with Chart (AD Instruments, version 5.5) and MATLAB (R2012a, The MathWorks, Inc.).

Hypothesis tests applied to two means

In chapters where the hypothesis tests applied to two means were needed, independent-samples *t*-tests were used when data met the assumptions of normality and homogeneity of variance. To verify the assumption of normality, the Kolmogorov-Smirnov test was used in conjunction with quantile-quantile (Q-Q) plots. Homogeneity of variance was investigated using the Levene's test, particularly in situations with unequal sample sizes (Howell, 2013). Data transformations (e.g. $\log_{10}$) were performed to deal with non-normality problems but no corrections for heterogeneity of variance were tried. When parametric approaches could not be used, the Mann-Whitney *U* test was used instead. Two-tailed (non-directional) tests were used by default, with a p-value $< .05$ being considered statistically significant.

ANOVAs

In studies where several means needed to be compared, an analysis of variance (ANOVA) using a general linear model (GLM) was performed on the data. Most studies in this thesis required repeated-measures designs, and the statistical tests commonly used were mixed ANOVAs. These tests include one or two between-subjects variables and one or two within-subjects variables (e.g. a two-way mixed ANOVA, with Genotype and CS type as factors). In addition, some chapters also include two-way between-subjects ANOVAs.

The ANOVA is, generally, a test robust against moderate departures of the assumption of normality (of scores within groups) and the assumption of homogeneity of variance, particularly when group sizes are equal (Glass et al., 1972; Field, 2013; Howell, 2013). Considering this and the fact that there is not an available equivalent non-parametric

test for mixed ANOVAs, there were occasions in this thesis where tests were conducted despite minor violations of assumptions. However, assumptions were always investigated. For instance, in the two-way mixed ANOVAs, this was done by verifying if the homogeneity of variances and normality assumptions were held for every level of the repeated-measures variable (Howell, 2013), by means of the Levene's test and the Kolmogorov-Smirnov test in conjunction with quantile-quantile (Q-Q) plots, respectively. It is important to note that, considering the big sample sizes used in some studies, these two tests could not always be entirely reliable (Field, 2013; Howell, 2013).

In addition, the sphericity assumption was verified when necessary by means of the Mauchly test. When this assumption was violated, degrees of freedom for the *F* values were adjusted via either the Greenhouse and Geisser correction or the Huynh and Feldt correction. The latter adjustment was only used in situations where the Greenhouse and Geisser estimate of sphericity (ε value) lied near or above .75 (Howell, 2013). However, in the interest of clarity, the original degrees of freedom of the ANOVA model were the ones reported in this thesis, not the adjusted ones.

A modified version of Keppel's (1982) notation was used to describe the GLM, in the form: $A_2 \times B_3 \times S_{24}$, where A is factor with 2 levels, B a factor with 3 levels and S_{24} indicates that 24 subjects in total (i.e. in the experiment) were used in the analysis (e.g. $Genotype_2$ x $ConditioningDay_3$ x S_{24}). The S_{24} element thus is not a factor of the ANOVA but just details an important aspect of the analysis, the sample (N) size used.

Data from male and female subjects were pooled if no significant interactions between genotype and sex were observed (Crusio et al., 2009). Therefore, unless otherwise stated, sex was not routinely included as a factor in the statistical analyses reported in this thesis. However, statistical results regarding interactions between genotype and sex for all the results discussed in this thesis including a mix of males and females can be found in

Appendix 24. Missing values were excluded from the analyses. ANOVA results were considered statistically significant at $p < .05$.

Analysis of simple main effects

Analysis of simple main effects were regularly investigated to interpret significant interactions, though there was a situation in this thesis where simple main effects were investigated despite an absence of a significant interaction (e.g. Chapter 5, section 5.4.4).

In two-way between-subjects ANOVAs, analyses of simple main effects were conducted by running standard one-way ANOVAs on the data of interest only (see Howell, 2013, pp. 422-423). In two-way mixed ANOVAs, this was also done with the between-subjects simple effects, but the within-subjects simple effects involved testing individual within-subjects contrasts against a pooled error term. When three-way ANOVAs with two between-subjects variables and one-within subjects variable were used, and the interaction of interest was between one of the between-subjects variable and the within-subjects variable, the *F* value of this interaction was reported but the simple main effects were investigated by running standard ANOVAs with the data of interest only. This was done to avoid working with the estimated marginal means and pooled standard errors produced by the three-way ANOVA.

All pairwise comparisons conducted in these analyses were performed using a Šidák correction (i.e. via a Dunn-Šidák test), which helps to control for the probability of Type I errors (Howell, 2013). The familywise error rate was set at $\alpha = 0.05$.

Chapter 3. Unconditioned anxiety and social behaviour in 5-HTTKO mice

3.1. Abstract

Previous studies have shown that 5-HTTKO mice exhibit increased anxiety-like behaviour in approach-avoidance tests such as the elevated plus maze (EPM). However, few studies have investigated social anxiety in 5-HTTKO mice. The aim of this chapter was to provide a comprehensive assessment of the anxiety profile of 5-HTTKO mice. To this end, two common conflict-based anxiety tests (EPM and hyponeophagia) were used to see if the previous finding of increased anxiety in 5-HTTKO mice could be replicated. In addition, two assays were used to determine whether 5-HTTKO mice exhibit altered social anxiety: the reciprocal social interaction test, and the three-chamber social approach test. This second assay also included a measure for social novelty preference.

Consistent with previous studies, 5-HTTKO mice exhibited increased anxiety in the EPM. Increased anxiety was also observed in the hyponeophagia test, a result that could not be explained by reduced locomotor activity which was a potential confound for the EPM. In the reciprocal social interaction test, 5-HTTKO mice interacted significantly less with a conspecific than WT mice, again indicative of increased anxiety. However, when tested in a less anxiogenic situation, such as the sociability phase of the three-chamber social approach task, 5-HTTKO mice did not differ from their WT controls on social investigation of a stimulus mouse. This suggests that when not forced to interact directly with another conspecific, 5-HTTKO mice show normal social investigation. Moreover, 5-HTTKO mice showed significantly enhanced social novelty preference when given a choice between investigating a novel mouse versus investigating a previously encountered mouse.

In summary, 5-HTTKO mice exhibited increased anxiety in conflict tests such as the EPM and hyponeophagia as well as an anxiogenic profile during a reciprocal social

interaction. However, social investigation per se was not impaired in 5-HTTKO mice and their heightened preference for investigating a novel conspecific may indicate enhanced recognition memory for previously encountered mice. This, in turn, may represent an increased sensitivity to social information in the environment, consistent with predictions advanced by the neuroplasticity theory of 5-HT function, namely that higher 5-HT availability increases sensitivity to environmental and social influences.

3.2. Introduction

It has been claimed that 5-HT and the 5-HTT are strongly involved in social behaviour, with evidence being provided from studies with humans, monkeys and rodents (Canli & Lesch, 2007; Kiser et al., 2012). This would result from differing levels of 5-HT availability influencing the sensitivity to social factors, a hypothesis stemming from the neuroplasticity theory of 5-HT function of enhanced sensitivity to environmental and social influences (Belsky et al., 2009; Branchi, 2011; Homberg & van den Hove, 2012).

Two of the most common reports involving the serotonergic system in social behaviour seem, however, somewhat contradictory. On one hand, some authors claim that 5-HT contributes to pro-social behaviour, improved perception of social cues and high levels of cooperation (Kiser et al., 2012); on the other hand, considerable research highlights the fact that social behaviour is impaired in many psychiatric disorders associated with alterations in the serotonergic system, such as autism and social anxiety disorders (Bartlett et al., 2005; Brune et al., 2006; Battaglia et al., 2005; Stoltenberg et al., 2013). Therefore, both benefits and detriments in social behaviour have been associated with 5-HT, which seems a contradiction. Additionally, some authors suggest that individual differences in social behaviour result from an interaction between psychosocial stress and different 5-HTT expression levels (Canli & Lesch, 2007; Bartolomucci et al., 2010). Still, according to a ‘for-

better-and-for-worse' view of these facts and claims, part of this apparent inconsistency could perhaps be explained by the existence of individual differences regarding sensitivity to environmental influences, which could be modulated by 5-HT. Accordingly, the neuroplasticity theory of 5-HT function predicts that individuals with different levels of 5-HT availability should differ in their sensitivity to social factors, with higher 5-HT associated with more sensitivity to those factors (Kiser et al., 2012).

5-HTTKO mice, 5-HTT heterozygous mice (5-HTTKO +/– mice) and their WT controls have been used to investigate the influence of the serotonergic system in social behaviour and social anxiety (i.e. avoidance of social situations; Murphy & Lesch, 2008; Kalueff et al., 2010; Kiser et al., 2012). In day-to-day social behaviours, such as aggression (e.g. biting), social-positive behaviour (e.g. allo-groom) and social exploration (e.g. body sniff), 5-HTTKO mice reportedly exhibit less aggression and more socio-positive behaviours than WT controls in a homo-genotype group with a well-established dominance hierarchy (Lewejohann et al., 2010).

Social anxiety and other alterations in social behaviour can also be investigated using tasks that include an anxiogenic factor. Specific symptoms of anxiety disorders need to be modelled with tasks different from exploration-based tasks of anxiety-like behaviour in mice, such as the elevated plus maze (EPM). In the case of anxiety elicited by social situations, this can be modelled by using tasks where subjects can show low physical interaction with an unfamiliar conspecific (Cryan & Holmes, 2005). This is the case with the reciprocal social interaction test (de Angelis & File, 1979; Lister & Hilakivi, 1988; File & Seth, 2003; see also Chapter 2, section 2.3.2). Briefly, this social assay consists of a free dyadic social interaction of the test subject with an unfamiliar conspecific in a neutral environment (i.e. not the homecage), with decreased exploratory social behaviour in the absence of locomotor deficits indicating greater (social) anxiety-like behaviour (Bailey & Crawley, 2009).

Subjects	Task/process	Effect/ results	Authors/year
5-HTTKO mice	Hetero-genotype social interaction test	↓ exploration	Kalueff et al., 2007a
	Analysis of nonaggressive social behaviours during a resident/intruder test	– social interest behaviours (↑ latency to attack ↓ # attacks)	Holmes et al., 2002a
	Three-chamber social approach test: habituation to the apparatus (10min) → sociability phase (10min) → preference for social novelty phase (10min)	Chamber measures: ↓ sociability – social novelty Sniffing measures: – sociability – social novelty	Moy et al., 2009
	Three-chamber social approach test: Saline injection (I.P.) → 30min → centre habituation (10min) → side chamber habituation (10min) → sociability (10min)	Chamber measures: – sociability Sniffing measures: – sociability	Moya et al., 2011
5-HTTKO mice (backcrossed to C57BL/6N)	Three-chamber social approach test: habituation to the apparatus (10min) → sociability phase (10min) → preference for social novelty phase (10min)	– sociability – preference for social novelty – sniffing	Sakakibara et al., 2014
5-HTTKO rats	Analysis of nonaggressive social behaviours during a resident/intruder test	– social interaction (↑ latency to attack ↓ offensive behaviour)	Homberg et al., 2007b
	Social play behaviour in peri-adolescent rats (~ social interaction test)	– social exploration ↑ following/chasing ↓ social play	Homberg et al. 2007c

Table 3.1. Studies investigating social behaviours in 5-HTTKO rodents. ↑, increased; ↓, reduced; –, no significant genotypic/group differences; #, number; ~, similar.

One study using a reciprocal social interaction test has reported reduced social interaction behaviours in 5-HTTKO mice due to increased anxiety (Kalueff et al., 2007a; see Table 3.1). However, this result might have been confounded by the hypoactivity also observed in the KOs. Additionally, other aspects of the methodology might have influenced the result (further discussed in section 3.7). For instance, contrary to what is typically done in this assay (Barkus et al., 2012; Sato, et al., 2013), researchers tested dyads of mice that

were not matched by weight (i.e. they were chosen randomly), and used hetero-genotype couples (i.e. a 5-HTTKO mouse versus one WT mouse). Although the use of hetero-genotype couples is a valid approach if the measures are focused in the KO subject, it has also been suggested that the serotonergic make-up of the conspecific can influence the social behaviour exhibited by the 5-HTTKO mice (Kiser et al., 2012; see also Lewejohann et al., 2010).

In addition, two studies reported that 5-HTTKO rodents do not exhibit altered social exploration compared with WT controls (Holmes et al., 2002a; Homberg et al., 2007b). However, these two studies did not use a reciprocal social interaction test but only analysed nonaggressive social behaviours during a resident/intruder test. Notably, one study has reported reduced social play in 5-HTTKO rats, but no changes in social exploration behaviour and even increased following and chasing behaviours were observed (Homberg et al., 2007c). This latter observation suggests that 5-HTTKO rats were still interested in the conspecific despite the reduction in social play.

In summary, the precise nature of the social phenotype of 5-HTTKO mice is still unclear. Therefore, this chapter aimed to run a reciprocal social interaction test addressing some of the limitations of the only study that has so far tested 5-HTTKO mice in this test (Kalueff et al., 2007a).

In addition, any account of altered social behaviour in 5-HTTKO mice must consider the anxiogenic phenotype of these mice. Therefore, as well as testing 5-HTTKO mice in the reciprocal social interaction test, two additional aims were set for this chapter: to (i) provide a comprehensive assessment of the anxiety profile of 5-HTTKO mice, and to (ii) use another social assay in which contact with the other conspecific was physically prevented but social investigation was still possible. If 5-HTTKO mice showed increased anxiety-like behaviour in other tests besides the reciprocal social interaction test, this would reveal consistency across different tests for a phenotype of increased anxiety in the KOs. Moreover, it would

make increased anxiety a likely source for reduced social exploration in the reciprocal social interaction test. Additionally, if increased anxiety was indeed affecting social interaction in this test, then it should be expected that these mice would exhibit normal levels of social exploration or investigation when tested in another less anxiogenic social assay (i.e. one in which physical contact is prevented).

Subjects	Task/process	Effect/ results	Authors/year
5-HTTKO mice	▪ Elevated plus maze ▪ Emergence test	↓ % OA time ↓ % OA entries ↓ OA entries and time – CA entries ↑ latency to exit shelter (males only) ↓ shelter ↔ open field transitions ↓ % time out of shelter	Holmes et al., 2003b
	Elevated plus maze	↓ % OA time ↓ OA entries (n.s.) ↓ CA entries ↓ total entries	Holmes et al., 2003a
	Elevated plus maze	↓ % OA time (trend) ↓ OA time (trend)	Kalueff et al., 2007b
	▪ Elevated plus maze ▪ Hyponeophagia	↓ % OA time ↓ % OA entries ↑ latency to enter OA (n.s.) – # entries into CA ↑ latency to eat	Line et al., 2011
	Food finding test	– latency to approach ↑ latency to touch/lick	Kalueff et al., 2007a
	Elevated plus maze	↓ % OA time (n.s.) ↓ % OA entries (n.s.) ↓ total entries (n.s.)	Moy et al., 2009
5-HTTKO mice (backcrossed to 129S6/SvEv)	Elevated plus maze Hyponeophagia	– OA and CA time – % entries into OA and CA ↓ total arm entries ↑ latency to begin eating	Lira et al., 2003
	Elevated plus maze	– % OA time – OA entries ↓ CA entries (males) ↓ total entries (males)	Holmes, et al., 2003a
	▪ Elevated plus maze ▪ Hyponeophagia Mice treated with saline from P4-21	– % entries into OA and CA ↓ total arms entries ↑ latency to begin eating	Ansorge et al., 2004

Table 3.2. Studies investigating anxiety-like behaviours in 5-HTTKO rodents. ↑, increased; ↓, reduced; –, no significant genotypic differences; OA, open arms; CA, closed arms; #, number; n.s., a statistically non-significant result.

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5-HTTKO mice (backcrossed to CD1 mice)	Elevated plus maze	↓ OA time ↓ total arms entries	Popa et al., 2008
5-HTTKO mice (backcrossed to C57BL/6N)	Elevated plus maze	↓ % time OA (n.s.) – % entries OA ↓ total arm entries	Sakakibara et al., 2014
5-HTTKO rats	▪ Elevated plus maze ▪ Hyponeophagia (after 24h of food deprivation) ▪ Home cage emergence task	↓ time in OA ↑ latency to eat (males only) ↑ latency to leave home cage	Olivier et al., 2008b
5-HTTOE mice	▪ Elevated plus maze (5min; walls 30cm; elevated 50cm placed at a distal end of a closed arm) ▪ Hyponeophagia box floor covered with food pellets	↑ time on the OA ↓ latency to enter OA – total arms entries ↓ latency to eat	Jennings, 2006
	▪ Elevated plus maze ▪ Hyponeophagia	↑ % OA time ↑ % OA entries ↓ latency to enter OA – #entries into CA – first contact ↓ latency to eat	Line et al., 2011

Table 3.2. Studies investigating anxiety-like behaviours in 5-HTTKO rodents. ↑, increased; ↓, reduced; –, no significant genotypic differences; OA, open arms; CA, closed arms; #, number; n.s., a statistically non-significant result.

Therefore, three more tests were used in this chapter besides the reciprocal social interaction test. The anxiety-like behaviour of 5-HTTKO mice was first validated in the EPM (Lister, 1987; Rodgers & Dalvi, 1997; see also Chapter 2, section 2.3.1). This was further investigated with two hyponeophagia tests (HPN; Deacon, 2011; Samuels & Hen, 2011; see also Chapter 2, section 2.3.1), one of them set in a more anxiogenic environment. The behavioural phenotyping of 5-HTTKO mice conducted on multiple genetic backgrounds has been well established in these two tests, in which the KOs typically show increased anxiety-like behaviours (see Table 3.2). Regarding the less anxiogenic social assay, the one used in

this chapter was the three-chamber social approach test (Moy et al., 2004; Nadler et al., 2004; see also Chapter 2, section 2.3.2).

Contrary to the reciprocal social interaction test, the three-chamber social approach test (hereafter the 'three-chamber test') was specifically designed to assess social interaction abnormalities in mice, being particularly used in studies investigating autism-related social deficits (Nadler et al., 2004). In the sociability phase of this test, a test mouse can choose to: (i) approach a cage enclosure containing an unfamiliar conspecific (a 'social stimulus') located in one of the three chambers of the apparatus; (ii) investigate a similar but empty cage enclosure placed in another chamber; (iii) not explore any of the cage enclosures. Accordingly, it is the experimental subject that chooses whether to investigate the social stimulus or not. The subject shows sociability (i.e. social approach or social investigation) by preferring to spend more time in the chamber containing the social stimulus than in the chamber containing only an empty cage enclosure. In addition to testing for sociability, the three-chamber test can also investigate social recognition memory, a form of memory which may reflect habituation to the recently investigated animal (Ferguson et al., 2002; Winslow, 2003). After the sociability phase, a novel social stimulus can be placed in the apparatus, in the opposite chamber to that containing the social stimulus used in the sociability phase, which becomes familiar at this point. The experimental subject shows social recognition memory when it discriminates between the two stimuli and shows a greater interest in the novel social stimulus over the familiar one.

Therefore, two social assays were conducted in this chapter: a reciprocal social interaction test conducted with homo-genotype dyads, and a three-chamber test, which also included a preference for social novelty phase. It was hypothesised that 5-HTTKO mice would exhibit reduced social interaction during the reciprocal social interaction test with a novel conspecific, and that this would be mostly due to anxiety. To support the latter hypothesis, 5-HTTKO mice subjected to the three-chamber test are predicted to exhibit

normal levels of interest and sociability towards a novel, same-sex conspecific held in a cage enclosure, a situation with considerably lower levels of anxiety than the previous social assay. Additionally, social recognition was also investigated in 5-HTTKO mice.

3.3. Elevated plus maze

3.3.1. Subjects, apparatus and procedure

Forty 5-HTTKO mice (female: n=20) and 42 WT mice (female: n=19) were tested in the EPM. Groups were matched by sex and age (around 5 months on average), with age at the day of testing varying from 6 weeks to nearly 8 months. Only two subjects were tested at the age of 6 weeks, with all other mice being at least 3 months old at the time of testing.

The maze used consisted of two sets of opposed arms (35×6 cm) perpendicularly united in a plus sign formation, and was elevated 70 cm above the floor (see Figure 2.1 in Chapter 2). The 'closed arms' were enclosed by (20 cm high) walls, whereas the 'open arms' were just bordered by a small raised edge, which mice could grip. The maze was entirely grey-coloured, with the open arm flooring having a lighter grey colour than the rest of the maze. The room luminance was set around 7 lux.

Mice were habituated to being handled, tail-marked and held in a temporary holding room for 1-2 hours per day during the 3 days before the testing session. Before the start of the session, mice from the same sex would be carried to the experimental room in their home cages and kept in the dark by means of cage cover. Then, four subjects at a time would be temporarily placed in individual cages on a shelf above the maze (i.e. away from the sight of the subject being tested). After an acclimatisation period ranging between 5 and 20 minutes, a subject would be placed at the end of a closed arm and facing way from the centre. This alternative to placing the mouse in the centre of the maze (as is commonly done) precluded the absence of data from a subject in case it did not leave the centre of the maze.

Subjects could then freely explore the maze for 5 mins. Two heterozygous mice for the 5-HTT gene were always tested first to give an odour to the maze, which was then cleaned with 10% alcohol between subjects. Data from these non-experimental mice were not analysed. The experimenter stayed in the room during the testing session.

Exploration of the maze was monitored by an automated video tracking system (ANY-maze Video Tracking Software, Sotelting Co., USA), which counted as an entry into an arm whenever 80% of the subject's body was inside that arm. The measures used for the data analyses were percentage of entries and time spent in open arms (e.g. ['time in open arms' / 'total time spent in all arms'] × 100), latency to first exit the closed arm, latency to enter an open arm, and number of closed arms entries. Distance travelled was also measured for the analysis of locomotor behaviour. The statistical tests used were independent samples *t*-tests and graphs show means and standard error of the means (SEM). When data did not meet parametric assumptions, transformations ($\log_{10}$) were utilised. Non-parametric data were analysed using a Mann-Whitney *U* test and medians are reported where appropriate. In the latter case, refined boxplots show not only the medians and inter-quartile ranges (IQRs) but also outliers, which were defined as data values exceeding the IQRs by 1.5 times. Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

3.3.2. Results

5-HTTKO mice exhibited increased anxiety-like behaviour during the EPM test

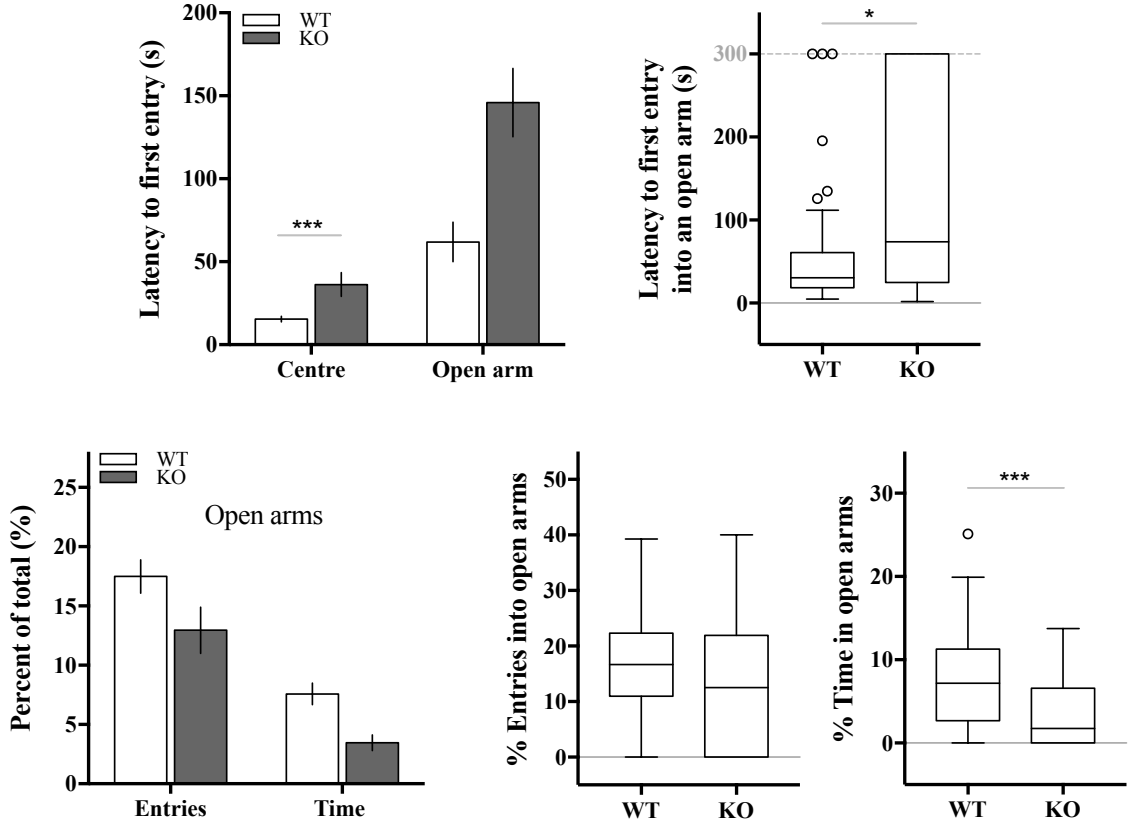


Figure 3.1. Top, left, Latencies to enter the centre of the EPM and an open arm (KOs: N=40; WTs: N=42). Mean ± SEM. ***$p < .001$ (independent *t*-test). **Top, right,** Boxplot for the data obtained with the previous two measures (white circles: outliers exceeding the IQR 1.5 times). *$p < .05$ (Mann-Whitney *U* test). **Bottom, left,** Percentage of entries into the open arms and percentage of time spent in the open arms for 5-HTTKO (N=40) and WT (N=42) mice. Mean ± SEM. **Bottom, centre and right,** Boxplots for the data obtained with the previous two measures (white circle: outlier exceeding the IQR by 1.5 times). ***$p < .001$ (Mann-Whitney *U* test).

5-HTTKO mice took significantly more time to exit from the closed arm used as starting point of the task into the centre of the EPM ($t(80) = -3.8$, $p < .001$), and then to enter an open arm ($U = 566.0$, $p = .011$; WTs median: 30.6; KOs: 73.8; see Figure 3.1, top), compared with WT controls. Moreover, compared with WT mice, 5-HTTKO spent a significantly reduced proportion of time in the open arms ($U = 445.5$, $p < .001$; WTs median: 7.2; KOs: 1.8; see Figure 3.1, bottom left and right). They also exhibited a smaller proportion

of entries into the open arms, although this just failed to reach significance (U = 641.5, p < .064; WTs median = 16.7; KOs: 12.5; see Figure 3.1, bottom left and centre).

5-HTTKO mice showed reduced locomotor activity in the EPM

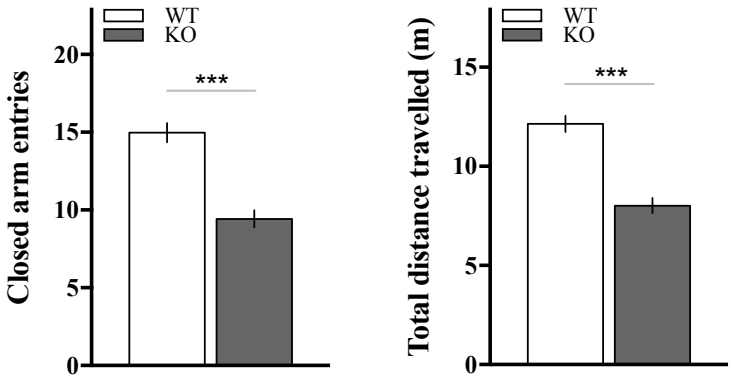


Figure 3.2. Left, Number of entries into the closed arms for 5-HTTKO (N=40) and WT (N=42) mice. **Right,** Total distance travelled by the same mice during the 5-minute-long EPM test. Mean ± SEM. ***p < .001 (independent-samples t-test).

Groups differed significantly regarding locomotor activity, with 5-HTTKO mice making significantly fewer entries into the closed arms (t(80) = 6.5, p < .001) and travelling less distance in the maze overall (t(80) = 7.3, p < .001), compared with the WT controls (see Figure 3.2).

3.4. Hyponeophagia tests

3.4.1. Subjects, apparatus and procedure

Twenty-five 5-HTTKO mice (female: n=13) and 25 WT mice (female: n=11) were tested in both hyponeophagia tests. All subjects had been tested on the EPM during the previous day to the first HPN test (HPN-1). Again, groups were matched by sex and age

around 5 months old on average). Mice waited a further three days before being tested on the second HPN test (HPN-2).

The apparatus used for the HPN-1 test consisted of a translucent plastic jug (12.5 cm high, 18 cm long, 13 cm at its biggest width) placed upside down on a white plastic base (30 cm^2) (Deacon, 2011; see Figure 2.2, left, in Chapter 2). A protruding spout at one extremity of the jug (2 cm long) worked as an alcove over a food well (0.9 cm high, 1.2 cm in diameter) attached to the base. Immediately before testing, the food well was filled with sweetened condensed milk (Nestle Carnation diluted 50:50 with water). For the HPN-2 test, a more anxiogenic environment was created by using the arm of an elevated Y-maze as the base of the 'apparatus', which was raised 79.5 cm above the floor, and by using higher illumination levels (see below; see also Figure 2.2, right, in Chapter 2). The arm flooring was black-coloured with a raised grey lip (0.5 cm). A food well (0.9 cm high, 1.2 cm in diameter) was attached at ~4.7 cm from the curved extremity of the arm, which had 9.4 cm as width. On the opposite extremity, 25 cm away, a grey wooden block (12.5 cm high, 9 cm long and 1 cm wider) blocked access to the rest of the maze. For this test, 6 sucrose pellets (20mg each, TestDiet, USA) were put in the food well before testing.

Each test was administered in a different experimental room, which were also different from the room where the EPM took place. Although both rooms had the luminance set around ~17 lux (HPN-1: yellow/warm light; HPN-2: white/cool light), this value would likely have been lower inside the translucent jug, this being an additional factor making the HPN-1 test less anxiogenic than HPN-2. Sessions were recorded with a video camera (LifeCam HD-3000, Microsoft, USA) placed near the apparatus, connected to a laptop computer located ~1.5m away from the maze. The experimenter stayed in the room during the testing session.

Subjects were food-deprived for 18-20h before being carried in their home cages to the experimental room. Their weight was checked just before the removal of the food from

the home cages, and then again before being tested on HPN-1 and HPN-2. Mice were then kept in the dark until a 5-20-minute-long acclimatisation period took place in a separate, individual cage far away from the apparatus. For the HPN-1 test, a subject was placed on the base facing away from the food well while the jug was gently lowered. In the HPN-2 test a mouse was placed in the Y-maze arm facing the wooden block.

A 180s-long trial was then given to each subject. The time taken to start continuous drinking/eating was recorded (~3s). If this did not happen during this cut-off period, the subject would return to the individual cage used during acclimatisation for 10-12mins, before then being tested again in the same conditions as the first trial. This was repeated another time if the subject failed to eat or drink during the second trial. Cumulative latencies to first contact (including sniffs) with food and to continuous consumption were recorded by the experimenter, who watched the recorded videos blinded to the genotype.

The maximum cumulative latency value was 540s, and was given to subjects that did not eat or drink at all. A small cohort of mice was only exposed to a 180s trial during HPN-2 test. Considering this, only those mice that drunk during this 180-second long period were included in the analyses of the HPN-2 test. The statistical tests used were independent samples *t*-tests and graphs show means and SEM. When data did not meet parametric assumptions, transformations ($\log_{10}$ or square root) were utilised. Non-parametric data were analysed using a Mann-Whitney *U* test, and medians are reported where appropriate. In the latter case, refined boxplots were used to depict the data. The boxplots show not only the medians and inter-quartile ranges (IQRs) but also outliers (i.e. data values exceeding the IQRs 1.5 times). Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

3.4.2. Results

5-HTTKO mice were significantly more anxious in both hyponeophagia tests

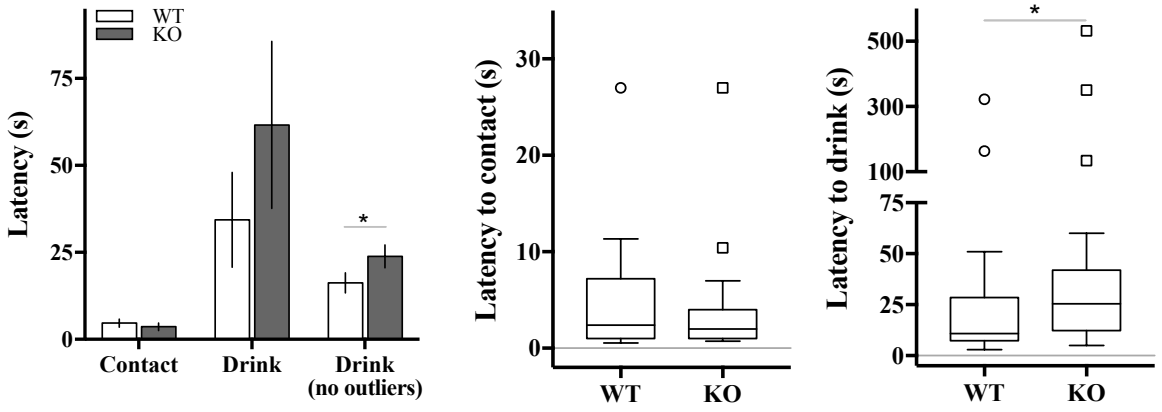


Figure 3.3. Results from the HPN-1 test. **Left,** Latencies to first contact with the milk and to continuous drinking (~3s) for all 5-HTTKO (N=25) and WT (N=25) mice (N's for the latency to continuous drinking excluding outliers: KOs = 22; WTs = 23). Mean ± SEM. **p* < .05 (independent *t*-test). **Centre and right,** Boxplots for the data obtained with the previous two measures (white circles and squares: outliers exceeding the IQR by 1.5 times). **p* < .05 (Mann-Whitney *U* test).

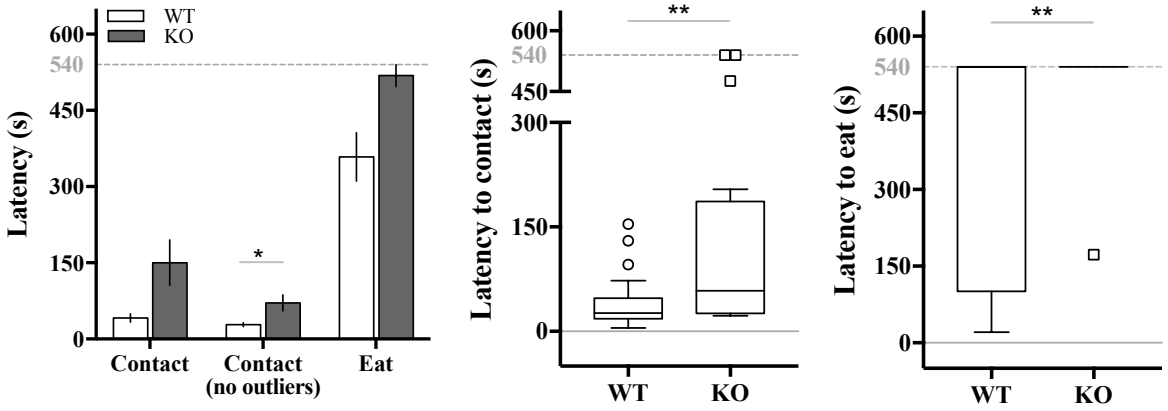


Figure 3.4. Results from the HPN-2 test. **Left,** Latencies to first contact with the sucrose pellets and to continuous eating (~3s) for all 5-HTTKO (N=17) and WT (N=22) mice (N's for the latency to first contact excluding outliers: KOs = 14; WTs = 19). Mean ± SEM. **p* < .05 (independent *t*-test). **Centre and right,** Boxplots for the data obtained with the previous two measures (white circles and squares: outliers exceeding the IQR by 1.5 times). ***p* < .01 (Mann-Whitney *U* tests). 540s was the maximum cumulative duration of the test.

In the HPN-1 test, 5-HTTKO mice (median = 25.5) were significantly slower to start drinking the sweetened condensed milk than WT mice (median = 10.9) ($U = 209.5$, $p = .046$;

$t(43) = -2.2$, $p = .037$, if 5 outliers are excluded from the data; see Figure 3.3). No significant genotypic difference was observed in the latencies to the first contact with the milk ($U = 2795$, $p = .521$). Groups did not significantly differ in the amount of percent weight lost due to food deprivation overnight ($t(48) = 1.4$, $p = .176$; WTs: ~6.8%; KOs: ~5.3%), arguing that this was not a factor in the genotypic differences in latency to drink.

In a potentially more anxiogenic environment during the HPN-2 test, genotypic differences were found with both measures. First, 5-HTTKO mice (median =58.4) were significantly slower than WT mice (median = 26.4) to make a first contact with the sucrose pellets ($U = 94$, $p = .008$; $t(31) = -3.3$, $p = .003$, if 6 outliers are removed from the data; see Figure 3.4). Strikingly, only one 5-HTTKO mouse did eat in a continuous manner (~3s), compared with 10 WT mice, thus leading to a significant genotypic difference regarding the latency to start eating ($U = 111$, $p = .007$; median for both WTs and KOs: 540).

It is worth noting that the results obtained with the HPN-2 test should be considered with caution given that WT mice seemed to have lost significantly more percent weight before being tested than 5-HTTKO mice (WTs: ~7.5%; KOs: ~5.5%; $U = 93.5$, $p = .008$; WTs: median = 7.1; KOs: 5.7).

3.5. Social interaction

3.5.1. Subjects, apparatus and procedure

Fourteen 5-HTTKO mice (female: n=6) and 16 WT mice (female: n=8) were used in this experiment. Groups were matched by sex and age (around 6.5 months on average), with age at the day of testing varying from 5 to 8 months. Male mice were tested 23 days after the EPM test previously described, whereas female mice waited 7 more days. Mice were again extensively handled (and tail-marked) during the four days before the first day of

testing. The mice were weighed prior to a daily exposure to the testing arena to habituate them to the testing context.

The apparatus consisted of a red-tinted Plexiglas arena (20×30 cm, with walls 15cm high; see Figure 2.3 in Chapter 2) with a video camera placed directly above, connected to a laptop computer located in the same room. The experimental room was always dimly lit (5 lux).

Testing day	1	2	3	4	5	6
Duration (mins)	5	5	5	5	5	5
Sessions	Habituation sessions				Test 1	Test 2
Subjects (e.g. A, B and C) in the arena	1 subject in the empty arena (e.g. A alone in the arena)				Dyad 1 (e.g. A + B)	Dyad 2 (e.g. A + C)

Table 3.3. Timeline of the reciprocal social interaction test.

Mice were individually habituated to the apparatus for four days, in 5-minute long daily sessions (see Table 3.3). Afterwards, they were subjected to a 5-minute test with an unfamiliar conspecific in each of the two following consecutive days (i.e. they were exposed to a different animal on each test). Subjects from one dyad would be placed at the same time in the apparatus at different extremities, each one facing the side, 30 cm distant from the opposite side. They would then be allowed to freely explore the chamber and the unfamiliar conspecific for the entire duration of the test.

Mice were matched by genotype, sex and, because they were over 4 months old, by weight. A within-pair weight difference of less than 5% is a good difference for matching by weight (Sato et al., 2013). However, this was not always possible to achieve in this study. Therefore, the pairs of mice used in this test were split into three groups: 8 pairs of mice had a within-pair weight difference up to 5%, 10 pairs had a difference between 5 and 10%, and 12 pairs had a difference greater than 10%. Care was also taken to avoid having dyads

sharing the same breeding parents, and in the two situations when this was not possible, it was assured subjects came from different litters and not only from different cages. Balancing of the order of genotype testing was ensured to control for effects of the time of the day. The starting position of the animals within the arena was also counterbalanced across groups.

Exploration levels of the apparatus were examined prior to the administration of the reciprocal social interaction test to check for differences in the rate of habituation and to ensure that no significant genotypic differences existed regarding this behaviour. This was achieved using the tracking software ANY-maze Video Tracking Software (Stoelting Co., USA), which was also used to film the reciprocal social interaction test. The measures used to quantify exploration of the apparatus were total distance travelled and time spent immobile. Social interaction consisted of instances of sniffing, grooming or following, but not passive body contact, within 1 cm of the unfamiliar conspecific. These social behaviours were manually scored by the experimenter (i.e. the author of this thesis) at a later time, blind to the subject's genotype. Each dyad counted as one unit for data analysis. The statistical tests used were a two-way and a three-way mixed ANOVA (with genotype, 'weight difference group' and habituation session as factors), and independent samples *t*-tests. Graphs show means and SEM. Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

3.5.2. Results

Exploratory levels were matched by the end of habituation to the apparatus

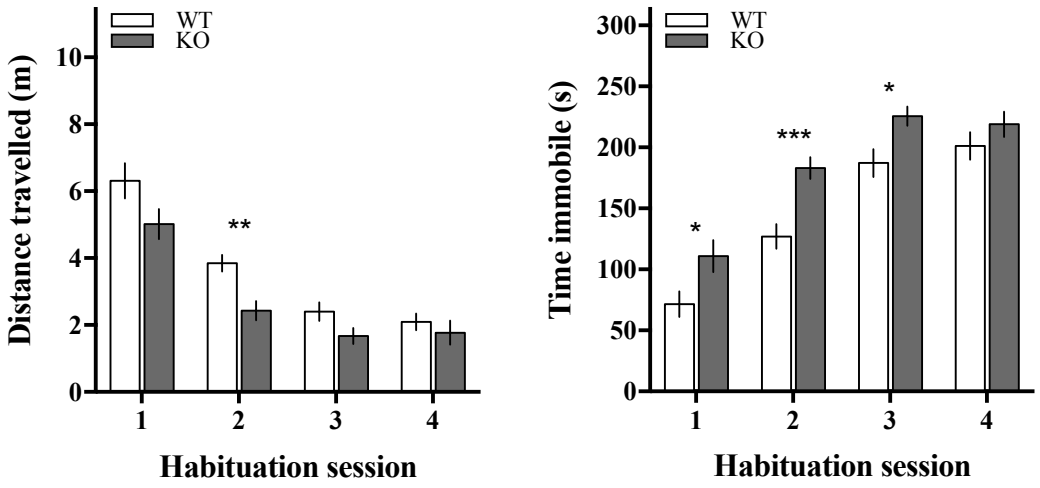


Figure 3.5. Left, Distance travelled by the experimental subjects on the 5-minute long habituation sessions administered during the four days prior to the first test session (KOs: N=14; WTs: N=16). **Right,** Time that the same subjects spent immobile during those habituation sessions. Mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ (simple effect of genotype on each habituation session).

Across the four days of the habituation phase, 5-HTTKO mice moved significantly less overall (main effect of genotype: $F(1,28) = 7.4$, $p = .011$), and spent significantly more time immobile than WT mice (main effect of genotype: $F(1,28) = 11.6$, $p = .002$; see Figure 3.5). Despite no significant interactions between genotype and day being registered with either measure, pairwise comparisons were used to demonstrate that groups were matched by the fourth day of habituation. This was indeed the case for both the distance travelled (simple effect of genotype on day 4: $p = .454$; $Genotype_2 \times Day_4$ interaction: $F(3,84) = 1.53$, $p = .228$), and time spent immobile (simple effect of genotype on day 4: $p = .261$; $Genotype_2 \times Day_4$: $F(3,84) = 1.79$, $p = .178$).

No significant interaction between genotype and sex ($F(1,26) = 1.7$, $p = .208$), or between genotype, sex and session ($F < 1$) were obtained regarding the distance travelled.

Additionally, no significant main effect of sex ($F(1,26) = 1.2$, p = .286) or interaction between genotype, sex and habituation session ($F < 1$) were detected regarding time spent immobile. However, a significant interaction between genotype and sex was detected ($F(1,26) = 6.3$, p = .018). Male KO mice spent significantly more time immobile than female KO mice and male WT mice over the 4 habituation sessions. This pattern of results was kept on day 4.

5-HTTKO mice exhibited significantly reduced social interaction

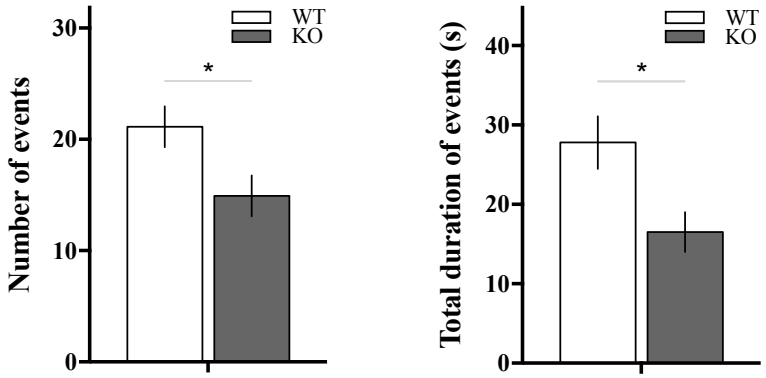


Figure 3.6. Left, Total number of social interaction events per pair (i.e. instances of sniffing, grooming or following) observed in 14 dyads of 5-HTTKO mice, and 16 dyads of WT mice. **Right,** Total duration of those social interaction events. Mean ± SEM. *$p < .05$ (independent samples *t*-test).

During the test phase, the dyads of 5-HTTKO mice showed significantly reduced interaction levels when compared with those of WT mice (see Figure 3.6). This was observed both when using the number of events (per pair) measure ($t(28) = 2.4$, $p = .024$), and when using the total duration that mice spent interacting ($t(28) = 2.7$, $p = .012$).

Although the dyads of 5-HTTKO mice had, on average, a greater weight difference than those of WT mice, this was not statistically significant ($U = 134.0$, $p = .377$; WT dyads: median = 8.7; KO dyads: 10.1). Moreover, no significant correlations were seen between weight differences and either the number of events or total duration of events for each

genotype (see Appendix 2). Furthermore, having three different range levels of weight difference across the dyads of mice (up to 5%, between 5 and 10%, and over 10% of weight difference) did not significantly influence the results of the reciprocal social interaction test (main effects 'weight difference group' and interactions between genotype and 'weight difference group' for number and duration of events: all F's < 1.1, all p's > 0.359). Therefore, weight difference within dyads did not seem to be a factor that could account for observed genotypic differences in social interaction.

3.6. Sociability and preference for social novelty in the three-chamber test

3.6.1. Subjects

This experiment used 23 5-HTTKO mice (female: n= 12) and 22 WT mice (female: n=9). Groups were matched by age (around 6 months on average), with age at the day of testing varying from 6 weeks to 11 months. The stimulus mice used consisted of male (n=10) and female (n=14) inbred adult CD-1 mice obtained from Harlan Laboratories, U.K. All mice were housed with littermates in small groups (2-6) of the same sex.

Apart from the stimulus mice, all subjects had been previously tested in the three anxiety tests described in this chapter, and under the same testing order (EPM, HPN-1 and, finally, HPN-2). However, the three cohorts of animals used in the current experiment differed in the amount of time elapsed since the end of the HPN-2 test. Specifically, cohort 1 waited 3-9 days to be tested, cohort 2 waited 22-26 days, and, finally, cohort 3 waited 105-107 days. Although the genotypes in each cohort were matched for age, the three cohorts differed with regard to the mean age at which they were tested (cohort 1: 4.4 months; cohort 2: 5.6 months; cohort 3: 9.8 months). All cohorts had genotypes matched for sex, apart from cohort 1 where the WT mice included 9 males and 2 females. Considering this, a factor of

cohort with three levels was included in all the ANOVAs used for the data analyses of this experiment.

3.6.2. Apparatus and procedure

The apparatus used was a rectangular, three-chamber box made of red-tinted Plexiglas, with two (15 cm high) walls dividing three (20×30 cm) adjacent compartments (see Figure 2.4 in Chapter 2). The dividing walls included manually retractable doors which could give access to the side chambers through a 10-cm passage when opened. Steel wire cage enclosures (8.1×8.1×21.8 cm), placed vertically inside the apparatus, were used to hold the stimulus mice during the test. They allowed each subject being tested to see, hear, smell, and even experience some limited direct interaction with the stimulus mice (e.g. sniffing each other), but prevented substantial contact (e.g. fighting). The experimental room was kept in very dim lighting conditions (5 lux), and a white noise generator was used in all stages of the experiment.

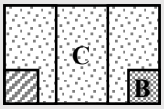
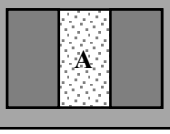
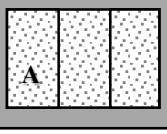
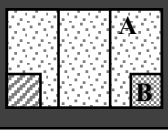
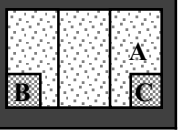
Day	1	2	3	4 (Testing day)			
Duration (mins)	~7	~7	~7	5	10	10	10
Phase	Habituation of stimulus mice to the arena and cage enclosures			Habituation to the central chamber	Habituation to the entire apparatus	Sociability phase	Preference for social novelty
Subjects (e.g. A, B and C) in the arena	Stimulus mice (e.g. B and C)			Test subject only (e.g. A)	Test subject only (e.g. A)	A + social stimulus B	A + social stimuli B and C
Scheme							

Table 3.4. Timeline of the three-chamber social approach test, which also included a preference for social novelty phase.

All mice were weighed and handled during the three days prior to the start of the experiment. In addition to this, and during this period, stimulus mice were acclimatised to the experimental room (30mins per day) and habituated to the apparatus (exploring it in small groups for 2mins), and also to the wire cage enclosures (individually for 1min, while other CD-1 mice were exploring the apparatus). On the day of testing, mice would be acclimatised to the experimental room for at least 30 minutes before the start of the experiment, with no possibility of seeing the apparatus when not being tested. The experimenter would remain in the room during the entire session, away from the subjects' sight. Males and females were tested on different days.

Four successive phases were used in this test: (i) a habituation phase to the central chamber only; (ii) a habituation phase to the entire apparatus; (iii) a sociability phase; and finally, (iv) a preference for social novelty phase. During the first phase, the subject was confined to the central chamber for 5mins. Afterwards, for phase (ii), the retractable doors would be drawn back, and the subject would be allowed to explore the entire apparatus, which was empty at this stage. After 10mins had elapsed, the subject would be confined to the central chamber before an empty cage enclosure was placed in one side chamber, and another containing a stimulus mouse placed in the other side chamber. The spatial location of the stimulus mouse alternated between the left and right side chambers across subjects (counterbalanced across genotypes) and, importantly, the empty cage enclosure used in this phase never held a social stimulus mouse throughout the experiment. The sociability phase (phase (iii)) would then start with the retractable doors drawn back, and the subject was allowed to explore freely the entire apparatus for 10 mins. Finally, for phase (iv), the subject would again be temporarily confined to the central chamber while the empty cage enclosure was replaced by a new one containing a novel stimulus mouse. Half of the animals in each group would have the familiar stimulus mouse in the same side chamber, and half would have the familiar animal moved to the opposite chamber in its (original) cage enclosure. The

subject could then explore the entire apparatus for the last 10 mins of the test. Accordingly, subjects were never removed from the apparatus during the test and were always free to choose what, or which stimulus mouse, to investigate. The apparatus and cage enclosures were cleaned between subjects with water and 70% alcohol, respectively.

Although part of the reason for administering this task was to compare the results obtained with those obtained with the 5-minute-long reciprocal social interaction test, the usual 10-minute periods used in this task for the last two phases were kept to ensure that subjects would have enough exposure time to the stimulus mice in each phase. These stimulus mice were re-used throughout the test but care was taken to ensure that each animal would have the maximum amount of time possible to rest between exposures. They would also be matched by sex and weight with each experimental subject being tested, and the novel social stimulus mouse used in phase (iv) would always come from a different home cage than the social stimulus mouse used during phase (iii).

A video camera attached to the ceiling directly above the apparatus recorded the sessions, with the subjects' movement being tracked by ANY-maze. The settings of this software associated 3 data points with each test subject (head, body and tail), and tracked their movement inside the three chambers, including time spent in close proximity (~2cm) to the cage enclosures. In addition, the video sessions from all mice tested in the three-chamber test were also manually scored by the experimenter (i.e. the author of this thesis) to ensure that the measures obtained with the tracking of head movements by ANY-maze were accurate.

3.6.3. Data analysis

Measures of the number of head entries into the proximity areas, and total time spent there, were collected during testing (see Figure 2.4, in Chapter 2). These measures are a more reliable, direct and valid measure of sociability in mouse models than time spent in the

respective chambers, reflecting true social investigation (Moy et al., 2004; Fairless et al., 2011). Afterwards, mean discrimination ratios (DR) for each measure were calculated (e.g. "time spent exploring the novel mouse" / ["time spent exploring the familiar mouse" + "time spent exploring the novel mouse"]). A mean value of 0.5 in a DR means that subjects did not show a preference for either proximity area during that phase; and the closer the mean value is to 1, the greater the extent to which that group exhibited a preference for exploring the social or novel stimulus over the non-social or familiar stimulus, respectively.

The relevant analyses were limited to the first 300s of the sociability and preference for social novelty phases, where most significant social approach behaviour usually occurs (Nadler et al., 2004). This focus on the first 5 mins would also facilitate comparisons with data obtained with the 5-minute long reciprocal social interaction test, which would be more compatible. However, raw data (i.e. absolute values) and DR results for the entire 600-second long period of the sociability and preference for social novelty phases can be found in Appendix 2. Raw data and DRs from the entire period of the sociability phase are important to ensure that genotypes did not differ in the amount of exposure to the social stimulus that would later become a familiar animal. That is, it is crucial that differences in exposure do not influence the performance during the preference for social novelty phase, because more exposure to the social stimulus in phase (iii) could potentially lead to faster habituation to the 'familiar animal' in phase (iv), i.e. more social contact during phase (iii) would increase the opportunity for learning and hence for recognition memory in phase (iv). The analysis of the raw data from the 600-second long sociability phase were also extended to the time spent in the chambers to ensure that no genotypic differences could be found regarding exposure even when mice were not near the proximity area (see Appendix 2).

Measures of distance travelled in the apparatus and total time spent immobile throughout the four phases of the test were also used. These measures could not only be used to investigate genotypic differences in locomotor activity, but could also be used as an index

of anxiety. These data were analysed with three-way ANOVAs, with genotype and cohort as between-subjects variables, and phase as within-subjects variable. Regarding the statistical analyses of the sociability and preference for social novelty phases, two-way between-subjects ANOVAs were used, with genotype and cohort as between-subjects variables. One-sample *t*-tests were also used to verify whether subjects showed statistically significant discrimination levels (i.e. a mean DR significantly above 0.5). In the situations where the assumption of normality failed to be met (e.g. significant results in normality tests), a non-parametric alternative to the one-sample *t*-test was used instead. This consisted of the sign test, which was conducted by means of chi-square goodness-of-fit (χ^2), where the H_0 = 0.5 was rejected if $\chi^2 \geq 3.84$ (i.e. the critical value of χ^2 for 1 degree of freedom; Howell, 2013). *P* values for the sign test result were calculated in the following website: www.graphpad.com/quickcalcs/pvalue1.cfm. However, non-parametric one-sample *t*-tests are also reported together with the sign test results. Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

One 5-HTTKO subject was excluded from all data analyses because it did not visit either side chamber during the entire sociability phase. Such a situation prevented this animal from having a familiar animal during the preference for social novelty phase rendering this test meaningless. Additionally, the tracking of one 5-HTTKO subject during habituation to the central chamber was of poor quality and thus its data were excluded from the analyses for this phase of testing only.

3.6.4. Results

5-HTTKO mice exhibited reduced locomotor behaviour during testing

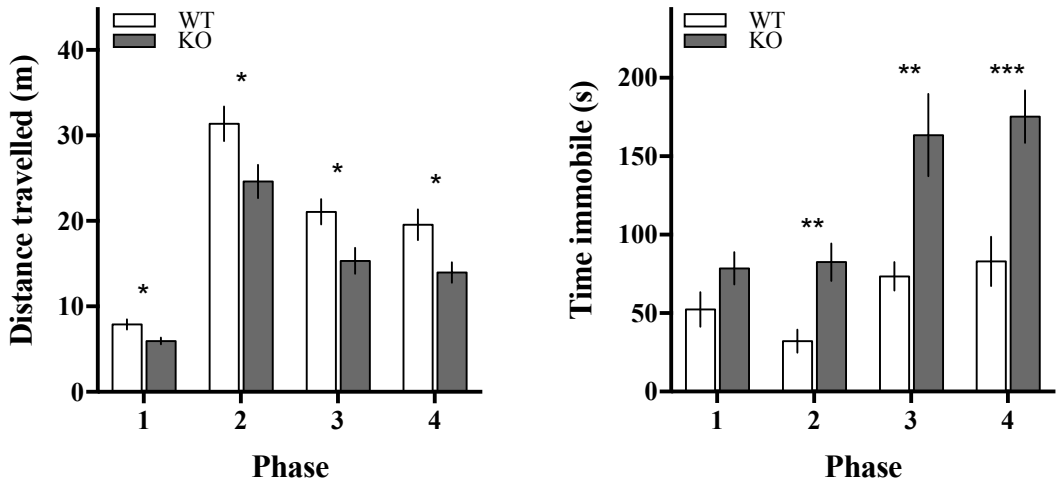


Figure 3.7. Left, Distance travelled during the four phases by 5-HTTKO (N=21) and WT (N=22) mice. **Right,** Time that the same mice spent immobile in each of the four phases. Mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ (simple effect of genotype on each phase).

In all four phases of the experiment 5-HTTKO mice travelled significantly less compared with WT mice (main effect of genotype: $F(1,37) = 11.3$, $p = .002$; $Genotype_2 \times Phase_4$ interaction: $F(3,111) = 4.1$, $p = .009$; see Fig. 3.7, left). Across the entire procedure, 5-HTTKO mice also spent significantly more time immobile than WT mice in all phases apart from the first one (main effect of genotype: $F(1,37) = 25.4$, $p < .001$; $Genotye_2 \times Phase_4$ interaction: $F(3,111) = 2.8$, $p = .045$; see Fig. 3.7, right). Therefore, taking both results together, 5-HTTKO mice moved significantly less than WT mice, which could reflect increased anxiety compared to the control group.

5-HTTKO mice exhibited normal levels of sociability but enhanced social recognition

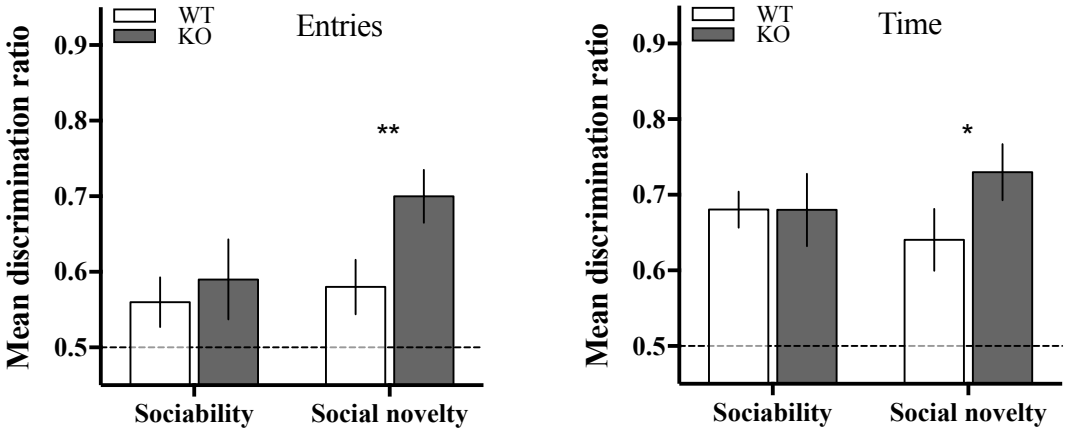


Figure 3.8. Left, Mean discrimination ratio for head entries into the proximity areas during the sociability and preference for social novelty phases for 5-HTTKO (N=22) and WT (N=22) mice. **Right,** Mean discrimination ratio for the duration of the head entries into the proximity areas during the same two phases for the same mice. Mean ± SEM. *$p < .05$, **$p < .01$ (main effect of genotype on the preference for social novelty phase).

During the sociability phase, both groups preferred exploring the cage enclosure containing the stimulus mouse compared to exploring the cage enclosure containing the empty cage (see Fig. 3.8). Although the mean DR for head entries into the proximity area was not significantly above the no preference level of 0.5 in both groups (WTs: $t(21) = 1.7$, $p = .098$; KOs: $t(21) = 1.8$, $p = .091$), the time spent exploring the social stimulus during this phase leaves no doubt about this preference (WTs, $\chi^2 = 14.7$, $p < .001$; KOs, $\chi^2 = 8.9$, $p = .003$; non-parametric one-sample *t*-test: WTs, $t(21) = 7.2$, $p < .001$; KOs, $t(21) = 3.7$, $p = .001$). No significant genotypic differences were observed for both the number of head entries into the proximity area, or time spent in the proximity area, during this phase (all *F*'s < 1).

Finally, during the preference for social novelty phase, both groups exhibited a significant preference for social novelty. This was seen with the mean DR for (i) the head entries into the proximity area (WTs, $\chi^2 = 8.9$, $p = .003$; KOs, $\chi^2 = 18.2$, $p < .001$; non-

parametric one-sample *t*-test: WTs, $t(21) = 2.1$, $p = .045$; KOs: $t(21) = 5.8$, $p < .001$), and (ii) the time spent there (sign test: WTs, $\chi^2 = 8.9$, $p = .003$; KOs, $\chi^2 = 8.9$, $p = .003$; non-parametric one-sample *t*-test: WTs, $t(21) = 3.4$, $p = .003$; KOs: $t(21) = 6.3$, $p < .001$). Strikingly, this preference was particularly strong in the 5-HTTKO mice, which exhibited a significantly greater number of head entries into the proximity area of the novel social stimulus over the proximity area of the familiar animal compared with the WT mice ($F(1,38) = 8.9$, $p = .005$), and also spent a significantly longer period of time there ($F(1,38) = 6.1$, $p = .018$; see Fig. 3.8 to visualise the comparison of the discrimination ratios). Importantly, these results were not influenced by any significant genotypic difference regarding exposure time to the social stimulus during the sociability phase (see Appendix 2).

3.7. Discussion

Summary of the results

The first three experiments of this chapter confirmed that 5-HTTKO mice exhibit increased anxiety-like behaviours in approach-avoidance tasks, such as exploring the open arms of the EPM significantly less than WT mice, and taking significantly more time to start consuming a desirable food in a novel environment than their controls. Additionally, reduced social interaction was observed in 5-HTTKO mice by using a free dyadic reciprocal social interaction test with homo-genotypes couples. Despite the decrease in social exploration in this assay, 5-HTTKO mice demonstrated equal levels of sociability to WT controls in the three-chamber test. Finally, and for the first time, it was shown that 5-HTTKO mice can exhibit increased preference to explore a novel social stimulus over a familiar one in the three-chamber test.

Validation of a mouse model for studying the effects of the 5-HTT on anxiety

5-HTTKO mice explored the open arms of the EPM significantly less than WT controls. Similar results were obtained by other studies showing increased anxiety-like behaviours in 5-HTTKO rodents in this test (e.g. Holmes et al., 2003b; Olivier et al., 2008b; see Table 3.2), which are opposite to those seen in mice overexpressing the 5-HTT (Jennings et al., 2006; Line et al., 2011). Notably, in addition to the longer latency to enter an open arm in the EPM, 5-HTTKO did take more time to leave the closed arm where they had been placed at the beginning of the test. This situation could be considered similar to an emergence test (Archer, 1973), and the result described here thus complements previous research using different forms of this test with 5-HTTKO rodents (Holmes et al., 2003b; Olivier et al., 2008b).

While significant genotypic differences were seen regarding locomotion with reduced activity levels in the KOs, this is common when testing 5-HTTKO mice in the EPM (e.g. Holmes et al., 2003b; Moy et al., 2009). Indeed, specific measurements of spontaneous locomotor activity in these mice show that they are less active than WT mice (e.g. Line et al., 2011). Nevertheless, it is probable that some inter-study variability regarding locomotor activity in the KOs tested on the EPM can be explained by differences in variables such as the presence of the experimenter during testing, open arm flooring, lighting levels and time of testing, to name a few (Rodgers & Dalvi, 1997).

Importantly, however, every new approach-avoidance test administered in this chapter complemented the previous one. The hyponeophagia tests compensated for the potential locomotor activity confounds seen during the EPM, and the hyponeophagia test conducted in a more anxiogenic environment produced greater anxiety-like behaviours in the 5-HTTKO mice than the one conducted in a more protected environment. Indeed, the hyponeophagia test is a good alternative to exploration-based tasks such as the EPM given that it is less dependent on locomotor activity (Andrew Holmes & Cryan, 2006).

In this chapter, the two hyponeophagia tests further validated that 5-HTTKO mice exhibit more anxiety-like behaviours than WT mice. In the HPN-1 test, it was possible to replicate previous findings (e.g. Line et al., 2011) by showing that 5-HTTKO mice take more time to consume a desirable food in a novel environment. Whereas no genotypic differences were seen in latency to the first contact with food in this first hyponeophagia test, administering another one in a more anxiogenic environment showed that even this first contact can be affected by anxiety in 5-HTTKO mice. Indeed, in the HPN-2 test, not only did groups differ in the amount of time taken to touch or sniff the food, but only a single 5-HTTKO mouse ate any of the food, compared with 10 WT mice. Even if the results of the HPN-2 test can be potentially confounded by group differences in percent of body weight lost, it is worth noting such differences cannot explain the results of HPN-1.

Further confirmation of reduced social interaction in 5-HTTKO mice

Regarding the social interaction behaviours, these were clearly diminished in the KOs in the reciprocal social interaction test. Importantly, the test used here was less confounded by other factors compared with the simple investigation of nonaggressive social behaviours during the resident-intruder test. This might explain why the current results do not match the absence of significant genotypic differences reported by some authors in 5-HTTKO rodents using the latter tests (e.g. Holmes et al., 2002a; Homberg et al., 2007b).

Furthermore, the results presented here confirm those obtained with Kalueff and collaborators (2007a). As previously mentioned in the introduction of this chapter, it was important to address some potential sources of confounds in their study, such as using hetero-genotype couples and randomly choosing subjects for the dyads regardless of their weight. The assessment of hypoactivity was not specifically addressed in this chapter but the habituation to the apparatus was greatly improved. Indeed, Kalueff et al. tested subjects in an ‘observation cylinder’ that had been used for several other tests and assays in the days

prior to testing (e.g. the sticky label and novel object tests). Although habituation to the apparatus is a common procedure in the social interaction test, such administration of other procedures in the same context could have compromised the neutrality and specificity of the context usually required for this task. Contrary to the study conducted by Kalueff et al., here mice were habituated to one context for four days and it was specifically ensured that the exploratory behaviour was matched between genotypes before testing started. Therefore, the present experiment refined the experiment of Kalueff and colleagues and further validated their claim of reduced social interaction behaviours in 5-HTTKO mice, at least under some conditions.

5-HTTKO mice exhibit normal sociability in a less anxiogenic situation than the reciprocal social interaction test

In contrast, 5-HTTKO mice exhibited equivalent levels of sociability compared to WT controls in the three-chamber test. Both groups of mice exhibited a similarly strong preference for investigating a social stimulus instead of an empty cage, thus replicating previous studies reporting no significant genotypic differences regarding sociability in this test (Moya et al., 2011; Sakakibara et al., 2014). This was particularly the case in the measures focused on the proximity areas to the cage enclosures (e.g. sniffing, in the other two studies).

Although Moy and collaborators (2009) reported decreased sociability in 5-HTTKO mice based on the time spent in the chambers, no differences in that particular measure were observed in the current experiment (see Appendix 2), and in the one conducted by Moya and colleagues (2011). Moreover, Moy and collaborators (2009) only observed decreased sociability in male 5-HTTKO mice, whereas the females spent *more* time near the social stimulus than near the empty cage (although this was not statistically significant). In addition, Moy and collaborators (2009) always used male mice as social stimuli despite

testing both males and females. Methodological differences might explain the inconsistency between this study and the reports from other studies, including this chapter. Therefore, overall, evidence points towards unaltered sociability levels in 5-HTTKO mice in non-anxiogenic situations.

5-HTTKO mice exhibit enhanced social recognition memory

For the first time, my data show that 5-HTTKO mice exhibit a stronger preference for investigating a novel social stimulus than a familiar social stimulus in the three-chamber test, compared to WT controls. This result suggests that 5-HTTKO mice have enhanced social recognition memory.

The result obtained here differs from those reported by two other studies, which reported no significant differences in social novelty preference between 5-HTTKO mice and WTs (Moy et al., 2009; Sakakibara et al., 2014). It is possible, however, that methodological differences might explain why other authors have not seen increased preference for social novelty in 5-HTTKO mice. For instance, the two other studies did not counterbalance the side of the familiar animal as was done in this chapter. This does not help to control for possible effects of contextual novelty on an (apparent) novelty preference (Pearson et al., 2010). Moreover, one study (Moy et al., 2009) did not match the sex of test and social stimulus subjects (i.e. they tested males and female KOs, but only used male social stimuli). Additionally, both studies relied on social stimulus mice from the same background strain used to backcross animals during the generation of the test subjects. Regarding this latter choice, it has been claimed that there are advantages in using mice from an outbred strain instead, such as the CD-1 mice used here (Arakawa et al., 2008; Pearson et al., 2010). For instance, the existence of genetic differences in major urinary proteins and major histocompatibility complex peptides expressed in mouse urine, which highly inbred strains do not have, might help to recognise a novel CD-1 mouse better than another C57BL/6J

mouse (e.g. via scent marking; Nevison et al., 2000; Hurst et al., 2001; Pearson, et al., 2010). Taking this latter point into account, the additional care taken here in using novel social stimuli from a different background strain, and from different home cages during the preference for social novelty phase, might have made it easier for the KOs to notice differences between the familiar animal and the novel animal, compared with other studies.

Another limitation seen in the study conducted by Moy and collaborators (2009) was that they did not pool data from males and females. Visual inspection of the data (see a copy their Figure 5 in Appendix 2) suggests that both male and female 5-HTTKO mice were sniffing the novel social stimulus more during the preference for social novelty phase, whereas male WT mice were not doing this. Although no statistically significant differences were reported for either sex, it would be interesting to perform a two-way ANOVA (with sex and genotype as factors) on DR data with a resulting larger sample size (Crusio et al., 2009).

Conclusion

As predicted, 5-HTTKO mice, which exhibit an anxiogenic profile in approach-avoidance tests such as the EPM and the HPN, showed reduced social interaction with a conspecific in the reciprocal social interaction test. Considering that these KOs did not exhibit alterations in social approach when tested in a social assay less anxiogenic than the previous test, it is likely that increased anxiety was a critical factor driving their behaviour during the reciprocal social interaction test. Therefore, the observation of altered social investigation in the 5-HTTKO mice depended on the context in which was tested. It is possible that these mice only exhibit altered social anxiety in anxiogenic situations, such as when they were forced to interact physically with a novel conspecific.

In addition, the results obtained with the three-chamber test, with no changes in sociability levels, and even an improvement in social recognition memory in mice with null

5-HTT expression, indicate that knocking out the 5-HTT does not affect sociability in mice per se, which is consistent with other studies (e.g. Lewejohann et al., 2010).

Finally, the results reported in this chapter also potentially support the neuroplasticity theory of 5-HT function, which predicts that subjects with low or null levels of 5-HTT expression should exhibit increased sensitivity to environmental social factors (Belsky & Pluess, 2009; Branchi, 2011; Kiser et al., 2012). First, altered social anxiety was only seen in an anxiogenic situation, and not in a less anxiogenic one. Second, the enhancement of social recognition memory in 5-HTTKO mice indicates an instance of enhanced recognition memory (Ferguson et al., 2002; Winslow, 2003). Subsequent chapters will investigate whether other types of memory are affected in 5-HTTKO mice.

Chapter 4. Processing of aversive cues in 5-HTTKO mice

4.1. Abstract

This chapter describes two experiments that investigated whether genetic knock-out of the 5-HTT affects aversive learning in mice. In Experiment 1, 5-HTTKO mice and WT controls performed a discriminative aversive learning task, where two cues were presented: one cue was followed by an aversive outcome (footshock) on every training trial (CS+ = 100% CS-US contingency) and the other cue was never followed by shock (CS– = no US). In Experiment 2, KOs and WTs were trained in a similar procedure but the CS+ was replaced with a cue that predicted footshock on 20% of the trials and was, therefore, an ambiguous cue (CSa = 20% CS-US contingency).

5-HTTKO mice exhibited enhanced discrimination in both experiments compared to WT controls, regardless of whether the CS+-US contingency was 100% or 20%. However, enhanced performance was found at different phases in the two experiments. During Experiment 1, 5-HTTKO mice exhibited facilitated cue discrimination during training, whereas during Experiment 2, when the ambiguous cue was used, 5-HTTKO mice showed enhanced fear memory recall. In addition, evidence of enhanced contextual fear learning was also observed with the 5-HTTKO mice.

These data show enhanced aversive learning in 5-HTTKO mice and support the neuroplasticity theory of 5-HT function which argues that increased 5-HT availability, which is found in 5-HTTKO mice, will enhance emotional learning.

4.2. Introduction

The neuroplasticity theory of 5-HT function argues that higher 5-HT levels produce increased responsiveness to positive and negative environmental experiences (Belsky et al., 2009; Branchi, 2011; Homberg & van den Hove, 2012). In addition to epidemiological and longitudinal data used to support this theory (see Chapter 1, section 1.3), researchers have also conducted laboratory experiments to investigate whether higher extracellular 5-HT levels can lead to increased adaptive behaviour in response to environmental changes.

This is particularly the case with studies testing low and high 5-HTT-expressing individuals in adverse learning situations by subjecting them to Pavlovian fear conditioning. First, low 5-HTT-expressing individuals (e.g. *s* allele carriers) are assumed to have increased extracellular 5-HT levels compared to individuals with higher 5-HTT expression levels (e.g. homozygotes for the *l* allele; Lesch et al., 1996; Homberg & van den Hove, 2012; see Chapter 1, section 1.2). Secondly, Pavlovian fear conditioning is a form of learning known to be modulated by serotonin (Homberg, 2012; Bauer, 2015). For example, tryptophan depletion in healthy participants can impair Pavlovian fear learning, which is accompanied by reduced amygdala processing and reduced autonomic responses to a cue predicting an aversive outcome (i.e. an aversive cue, or CS+; Attar et al., 2012). Therefore, testing individuals with (putative) different 5-HTT expression levels on a task dependent on serotonergic modulation can work as an approach to test if higher extracellular 5-HT levels can lead to increased behaviour such as enhanced aversive learning.

Subjects	Task/process	Effect/ results	Authors/ year
Healthy participants	Habituation → discriminative fear conditioning [6×CS+/6×CS-] US = electric shock measurements: SCRs	↑ discrimination is correlated with lower 5-HTT availability in the amygdala	Åhs et al., 2015
5-HTTLPR *s*/*s* or *s*/*l* individuals	Habituation → discriminative fear conditioning [8×CS+/8×CS-] → extinction [8×CS+/8×CS-] US = electric shocks measurements: SCRs	↑ discrimination acquisition ↑ expression (n.s.)	Garpenstrand, et al., 2001
	Discriminative fear conditioning [16×CS+/16×CS-] → reminder → 10mins/6h → extinction [8×CS+/8×CS-] → reacquisition [4×CS+/4×CS-] US = electric shock measurements: SCRs	– discrimination acquisition ↓ extinction ↑ reacquisition within the group subjected to extinction outside the reconsolidation period (the 6h group)	Agren et al., 2012
	Discriminative fear conditioning [9×CS+/9×-] + discriminative fear-potentiated startle → extinction [18×CS+/18×CS-] CS+ → electric stimulation measurements: startle blink Rs and SCRs	↑ acquisition of potentiated startle R to the CS+ ↑ acquisition of CS+/CS- startle potentiation discrimination (comparing within-group analyses; no sig. genotypic effects on SCRs) – fear extinction	Lonsdorf et al., 2009
	Observational fear conditioning (5×CSa/5×CS-) → test US = electric stimulation measurements: SCRs	↑ SCRs to CS+ and US during learning (n.s.) ↑ SCRs to CS+ during test ↑ cue discrimination (statistics NA)	Crişan et al., 2009

Table 4.1. Selection of studies investigating the effects of different 5-HTT expression levels on fear learning with human participants. ↑, enhanced; ↓, diminished; –, no changes; SCRs, skin conductance responses; Rs, responses; US, unconditioned stimulus; n.s., a statistically non-significant result; →, followed by;

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	Discriminative fear-potentiated startle CS+ → electric shock threat measurements: startle blink Rs	↑ CS+/CS- startle potentiation discrimination – extinction of fear after the offset of CS+	Klumpers et al., 2012
	Discriminative fear conditioning [20×CS+/20×CS-] US = aversive pictures measurements: SCRs	– cue discrimination acquisition	Klucken et al., 2013a
	Discriminative emotional startle paradigm Acoustic startle probes are administered alone or during viewing of emotional pictures (e.g. positive, negative or neutral) aversive US = unpleasant pictures measurements: startle blink Rs	– fear/unpleasant potentiation startle	Brocke et al., 2006; Armbruster et al., 2009; Larson et al., 2010; Pauli et al., 2010;

Table 4.1. Selection of studies investigating the effects of different 5-HTT expression levels on fear learning with human participants. ↑, enhanced; –, no changes; SCRs, skin conductance responses; Rs, responses; US, unconditioned stimulus; n.s., a statistically non-significant result; →, followed by;

Individuals with putatively lower 5-HTT expression exhibit enhanced conditioned fear learning (see Table 4.1). This has been shown using discriminative fear conditioning procedures (e.g. Åhs et al., 2015; Garpenstrand et al., 2001), and procedures measuring conditioned fear with fear-potentiated startle (e.g. Lonsdorf et al., 2009; Klumpers et al., 2012). In studies using either type of procedure, low 5-HTT-expressing participants showed better discrimination between an aversive (CS+) and a non-aversive cue (CS–), which predicted the absence of the aversive unconditioned stimulus (US; e.g. electric shock). In discriminative fear conditioning procedures, the CS– controls for reactivity differences that are not related to fear conditioning (Åhs et al., 2015), although some researchers argue that it can also function as a safety signal (e.g. Lissek et al., 2005). Furthermore, compared to homozygotes for the *l* allele, *s* allele carriers exhibit enhanced observational fear conditioning (Crisan et al., 2009), a form of learning that occurs through indirect, social means (e.g. mere observation of an unfamiliar individual being fear conditioned).

Despite the above-mentioned evidence, not all studies report results consistent with the predictions of the neuroplasticity theory of 5-HT function (e.g. Klucken et al., 2013a; see Table 4.1), though it is possible that methodological differences could explain some of these inconsistencies. For instance, the use of unpleasant/aversive pictures as the aversive US instead of electric shocks/stimulation may be a factor (e.g. Brocke et al., 2006; Armbruster et al., 2009; Larson et al., 2010; Pauli et al., 2010). These different types of US can certainly lead to different anxiety states (Lissek et al., 2007), and it is thus possible that relatively less threatening procedures such as those using unpleasant pictures might not be as sensitive as those using physical discomfort/pain to detect the effects of the 5-HTTLPR polymorphism on conditioned fear. Additionally, one study using a discriminative fear conditioning procedure claimed that *s* allele carriers exhibited enhanced fear reacquisition, but no altered acquisition (Agren et al., 2012). Notably, however, low 5-HTT-expressing individuals exhibited better acquisition of the CS+/CS– discrimination within the subset of participants where enhanced fear reacquisition was later observed (Agren et al., 2012; supplementary tables 3 and 4; statistics not provided).

Subjects	Task/process	Effect/ results	Authors/ year
5-HTTKO mice	Cued fear conditioning [3×CS+] → extinction training [40×CS+] → extinction recall [40×CS+]	– Rate of fear acquisition – Fear expression – Extinction learning ↓ extinction recall	Wellman et al., 2007
	Habituation [5×CS-] → cued fear conditioning [2×(3×CS+)] → extinction training [6×(4×CS- and 4×CS+)] → extinction recall [4×CS- and 4×CS+]	Rate of fear acquisition: NA – Fear expression – Extinction learning (– CS- Δfreezing) ↓ extinction recall	Narayanan et al., 2011
	Single-cue fear conditioning [6 × 30s CS+] → FMR session [1 × 1min CS+]	– Rate of fear acquisition ↑ Fear expression	Pang et al., 2011
5-HTTKO mice (129S6/SvEv background)	Cued-fear conditioning [2days×(3×CS+)] → FMR [3×CS+]	Rate of fear acquisition: NA ↓ freezing to context – Fear expression	Muller et al., 2011
5-HTTKO rats	Cued-fear conditioning [4×CS+] → extinction 1 [10×CS+] → extinction 2 [10×CS+]	Rate of fear acquisition: NA Fear expression: NA ↓ fear extinction	Nonkes, et al., 2012
	Cued-fear conditioning [5×CS+] → extinction [24×CS+]	Rate of fear acquisition: NA – Fear expression ↓ fear extinction	Shan et al., 2014
5-HTTOE mice	▪ Single-cue fear conditioning [2×CS+] → FMR session [2×CS+] ▪ Pre-exposure → Discriminative fear learning conditioning [3days×(5CS+/5CS-)] → FMR session [5CS+/5CS-]	▪ Rate of fear acquisition: NA ↓ fear expression ▪ ↓ rate of fear discrimination learning ~ Fear expression	Barkus et al., 2014
	Cued-fear conditioning [2×CS+] → a cue [2×CS+] and context testing session (test order counterbalanced)	Rate of fear acquisition: NA ↓ fear expression ↓ freezing to context	Line et al., 2014
	Pre-exposure → Discriminative fear learning conditioning [3days×(5CS+/5CSa/5CS-)] → FMR session [5CS+/5CSa/5CS-]	↓ freezing to the CSa (learning & FMR) ↓ freezing to context	McHugh et al., 2015

Table 4.2. Selection of studies investigating the effects of different 5-HTT expression levels on fear learning with 5-HTTKO rodents. ↑, enhanced; ↓, diminished; –, no changes; ~, similar; NA, not available; CS+, cue paired with a footshock; CS–, cue predicting the absence of footshock delivery; CSa, cue predicting a footshock in 20% of trials (i.e. an ambiguous cue); FMR, fear memory recall; →, followed by;

Additionally, as it is possible to see in table 4.1, only one study actually measured the 5-HTT availability of the participants subjected to fear conditioning (Åhs et al., 2015). As previously discussed (Chapter 1, section 1.2), there is controversy about the lower transcriptional potential of the 5-HTT gene in *s* allele carriers compared to homozygotes for the *l* allele. Moreover, human (and monkey) collected in studies using various different indirect indices (e.g. measurements of 5-HT metabolites in the cerebrospinal fluid) suggesting that the occurrence of increased brain 5-HT function in putatively low 5-HTT-expressing individuals is also controversial (Heils et al., 1996; Lesch et al., 1996; Hanna et al., 1998; Greenberg et al., 1999; Whale et al., 2000; Reist et al., 2001; Williams et al., 2001; Bennett et al., 2002; Barton et al., 2008).

One way to clarify whether higher 5-HT availability can affect aversive learning is to test 5-HTTKO rodents, which reliably have significantly higher extracellular 5-HT levels than their WT counterparts (Fabre et al., 2000; Mathews et al., 2004; Shen et al., 2004; Homberg et al., 2007a; Olivier et al., 2008b; Jennings et al., 2010). Accordingly, several studies have investigated whether aversive learning is altered in these animals (see Table 4.2), but the results obtained so far have not been entirely consistent with the human data. Although one study has reported increased fear expression with 5-HTTKO mice (Pang et al., 2011), several other studies have not reported any enhancement of fear learning in 5-HTTKO rodents compared with WT controls. That is, in contrast to what studies with humans suggest, higher extracellular 5-HT availability has not generally been associated with altered fear conditioning in rodent studies. Indeed, the studies testing 5-HTTKO mice that do provide data for the conditioning (i.e. acquisition) phase report no significant differences regarding fear acquisition compared with the WTs (Wellman et al., 2007; Pang et al., 2011). Additionally, no alterations regarding extinction learning were registered in 5-HTTKO mice, although 5-HTTKO rats do exhibit impaired extinction learning (Nonkes et al., 2012; Shan et al., 2014). Although conflicting results exist regarding changes in fear expression, two

studies reported an impairment in fear extinction recall in 5-HTTKO mice (Wellman et al., 2007; Narayanan et al., 2011). This has been interpreted by some researchers as reflecting increased associative learning despite an absence of genotypic differences during acquisition (e.g. Kalueff et al., 2010).

Given that so far alterations in aversive learning have yet to be seen unequivocally in 5-HTTKO rodents, clear support for the prediction of the neuroplasticity theory of 5-HT function that higher 5-HT availability facilitates learning of aversive experiences is lacking. This chapter thus aimed to further test this prediction by subjecting 5-HTTKO mice to a discriminative fear conditioning procedure including both CS+ and CS– cues. This is the procedure that is typically used in studies with humans investigating the effects of different 5-HTT expression levels on fear learning (see Table 4.1), but rarely used with rodent models investigating the same effects (which tend to only include the CS+ cue).

In Experiment 1 of this chapter two cues were presented: a fully predictive aversive cue (CS+ = 100% CS-US contingency) and a fully predictive non-aversive cue (CS– = no US). Experiment 1 constitutes an important contribution to studies investigating conditioned fear with 5-HTTKO rodents given that only 'single-cue' fear conditioning procedures were used until now. Considering how fast these single cue procedures can produce a strong fear response (freezing behaviour; Fanselow & Ponnusamy, 2008), it can be argued that there is a reduced chance of detecting genotypic differences due to possible ceiling effects, particularly if the shock intensity is high (Jaffard et al., 2001). Furthermore, forcing a subject to pay attention to the specific sensory features of the cues used in a discriminative aversive conditioning procedure might increase the sensitivity of the learning task, and thus the chance to detect genotypic differences.

In addition, this chapter also aimed to investigate if altered fear learning would occur when the aversive cue was only partially reinforced. The reason for using such a cue was based on a previous study conducted in the laboratory showing that aversive conditioning to

an ambiguous cue was more sensitive to variation in 5-HTT expression than conditioning to the fully predictive aversive cue. Specifically, 5-HTTOE mice showed a selective reduction in fear to an ambiguous aversive cue (CSa) predicting footshock on only 20% of trials in a procedure where subjects were required to discriminate between that CSa, a fully predictive CS+ and a fully predictive CS− (McHugh et al., 2015). WT mice feared the CSa in a similar way as the CS+ while exhibiting minimal fear to the CS− cue. 5-HTTOE mice showed high fear only to the CS+, and minimal fear to both the CS− and CSa. Therefore, it was hypothesised that 5-HTTKO mice could differ to a greater extent from their WT controls when the discriminative fear conditioning procedure used a CSa cue instead of a CS+. Specifically, it was expected that increased 5-HT availability would make the KOs more sensitive to the few pairings of an aversive US with a discrete cue than the WTs, which could affect discrimination learning in Experiment 2.

Accordingly, in Experiment 2, näive 5-HTTKO mice and WT controls were subjected to the same procedure used in Experiment 1 but the CS+ was replaced with a CSa that predicted footshock on 20% of the trials. In a task using such a partially reinforced aversive cue, cue discrimination should still be possible if the probability of the US presentation following the CSa is always greater than the non-reinforced CS, and this is perceived by the subject (Zielinski, 1979).

Additionally, other forms of associative learning were also tested with 5-HTTKO mice. Fear extinction learning was further investigated after the fear memory recall session in both Experiments 1 and 2 of this chapter. Although previous research has suggested that 5-HTTKO mice do not show altered extinction learning, it is important to verify this claim with a discriminative procedure, particularly because differences have been reported in 5-HTTKO rats in this type of learning. Different rates of extinction learning were potentially expected for this phase in experiments 1 and 2 considering the partial reinforcement

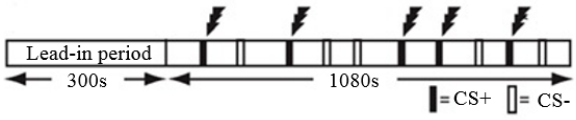
extinction effect (Wagner et al., 1967), which predicts that subjects in Experiment 2 would likely show more resistance to extinction than those used in Experiment 1. Furthermore, contextual fear learning was also investigated.

4.3. Experiment 1: Processing of predictable aversive cues

4.3.1. Methods

Nineteen 5-HTTKO mice (female: n=9) and 19 WT controls (female: n=9) were used. Mice were between 4 and 10 months old at the start of the experiment, with 5-HTTKO mice being slightly older than WT mice (6.5 and 5.2 months old on average, respectively). Subjects were group housed with littermates in a temperature-controlled colony room on a 12/12h light/dark cycle (lights on at 7 am), with food and water always available.

Mice were subjected to virtually the same routine and session parameters on each day of testing. At the start of each session, two mice were placed in individual cages and taken to a quiet and dimly-lit experimental room (~100 lux). After 10 minutes of acclimatisation, they were placed in the conditioning chambers in the experimental room. The two mice were tested simultaneously under the same session settings (i.e. both mice would have the same allocation of the auditory cues to the CSs, and thus would be shocked at the same time).



Testing day	1	2	3	4	5
Session	C1	C2	FMR	E1	E2
Phase	Conditioning		FMR/Extinction		
Context	A		B		

Figure 4.1. Left, A diagram of a conditioning session used in Experiment 1. The black lightning marks indicate the 0.3mA footshocks. No shocks were given during the fear memory recall (FMR) or extinction sessions. Adapted from McHugh et al. (2014), under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014. **Right,** 5-day procedure used in Experiment 1. The 2-day conditioning phase consisted of two conditioning sessions (C1 and C2). This was followed by a 3-day FMR/Extinction phase. Technically, FMR, E1 and E2 were all extinction sessions. These were administered in a context different from that used during the conditioning phase (e.g. context B, if conditioned in context A).

Mice were then subjected to a discriminative fear conditioning procedure, in which two auditory cues were pseudo-randomly presented, one of them co-terminating with a 0.5s mild (0.3 mA) footshock (CS+), but the other one not followed by footshock (CS–) (see Figure 4.1, left). The cues consisted of two 30s-long, 72 dB auditory cues: a 2900 Hz continuous tone and a white noise. The allocation of each type of auditory cue to the CSs was counterbalanced across subjects, as well as with two distinct orders of presentation of the cues. The CS presentations were interspersed with a mean inter-cue interval of 80s (ranging from 60 to 100s). In addition, every session started with a 300s lead-in period prior to the first cue presentation, and ended with a 60s lead-out period after the last cue presentation, thus lasting 1980s in total.

After a 2-day conditioning phase (conditioning sessions 1 and 2, or C1 and C2, respectively), mice underwent three extinction sessions (see Figure 4.1, right). In this thesis the first of these sessions is called the fear memory recall (FMR) testing session, whereas the following two are called extinction sessions 1 and 2 (E1 and E2, respectively).

Information about the apparatus, the different contexts used, and on how freezing behaviour was measured, can be found in Chapter 2 (section 2.3.4).

ANOVAs using a general linear model (GLM) were performed on the data, which were followed by investigation of simple main effects using pairwise comparisons with one-way ANOVAs (see Chapter 2, section 2.6). Student's *t*-tests were used for the analysis of the first cue discrimination of the FMR session (i.e. the first trial, or T1). The one sample *t*-tests used for the discrimination index analysis were rejecting H_0: $\mu = 0$. Prior to the data analyses performed on the conditioning and extinction data, analyses were also conducted on the raw pre-CS freezing responses across the five days. The results of these data analyses can be found in Appendix 3. Figures showing raw freezing during 30s-long CS periods and freezing levels during 30s-long pre-CS periods across all trials of Experiment 1 can be found in Appendix 4. Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

4.3.2. Results

5-HTTKO mice exhibited facilitated discriminative aversive learning

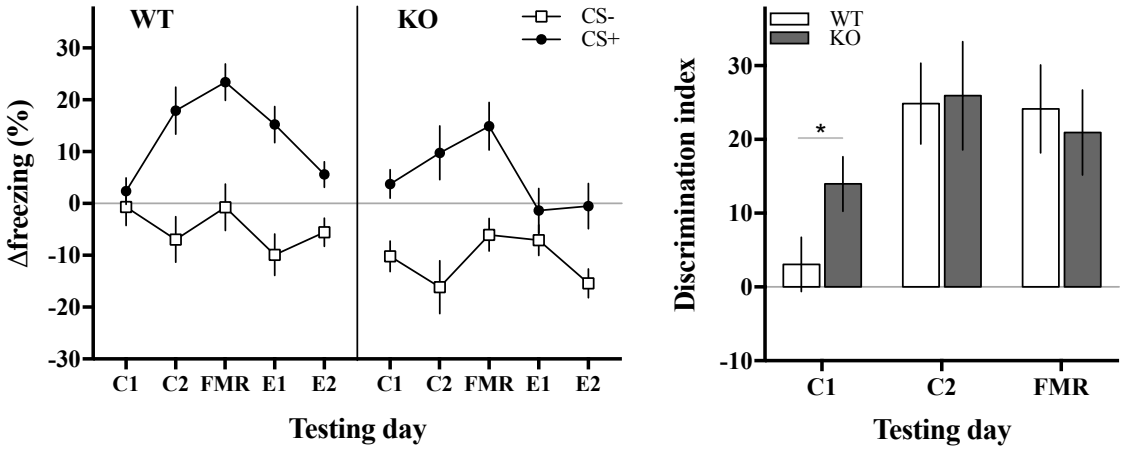


Figure 4.2. Left, Cue-evoked Δfreezing on the conditioning (C1, C2) and extinction sessions (FMR, E1 and E2) for the 5-HTTKO (N=17) and WT (N=18) mice used in Experiment 1. Both groups learned to significantly discriminate between the CS+ and the CS- cues, with the former evoking an increase in freezing compared to the pre-CS period, and the latter either a decrease in freezing or no change at all. **Right,** Discrimination index (CS+ Δfreezing – CS- Δfreezing) during the conditioning phase (C1, C2) and the FMR session for the same mice. Discriminative aversive learning was faster in the 5-HTTKO mice than WT mice. Mean ± SEM. **p* < .05 (simple effect of genotype on C1).

Both groups learned to discriminate between the two cues (main effect of CS across the 5 sessions: $F(1,33) = 43.2$, $p < .001$), with the CS+ evoking more Δfreezing than the CS–, particularly during the conditioning phase and the FMR session. However, the groups differed significantly in how they responded to the two cues over the 5-day procedure ($Genotype_2 \times Day_5 \times CS_2$ interaction: $F(4,132) = 3.9$, $p = .005$; see Figure 4.2, left). Notably, 5-HTTKO mice showed significantly stronger fear discrimination on C1 compared to WT mice (simple effect of genotype on C1: $p = .045$; see Figure 4.2, right). Afterwards, groups showed similar discrimination levels on C2 ($p = .907$) and FMR ($p = .702$).

Enhanced fear discrimination in 5-HTTKO mice on C1 was driven by differences in CS– rather than CS+ responses

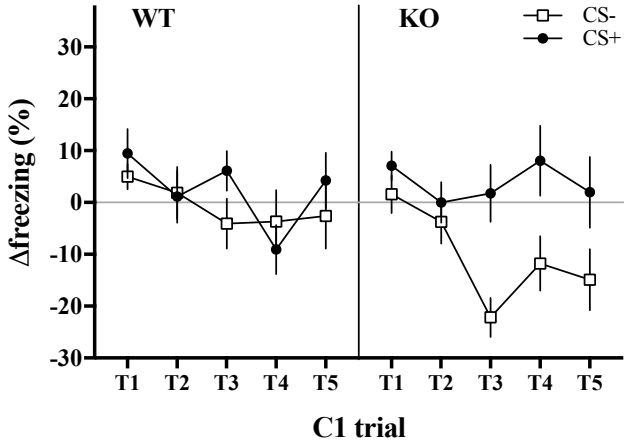


Figure 4.3. Cue-evoked Δfreezing for each successive pair of CS+ and CS– presentations (i.e. for each trial) during C1 obtained with the 5-HTTKO (N=17) and WT (N=18) mice used in Experiment 1. Only the 5-HTTKO mice learned to discriminate between the two cues during C1, which occurred in a statistically significant way from the third trial onwards (simple effect of cue within T3: p = .001; T4: p = .029; T5: p = .014; all corresponding p values for the WTs were statistically non-significant). The enhanced discrimination was mostly driven by a decrease in freezing during the CS– presentations compared to the pre-CS periods. Mean ± SEM.

To investigate whether the above-mentioned difference in aversive discrimination learning seen on C1 developed throughout conditioning training, data analyses were conducted on a trial by trial basis. That is, comparisons were made between the pairs of CS+ and CS– cues in the same order of presentation (e.g. the second CS+ of the session was compared to the second CS– to be presented). Such pairing reflects the structure of the session, given that every two CSs were either an aversive cue or a control cue due to the rules for the sequences of CS presentations used (see Chapter 2, section 2.3.4).

As it is possible to see in Figure 4.3, both groups exhibited a similar absence of cue discrimination at beginning of C1, that is, during trials 1 and 2 (T1-T2). During the third and last two trials of the session (T3-T5), however, 5-HTTKO mice exhibited a strong cue discrimination, whereas WT mice did not. Specifically, 5-HTTKO mice clearly showed

reduced freezing (i.e. more movement compared with a pre-CS baseline period) during the CS– presentations, something that WT mice did not show at all in this session. Notably, statistical analyses of the cue-evoked Δfreezing across the five trials (T1-T5; $Genotype_2 \times CS_2$ interaction: $F(1,33) = 4.3$, $p = .045$) revealed that the greatest genotypic difference was seen in the responses to the CS– (simple effect of genotype within CS–: $p = .050$), rather than the CS+ ($p = .713$). In short, the enhanced cue discrimination learning did develop throughout the session and was mainly driven by differences regarding the CS– Δfreezing responses.

5-HTTKO mice exhibited facilitated fear extinction learning

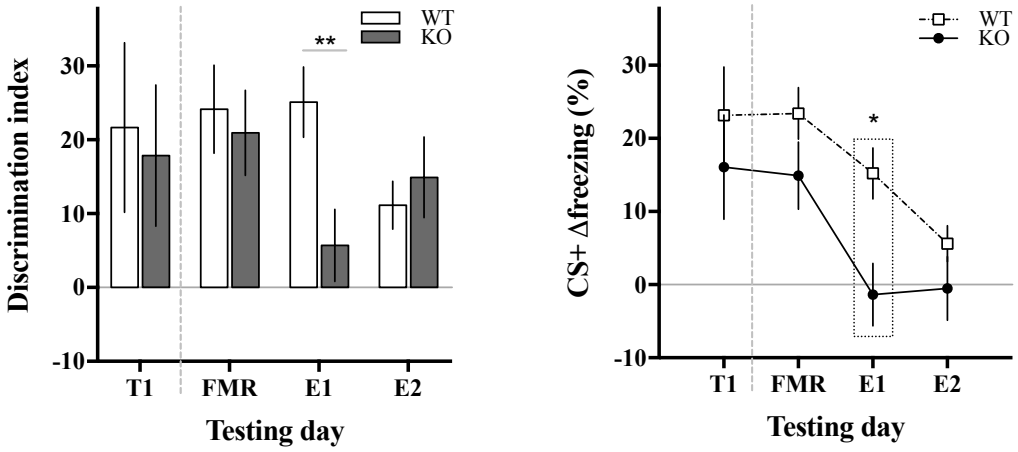


Figure 4.4. Left, Discrimination index (CS+ Δfreezing – CS– Δfreezing) during the extinction sessions (FMR, E1 and E2) for the 5-HTTKO (N=17) and WT (N=18) mice used in Experiment 1. T1 is the first pair of CS+ and CS– presentations (i.e. trial 1) of the FMR session. **Right**, CS+ Δfreezing during T1, FMR, E1 and E2 sessions. These results indicate that 5-HTTKO mice seem to learn faster than WT mice that the CS+ is no longer followed by footshock, which contributed to a decrease in the discrimination level seen from FMR to E1. Mean ± SEM. *$p < .05$, **$p < .01$ (simple effect of genotype on E1).

5-HTTKO mice were faster to learn to discriminate between the CS+ and the CS– during conditioning but the discrimination levels and CS+ (evoked) Δfreezing did not significantly differ between groups during the FMR session. There were no significant genotypic differences during the first trial (T1) of the FMR session (i.e. by the end of the

conditioning sessions; discrimination index: $t(33) = 0.3$, $p = .801$; CS+ Δfreezing: $t(33) = 0.7$, $p = .472$; see Figure 4.4).

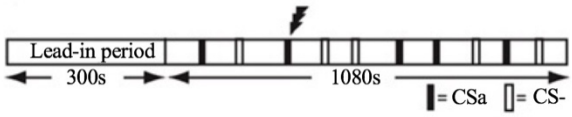
Although equivalent discrimination levels were seen in WT and 5-HTTKO mice during FMR, 5-HTTKO mice exhibited significantly less discrimination than WTs during E1 ($Genotype_2 \times Day_3$ interaction: $F(2,66) = 5.0$, $p = .010$; simple effect of genotype on E1: $p = .007$; see Figure 4.4 left). These results suggest enhanced extinction learning in 5-HTTKO mice, who exhibited a greater reduction in cue discrimination after two extinction sessions (FMR and E1) compared to WT controls.

Underlying this difference in the rate of extinction learning were differences in the way groups changed their responses to the CS+ from FMR to E1. This is particularly clear in the significantly lower CS+ Δfreezing exhibited by the 5-HTTKO mice during E1 compared to WT mice (simple effect of genotype on E1: $p = .005$; see Figure 4.4., right).

4.4. Experiment 2: Processing of ambiguous aversive cues

4.4.1. Methods

Twenty-three 5-HTTKO mice (female: n=12) and 23 WT mice (female: n=5) were used in Experiment 2. Subjects were aged 1.7 to 7 months old (5-HTTKO mean = 3.4; WT mean = 4.2 months old). Mice were experimentally naïve. They were kept in the same colony room as those used in Experiment 1, and were subjected to the same handling and acclimatisation procedures as mentioned above.



Testing day	1	2	3	4	5
Session	C1	C2	FMR	E1	E2
Phase	Conditioning		FMR/Extinction		
Context	A		B		

Figure 4.5. Left, A diagram of a conditioning session used in Experiment 2. The black lightning mark indicates the 0.3mA footshocks administered in 20% of the ambiguous cue (CSa) trials. No shocks were given during the fear memory recall (FMR) or extinction sessions. Adapted from McHugh et al. (2014). Reprinted under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014. **Right,** 5-day procedure used in Experiment 2. The 2-day conditioning phase consisted of two conditioning sessions (C1 and C2). This was followed by a 3-day FMR/Extinction phase. Technically, FMR, E1 and E2 were all extinction sessions. These were administered in a context different from that used during the conditioning phase (e.g. context B, if conditioned in context A).

Mice were subjected to the exact same procedure, session settings and parameters as those used in Experiment 1, but now the CS+ was replaced with a cue that only predicted footshock on 20% of the trials (see Figure 4.5). Footshock deliveries were paired with the third CSa presentation of C1, and the second one of C2. The CS– was never paired with footshock in either experiment.

The same measures and data analyses used in Experiment 1 were also obtained and conducted, respectively, in Experiment 2. However, given that subjects did not have many opportunities to learn about the meaning of the CSa during C1, because they were only subjected to one footshock delivery for five cue presentations, data from this session were shown in the graphs but were excluded from the statistical analyses. Prior to the data analyses performed on the conditioning and extinction data, analyses were also conducted on the raw pre-CS freezing responses across the four days. The results of these data analyses can be found in Appendix 3. Figures showing raw freezing during CS periods and freezing levels during pre-CS periods across all trials of Experiment 2 can be found in Appendix 5.

Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

4.4.2. Results

Only 5-HTTKO mice maintained cue discrimination from C2 to FMR

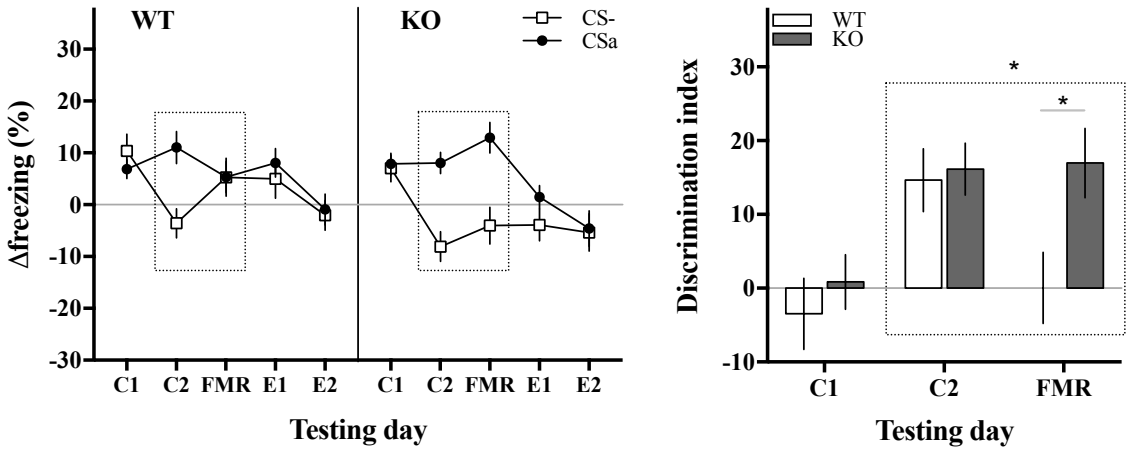


Figure 4.6. Left, Cue-evoked Δfreezing on the conditioning (C1, C2), FMR and extinction sessions (FMR, E1 and E2) for the 5-HTTKO (N=23) and WT (N=23) mice used in Experiment 2. **Right,** Discrimination index (CSa Δfreezing – CS– Δfreezing) during the conditioning phase (C1, C2) and the FMR session for the same mice. Both groups learned to significantly discriminate between the CSa and the CS– cues by C2, but only 5HTTKOs exhibited cue discrimination during FMR. Mean ± SEM. *$p < .05$ (Data analyses over C2 and FMR revealed a significant interaction between genotype and session. Although the cue discrimination levels were similar on C2, a significant simple effect of genotype was seen on FMR).

After only one pairing of the CSa with a footshock (i.e. during C1) neither genotype could discriminate this cue from the CS– (see Figure 4.6, left and right). Afterwards, both groups exhibited equivalent significant cue discrimination levels by C2 but only the 5-HTTKO mice kept a similar level of discrimination from this session to the FMR session (discrimination index: $Genotype_2 \times Day_2$ interaction: $F(1,44) = 4.5$, $p = .039$). This led to a strong, significant genotypic difference being seen during the FMR session (simple effect of genotype on FMR: $F(1,44) = 6.3$, $p = .016$; see Figure 4.6, right). In this FMR session, the

CSa and CS– cues evoked equivalent levels of Δfreezing in WT mice but strong discrimination in the KOs (simple effect of CS within levels of genotype during FMR: WTs: $p = .995$; KOs: $p = .001$; see Figure 4.6, left). The CS+/CS– discrimination of WTs during FMR differed from what they had shown on the previous session (simple effect of CS within levels of genotype during C2: WTs: $p = .001$; KOs: $p < .001$). The more noticeable change underlying the WTs response was the CS– Δfreezing, which increased significantly from C2 to FMR (simple effect of day within CS–: $p = .028$; CS+: $p = .141$).

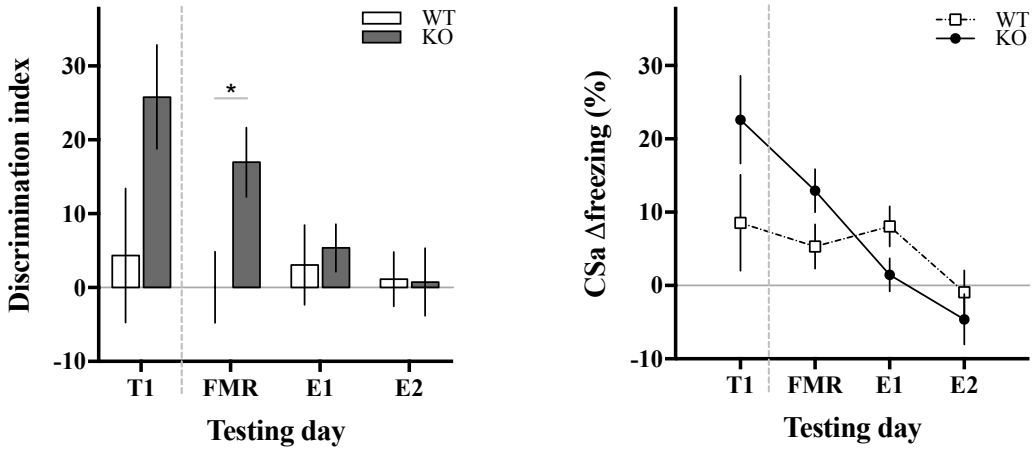


Figure 4.7. Left, Discrimination index (CSa Δfreezing – CS- Δfreezing) during the extinction sessions (FMR, E1 and E2) for the 5-HTTKO (N=23) and WT (N=23) mice used in the Experiment 1. T1 is the first pair of CSa and CS- presentations (i.e. trial 1) of the FMR session. The lack of discrimination exhibited by the WT mice on FMR was not due to faster extinction learning. **Right**, CSa Δfreezing during T1, FMR, E1 and E2 sessions. 5-HTTKO mice exhibit extinction learning regarding their response to the CSa. Mean ± SEM. *$p < .05$ (simple effect of genotype on FMR).

The result exhibited by WT mice on FMR was not due to faster extinction learning, because WTs showed no significant cue discrimination even during the first trial of FMR (one-sample *t*-test for the discrimination index versus chance exhibited during T1: WTs: $t(22) = .006$, $p = .995$; KOs: $t(22) = 3.6$, $p = .002$; see Figure 4.7, left). Due to the absence of cue discrimination in the WTs during FMR, it was not possible to investigate the effect of 5-HTT knock-out during the extinction sessions.

However, it is worth noting that 5-HTTKO mice exhibited extinction learning from FMR to E2, as it is possible to see in the reduction of cue discrimination levels over the 3-day FMR/Extinction phase (repeated measures ANOVA: $F(2,44) = 5.6$, $p = .007$). Analysis of the Δfreezing evoked by the CSa cue showed that 5-HTTKO mice did reduce their response to the ambiguous aversive cue in a significant way during this 3-day period (repeated measures ANOVA: $F(2,44) = 11.0$, $p < .001$; see Figure 4.7, right), particularly from FMR to E1 ($p = .008$).

4.5. Unconditioned response and reactivity to footshocks

To investigate whether the effects found in Experiments 1 and 2 could result from altered footshock sensitivity in the 5-HTTKO mice, compared to WT mice, a footshock reactivity test was administered in naïve mice. This analysis was complemented with further analyses of data obtained from 5-HTTKO mice and WT controls subjected to cued fear conditioning in this thesis. Specifically, it was investigated if genotypic differences could be seen regarding (i) the unconditioned response (UR) to the very first footshock presentation, and (ii) the reactivity to footshock when more shocks were delivered during C1.

4.5.1. Methods

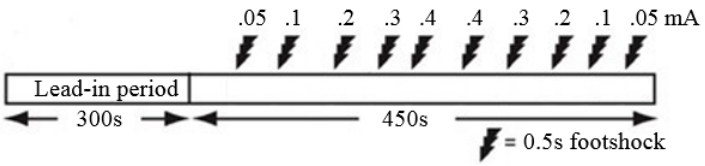


Figure 4.8. Diagram of the experimental procedure used for the footshock reactivity test. The black lightning marks indicate the 10 footshocks administered during the test. The respective intensity of the shock (in mA) is indicated above each mark. The order of presentation of the different footshock intensities followed the one indicated in this diagram.

The footshock reactivity test was performed on 5 KOs (female: n=3) and 10 WTs (female: n=5), approximately matched for age (3.4 and 3.9 months old on average, respectively, with ages ranging between 2 and 5 months). The test was administered in the same apparatus used for experiments in this chapter. Ten footshocks were given, with the first five trials administered in an ascending order of intensity, and the last five in a descending order (see Figure 4.8). All footshocks lasted 0.5s, and the intensities used were 0.05, 0.1, 0.2, 0.3 and 0.4 mA. The 810-second long session started with a 300s lead-in period prior to the first footshock delivery, and ended with a 60s lead-out period after the last footshock delivery. A mean inter-shock interval of 50s was used (range 30-70s).

Additional analyses investigating genotypic differences on UR to the footshock and footshock reactivity were conducted on C1 data collected from all 5-HTTKO mice and WT controls subjected to cued fear conditioning in Chapters 4 and 5 of this thesis, plus two additional cohorts of mice used for pilot studies with similar procedures. These two additional cohorts of mice were subjected to cued fear conditioning procedures with the same 30s long auditory cues, footshock intensity (0.3 mA), and number of CS presentations per session (5 of each type), all provided in the same apparatus. The additional data analyses thus included data from 96 5-HTTKO mice and 98 WT mice. However, whereas analyses related to the UR to the footshock (i.e. the first footshock of the C1) included all mice tested (n=194), analyses related to reactivity to additional footshocks excluded the subjects used in Experiment 2 of this chapter (n= 194 – 46 = 148). This was done because subjects tested in Experiment 2 only received one footshock during C1. Information regarding sample sizes, sex and age of subjects at the start of testing can be found in Appendix 6.

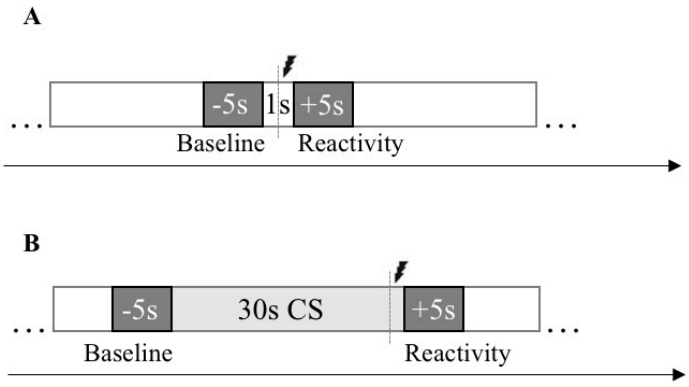


Figure 4.9. Diagrams of the data retrieved from the session used to calculate the activity evoked by a footshock delivery (i.e. Δactivity), for the footshock reactivity test **(A)** and for the cued fear conditioning procedures **(B)**. In the former case, the local baseline period chosen occurred immediately before the 1s-long bin containing the footshock delivery, whereas in the latter case it was retrieved from the inter-cue period immediately before the CS that co-terminated with the footshock delivery.

The percentage of changed pixels in each 1s-long bin processed by the image analysis software to measure freezing behaviour (see Chapter 2, section 2.3.4) was used as an index of activity levels. Reactivity (and UR) to the footshock was then analysed by calculation of the change in activity levels evoked by a footshock delivery (Δactivity). The Δactivity was obtained by subtracting the mean percentage of changed pixels of a 5s-long local baseline period from the mean percentage of changed pixels of a 5s-long post-footshock period (Δactivity (%) = % pixels changed during the 5s-long post-footshock period minus % pixels changed during a 5s-long period before the footshock). However, the local baseline period used differed for the data obtained from the footshock reactivity test and the data obtained from the cued fear conditioning procedures (see Figure 4.9). In the former case, the local baseline period chosen occurred immediately before the 1s-long bin containing the footshock delivery. In the latter case, the local baseline period was retrieved from the inter-cue period immediately before the CS that co-terminated with the footshock delivery. This selection more closely resembles the baseline periods used in the footshock reactivity test, which were not affected by auditory cues.

In the results of the footshock reactivity test, the data are presented as the mean response to the two footshock presentations of each intensity. Additionally, raw activity levels during only a 5s-long post-footshock period were also analysed with the data from the footshock reactivity test (Activity levels = % of changed pixels during each second of 5s-long post-footshock period). ANOVAs were conducted as previously described (see Chapter 2, section 2.6), and a factor of Experiment was also included in all the ANOVAs performed on data obtained across several experiments. Results from data analyses conducted on pre-US activity levels can be found in Appendix 16. Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

4.5.2. Results

No genotypic differences were observed in the footshock reactivity test

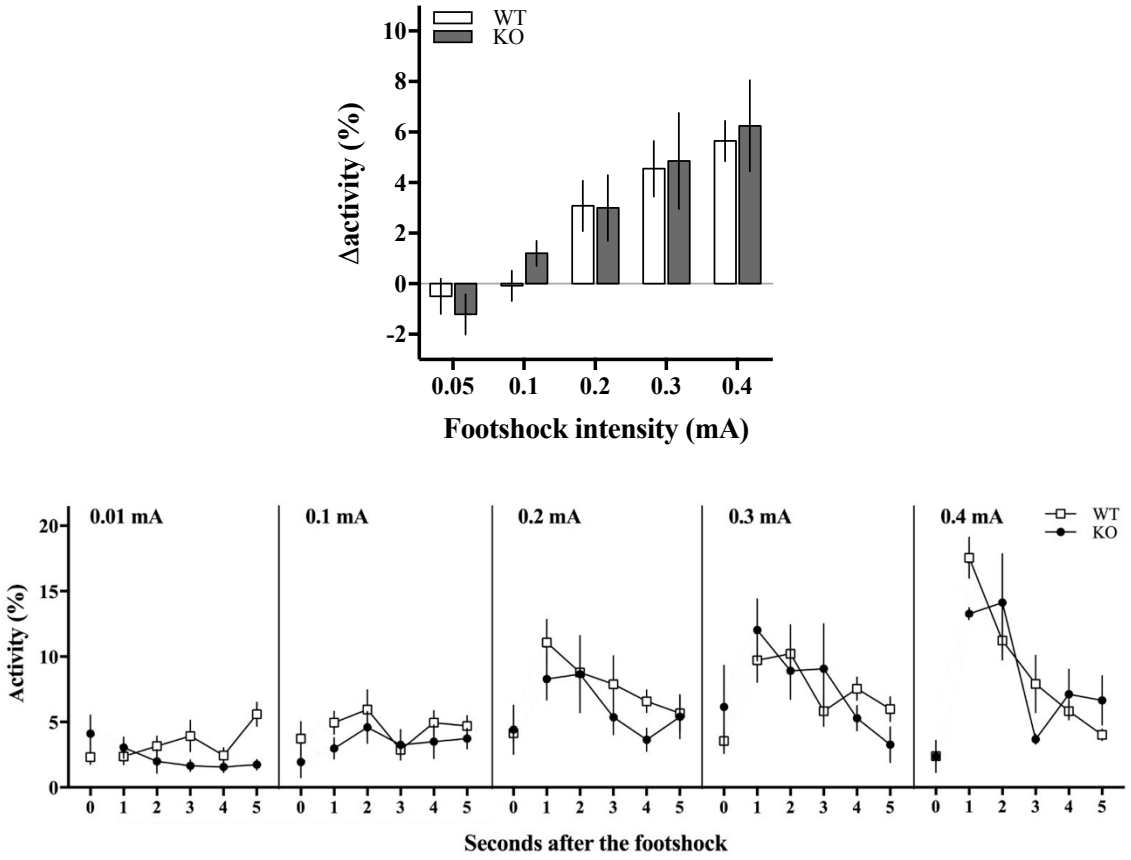


Figure 4.10. Top, Changes in activity levels (Δactivity (%) = % pixels changed during the 5s-long post-footshock period minus % pixels changed during a 5s-long period before the footshock) during the footshock reactivity test for 5-HTTKO (N=5) and WT (N=10) mice. **Bottom,** Patterns of reactivity after footshock deliveries at five different intensities for the same subjects. Activity levels (Activity levels = % of changed pixels during each second of 5s-long post-footshock period). Genotypes did not significantly differ on any of five footshock intensities tested. Mean ± SEM.

No significant genotypic differences were found in the footshock reactivity test. Higher intensities of footshock elicited greater Δactivity in both genotypes (main effect of intensity: $F(4,52) = 22.0$, $p < .001$; see Figure 4.10, top), particularly for shocks above 0.1 mA. No significant genotypic differences were found at any footshock intensity used (main effect of genotype: $F < 1$; $Genotype_2 \times Intensity_5$ interaction: $F < 1$). A significant interaction between genotype, sex and intensity was obtained ($F(4,44) = 3.0$, p = .027. WT males

showed significantly lower reactivity for the first two intensities, whereas male KOs showed significantly lower reactivity for the highest intensity than female KOs.

Data analyses on raw activity levels during the 5s-long post-footshock period point towards similar conclusions. Accordingly, higher activity levels were recorded when greater footshock intensities were delivered (main effect of intensity: $F(4,52) = 24.1$, $p < .001$), but no significant genotypic differences were found (main effect of genotype: $F(1,13) = 1.4$, $p = .251$; $Genotype_2 \times Intensity_5$ interaction: $F < 1$; see Figure 4.10, bottom). A similar pattern of unconditioned response to the footshocks could be seen clearly between groups ($Genotype_2 \times Intensity_5 \times Bin_5$ interaction: $F(16, 208) = 1.6$, $p = .102$).

To summarise, both data analyses indicated that 5-HTTKO mice do not exhibit altered sensory processing of the footshocks compared to their WT controls.

No genotypic differences were found regarding the UR to the first footshock, but 5-HTTKO mice exhibit greater footshock reactivity during fear conditioning

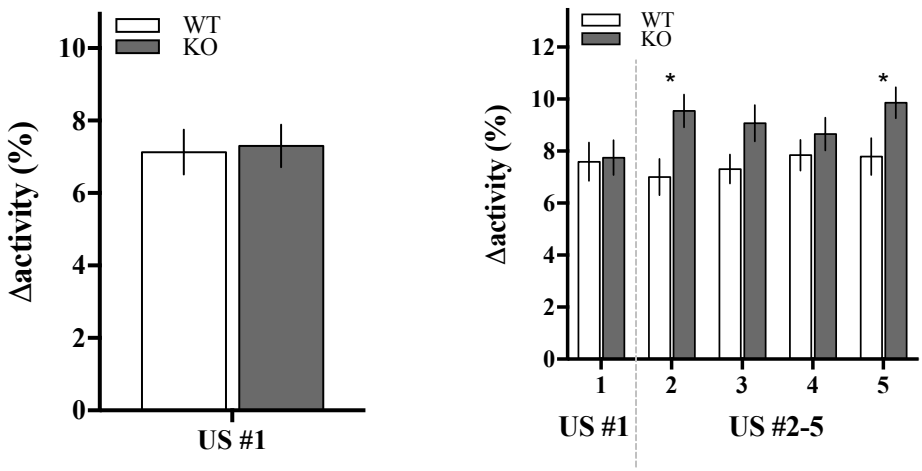


Figure 4.11. Left, Changes in activity levels (Δactivity (%) = % pixels changed during the 5s-long post-US period minus % pixels changed during a 5s-long period before the footshock-predicting CS) for the first footshock delivery (US #1) of C1 administered to 5-HTTKO (N=96) and WT (N=98) mice during fear conditioning. **Right,** Changes in activity levels for the five US deliveries (US #1-5) of C1 administered to 5-HTTKO (N=73) and WT (N=75) mice during fear conditioning. Mean ± SEM. *$p < .05$ (simple effect of genotype within different levels of footshock intensity).

Analyses of the UR to the first footshock were obtained by analysing the Δactivity elicited by the very first footshock ('US #1') delivery during the C1 session administered to 5-HTTKO and WT mice during fear conditioning (see Figure 4.11, left). No overall significant differences were seen between 5-HTTKO and WT mice (main effect of genotype: $F < 1$).

To investigate whether 5-HTTKO mice became more reactive to the US as the session progressed and they had received further footshocks, the analyses conducted with the 'US #1' were extended to the following four footshock deliveries of C1 ('US #2', 'US #3', 'US #4', 'US #5'; see Figure 4.11, right). After a similar UR to the first footshock presentation of C1 (main effect of genotype: $F < 1$), 5-HTTKO mice exhibited greater Δactivity levels than WT mice across the remaining four footshocks (main effect of genotype during US# 2-5: $F(1,140) = 9.4$, $p = .003$). However, this genotypic difference did not develop throughout the session ($Genotype_2 \times Trial_5$ interaction: $F(4, 560) = 1.1$, $p = .348$; main effect of trial US# 1-5: $F(4,560) = 1.4$, $p = .248$), with significant genotypic differences being found already with 'US #2' but not with the 'US #4', for instance.

Additional analyses using a 5s-long local baseline period immediately before the bin containing the footshock delivery revealed a similar pattern of results (main effect of genotype during US#1: $F < 1$; main effect of genotype during US# 2-5: $F(1,140) = 4.8$, $p = .031$; session $Genotype_2 \times Trial_5$ interaction: $F(4, 560) = 1.6$, $p = .163$).

4.6. Contextual fear conditioning

Contextual fear conditioning was investigated by extending the procedure used for the footshock reactivity test with an additional experimental session 24h later (see below). Additionally, three analyses were conducted with data obtained from the footshock reactivity

test and Experiments 1 and 2 of this chapter to investigate possible genotypic differences on freezing to the background context.

4.6.1. Methods

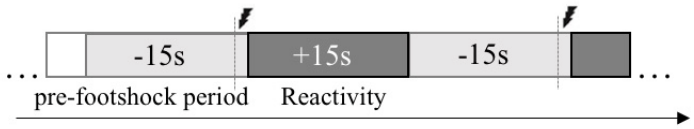


Figure 4.12. To investigate contextual freezing throughout the footshock reactivity test, freezing was measured during the 15s-long periods leading up to, and co-terminating with, each of the 10 footshock deliveries.

The first analysis was conducted on the 15s-long periods leading up to, and co-terminating with, each footshock delivery during the footshock reactivity test (i.e. a 15s-long pre-US period; see Figure, 4.12). This was done to investigate if one group was learning to associate the context and the footshocks faster than the other group. The selection of these data resembles what was done in Experiments 1 and 2, where a CS period included the bin containing the footshock because the reactivity to the shock is only detected in the following bin. Given that the shortest inter-shock duration used in this task was 30s, the duration used for analysis was only 15s to prevent contamination from a previous footshock reactivity period.

The second analysis consisted of investigating contextual fear conditioning in the mice subjected to the footshock reactivity test in extinction. Twenty-four hours after the test was administered, subjects were placed again for 300s in the chamber where they had previously received footshocks. Contextual fear conditioning was investigated by measuring freezing behaviour during that period, and then comparing it to the freezing behaviour measured during the 300s-long lead-in period prior to the first footshock delivery used on the previous day.

Finally, a third analysis was done focusing on the conditioning to the background context during the fear conditioning phase of Experiments 1 and 2. This was done by comparing the freezing behaviour exhibited during the 300s lead-in periods prior to the first CS presentation of the C1 and C2 sessions.

Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

4.6.2. Results

5-HTTKO mice show enhanced association of the context with the footshocks

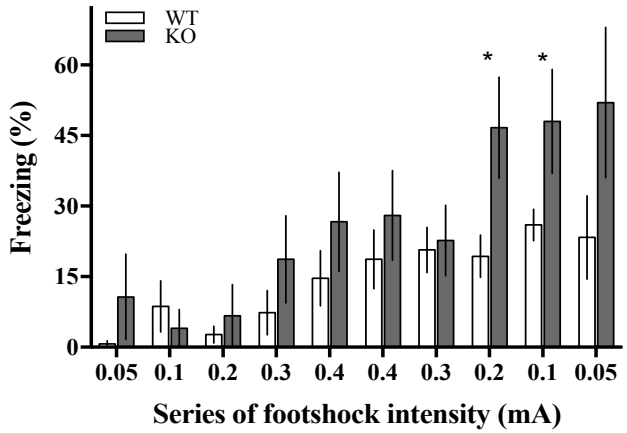


Figure 4.13. Mean levels of freezing behaviour registered during the 15-second long periods of time leading up to a footshock during the footshock reactivity test for 5-HTTKO (N=5) and WT (N=10) mice. Mean ± SEM. *$p < .05$ (simple effect of genotype within different trials).

Looking at (15s long) periods of time just before the delivery of each footshock during the footshock reactivity test, it is possible to see that 5-HTTKO mice were associating the context with the footshocks in a stronger way than WTs ($Genotype_2 \times Trial_{10}$ interaction: $F(9,117) = 2.8$, $p = .033$; see Figure 4.13). This was evident towards the end of the session, when they were freezing much more to the context, particularly in the absence of very noticeable footshocks.

5-HTTKO mice exhibited stronger contextual fear conditioning

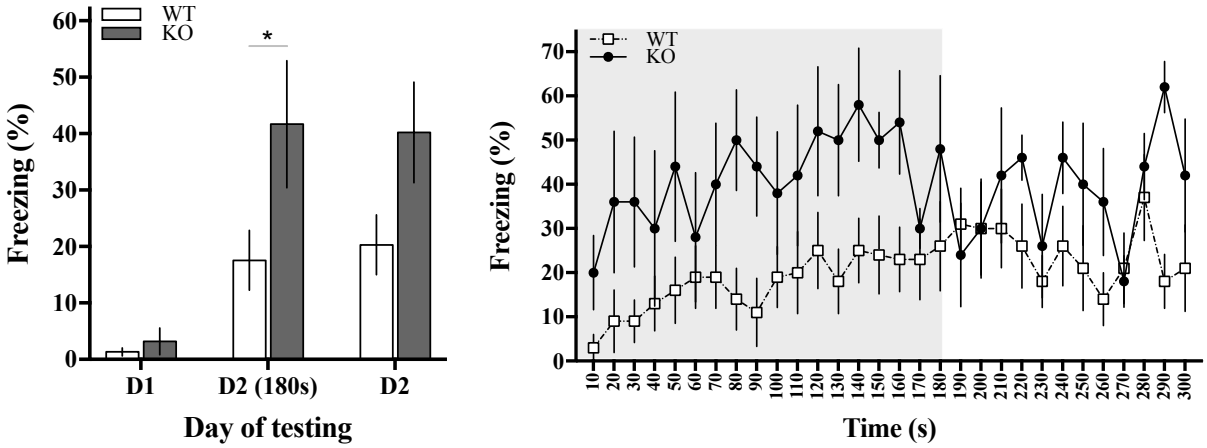


Figure 4.14. Left, Mean levels of freezing behaviour registered during the lead-in period of the footshock reactivity test (day 1, or D1), the second exposure to the chamber 24h later (day 2, or D2), and the first 180s of the latter session ('D2 (180s)') for 5-HTTKO (N=5) and WT mice (N=10). **Right,** Mean levels of freezing behaviour registered during D2 in 10s-long bins. The shaded area delimits the first 180s of D2. Mean ± SEM. *$p < .05$ (main effect of genotype).

Compared with the WTs, 5-HTTKO mice exhibited stronger contextual fear conditioning when they were re-exposed to the same context where they had been subjected to a footshock reactivity test 24h earlier. Although it was numerically clear that this occurred during the 300s-long session of re-exposure to the chamber, this just failed to reach statistical significance (main effect of genotype: $F(1,13) = 4.2$, $p = .062$; $Genotype_2 \times Bin_{30}$ interaction: $F(29,377) = 1.9$, $p = .176$; see Figure 4.14). An analysis limited to the first 180s revealed a significant genotypic difference regarding contextual fear conditioning (main effect of genotype: $F(1,13) = 5.0$, $p = .044$; see 'D2 (180s)' in Figure 4.14, left; see also shaded area in Figure 4.14, right). No significant genotypic differences were seen during the 180s ($U = 23.5$, $p = .859$) and 300s periods ($U = 18.0$, $p = .440$) on day 1 (i.e. before footshocks were delivered).

It could be argued that an analysis focused on the first 180s might not be strictly appropriate due to, for instance, the lack of a significant interaction between genotype and bin across the entire 300s period when the data is binned in 10s-long bins. However, what

was observed during a 180s period on average does not misrepresent what was observed throughout the 300s period (see Figure 4.14, left).

Conditioning to the background context during fear conditioning was equivalent in both genotypes

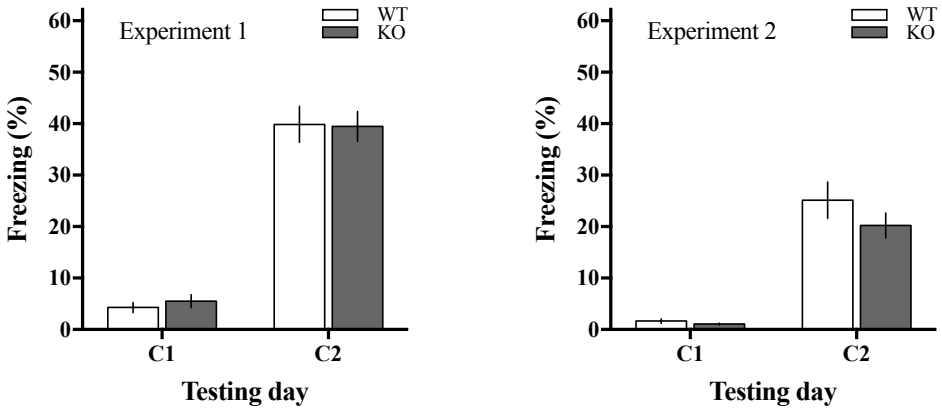


Figure 4.15. Left, Mean levels of freezing behaviour registered during the lead-in period of C1 and C2 for the 5-HTTKO (N=17) and WT mice (N=18) of Experiment 1. **Right,** Mean levels of freezing behaviour registered during the lead-in period of C1 and C2 for the 5-HTTKO (N=23) and WT mice (N=23) of Experiment 2. Mean ± SEM.

In Experiment 1, both groups exhibited significant freezing to the background context from C1 to C2 (main effect of day: $F(1,33) = 249.1$, $p < .001$; see Figure 4.15, left), and no genotypic differences were detected on either day ($Genotype_2 \times Day_2$ interaction: $F < 1$). A similar pattern of results to these was found in Experiment 2 (main effect of day: $F(1,44) = 105.2$, $p < .001$; $Genotype_2 \times Day_2$ interaction: $F(1,44) = 1.1$, $p = .303$; see Figure 4.15, right). Notably, a greater number of US presentations leads to significantly stronger freezing to the background context in Experiment 1 compared to Experiment 2 (e.g. main effect of experiment on C2: $F(1,79) = 29.1$, $p < .001$).

4.7. Discussion

Summary of the results

In this chapter, 5-HTTKO mice showed enhanced discriminative learning compared to WTs, when either fully predictive aversive cues (Experiment 1) or ambiguous aversive cues (Experiment 2) were used (versus a control cue). However, this enhancement exhibited a different form in each situation. Specifically, in Experiment 1 the enhanced learning was seen as earlier discrimination during training, whereas in Experiment 2 it was seen as stronger fear memory recall. Additionally, there was some evidence of enhanced extinction learning in 5-HTTKO mice in Experiment 1. Finally, 5-HTTKO mice exhibited stronger contextual fear conditioning compared to WT controls.

5-HTTKO mice exhibited enhanced aversive discrimination learning

In Experiment 1, 5-HTTKO mice exhibited facilitated cue discrimination during fear conditioning training. Specifically, they had significantly stronger cue discrimination by the end of the first conditioning session, whereas the WT needed one more session to show equivalent learning. This result contrasts with those reported by other researchers when testing 5-HTTKO mice in single cued fear conditioning procedures (Pang et al., 2011; Wellman et al., 2007). However, these studies used considerably stronger USs compared with the ones used in this thesis (Wellman et al.: 2s, 0.6 mA scrambled footshock; Pang et al.: 1s, 0.5 mA footshock). Higher footshock intensities lead to faster fear acquisition and stronger fear conditioned responses, including conditioned freezing (Annau & Kamin, 1961; Morris & Bouton, 2006). For this reason, lower levels of footshock intensity are usually chosen, together with other changes in the procedures, to ensure that control animals will exhibit a smaller percentage (e.g. 50%) of their maximal level of freezing, and thus ensure the detection of treatment effects (i.e. decreases and increases) in conditioned freezing (e.g.

Burghardt et al., 2004). Therefore, it is possible that very strong footshock administrations might have led to high levels of freezing very quickly in both 5-HTTKO mice and WTs in the aforementioned studies, thus making it difficult to detect genotypic differences during conditioning training due to possible ceiling effects (Jaffard et al., 2001).

The enhancement in discriminative fear learning reported here does not seem to result from alterations in basic sensory processing in the KOs. 5-HTTKO mice and WTs showed equivalent levels of reactivity to the five shock intensities administered during a footshock reactivity test, which is in line with a previous report (Lira et al., 2003). Consistent with this, analyses of the UR to the first footshock delivery in mice subjected to fear conditioning in this thesis showed an absence of significant differences between KOs and WTs. Genotypic differences regarding URs to the auditory cues could not be investigated as there was no measurable UR (see Appendix 6).

Notwithstanding the absence of genotypic differences in the UR to the footshock, the additional administration of subsequent footshocks during the first fear conditioning session elicited stronger reactivity in the KOs compared to WT controls. It is thus possible that the US became more aversive to the KOs during C1, which could contribute to the enhanced learning observed during that session. However, it should be noted that the KOs did not freeze more to the aversive cue during this session compared to WTs. Instead, what seems to have been crucial in driving the enhanced discrimination during C1 was a greater reduction of freezing (compared to pre-CS levels) in the presence of the non-aversive CS– cue by the KOs compared with WT mice. Moreover, during C2 and FMR, 5-HTTKO mice exhibited lower freezing levels during the presentation of the CS– cue compared with WT mice. Therefore, the knock-out of 5-HTT led to bidirectional effects on the processing of emotionally-relevant cues, such that 5-HTTKO mice froze more during the presentation of an aversive CS+ compared to a pre-CS period, but froze less during the presentation of the CS– (i.e. this cue made them move more compared to a baseline level).

It is possible that a discriminative aversive learning procedure such as the one used in Experiment 1 could have made the CS– work as conditioned inhibitor. In a bidirectional response system like the one used, where freezing could go either above or below a baseline level (obtained during a local pre-CS period), a conditioned inhibitor is easily detected given that it produces an opposite response to that evoked by the conditioned excitor (i.e. CS+; Bouton, 2007). The results obtained in Experiment 1 show that whereas a CS+ signalling footshock increased freezing behaviour in mice, a CS– signalling a decrease in the probability of footshock led to decreased freezing. In this situation, it is possible that the CS– "inhibits fear, signals safety, or causes 'relief'" (Bouton, 2007, p.89). Indeed, several authors consider that the CS– can work as a safety signal (Lissek et al., 2005; Grupe & Nitschke, 2013; Likhtik & Paz, 2015).

It is therefore possible that low or null 5-HTT expression levels can make subjects not only more sensitive about aversive events, such as the repetition of footshock, but also more sensitive to relatively positive events, such as a safety signal predicting a temporary absence of an aversive event. Consistent with this hypothesis, the WT controls for the 5-HTTOE mice tested in a similar discriminative aversive procedure as the one used in this chapter also exhibited numerically stronger reduction of freezing during the CS– presentations (compared to pre-CS levels) at the end of conditioning training (Barkus et al., 2014; see results for "Training day 3" in their Figure 3. A copy of this figure can be found in Appendix 18).

Importantly, the enhanced discriminative aversive learning seen here with 5-HTTKO mice is consistent with claims that 5-HTTOE mice exhibit impairments in conditioned fear in single cue fear conditioning tasks (Line et al., 2014) and a reduced rate of learning in discriminative fear learning tasks (Barkus et al., 2014).

Genotypes do not differ on FMR when a fully predictive aversive cue is used

The absence of significant genotypic differences during the FMR session in terms of cue discrimination levels likely reflects equivalent learning in both KOs and WTs by the end of conditioning training. Additionally, no significant differences were seen regarding the freezing response evoked by the CS+ in this session.

5-HTTKO mice exhibit enhanced discrimination between an ambiguous aversive cue and a control cue

5-HTTKO mice learned better than WT mice to discriminate between an ambiguous aversive cue and a non-aversive, control cue. However, changes in the conditioning procedure from Experiment 1 to Experiment 2 seemed to have affected how and when the enhanced discrimination learning was shown. Specifically, with the ambiguous cue no genotypic differences were seen during the acquisition phase, but only during the FMR session. This could reflect the number of footshocks received, which occur at different phases across the two experiments.

Additionally, 5-HTTKO mice did not interpret an ambiguous aversive stimulus as any more threatening compared with WT mice. Instead, they increased their freezing response during the presentation of the CSa to the same extent as the WTs, but reduced it during the CS− presentations in a relatively more pronounced way than their controls. This bidirectional effect of the processing emotionally-relevant cues resulting from the lack of 5-HTT is similar to that observed in Experiment 1. Despite an absence of significant genotypic differences during the conditioning phase, the KOs exhibited significantly stronger fear memory recall. More precisely, 5-HTTKO mice kept the same cue discrimination level from C2 during the FMR session, but the WT mice failed to show any discrimination at all during FMR.

One possible explanation for this FMR result could be that the WT might have been more sensitive to the change of context than the KOs. However, this might predict a significant reduction in freezing to the CSa in the WTs, which was not observed with the CSa-evoked Δfreezing (and raw freezing too; see Appendix 5). On the contrary, despite a slight decrease in freezing towards the CSa, WTs significantly increased their freezing to the CS– from one session to the other, leading to a situation of matched freezing levels between the CSa and the CS– cues (see Figure 4.6, left). Data analyses on the UR to the footshock restricted to the sample of Experiment 2 did not provide a different result to those obtained with all mice fear conditioned in this thesis. Therefore, altered URs to the cues and shocks do not explain the WT behaviour. Overall, the FMR result suggests that the cue discrimination learning was not as well learned/consolidated in the WTs as with the KOs. It is possible that 5-HTTKO mice only needed two CSa-US pairings out of 10 CSa presentations to develop and consolidate a memory for the CSa/CS– discrimination, whereas the WT mice would need more CSa-US pairings to achieve a similar degree of learning/consolidation before the FMR session.

5-HTTKO mice show enhanced fear extinction learning

Evidence of enhanced extinction learning in KOs was found in Experiment 1, both in the form of reduced cue discrimination levels and of reduced CS+ Δfreezing from FMR to E1. One explanation for what occurs during fear extinction learning is that the CS-US association formed during conditioning training is not destroyed but, instead, a second inhibitory association between the CS+ and the US is gradually acquired during extinction (Bouton, 2007). After extinction learning, the CS+ has thus two associations with the US: one excitatory association (i.e. the original CS-US), and another association that suppresses the learned conditioned response by inhibiting the activation of the US representation in the

extinction context. One way of interpreting this is by considering that the "excitatory association provides a 'danger' meaning, whereas the inhibitory association provides a 'safety' meaning" (Vervliet et al., 2013, p. 227). Indeed, many authors do conceptualise extinction learning as the acquisition of a new association between the CS and safety (e.g. Homberg, 2012; Grupe & Nitschke, 2013; Likhtik & Paz, 2015). Importantly, some also consider that after fear extinction training, the aversive CS can be a safety signal as much as the CS– is during the discriminative fear learning (e.g. Likhtik & Paz, 2015). Following this interpretation, then two instances of enhanced learning about safety signals were seen in the KOs tested in Experiment 1, because in both situations they exhibited a more pronounced reduction in freezing during the 'safety signal' (i.e. CS–) presentations compared to WTs (after the same number of presentations). Such learning would certainly contribute to enhanced cue discrimination when this was relevant (i.e. during conditioning), and a faster decrease in cue discrimination when differentiation of the cues was no longer biologically relevant (i.e. during extinction).

Previous results obtained with 5-HTTKO mice during extinction learning had indicated no differences (Wellman et al., 2007; Narayanan et al., 2011). However, the absence of effects on fear extinction learning due to the knock-out of the 5-HTT is challenged both by data showing impaired fear extinction learning in 5-HTTKO rats (Nonkes et al., 2012; Shan et al., 2014), and by the instance of enhanced extinction learning described in this chapter.

Considering the research conducted with 5-HTTKO mice on fear extinction learning, differences in the methods used should be considered when interpreting the different results produced by different research groups. For instance, the use of considerably stronger USs compared with the ones used in this thesis (Wellman and collaborators: 2s, 0.6 mA; Narayana and collaborators: 1s, 0.4mA) could have led to ceiling effects at the end of acquisition. If a situation like this occurred, this could hide 'over-conditioned' KOs and

partly mask faster extinction learning in these mice compared to WT controls. Additionally, Wellman et al. used a massed-trial procedure (40 CS presentations with a 5s inter-stimulus interval), whereas Narayanan and colleagues used a mixture of a massed-trial procedure (trials of 4 CS– and 4 CS+) and spaced-trial procedure (6 consecutive sessions separated by an inter-trial interval of 30min). However, both research groups administered the extinction training for only one day, whereas in Experiment 1 of this chapter intervals of 24h were used between the three extinction sessions administered. Different procedures might have differently affected processes such as those of protein synthesis underlying extinction learning (Lin et al., 2003; Santini et al., 2004), with a 24h interval after only five CS-no US pairings allowing us to see genotypic differences on the following day that a massed-trial procedure could not show. Additionally, it might have happened that having already learned about a safety signal during conditioning training (CS–), this could help the acquisition of a new safety signal during extinction (i.e. the inhibitory association between the CS+ and the US). If Narayanan et al. had used a discriminative aversive learning procedure during training, perhaps it would be possible to see genotypic differences as those reported here during fear extinction learning.

5-HTTKO mice exhibit enhanced contextual fear conditioning

After being subjected to several footshocks in a conditioning chamber during a footshock reactivity test, 5-HTTKO mice exhibited enhanced contextual fear conditioning when they were again exposed to the same context 24h later. No other study testing 5-HTTKO mice with Pavlovian fear conditioning has yet reported this result. Additionally, 5-HTTKO mice were also freezing more during the inter-shock intervals towards the end of the footshock reactivity test compared with WT controls. Although a different task was used in their study (i.e. single cued fear conditioning), it is noteworthy to mention that Line and colleagues (2014) reported that 5-HTTOE mice froze significantly less than WTs to the

background context after receiving two footshocks. This is consistent with the result found here with 5-HTTKO mice.

No genotypic differences were seen regarding freezing to the background context during discriminative fear conditioning

Despite this result at the end of the footshock reactivity test, 5-HTTKO mice did not show enhanced freezing to the background context during discriminative fear conditioning, either in Experiment 1 or 2. However, it is important to realise that the latter method usually leads to low levels of contextual conditioning due to competition that occurs between the context and the CS+ for association with the US (Jaffard et al., 2001; Curzon et al., 2009).

It is worth noting however that the results seen in Experiments 1 and 2 of this chapter regarding freezing to the background context are consistent with the absence of genotypic differences regarding the CSa-evoked Δfreezing in Experiment 2. Indeed, like the ambiguous aversive cue in Experiment 2, the background context of a cued fear conditioning procedure predicts both the delivery and the absence of footshock. Therefore, it is also an ambiguous cue. In their experiment with 5-HTTOE mice, McHugh and colleagues (2015) showed that WT controls not only feared the CSa in a similar way to the CS+, but they also exhibited significantly higher freezing to background context during conditioning training (e.g. C2) than their 5-HTTOE counterparts. Therefore, in this thesis, 5-HTTKO mice and their WT controls dealt with two types of ambiguous aversive cues in a similar way during discriminative fear conditioning training.

Conclusion

This chapter aimed to verify whether altering 5-HT availability by means of genetic manipulation of 5-HTT expression levels in mice could increase sensitivity to aversive and positive events in the environment, and lead to alterations in associative learning processes.

Importantly, evidence for enhanced sensitivity to aversive and positive events in the environment was obtained, given that 5-HTTKO mice showed enhanced reactivity to the successive presentation of footshocks, and inhibition of the fear conditioned response during the presentation of the CS– cue. Additionally, some evidence for enhanced fear extinction learning in 5-HTTKO mice was also obtained. This result is particularly relevant if one considers that by the end of extinction training the CS+ works as safety signal given that it no longer elicits a fear conditioned response (see Likhtik & Paz, 2015). This would mean that two instances of enhanced sensitivity to a positive stimulus in the environment were found, and not just one. Moreover, evidence for another instance of enhanced learning was seen regarding the contextual fear learning. However, this latter finding needs to be further investigated on its own, that is, with the right experimental procedure and control groups (see Chapter 8, section 8.5).

Importantly, the above-mentioned results were achieved with procedures that are amenable to be combined with invasive recordings of neural and haemodynamic activity from the amygdala (Barkus et al., 2014). Specifically, the use of a discriminative fear learning procedure controls for non-associative sensitisation processes that could affect the responsivity of the amygdala to any auditory cue, thus allowing the investigation of CS– specific changes in the amygdala (Collins & Paré, 2000; Maren & Quirk, 2004; Kim & Cho, 2017). This means that the neuronal substrates of the behavioural phenotype can be investigated. Indeed, this was the aim of the experiment that will be presented in Chapter 5.

In sum, this chapter provides support for the neuroplasticity theory of 5-HT function, which argues that increased 5-HT availability, which is found in 5-HTTKO mice, will enhance emotional learning. Importantly, the results here presented and discussed challenge one of the most commonly reported findings obtained with 5-HTTKO rodents, which is that they do not exhibit altered fear learning. Moreover, they are consistent with previous observations of impaired fear learning in 5-HTTOE mice (Line et al., 2014; Barkus et al.,

2014). Together, these results strengthen the neuroplasticity theory of 5-HT function given that they suggest conditioned fear learning can be influenced across a continuum of 5-HTT expression levels, which in turn are associated with varying extracellular 5-HT levels.

Chapter 5. Fear-evoked amygdala responses in 5-HTTKO mice

5.1. Abstract

This chapter aimed to investigate a neuronal substrate of the enhanced aversive discriminative learning observed in 5-HTTKO mice reported in Chapter 4. The brain region of interest was the amygdala, given its importance on the processing of aversive stimuli and on emotional behaviour, but also due to data from human research that individuals with low 5-HTT-expression levels show altered amygdala function.

Here, 5-HTTKO mice and WT controls were trained in a discriminative Pavlovian fear conditioning procedure using fully predictive cues (i.e. a CS+ that was always followed by shock during training and a CS– that was never followed by shock). During fear conditioning, I recorded tissue oxygen (TO_2) signals, which provide an index of brain activity analogous to BOLD signal, and local field potentials (LFPs), from the basolateral amygdala (BLA).

5-HTTKO mice exhibited superior discriminative aversive learning compared to WT mice, and this was accompanied by significantly larger TO_2 responses to the shock, the CS+ and, to a lesser extent, the CS–. Additionally, the CS+ came to evoke stronger theta-frequency oscillations than the CS– in both groups throughout testing, but 5-HTTKO mice showed this discrimination earlier than WT controls.

In conclusion, enhanced discriminative aversive learning in 5-HTTKO mice is accompanied by enhanced BLA haemodynamic responses to the unconditioned stimulus and conditioned cues, and a faster development of stronger theta-frequency neuronal oscillations to the aversive cue compared to the control cue.

5.2. Introduction

Several studies have reported altered amygdala function during emotional processing as a consequence of 5-HTTLPR genotype (Hariri et al., 2002, 2005; Munafò et al., 2008; see Chapter 1, section 1.2.2). For example, during an emotional-face-matching task with aversive stimuli (e.g. angry/fearful faces), 5-HTTLPR *s* allele carriers (i.e. putatively low 5-HTT-expressing individuals) were reported to exhibit increased amygdala reactivity, compared to *l*/*l* individuals. One account of this finding is that s carriers have increased sensitivity (or hyper-responsiveness) to emotionally significant environmental stimuli (Hariri et al., 2006; Williams et al., 2009; Jonassen & Landrø, 2014). However, it is important to note that the effects of 5-HTTLPR genotype on amygdala reactivity are mixed (e.g. Munafò et al., 2008; Murphy et al., 2013b; Bastiaansen et al., 2015).

Nevertheless, support for the hypothesis of an altered amygdala function modulated by variation in 5-HTT availability has been provided by researchers employing other techniques apart from fMRI. For example, Rhodes and collaborators (2007; see Table 5.1) used PET to measure 5-HTT availability in healthy participants who later had their amygdala activity measured with fMRI BOLD signal while being subjected to an emotional-face-matching task with aversive stimuli. Such a combination of PET with fMRI allowed these researchers to clearly show the existence of a significant negative correlation between amygdala 5-HTT availability and amygdala reactivity to negative emotional stimuli. This result was thus a significant contribution to human imaging studies that were relying on the assumption that *s* allele carriers had low 5-HTT expression levels compared to homozygotes for the *l* allele.

Subjects	Task/process and imaging method(s)	Effect/ results	Authors/year
Healthy participants	Emotional-face-matching task [^{11}C]-DASB PET imaging and fMRI	↑ amygdala reactivity in participants with ↓ amygdala 5-HTT availability	Rhodes et al., 2007
	Habituation → cued-fear conditioning [6×CS+/6×CS-] US = electric shock; CSs: pictorial stimuli measure: conditioned SCRs [^{11}C]-DASB PET imaging	↑ discrimination in participants with ↓ amygdala 5-HTT availability (peak correlation in the BLA)	Åhs et al., 2015
5-HTTLPR /rs25531 SS, SL_G, L_GL_G individuals	Cued-fear conditioning [20×CS+/20×CS−] US = highly aversive pictures (e.g. mutilations; 4s); CSs: distinct squares; measures: SCRs; fMRI	− cue discrimination acquisition ↑ amygdala reactivity for the contrast CS+ > CS−	Klucken et al., 2013a
5-HTTLPR *s*/*s* and *s*/*l* individuals	Cued-fear conditioning [16×CS+/16×CS−] US = electric shock; CSs: coloured squares; measures: conditioned SCRs; fMRI	− cue discrimination acquisition ↑ amygdala reactivity for the contrast CS+ > CS−	Klucken et al., 2015
5-HTTLPR /rs25531 SS, SL_G, L_GL_G, SL_A, L_GL_A individuals	Cued-fear conditioning [20×CS+/20×CS−] US = electrical stimulation CSs: two geometrical figures measures: SCRs fMRI ↑ number of traumatic life events (i.e. >1): 29.7% of the <u>high functioning</u> group (L_AL_A) and 37.8% of the <u>low functioning</u> group (i.e. non L_AL_A)	<u>Acquisition results</u>: ↑ CRs in participants with ↑ number of TLFs <u>Conditioned Rs</u>: − amygdala react. ↑ insula reactivity <u>Rs to the US:</u> ↑ insula activation	Hermann et al., 2012
rh5-HTTLPR S carriers rhesus macaques	Ethologically-relevant stressful situation Separated from cage mates and relocated to a test cage in a testing room for 30mins [^{18}F] fluoro-2-deoxy-D-glucose (FDG) microPet scanning	↑ amygdala reactivity during the relocation period compared to a baseline period (alone in the home cage)	Kalin et al., 2008

Table 5.1. Imaging studies investigating how variation in 5-HTT expression levels modulates amygdala reactivity to environmental emotional stimuli or stressful situations in humans and monkeys. ↑, enhanced; ↓, diminished; −, no changes; ~, similar; Rs, responses; CRs, conditioned responses; TLFs, traumatic life events; SCRs, skin conductance responses; →, followed by.

Consistent with the predictions of the neuroplasticity theory of 5-HT function (see Chapter 1, section 1.3), Åhs and collaborators (2015) later showed that healthy participants with lower levels of 5-HTT availability in the amygdala (measured with PET) also exhibit better discriminative aversive learning between an aversive cue and a control cue, compared to high 5-HTT-expressing participants. Additionally, other researchers showed that *s* allele carriers, compared to participants homozygotes for the *l* allele, exhibit increased amygdala responses during discriminative aversive learning (Klucken and collaborators 2013a, 2015; see Table 5.1). Specifically, they exhibited significantly increased reactivity to the CS+ as compared to the CS– (i.e. contrast CS+ > CS–) compared to the *l*/*l* carriers in both studies.

Considering all the above-mentioned studies together, there is strong support for a role of 5-HTT function in emotional processing and learning via amygdala modulation, possibly driven by increased extracellular 5-HT levels. Consistent with this hypothesis, another study also showed increased amygdala reactivity to emotionally negative stimuli in healthy participants with low 5-HT_{1A} autoreceptor density levels (Fisher et al., 2006), which is assumed to increase 5-HT neurotransmission. Moreover, in a more ethologically-relevant stressful situation, rhesus monkeys carrying the rh5-HTTLPR *s* allele exhibited enhanced amygdala activity in response to the stress of relocation compared to their *l*/*l* counterparts (measured with microPET [^{18}F]fluoro-2-deoxy-D-glucose scanning ~30mins after testing; Kalin et al., 2008).

Taking the previously mentioned research with humans and monkeys into account, and the enhanced discrimination learning seen in the 5-HTTKO mice subjected to a discriminative fear conditioning procedure with fully predictive aversive (CS+) and non-aversive (CS–) cues reported in Chapter 4, it is worth considering the hypothesis that an altered amygdala function could underlie this behavioural phenotype. Particularly, an altered function of the basolateral amygdala (BLA) could either track or parallel the enhanced differential behaviour to the CS+ or CS– cues exhibited by the KOs.

Although it is not the only structure capable of forming a CS-US association, and some forms of aversive learning can occur without its contribution (Killcross et al., 1997; Vazdarjanova & McGaugh, 1998), the BLA is generally necessary for acquisition and expression of conditioned fear responses (Fanselow & LeDoux, 1999; Sigurdssonet al., 2007; Pape & Paré, 2010; Duvarci & Paré, 2014; Gründemann & Lüthi, 2015; Bocchio et al., 2016; Bocchio et al., 2017). The role of the BLA in the formation of CS-US associations has been demonstrated, for instance, with lesions studies (LeDoux et al., 1990; Goosens & Maren, 2001) and disruption of neural activity during conditioning (Muller et al., 1997; Lee & Kim, 1998). Importantly, the BLA (particularly its input region, the lateral amygdala) is a site where associative plasticity occurs during cued fear conditioning (Quirk et al., 1995; Rogan et al., 1997; Collins & Paré, 2000; Goosens et al., 2003; Nabavi et al., 2014).

Relevant to this thesis, the amygdala is profusely innervated by serotonergic neurons, and serotonergic transmission is thought to modulate information flow in amygdala circuits (Steinbusch, 1981; Sur et al., 1996; Asan et al., 2013). This is particularly true for the BLA (Vertes, 1991; Abrams et al., 2005; Lowry, et al., 2005; Muller et al., 2007; Hale et al., 2008; Bocchio, et al., 2016). Additionally, subjecting rodents to Pavlovian fear conditioning can raise both extracellular 5-HT levels and local cerebral blood flow (CBF) in the amygdala (LeDoux et al., 1983; Holschneider et al., 2006; Yokoyama et al., 2005; Zanoveli et al., 2009). Moreover, the BLA contributes to freezing responses via its actions on the central nucleus of the amygdala (Sigurdsson et al., 2007; Li et al., 2013; Penzo et al., 2014), and these freezing responses can be modulated by serotonergic modulation of the BLA (Izumi et al., 2012; Johnson et al., 2015). Therefore, recording haemodynamic responses from the BLA during the fear conditioning of 5-HTTKO mice is a reliable way to investigate the neuronal correlates of the enhanced discriminative fear learning seen in those animals in Chapter 4.

Accordingly, the research described in this chapter aimed to investigate the neuronal correlates of the effects of null 5-HTT activity on discriminative aversive learning by (i) subjecting 5-HTTKO mice to a similar task used in chapter 4 (Experiment 1) and (ii) simultaneously recording haemodynamic responses and local field potentials (LFPs) from the BLA. The method used to detect haemodynamic responses during fear conditioning in freely, behaving animals was tissue oxygen (TO_2) amperometry. This is an invasive, *in vivo* voltammetric technique sensitive to local changes in TO_2 levels that provides an index of brain activity analogous to the BOLD signal (Lowry et al., 1997; Lowry et al., 2010; Bolger et al., 2011; see Chapter 1, section 1.2.4). Like the fMRI BOLD signal, TO_2 responses follow neuronal activity and can be used to detect the different patterns of activity evoked by a CS+ and a CS− during fear conditioning (Li et al., 2011; McHugh et al., 2013). Notably, TO_2 amperometry can be combined with simultaneous electrophysiological recordings, such as measurements of LFPs (McHugh et al., 2014; Barkus et al., 2014). This allows the tracking of potential neuronal mechanisms by which altered extracellular 5-HT levels influence BLA responsivity during conditioned fear learning.

5.3. Methods

5.3.1. Subjects

This study used 29 5-HTTKO mice (female: n=14) and 31 WT mice (female: n=16), which were aged 4.5 to 8.5 months on the first day of testing (KOs: M=6.8 months; WTs: M=6.9 months). Brain recordings were made from a subset of these mice, consisting of 14 KOs (female: n=7) and 14 WTs (female: n=8). Both implanted and non-implanted mice had been subjected to a small number of ethological, unconditioned tests of anxiety (see Chapter 3) prior to fear conditioning. Mice were housed with (1-5) littermates throughout the

experiment, with food and water available all the time. However, after surgery, implanted animals were single housed to prevent damage to the 'pedestal' plug.

5.3.2. Surgery

Each subject was chronically implanted with six intra-cranial electrodes by means of stereotaxic surgery under isoflurane anaesthesia: a carbon paste electrode (CPE, gifted by Lilly UK, Surrey, UK) was implanted into the BLA of one hemisphere for measuring changes in the TO_2 concentration; a silver wire electrode was implanted into the BLA of the contralateral hemisphere for the LFP measurements; and two reference electrodes and two auxiliary electrodes were implanted into the parietal cortex (~2mm), to complete the circuits for each signal recording. Coordinates for BLA implantations were -1.35 mm anterior/posterior, ±3.10 mm medial/lateral and -5.00 mm dorsal/ventral, relative to bregma. The TO_2 and LFP electrodes were implanted in different hemispheres to minimise tissue damage in the BLA. The hemisphere that would receive the CPE electrode was counterbalanced across the subjects of each group.

CPEs (Teflon-coated 200µm silver wire; ~270µm coated diameter, Advent Research Materials, Oxon, UK) were constructed as previously described (O'Neill et al., 1982; Lowry et al., 1997). This involved pulling the insulation down the wiring, filling the resulting 2mm cavity with carbon paste, and smoothing it. The carbon paste used consisted of 2.83g of carbon powder and 1.0ml of silicone oil.

The LFP electrodes were made from 125µm diameter silver wire (~177µm coated diameter, Advent), whereas the reference and auxiliary electrodes were made with non-coated, silver wires with a larger diameter (200µm). Each electrode was inserted into a gold pin and then inserted into a six-hole 'pedestal' plug, which was cemented on the skull.

Specifics about the surgical procedure can be found in chapter 2 (section 2.4). Subjects were allowed to recover for at least seven days after surgery.

5.3.3. Tissue oxygen and local field potential recordings

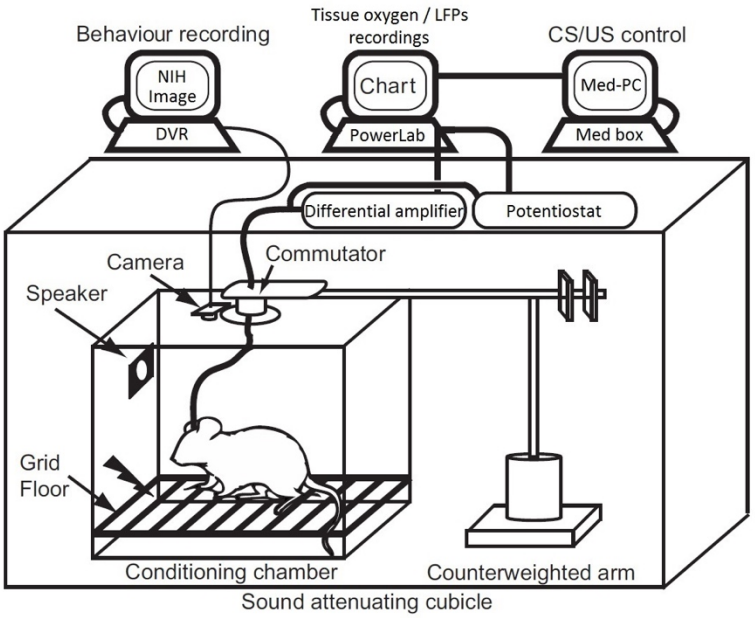
Changes in TO_2 were measured using constant potential amperometry (CPA), a subtype of voltammetry which has been previously described in detail (Lowry et al., 2010; Bolger et al., 2011; Li et al., 2011; McHugh et al., 2011; McHugh et al., 2013; see also Chapter 1, section 1.2.4). This technique involved using a potentiostat ('Biostat,' ACM Instruments, Cumbria, UK) to induce an electrical current flow from the CPE to the reference electrode, and, in this way, reducing the O_2 at the surface of the CPE. Specifically, this electrode was held at a constant negative potential (-650mV) relative to the reference electrode, a process resulting in an electrochemical reduction of the O_2 via a two-electron process producing H_2O_2 ($O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$; $H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$) (Bolger et al., 2011). This acceptance of electrons by the O_2 produces a measurable change in the measured Faradaic current, with the magnitude of change being proportional to the local concentration of O_2 available (Hitchman, 1978). The area of sensitivity (~400μm in this case) is considered to be a sphere with a diameter two times larger than the electrode surface diameter (Li et al., 2011). The auxiliary electrode's contribution to the circuit was to eliminate the 'Ohmic drop' whenever it occurred (Amatore et al., 2000).

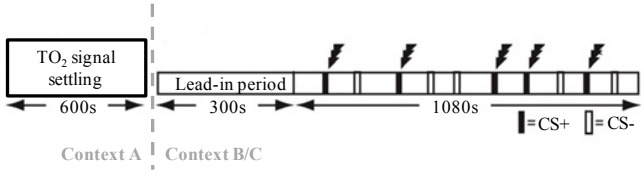
With respect to the LFP signal, a differential amplifier (DP-301, Warner Instruments, CT, USA) compared the signal recorded by the working electrode in the BLA to the one obtained by the reference electrode in the parietal cortex, which was electrically inactive. The amplifier then measured the potential difference between the two electrodes and removed any signal common to them (i.e. the 'differential'). This helped to subtract any electrical contamination (i.e. noise) between the signals (Reed et al., 2015). Finally, the

resulting LFP signal of interest was amplified (100 times) and filtered between 1Hz and 3 kHz.

Both signals were transmitted to a digital acquisition interface (PowerLab 8/30 interface, AD Instruments Ltd, Oxon, UK), which converted the analogue form of the signal to a digital one. This device also amplified the TO_2 signal (100 times) and filtered it between 47-53Hz, a process that helped to remove electrical noise interference. TO_2 and LFP data were then sampled continuously at 4 kHz on a computer running Chart software (AD Instruments, version 5.5).

5.3.4. Apparatus and procedure





Testing day	1	2	3	4	5
Session	**PreExp**	**C1**	**C2**	**C3**	**FMR**
Phase	Pre-exposure	Conditioning			Recall
Context	A	B			C

Figure 5.1. Top, Diagram of the recording setup. **Bottom, left,** A diagram of a conditioning session used in the experiment. The black lightning marks indicate the 0.3mA footshocks. No shocks were given during the Pre-exposure (PreEx) and fear memory recall (FMR) sessions. **Bottom, right,** 5-day procedure used in the experiment. A PreEx session on day 1 was followed by a 3-day conditioning phase (C1, C2 and C3), which was then followed by a fear memory recall (FMR) session on day 5. The PreEx session was administered in the same context for all animals (i.e. context A). The FMR session was administered in a context different from that used during the conditioning phase (e.g. context C, if conditioned in context B). The Top and Bottom, left, Figures were adapted from McHugh et al. (2014). Reprinted under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014.

Three experimental chambers (ENV-307A, Med Associates Inc., Lafayette, IN, USA) were used in this experiment: one was used as a 'neutral context' (context A) and two were used as contexts for fear conditioning and FMR sessions (contexts B and C). Contexts B and C were placed inside sound-attenuated cubicles, which were standing side by side. Different

visual and olfactory cues in the interior of these cubicles provided different contexts, as described in chapter 2 (section 2.3.4). Context A was not enclosed inside a cubicle but had its lateral polycarbonate walls painted black. All chambers had a transparent polycarbonate top that included a hole in its centre to allow cables to go inside the chambers. Placed inside the cubicle, a counterweighted arm (PHM-110P1, Med Associates) held a 6-channel rotating commutator (SL6C, Plastics One) where two screened cables (363-363 6TCM, Plastics One) were connected, one on each side of the commutator (see Figure 5.1, top). One cable (6 channels) would enter the chamber throughout the hole in the chamber's ceiling and connect the commutator to the 'pedestal' plug of a subject before the start of an experimental session. The other cable would connect the commutator to the potentiostat (3 channels) and to the differential amplifier (3 channels). This arrangement ensured that a subject could move freely inside the chamber when the multi-channel cable was connected to the 'pedestal' plug of the subject (see Figure 5.1, top).

One week prior to the start of the experiment, implanted animals were subjected to two hours of having potentials applied to the electrodes in (neutral) context A. The initial response properties of the CPEs can be considerably variable but this 'conditioning' of the electrodes is enough to modify them and produce a more stable amperometric signal, possibly because oil is removed from the surface of the CPE (Vanderkooi et al., 1991; Lowry et al., 1996; Kane & O'Neill, 1998). A cardboard insert was placed on the top of the floor to avoid contact with the rods, and some bedding was added to the chamber during this period. One mouse at a time would be taken to the experimental room, connected to the multi-channel cable and left to rest/sleep for 2 hours while the brain recordings were made.

In addition to this, a potential would be applied to the CPE for a 10-minute period in context A just before each experimental session to ensure that the TO_2 signal was stable on each recording day ('TO_2 signal settling' period). This was done to counteract an initial large capacitance current that occurs as soon as the potential is applied to the CPE.

One week later, the experiment began. On the first day ('pre-exposure', PreEx), mice were exposed to 10 auditory cues (5 × white noise, 5 × 2900Hz tone; all 72dB). This session followed a similar structure to those administered in the fear conditioning experiments reported in Chapter 4, and to those that would be administered in the following days: there was a 300s lead-in period, followed by a pseudorandom presentation of two types of 30s-long auditory cues, interspersed with a mean inter-cue interval of 80s (ranging from 60 to 100s), and ending with a 60-second lead-out period. However, no footshocks were administered on this session, which took place in context A. The purpose of PreEx was to investigate if there were any genotypic differences on behavioural, haemodynamic, and electrophysiological responses to the cues before conditioning.

On the following days (C1, C2 and C3), a conditioning protocol was administered. After an initial 'TO_2 signal settling' period in the neutral context, mice would be placed in one of the enclosed experimental chambers, where one of the cues was contingently paired with a (0.3 mA, 0.5 s) footshock (see Figure 5.1, bottom left). The reason why the settling period was administered in a different context was to avoid having subjects habituating for too long to the conditioning context, which could confound the behavioural measure (i.e. freezing).

Finally, a fear memory recall (FMR) session was administered in the alternative enclosed experimental chamber (e.g. context C, if conditioned in context B), with no administration of footshocks. Moreover, subjects now had no contact with the grid floor because a cardboard insert was placed on the top of the floor (as previously done in a study conducted with 5-HTTOE mice; see Barkus et al., 2014). Apart from the 2h rest/sleep session and being tethered during the sessions, non-implanted mice were subjected to the same conditions and procedures as the implanted subjects. That is, they were also tested one at the time and spent 10mins in the 'neutral context' before being moved to the experimental chamber.

Further information about the apparatus and the different contexts used can be found in Chapter 2 (section 2.3.4). The events administered in the conditioning chambers were controlled by a script written in Med-PC IV software programme. This software also delivered TTL (transistor-transistor logic) pulses to the PowerLab interface for the main events, such as the start and end of cue presentation periods. This allowed for the time-locking of the TO_2 and LFP signals to those events in the Chart software at 1ms resolution.

5.3.5. Data analyses

Behaviour

Information on how freezing behaviour was measured and analysed can be found in Chapter 2 (section 2.3.4). Statistical results regarding the non-significant interactions between genotype and sex can be found in Appendix 24.

Tissue oxygen responses

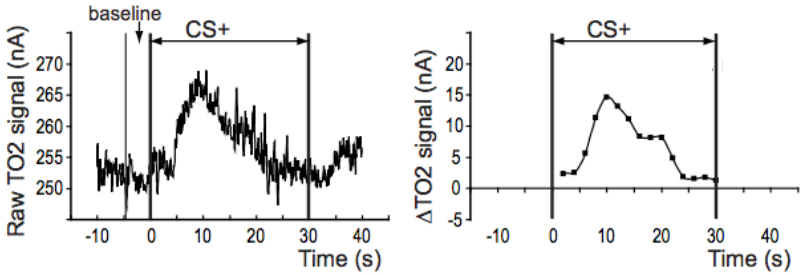


Figure 5.2. Left, Example of raw TO_2 data with the presentation of a CS+ (0-30s) time-locked onto the trace. The mean value of TO_2 signal in the 5s-long period prior to the CS+ period was used as a baseline signal to detect whether the CS-evoked TO_2 response consisted of an increase or decrease of the local TO_2 concentration. **Right,** TO_2 data transformed into ΔTO_2 signal after the baseline was subtracted from every data point of the CS+ period and the data were binned into (15) 2s-long epochs (i.e. each data point is the mean value of the ΔTO_2 signal during two consecutive seconds). Reprinted and adapted from McHugh et al. (2014), under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014.

Given that it is not possible to measure absolute TO_2 concentrations with CPA, it was necessary to get a local baseline around the relevant periods of the TO_2 signal to be analysed (e.g. CSs) to detect a relative increase or decrease of the TO_2 concentration (ΔTO_2) (see Figure 5.2, left). After down-sampling the TO_2 signal to 100Hz, cue-evoked TO_2 responses were calculated with the following steps: selection of the cue periods of the raw TO_2 signal trace together with the signal trace from the 5s before the cue onset (i.e. the baseline); the mean TO_2 signal value for this 5 s period was then used to transform the data of interest by subtracting that value from every data point contained in the selected cue period; afterwards, the data were binned into 15 2-second epochs, and graphs were made by connecting the resulting data points by linear interpolation (see Figure 5.2, right). Finally, TO_2 responses evoked by each cue type in each session were averaged together, firstly for each subject and then across groups. All these procedures were also used for the US periods (i.e. the 30s-long period following the administration of footshock) and 'no US' periods (i.e. the 30s-long period following the absence of footshock), with the baseline period consisting of the last 5s of the preceding cue.

This method of data processing counteracts some specific issues associated with the raw TO_2 signal, such as the inter-subject variation in the raw baseline signals (i.e. the background current), and the occasionally observed drift in that raw signal within a session, which is not always linear (Barkus et al., 2014). In addition to these situations, TO_2 signals can contain non-physiological noise, caused by cable artefacts and other electrical issues. Often these problems affect a very short period of time (1 or 2s out of 30s) in a trial but they can have a large effect on group means for that particular epoch, even when standard smoothing methods are used (e.g. averaging based on values of nearest neighbours). Rather than excluding these trials from analysis altogether, a method was used to remove clear outliers and replace these with representative values. To this end, data for each trial were meaned across all subjects in each genotype (i.e. all data regardless of the presence of noise).

Then, for each data point in the time series, if any animal was contributing a data point that was +/- 3 standard deviations from the group mean, this data point was replaced with the group mean. Fewer than 1.5% of data points were adjusted in this way.

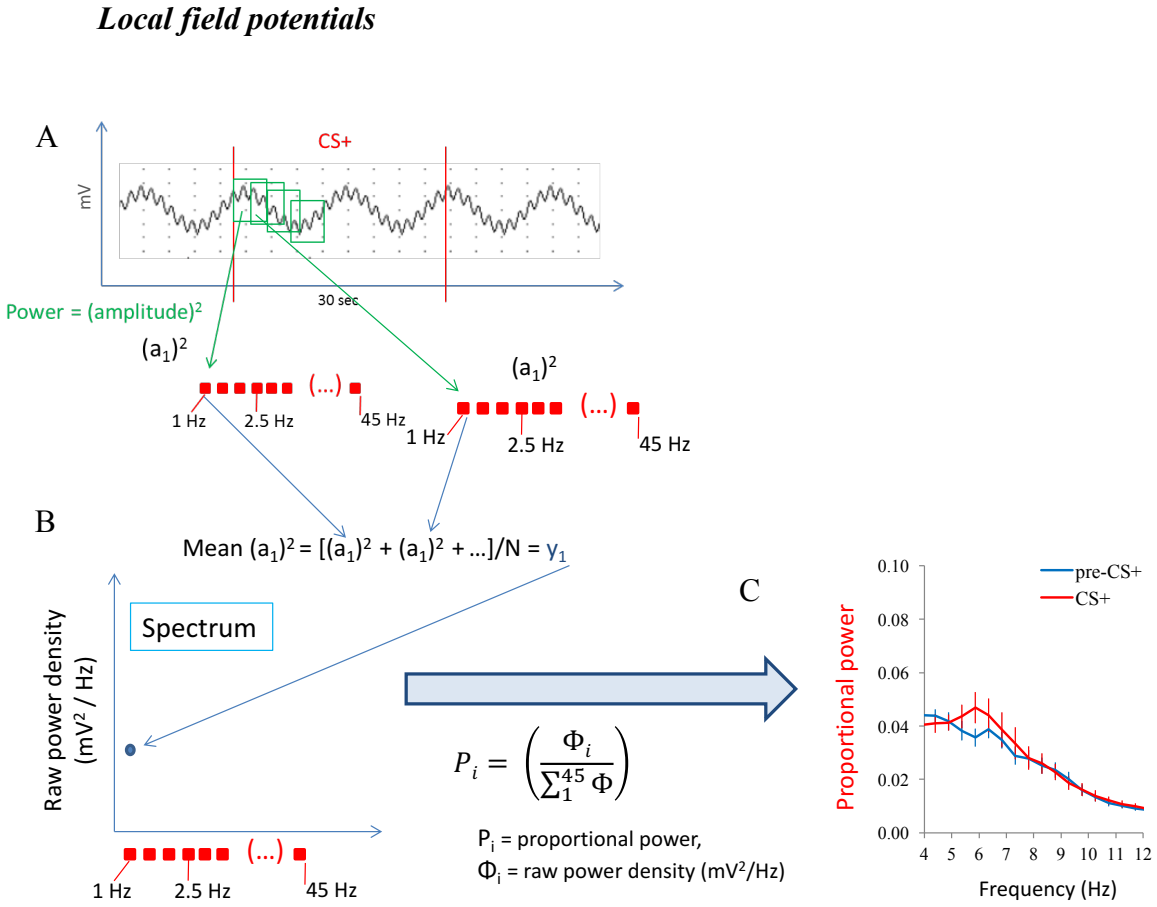


Figure 5.3. Diagram illustrating how the LFP data were processed. **A)** First, a Fast Fourier Transform was used to separate the relevant parts of the trace into a set of frequency components. **B)** Then a power spectral analysis was used to calculate the strength of each of those components. **C)** Afterwards, the raw power in each of these components was transformed to be expressed as a proportion of the total (i.e. summed) power between 1 and 45Hz. This was done for the cue periods (e.g. red curve) and for the pre-cue periods (e.g. blue curve). Later, the summed theta (θ) power between 5-10Hz of the blue curve was subtracted from the equivalent data of the red curve, to calculate the (summed) θ power evoked by that cue. Mean ± SEM.

For the analyses of the LFPs, a digital band-pass filter in Chart was first used to show only the output signal trace corresponding to frequencies between 1 and 45 Hz, thus reducing noise and providing a smoother signal. Afterwards, the relevant parts of the (waveform) trace

(e.g. CSs periods) were decomposed into a set of frequency components (i.e. waves of different amplitudes) by means of a Fast Fourier transform, and the strength of the constituent wavelengths of each selection evaluated by means of a power spectrum analysis. This was done by using the Spectrum function on the Chart software (FFT size of 8192 points, with a Hamming window, 50% overlap). This process divided the signal into several segments and produced a spectrum with 93 frequency bins from each one of them (minimum value: 1.46Hz; maximum value 44.92Hz) (see Figure 5.3, A). Afterwards, the power values obtained for a given frequency bin in all segments for each of the 93 frequencies available were averaged, and a final spectrum with the raw power spectral density (mV^2 / Hz) in each frequency bin was obtained for the relevant period (see Figure 5.3, B). Each of these 93 values was then transformed to be expressed as a proportion of the total (i.e. summed) power between 1 and 45Hz (see Figure 5.3, C).

Afterwards, for each subject, an average spectrum for each CS type on each session was generated by averaging the five obtained values for each of the frequency bins between 1-45Hz. The same was done for each pre-CS period. The duration of the periods selected was 29s to avoid artefacts due to footshock administration at the end of the CS+ cues. Finally, cue-evoked theta (θ) power was calculated for every subject in each session by subtracting the summed θ power (∑5-10Hz) during the pre-cue period' from the 'summed θ power during the cue period'. This was the equivalent to subtracting the sum of all raw power spectral density values between 5-10Hz of the blue curve from the equivalent data of the red curve in the graph depicted in figure 5.3, C. These data were averaged together across subjects of the same genotype for every session.

Despite the focus on the oscillations occurring in the theta range in this chapter (see section 5.4.4), it should be noted that it is not a raw measure of theta that is being reported here. Instead, the proportional measure used takes into account all power of several frequencies (between 1-45Hz), including those in the delta range (1-4Hz). Therefore, an

increase in theta power in the measures here reported is accompanied by a decrease in delta power. Nevertheless, an additional figure depicting another measure of theta power relative to delta power can be found in Appendix 25.

Histology

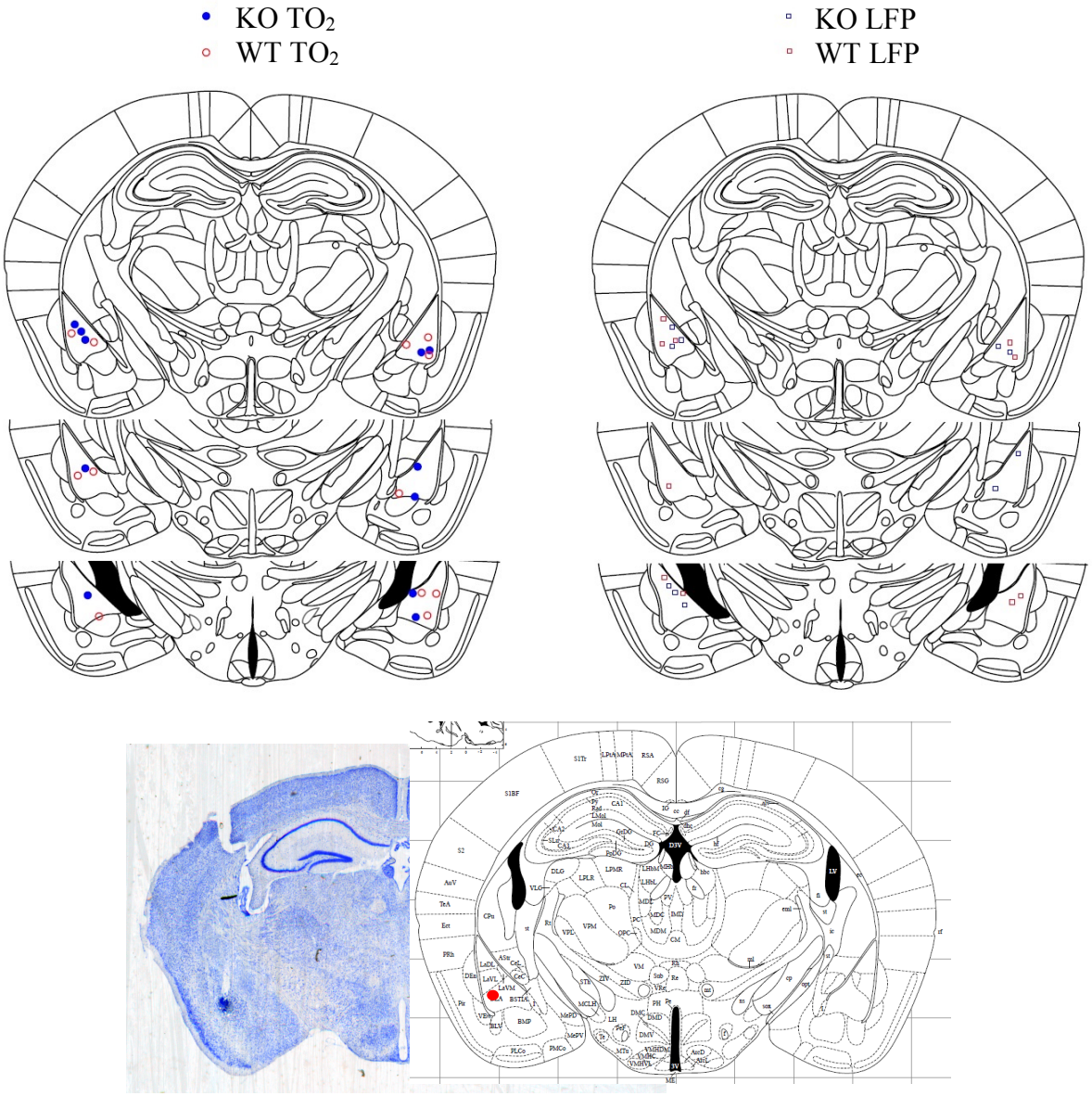


Figure 5.4. Top, Schematic representations of the location of the tip of the tissue oxygen (TO_2) and local field potential (LFP) electrodes in the 5-HTTKO mice and WT mice. **Bottom,** Photomicrograph from one mouse showing the electrode location in the BLA and a schematic representation of that placement. Source of the stereotaxic atlas pictures: Paxinos & Franklin, 2001. Reprinted and adapted from The Mouse Brain in Stereotaxic Coordinates, Second Edition, George Paxinos and Keith B.J. Franklin, Figures, pp. 95-97 and 99 (4 Pages), Copyright (2001), with permission from Elsevier.

The histological technique used for determination of electrode placements is described in Chapter 2 (section 2.5). Only subjects in which the tip of the electrodes was within the BLA boundaries were included in the haemodynamic and electrophysiological

analyses. This meant that 2 KOs and 2 WTs were excluded from the data analysis on TO_2 signal responses, and 3 KOs and 3 WTs were not used in the investigation of LFPs. Therefore, 12 5-HTTKO mice and 12 WTs were used for the analyses of TO_2 responses, and 10 mice of each genotype were used for the analyses of LFPs.

Statistical analyses

ANOVAs using a general linear model (GLM) and t-tests were performed on the data, as previously described in Chapter 2 (section 2.6). The one sample *t*-tests used for the discrimination index analysis were rejecting H_0: $\mu = 0$. Prior to the data analyses performed on the pre-exposure, conditioning and FMR data, analyses were also conducted on the raw pre-CS freezing responses across the five days. The results of these data analyses can be found in Appendix 3. Figures showing raw freezing during CS periods and freezing levels during pre-CS periods across all trials of Experiment 1 can be found in Appendix 7.

One 5-HTTKO mouse had its auditory cue allocation to the CSs reversed by C3 and, therefore, its data were excluded from all data analyses. However, one exception was made during an analysis of the US-evoked ΔTO_2 responses during C1, when the signals of this mouse were still valid. Additionally, one WT mouse was also excluded from the analysis of LFPs as no signals could be obtained during FMR.

5.4. Results

5.4.1. Pre-exposure results

Groups did not differ during pre-exposure to the auditory cues

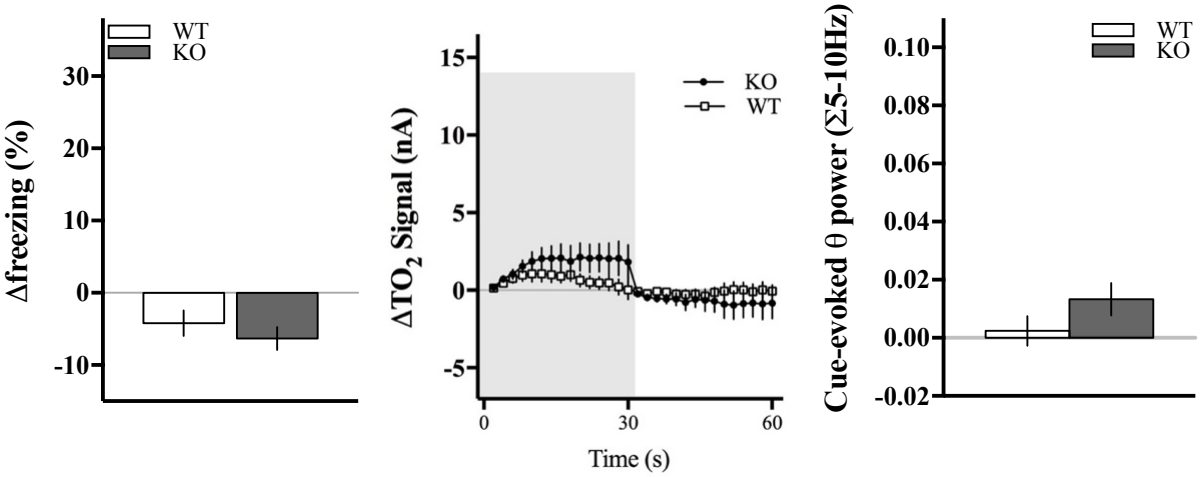


Figure 5.5. Left, Δfreezing (%) evoked by the 10 cues presented on the pre-exposure (PreExp) session for the 5-HTTKO (N=28) and WT (N=31) mice. **Middle,** BLA TO_2 signal responses during the presentation of those cues (shaded area) and the following 30s-long periods (KOs: n=11; WTs n=12). **Right,** Mean theta (θ = 5-10Hz) power evoked by the same cues (i.e. Cue θ power minus pre-Cue θ power; KOs: n=10; WTs: n=10). Mean ± SEM.

Analyses of behavioural responses (Δfreezing) to the auditory cues during the PreExp session showed that both groups exhibited a reduction of freezing (i.e. movement) during the presentation of the 30s-long auditory cues compared with a 30s-long pre-cue period (see Figure 5.5, left). Statistical analyses (ANOVA model: Genotype $_{(WT, KO)}$ × 'Cue Allocation to CS+'$_{(white\ noise,\ tone)}$ × 'Auditory Cue'$_{(white\ noise,\ tone)}$) revealed no significant differences between genotypes, or between the cue allocation to the CS+, or a significant interaction between genotype and cue allocation (main effects of genotype and interaction between genotype and cue allocation: all Fs < 1). A statistically significant effect was detected between genotype and auditory cue ($F(1,55) = 19.3$, $p < .001$), with WT mice freezing more

in the presence of the white noise than the tone, and the KOs exhibiting an opposite pattern. However, no significant differences were detected regarding the responses to the 'to-be-allocated' CS types (i.e. the future CS+ and CS–; main effect of CS: F <1; $Genotype_2 \times CS_2$ interaction: $F(1,57) = 1.8$, $p = .180$). During the PreEx session, a significant interaction between genotype and sex was obtained ($F(1,51) = 5.9$, $p = .019$. Female WT mice froze significantly less than male WTs, and these mice froze significantly more than male KOs.

Analyses of the UR to the very first presentation of the auditory cues on the pre-exposure session were performed by comparing the freezing behaviour during a 5s-long pre-cue period ('pre-Cue phase') to the freezing behaviour during a 5s-long period after cue onset ('Cue phase'). Analyses of the freezing responses revealed no observable UR to the first auditory cue presented in both genotypes (main effect of phase $F < 1$). A two-way between-subjects ANOVA, with genotype and auditory cue as factors, indicate that this was the case for both auditory cues (main effects of auditory cue and genotype: Fs < 1; $Genotype_2 \times Cue_2$ interaction: $F(1,55) = 1.1$, $p = .293$). The latter result cannot be explained by differences in pre-Cue freezing levels (main effect of genotype and $Genotype_2 \times preCue_2$ interaction: Fs < 1).

Analyses of the haemodynamic responses to the 30s-long auditory cues presented on the PreExp session revealed a small increase of TO_2 responses during the presentation of the auditory cues in both groups, with a slight suggestion for bigger TO_2 responses in the 5-HTTKO mice (see shaded area of Figure 5.5, middle). However, statistical analyses (ANOVA model: $Genotype_2 \times$ 'Auditory Cue'$_{(white\ noise,\ tone)} \times Bin_{15}$) revealed no significant differences between genotypes (main effect of genotype: $F(1,21) = 1.9$, $p = .177$; $Genotype_2 \times Cue_2$ interaction: $F(1,21) = 1.5$, $p = .238$), or between the effects of the auditory cues (main effect of cue: $F < 1$). Additionally, no significant differences in the responses to the 'to-be-allocated' CS types were registered (main effect of CS: $F < 1$; $Genotype_2 \times CS_2$

interaction: $F(1,21) = 2.1$, $p = .159$). A significant interaction between genotype, auditory cue and sex was obtained ($F(1,19) = 5.4$, $p = .032$). Male KO mice exhibited significantly greater mean TO_2 signals than male WT mice ($p = .037$).

Finally, analyses of the evoked theta (θ) power by the auditory cues on pre-exposure suggested bigger θ responses in the KOs, compared to WT controls (see Figure 5.5, right). However, no statistically significant differences between genotypes were found (ANOVA model: $Genotype_2 \times Cue_2$; main effect of genotype: $F(1,18) = 2.1$, $p = .166$; $Genotype_2 \times Cue_2$ interaction: $F < 1$), nor between the effects of the auditory cues (main effect of cue: $F(1,18) = 2.1$, $p = .160$). No significant differences in the responses to the 'to-be-allocated' CS types were registered (main effect of CS: $F < 1$; $Genotype_2 \times CS_2$ interaction: $F < 1$). This result cannot be explained by differences in the pre-CS θ power (main effect of genotype and $Genotype_2 \times pre\text{-}CS_2$ interaction: Fs < 1).

In short, no significant genotypic differences were found during the PreEx session to the auditory cues regarding behavioural, haemodynamic and electrophysiological responses.

5.4.2. Behavioural responses

5-HTTKO mice exhibited enhanced discriminative aversive learning

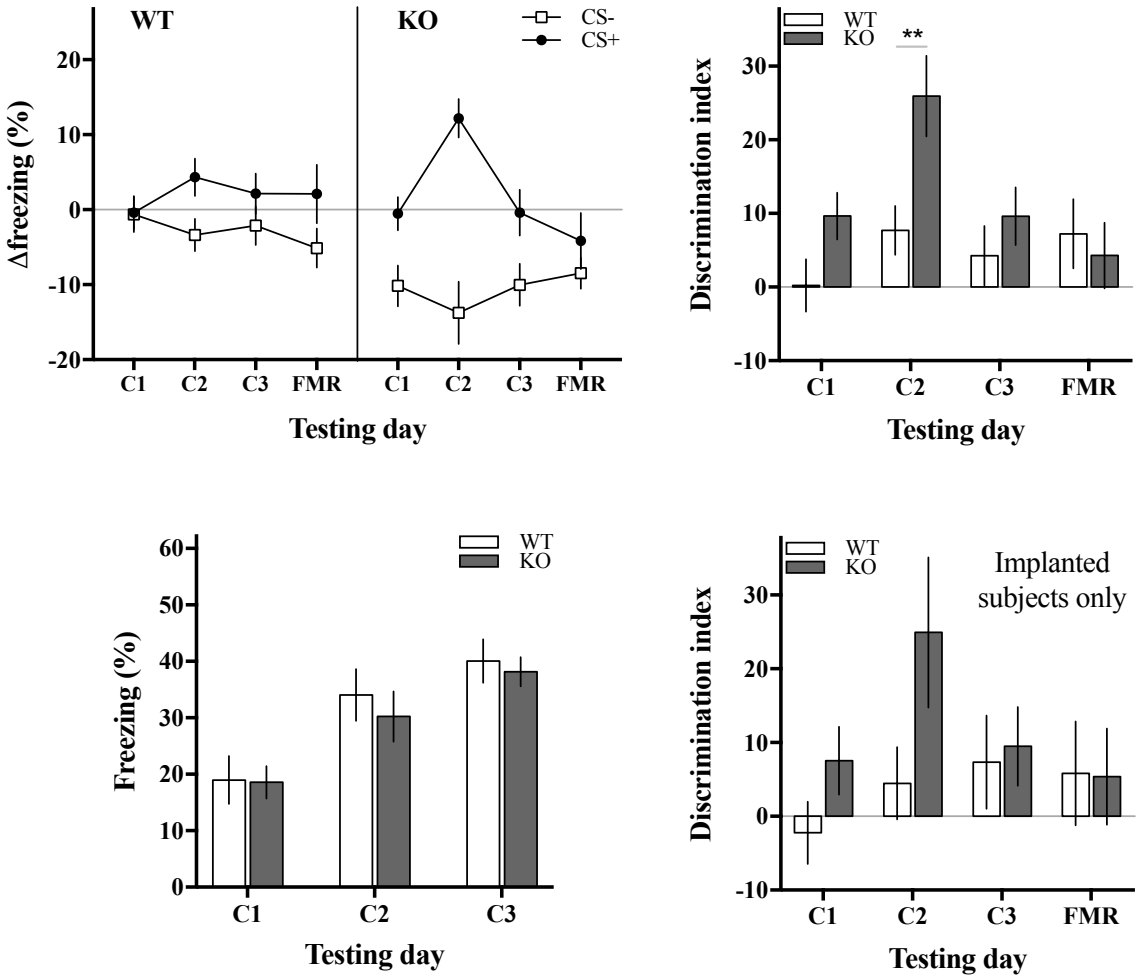


Figure 5.6. Top left, Cue-evoked Δfreezing for CS+ and CS– on the conditioning (C1, C2, C3) and FMR sessions for the 5-HTTKO (N=28) and WT (N=31) mice. **Top right,** Discrimination index (CS+ Δfreezing – CS- Δfreezing) during the same sessions for the same mice. Discriminative aversive learning was faster in the 5-HTTKO mice than WT mice. **Bottom left,** Mean levels of freezing behaviour registered during the lead-in period of C1, C2 and C3 for the 5-HTTKO (N=28) and WT mice (N=31). Freezing to the background context during fear conditioning was equivalent in both genotypes. **Bottom right,** Discrimination index during the conditioning and FMR sessions for the subjects with implanted electrodes that provided the BLA neuronal and/or hemodynamic responses presented in this chapter (KOs: n=13; WTs: 12). Implanted mice exhibited a similar pattern of behaviour to that exhibited by non-implanted mice. Mean ± SEM. ** $p < .01$ (simple effect of genotype on C2).

Analyses of Δfreezing (%) revealed that subjects learned to freeze more during the presentations of the CS+ compared to the presentations of the CS– over the 4-day period (main effect of CS: $F(1,57) = 18.2$, $p < .001$; see Figure 5.6, top left). However, genotypes significantly differed on their discrimination levels over the conditioning and FMR sessions ($Genotype_2 \times Day_4 \times CS_2$ interaction: $F(3,171) = 3.3$, $p = .023$; see Figure 5.6, top right). Specifically, 5-HTTKO mice showed stronger cue discrimination levels than WT mice already during the first conditioning session (simple effect of genotype on C1: $p = .055$). Although WT mice then showed significant cue discrimination on the following day (C2; one sample *t*-test for the discrimination index exhibited during C2: $t(30) = 2.3$, $p = .027$), the KOs exhibited an even stronger cue discrimination level than that shown on the previous day. There was a significant genotypic difference regarding cue discrimination on C2 (simple effect of genotype on C2: $p = .005$). In short, 5-HTTKO mice exhibited facilitated discriminative aversive learning compared to WTs during the first two days of conditioning, replicating the main finding in Chapter 4.

WTs and 5-HTTKO mice showed equivalent levels of cue discrimination during C3 and FMR. Surprisingly, however, the discrimination levels were unusually low during that period for both groups of mice. Indeed, WT mice failed to show significant cue discrimination both on C3 and FMR (one sample *t*-test for the discrimination index exhibited during C2: $t(30) = 1.1$, $p = .302$; FMR: $t(30) = 1.5$, $p = .135$). 5-HTTKO mice, however, still showed significant discrimination on C3, though not on FMR (C3: $t(27) = 2.5$, $p = .021$; $t(27) = .962$, $p = .345$).

Both genotypes froze significantly more to the background context during the initial 300s of the C2 and C3 sessions compared with the same period of C1 (main effect of day: $F(2,114) = 16.8$, $p < .001$; pairwise comparison between C1 and C2: $p = .009$; C1 versus C3: $p < .001$; see Figure 5.6, bottom left). No genotypic differences were seen regarding

increased freezing to the background context (main effect of genotype and $Genotype_2 \times Day_3$ interaction: both $Fs < 1$).

5.4.3. Tissue oxygen responses

Haemodynamic responses to the CS+ and CS− were larger in 5-HTT KO mice

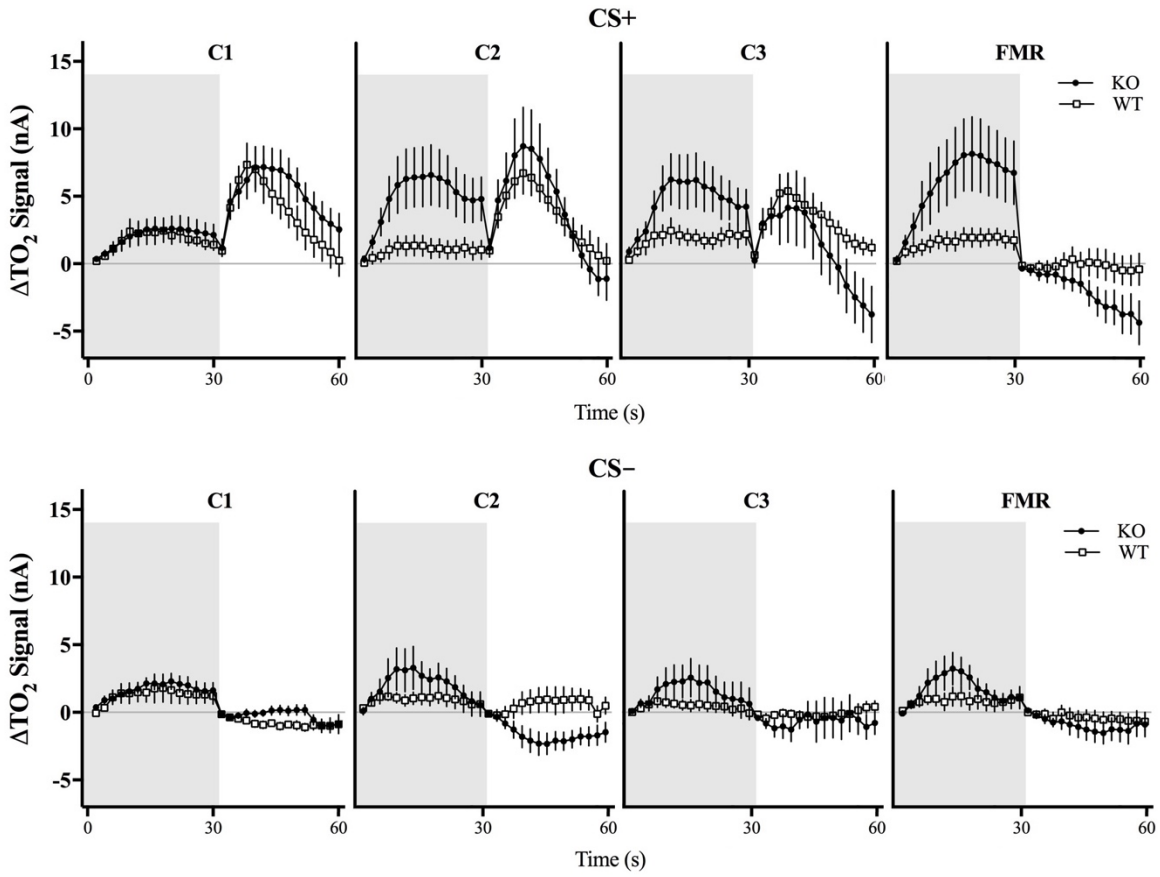


Figure 5.7. Top, BLA TO_2 signal responses during the 30s-long CS+ period and the following 30s-long ('US') period during the conditioning (C1, C2, C3) and FMR sessions for the 5-HTTKO mice (N=11) and WT mice (N=12). **Bottom,** BLA TO_2 signal responses during the 30s-long CS− period and the following 30s-long non-US (i.e. absence of footshock) period during the same sessions for the same subjects. 5-HTTKO mice exhibited larger haemodynamic responses to the CS+ and, to a lesser extent, the CS−. Mean ± SEM.

Analysis of the stimuli-evoked TO_2 responses (CS−, CS+, US) revealed that the two genotypes responded to stimuli differently during training/recall ($Genotype_2 \times Day_4 \times Stimuli_3$

interaction: $F(6,126) = 2.7$, $p = .017$; $Genotype_2 \times Day_4 \times Stimuli_3 \times Bin_{15}$ interaction: $F(84,1764) = 1.8$, $p < .001$; see Figure 5.7). At an early stage of conditioning (C1), 5-HTTKO mice and WT controls exhibited equivalent TO_2 responses to the CS+ and CS– cues, although 5-HTTKO mice exhibited larger TO_2 responses to the US. During C2, the KOs exhibited larger TO_2 responses to both the CS+ and CS– than the WT mice, although only the CS+ ΔTO_2 responses were significantly different between genotypes (simple effect of genotype for the CS+: $p = .029$; CS–: $p = .273$). This pattern remained during C3, and FMR, where a significant genotype difference was again seen in the CS+ evoked TO_2 responses (FMR results: CS+: $p = .043$; CS–: $p = .120$).

Additionally, 5-HTTKO mice clearly exhibited greater TO_2 responses to the CS+ than to the CS– from C2 onwards, though only on FMR were these responses significantly different (simple effect of CS type in the KOs: $p = .018$; WTs: $p = .929$). WT mice only showed considerable discrimination between the CSs with their TO_2 responses during C3 (a repeated measures ANOVA during C3 ($CS_2 \times Bin_{15}$) for the WT data revealed the following main effect of CS: $F(1,11) = 4.6$, $p = .055$). In sum, both groups showed greater TO_2 responses to the CS+ than to the CS– during conditioning training, though WT mice took longer to exhibit such a pattern and their TO_2 signals showed weaker discrimination compared to the KOs.

Larger CS+ evoked TO_2 responses in the KOs on C2 were preceded by larger US-evoked TO_2 responses on the previous session

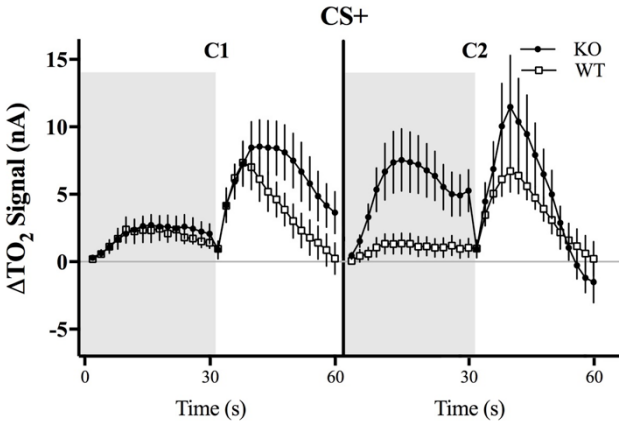


Figure 5.8. BLA TO_2 signal responses during the 30s-long CS+ period and the following 30s-long ('US') period during conditioning (C1 and C2) for the 5-HTTKO mice (N=12) and WT mice (N=12). This graph includes data from an additional 5-HTTKO mouse (see methods section). US-evoked TO2 responses were significantly larger in the 5-HTTKO mice than in WT mice during C1 (see unshaded area). Mean ± SEM.

During the conditioning phase, administration of footshock evoked a marked increase in the TO_2 responses, which was evident for ~30s after shock termination. No such TO_2 response was seen in the corresponding periods following the CS– cue (main effect of stimulus, i.e. US vs. 'no US', across C1, C2 and C3: $F(1,21) = 26.6$, $p < .001$). Analyses focused only on the US periods revealed that the TO_2 responses during these periods varied between the genotypes ($Genotype_2 \times Day_3 \times Bin_{15}$ interaction: $F(28,588) = 2.4$, $p < .001$; see Figure 5.7, unshaded areas). These US-evoked TO_2 responses were significantly larger in the 5-HTTKO mice than in WT mice during C1 but not C2 ($Genotype_2 \times Bin_{15}$ interaction on C1 only: $F(14,308) = 2.1$, $p = .012$; C2: $F(14,308) = 1.5$, $p = .099$; see Figure 5.8). In short, KOs had larger TO_2 responses to footshock but only during C1.

5.4.4. Local field potentials

5-HTTKO mice exhibit faster development of stronger CS+ evoked θ power

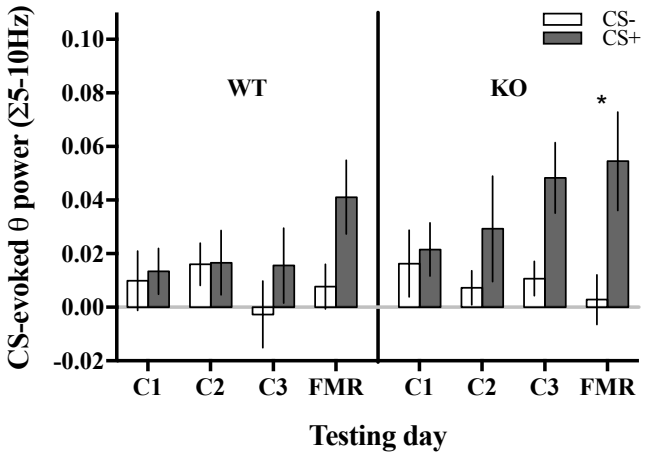


Figure 5.9. CS-evoked theta (θ) power (i.e. CS θ power minus pre-CS θ power) between 5 and 10 Hz during the conditioning (C1, C2, C3) and FMR sessions for 5-HTTKO mice (N=10) and WT mice (N=10). The CS+ comes to evoke higher levels of theta power than the CS– in both groups with training, but this develops faster in the 5-HTTKO mice. Mean ± SEM. *$p < .05$.

Although no statistically significant interactions were detected ($Genotype_2 \times Day_4 \times CS_2$ interaction: $F < 1$), investigation of how the theta power (θ = 5-10Hz) evoked by the CSs varied in each genotype across the conditioning phase and FMR session was justified based on previous findings of reduced theta oscillations in mice with increased 5-HTT expression (Barkus et al., 2014; see discussion below). Additionally, no significant genotypic differences were observed during the pre-CS periods (main effect of genotype, $Genotye_2 \times preCS_2$ interaction and $Genotype_2 \times Day_4 \times preCS_2$ interaction: Fs < 1; $Genotype_2 \times Day_4$ interaction: $F(3,54) = 1.6$, $p = .204$).

In the first conditioning session, both CSs evoked similar levels of theta power in each group (see Figure 5.9). However, from C2 onwards the CS+ evoked higher levels of theta power compared with the CS– in the 5-HTTKO mice. On C3, a trend could be registered in this group ($p = .081$), and in the following FMR day a significant difference

between the theta power evoked by each CS was registered (p = .010). A similar evolution of increased theta power evoked by the CS+ compared with the CS− was also observed in the WT mice, although it took them longer to exhibit this effect. On FMR, a trend in data could be registered in the WT mice regarding the difference in theta power evoked by each of the CSs (p = .080). No significant genotypic difference was observed for any of the cues in any of the conditioning and FMR sessions.

In summary, the CS+ came to evoke higher levels of theta power in both groups with training whereas the CS− did not. Notably, this development occurred earlier in the 5-HTTKO mice compared with the WT controls. However, some caution is merited in this observation given that statistically significant interactions between day, cue and genotype were not found.

5.5. Discussion

Summary of the results

Compared to WT mice, 5-HTTKO mice exhibited enhanced discriminative aversive learning by exhibiting greater cue discrimination levels early in the conditioning training, consistent with the findings in Chapter 4. Moreover, 5-HTTKO mice exhibited significantly larger haemodynamic responses to the CS+ and, to a lesser extent, the CS–, during training and this pattern was maintained during the FMR session. Notably, the larger TO_2 responses evoked by the CS+ on the second session of conditioning were preceded by larger TO_2 responses to the aversive stimulus on the previous session. Additionally, during training, the CS+ came to evoke stronger theta oscillations than the CS− in both groups, but this change occurred earlier and more robustly in the 5-HTTKO mice than WT controls. Importantly, these genotypic differences were not observed during the PreEx session, suggesting that the

distinct patterns of BLA haemodynamic and oscillatory responses to the CSs were a consequence of the aversive learning task.

Subjects	Task/process and imaging method(s)	Effect/results	Authors/year
5-HTTKO mice	Single-cue fear conditioning [6 × 30s CS+] → FMR session [1 × 1min CS+] rCBF (perfusion autoradiography with [^{14}C]-iodoantipyrine, followed by 3-D reconstruction of the digitised autoradiographs)	– Rate of fear acquisition ↑ Fear expression ↑ rCBF in the amygdala, particularly the LA ↑ freezing correlated with ↑ rCBF in the BLA	Pang et al., 2011
	Habituation [5×CS-] → cued fear conditioning [2×(3×CS+)] → extinction training [6×(4×CS- and 4×CS+)] → extinction recall [4×CS- and 4×CS+] LFPs electrodes implanted into the LA and infralimbic of the mPFC	– fear expression – fear extinction ↓ extinction recall ↑ amygdala-PFC θ synchrony during extinction and recall (CS+ only), particularly during freezing episodes	Narayanan et al., 2011
	cued fear conditioning [3×CS+] → extinction training [40×CS+] → extinction recall [40×CS+] spine density and dendritic morphology of Golgi-stained BLA pyramidal neurons	– fear acquisition, expression, extinction; ↓ extinction recall ↑ spine density – dendritic morphology	Wellman et al., 2007
5-HTTOE mice	Pre-exposure to the auditory cues → cued fear conditioning [3days×(5×CS+/5×CS-)] → fear memory recall [5×CS+/5×CS-] TO_2 voltammetry and LFPs recordings from the BLA	↓ rate of fear discrimination learning ~ Fear expression ↓ TO_2 responses and ↓ θ oscillations to the CS+	Barkus et al., 2014

Table 5.2. Studies investigating altered amygdala function and structure in genetically modifying mice with altered levels of 5-HTT expression. ↑, enhanced; ↓, diminished; – , no changes; ~, similar; rCBF, regional cerebral blood flow; θ, theta; →, followed by; CS+, cue paired with a footshock; CS–, cue predicting the absence of footshock delivery.

Facilitated discriminative aversive learning in 5-HTTKO mice

5-HTTKO mice exhibited better fear discrimination learning than WT mice, which replicates the findings in Chapter 4. Notably, strong genotypic differences were seen early on during conditioning training in both experiments. However, whereas a significant genotypic difference was seen at the end of C1 in the previous experiment (see Chapter 4, section 4.3.2), a statistically significant difference was only registered on C2 in the present experiment. It is possible that latent inhibition resulting from the exposure to the auditory cues during the PreEx session of the present experiment might have slowed the rate of conditioning and thus contributed to this delayed statistically significant result. As previously seen, it also took another day of conditioning for WT mice to catch up with the discrimination level that the 5-HTTKO mice were exhibiting by the end of C1.

Importantly, significant genotypic differences were also seen on C2 in a previous study testing 5-HTTOE mice and their WT controls in the same discriminative fear learning procedure used in this chapter (Barkus et al., 2014; see Table 5.2 and Appendix 18). It is thus possible that altered extracellular 5-HT availability only affects the rate of learning of discriminative fear conditioning. Notably, the opposing effects of high versus low extracellular 5-HT availability reported during FMR sessions in single-cue fear conditioning procedures also occurred 24 hours after the first and only conditioning session (i.e. during FMR; Barkus et al., 2014; Line et al., 2014; Pang et al., 2011; see Chapter 4, Table 4.2).

Considering the low levels of CS+ Δfreezing exhibited by the WT mice throughout, particularly when compared to those seen in the Chapter 4 (see section 4.3.2), it is worth noting that WT counterparts of 5-HTTOE mice tested by Barkus and colleagues (2014) also exhibited similar low levels of freezing (i.e. below 10% on average from C2 to FMR; see Appendix 18). Still, WT mice clearly learned to behaviourally discriminate the cues in a significant way in the current experiment, as they had previously done in Experiment 1 of Chapter 4.

Both groups showed similar cue discrimination levels on the last conditioning day (C3), and when tested in a FMR session. Strikingly, however, notwithstanding that the discrimination levels were clearly positive (i.e. CS+ > CS–), they were considerably lower during C3 for 5-HTTKO mice than they had exhibited behaviourally on C2 (see Figure 5.6. top left). Although they were still capable of showing significant cue discrimination by C3, there was a dramatic reduction in their discrimination performance from C2 to C3 that mostly reflected a decrease in freezing to the CS+. This pattern was seen both in implanted and non-implanted subjects, but crucially only in the 5-HTTKO mice. It is worth mentioning, however, that reductions in freezing throughout conditioning training are not unprecedented. Indeed, when subjected to the same procedure used in this chapter, WT controls for 5-HTTOE mice exhibited a drop of nearly 50% of CS+ Δfreezing from C2 to C3 in the study conducted by Barkus and colleagues (2014; see Appendix 18).

This study administered 15 CS-US pairings, which is considerably more compared with other studies that have had 5-HTTKO mice fear conditioned, which used between 3 to 6 CS-US pairings (see Table 5.2). It is possible that with so many pairings the KOs might have found that freezing to the CS+ was not adaptive, given that they would still be shocked at the end of the cue presentation. Thus, one possible explanation for the reduction in CS+ Δfreezing could be that footshocks were "punishing" freezing behaviour. Visual inspection of the raw data does suggest that the response to the CS+ was decreasing gradually in the KOs from the beginning of C2 to the end of C3 (see Appendix 7). However, a more quantitative analysis failed to support this hypothesis. Indeed, freezing at the time of the footshock delivery during C2, and particularly during the last footshock presentation, predicts more CS+ Δfreezing on C3 in the KOs, and not less (see Appendix 17). Moreover, no overall decrease in freezing was seen during C3, as visual inspection of the pre-CS freezing levels indicates (see Appendix 7).

Nevertheless, overtraining (i.e. many conditioning trials) might be the most likely explanation for the changes in CS+ Δfreezing observed in the KOs from C2 to C3. Indeed, one study conducted by Laxmi and colleagues (2003) provides some evidence for this hypothesis. In their study of cued fear conditioning, different groups of C57Bl/6J mice were subjected to different training intensities, varying both the US intensity (e.g. 0.4 or 0.6 mA, for 1s) and the number of CS-US pairings delivered (e.g. 3 or 10). Focusing on the groups receiving a 0.4mA footshock, they showed that the amount of freezing behaviour to the CS+ declined considerably when 10 pairings were given instead of 3 (see a copy of their Figure 2.A in Appendix 18). Therefore, it is not implausible to imagine that mice receiving 15 CS-US pairings instead of 10 like in Experiment 1 of Chapter 4 could reduce their expression of freezing to the CS+ by C3, possibly because they would engage in other defensive behaviours (Laxmi et al., 2003; Maren, 2008). Considering why only the KOs would exhibit such a reduction in freezing, it is worth referring to the results shown in Chapter 4 about an increased reactivity to footshock seen in the KOs when several footshocks are successively presented during C1. Despite both groups receiving five shocks, this was more aversive to the KOs with repetitive presentations (see Chapter 4, section 4.5.2). Although this may have facilitated learning in the early stages of conditioning for the 5-HTTKO mice, it might have accelerated a reduction in CS+ Δfreezing seen at a later period of conditioning training.

Finally, no significant genotypic difference was seen regarding conditioned freezing to the background context during fear conditioning training. This result replicates the finding reported in Chapter 4.

The enhanced discrimination learning in 5-HTTKO is accompanied by larger BLA haemodynamic responses

During the conditioning phase, TO_2 responses to the CS+ and, to a lesser extent, to the CS− were larger in the 5-HTTKO mice than in WT mice. Moreover, the CS+ ΔTO_2

responses exhibited by the 5-HTTKO mice were considerably larger than those evoked by the CS–, which mirrored the discrimination learning shown by these mice (i.e. CS+ Δfreezing > CS– Δfreezing), particularly during the conditioning phase. Regarding the WT mice, they also showed some differential TO_2 responses to the CS+ and CS– (on C3) that mirrored the direction of the positive (i.e. CS+ > CS–) cue discrimination learning shown during conditioning training (e.g. on C2). However, this was less consistent than exhibited by the KOs. It is worth noting, however, that other studies subjecting WT mice to a similar procedure to the one used in this chapter have not reported significant CS+/CS– discrimination in TO_2 responses for these mice on individual conditioning days (McHugh et al., 2014; Barkus et al., 2014).

These findings are consistent with one study conducted by Pang and collaborators (2011), who showed, by means of perfusion autoradiography, that one session of cued fear conditioning increased regional CBF in the amygdala of both 5-HTTKO mice and WT controls during a FMR session administered 24h later. Importantly, these researchers also showed that the enhanced fear expression exhibited by the 5-HTTKO mice during FMR was accompanied by a significantly greater increase in regional CBF in the amygdala (particularly in the lateral amygdala) compared to WT mice.

Additionally, the findings reported here complement and further validate the findings obtained with 5-HTTOE mice subjected to the exact same procedure. Barkus and colleagues (2014) showed that 5-HTTOE mice exhibit reduced BLA TO_2 responses to the CS+ compared with their WT controls during discriminative fear conditioning. Notably, the BLA TO_2 responses obtained with 5-HTTKO mice discriminate the CS+ and CS– in a similar but more pronounced way to what the WT controls for the 5-HTTOE mice exhibited (see Appendix 19). Additionally, whereas the mean TO_2 response evoked by the aversive cue was significantly higher in the WTs than the 5-HTTOE mice at the end of training (C3) in

the Barkus et al. study, in the current study 5-HTTKO mice showed significantly higher TO_2 responses during the CS+ presentations during C2 (and on the FMR session). Therefore, the cue-evoked haemodynamic responses recorded from the BLA of 5-HTTKO mice differed from those exhibited in their WT controls in an opposite pattern to that exhibited by 5-HTTOE mice in relation to their WT counterparts.

The relatively larger TO_2 responses evoked by the CS− in the 5-HTTKO mice, compared to WT controls, could also be a consequence of augmented BLA function. Research using discriminative aversive learning procedures has shown that the BLA has neurons which specifically code a (relatively) safe cue such as the CS− (Sangha et al., 2013; Genud-Gabai et al., 2013). Notably, these cells can develop strong responses simultaneously to others developing strong responses to the CS+. In addition, the BLA is a structure connected with multiple other circuits likely to be involved in the processing of safety signals. For instance, the insular cortex has projections to the basal amygdala and it has been shown that either pre-training lesions or pharmacological inactivation of the (posterior) insula can eliminate the effects of safety signals on behaviour (Christianson et al., 2008). Given that the insula is a brain area that has been shown to be, in general, significantly more activated in 5-HTTKO mice than WT controls (Pang et al., 2011), altered connectivity between these two structures could underlie the genotypic differences seen here regarding BLA activity during the CS− presentation. Notably, healthy human participants with (putatively) low levels of 5-HT availability have been shown to exhibit stronger activation in the insula during discriminative fear learning in response to the CS+ versus CS− cues (Hermann et al., 2012).

Larger BLA haemodynamic responses in the 5-HTTKO mice could reflect increased attention to emotionally relevant stimuli

One plausible explanation of larger BLA TO_2 responses evoked by both the CS+ and CS− in the 5-HTTKO mice could be that it reflects increased attention paid to both the cues by the KOs compared to WT mice. Several researchers have argued that the amygdala plays a crucial role in attention and vigilance (Kapp et al., 1992; Holland & Gallagher, 1999; Davis & Whalen, 2001). Although the focus of those researchers has usually been the central nucleus of the amygdala and its output to subcortical areas, the BLA is also thought to play a role in increasing attention to stimuli and thus modulating stimulus processing (Roesch et al., 2010; Roesch et al., 2012; Janak & Tye, 2015). After encoding associate information, the BLA could augment the allocation of attentional recourses to drive acquisition of that information by other downstream areas requiring BLA for associative encoding (Roesch et al., 2010). This could make, for instance, the stimuli more associable or salient in downstream regions after several presentations (Roesch et al., 2012). This increased salience promoted by the BLA could lead to enhanced memory storage in downstream regions and, probably, enhanced learning (Janak & Tye, 2015).

Importantly, the BLA can represent both aversive and rewarding USs (e.g. Gore et al., 2015), and if one considers the absence of a footshock (i.e. safety) following a CS− as rewarding (e.g. Likhtik & Paz, 2015), then this could explain that an altered attentional signalling in the BLA of 5-HTTKO mice could lead to larger TO_2 responses to both the CS+ and CS−. Indeed, it has been shown that a subset of BLA neurons that respond to the CS− also fire to a CS predicting reward, suggesting that safety and reward cue processing involve overlapping circuitry (Sangha et al., 2013).

Consistent with the hypothesis of altered attentional signalling in the BLA of 5-HTTKO mice, the higher TO_2 responses to the US seen in these mice on C1 (see Figure 5.8)

could reflect enhanced attention to the initially surprising administration of that US. This could then promote the stronger cue discrimination learning seen in the KOs on C2 if the US were acting as teaching signals. This could be achieved if the USs triggered plasticity at the CS input synapses into the BLA (McNally et al., 2011), with the first presentations of the US likely leading to greater synaptic plasticity than the later ones (see also Li & McNally, 2014). Notably, this enhanced attention to footshock could parallel the increased behavioural reactivity to the successive US presentations that the 5-HTTKO mice exhibit on C1 compared to WT mice (see Chapter 4, section 4.5.2).

Also consistent with the above-mentioned hypothesis of the BLA's role in attention and vigilance, it has been shown that BLA neurons can respond more robustly to aversive USs when they are presented unexpectedly rather than when they are predicted by a CS (Belova et al., 2007; Johansen et al., 2010). This could have happened early in conditioning (i.e. C1), but also during the FMR (i.e. early in extinction learning; see Quirk et al., 1997). Indeed, situations where stimulus contingencies are changed are thought to generate greater attention levels from the amygdala, and so are those introducing unexpected sources of safety or a more ambiguous stimulus, such as extinction (Davis & Whalen, 2001; McNally et al., 2011). Notably, 5-HTTKO mice exhibit a significantly larger response to the CS+ during the FMR than WT mice during the FMR session (which is an extinction session). Moreover, despite the inter-subject variability seen in the KOs regarding CS+ ΔTO_2 response on FMR, this response seems to have remained at high level for a longer period during FMR than what was seen in the same mice on C3.

Faster acquisition of stronger CS+ evoked theta power in the 5-HTTKO mice

The CS+ acquired the capacity of evoking relatively stronger levels of theta power (5-10Hz) compared to the CS− in both genotypes. Notably, however, this development occurred earlier in 5-HTTKO mice than in WT controls. The first noticeable changes

occurred on the days when each group showed for the first time a stronger CS+ TO_2 evoked response (C2, for the KOs; C3, for the WTs). Importantly, these results indicate that the BLA responsivity was increased selectively to the CS+, as other studies have shown (Collins & Paré, 2000; Goosens et al., 2003; Seidenbecher et al., 2003). This might reflect selective synaptic potentiation, which has been shown to occur in CS+ pathways to the BLA during discriminative fear conditioning, but not in CS– pathways (Kim & Cho, 2017).

Synchronised oscillatory activity in the theta frequency in the BLA is thought to promote synaptic plasticity and facilitate the consolidation of emotional memories (Paré et al., 2002; Pape & Paré, 2010). Moreover, several studies have provided evidence for the importance of theta oscillations in the processing of aversive stimuli. For instance, Paré and Collins (2000) have shown in cats that not only the firing rate of lateral amygdala neurons doubled during the presentation of an aversive CS+, but the probability of synchronisation also doubled during this period. By analysing data obtained with single-unit recordings from multiple pairs of simultaneously recorded LA neurons, these researchers detected that the activity of those neurons synchronised at the theta frequency range (4-7Hz) during the anticipation period. Seidenbecher and collaborators (2003), complemented these findings by showing in mice that periods of retrieval and/or expression of conditioned fear were specifically associated with a shift in LA neuronal activity with no predominant frequency towards a pronounced rhythmic activity at the theta frequency (~5Hz). Later, Pape and colleagues (2005) showed that this pattern of neuronal activity was specifically related to long-term (i.e. 24 h) fear memory recall and not, for instance, the expression of conditioned fear behaviour *per se*. Moreover, they also reported increased theta synchronised activity (4-8Hz) between the LA and CA1 during the presentation of the aversive cue during a FMR session administered 24 h after conditioning (Pape et al., 2005).

Theta oscillations have been hypothesised to synchronise activity across the fear memory network (i.e. amygdala-hippocampus-mPFC), thus increasing the communication

between its constituents via facilitated synaptic interactions in co-active structures (Paré et al., 2002; Seidenbecher et al., 2003; Pelletier & Paré, 2004). Theta oscillations are seen as an efficient mechanism that makes BLA neurons synchronise first with each other and then with afferent neurons (e.g. from the hippocampus), with minimal change in their firing rates (Pape & Paré, 2010). This coincident activity induces synaptic plastic locally, but can also, in an efficient way, enhance the BLA activity output on target structures (Paré et al., 2002). Such a mechanism could promote and consolidate fear memories. However, other theories exist about the function of theta oscillations. For instance, theta oscillations could facilitate learning via attentional modulation of the flow of information about stimuli, instead of representing them (Sejnowski & Paulsen, 2006). In this case, they could regulate neuronal communication by filtering the salient and significant inputs to the network.

The results obtained in this chapter are consistent with a study reporting enhanced theta-frequency (4–8 Hz) activity in the amygdala-PFC network of 5-HTTKO mice during the presentation of an aversive CS+ (Narayanan et al., 2011). However, this study measured theta activity only during fear extinction and extinction recall, and no indication of the neuronal activity measured specifically from the amygdala was reported (i.e. it was not clear whether amygdala theta oscillations were larger per se). Importantly, 5-HTTOE mice have been shown to exhibit significantly lower CS+ evoked theta power than their WT controls during discriminative fear conditioning training and FMR (Barkus et al., 2014). Although no major significant differences were seen between 5-HTTKO mice and WT controls in this chapter regarding CS+ evoked theta power, it is worth noting that, at least numerically, the CS+ evoked higher levels of theta power in the KOs than WTs during training (e.g. C3). Taking these results together, it seems that there exists a modulatory effect of different extracellular 5-HT levels on amygdala neuronal oscillations, particularly in the theta-frequency range.

Although studies have shown that changes in BLA neuronal activity can precede behavioural changes (Repa et al., 2001; Herry et al., 2008), this was not the case in the current study. Indeed, only after some behavioural cue discrimination had been seen, was it possible to see CS+/CS− discrimination in the cue-evoked theta power. However, this is consistent with one study conducted by Narayanan and colleagues (2007) who showed that CS+ presentations 24h after a conditioning session could originate theta activity, but not at 2h later.

Regarding the occasional CS− evoked theta activity registered during conditioning in the present study, this does not fit with other reports indicating that BLA responsivity should decrease with discriminative fear learning (Collins & Paré, 2000) or safety learning (Rogan et al., 2005). However, some of these studies recorded specifically from the lateral amygdala, whereas this one did not. Importantly, these brain areas have distinctive connections with various areas (Duvarci & Paré, 2014), which might explain part of this inconsistency between studies. Another reason might be related to methodology or the data analyses conducted. For instance, McHugh and colleagues (2014) have shown a decrease in theta evoked by the CS− in WT mice subjected to a discriminative fear learning procedure like the one used in this chapter by analysing peak theta power during the CS− presentations. Nevertheless, they did observe CS− evoked theta activity during conditioning. Moreover, Seidenbecher and colleagues (2003) also reported a non-significant but partial increase in theta synchronisation (4-8Hz) during CS− presentations.

Additionally, Likhtik and colleagues (2014) have shown that BLA-mPFC synchronisation in the theta range is observed during both fear discrimination and safety learning (Likhtik et al., 2014). Particularly, switches from a fearful to a safety state occur in the BLA oscillations, which become more coupled with theta input from the mPFC during the presentations of the CS−. These researchers also observed that only those animals

showing behavioural discrimination during training exhibited larger increases in theta power during the CS+ compared to the CS−. Notably, the data obtained with 5-HTTKO mice in this chapter fits with this result, given that these mice not only showed strong CS+/CS− discrimination in their behavioural and haemodynamic responses at an early stage of conditioning, but also larger increases in theta power during the CS+ compared to the CS−.

BLA responsivity does not provide a simple readout of freezing behaviour

In addition to the above-mentioned findings, Pang and collaborators (2011) also reported that increased freezing scores were correlated with increased regional CBF specifically in the BLA, in both 5-HTTKO mice and WT controls. McHugh and colleagues (2013) have shown that a TO_2 difference score (i.e. CS+ minus CS− evoked TO_2 responses) was significantly correlated with the magnitude of the CS+/CS− discrimination when subjecting rats to a similar procedure to the one used in this chapter. Additionally, Narayanan and colleagues (2011) showed that a reduction in conditioned freezing during extinction and extinction recall was paralleled by reduced theta synchronisation between the lateral amygdala and mPFC in WTs, and high freezing levels exhibited by 5-HTTKO mice during the latter session showed highly synchronized LA- mPFC theta during freezing episodes. Taking these reports together (and other studies, such as Seidenbecher et al., 2003), it seems that BLA responsivity could work as a readout of freezing behaviour.

However, this was not the case in the current study. Indeed, changes in behaviour, TO_2 responses and theta oscillations did not occur at similar states. Indeed, whereas changes in behaviour were seen on the first two days, changes in the BLA responses were mainly occurring on days 2 and 3. One possible reason for this could be that multiple brain regions might compete with the BLA for controlling the behaviour during the initial states of fear learning in a task such as the one used in this chapter (e.g. lateral central amygdala; see, for

instance, (Tovote et al., 2015). Furthermore, it is not uncommon to see such temporal differences between changes in behaviour and BLA activity. This is in line with the study conducted by Barkus and collaborators (2014), who reported that despite similar levels of behavioural discrimination by the end of conditioning (C3 and FMR), the BLA haemodynamic responses in the 5-HTTOE mice and WT controls were significantly different (see Appendix 19). Importantly, other studies in mice have shown that BLA neurons can respond to a CS-US association irrespective of a behavioural expression of fear (Goosens et al., 2003; Pape et al., 2005; Narayanan et al., 2007), thus strengthening the claims that plasticity in the BLA is of an associative nature (Maren & Quirk, 2004; Pape & Paré, 2010). Therefore, the pattern of BLA responsivity does not necessarily provide a simple readout of freezing behaviour. Crucially, this also rules out the possibility that genotypic differences in behaviour could explain the differences seen in the BLA responses to the cues in this chapter.

Conclusion

This chapter aimed to investigate neural correlates of the enhanced discriminative aversive learning previously observed with 5-HTTKO mice in Chapter 4. 5-HTTKO mice and WT controls were thus tested in a similar cued fear conditioning procedure, with a subset of them simultaneously subjected to invasive haemodynamic and LFP recordings from the BLA during pre-exposure, conditioning and fear memory recall sessions. Enhanced fear discrimination learning was seen in 5-HTTKO mice and this was accompanied by altered BLA responsivity. These mice exhibited heightened haemodynamic responses to the conditioned cues, particularly to the aversive CS+ cue, and also to the aversive US. Both groups showed higher TO_2 responses to the CS+ compared to the non-aversive CS– during conditioning training, but the KOs exhibit this pattern first. Compared to WT mice, the KOs

also exhibit a faster development of stronger theta-frequency neuronal oscillations to the CS+ compared to the CS−, although this was not statistically robust.

These findings are in line with the prediction that 5-HTTKO mice would exhibit the opposite pattern of behavioural, haemodynamic and neuronal responses seen with 5-HTTOE mice during discriminative fear conditioning (Barkus et al., 2014). Therefore, the hypothesis that altered 5-HTT expressions levels in the BLA alter its functioning and that this underlies altered discriminative fear learning was further strengthened with evidence of bidirectionality in the three measures registered: less extracellular 5-HT levels leads to decreased BLA reactivity and slower discriminative aversive learning (and impaired simple fear conditioning; data from 5-HTTOE mice), and more extracellular 5-HT levels leads to enhanced BLA reactivity and faster/enhanced discriminative aversive learning (data from 5-HTTKO mice). This also supports the claims advanced by human neuroimaging studies that participants with (putatively) higher levels of 5-HT availability should exhibit increased amygdala activation towards aversive stimuli, reflecting increased sensitivity to emotionally significant environmental stimuli (Hariri et al., 2006; Williams et al., 2009; Jonassen & Landrø, 2014).

However, there are some limitations that advise caution with these conclusions. In particular, there is a possibility that the changes seen in the neuronal oscillations of the BLA might be volume conducted from other brain regions (Kajikawa & Schroeder, 2011), even though some researchers claim that these oscillations can be generated locally in the amygdala (e.g. Pape & Paré, 2010). Additionally, as noticed by Pang and colleagues (2011), it is also possible in theory that increased 5-HT due to the absence of 5-HTT might alter blood pressure, which could affect regional CBF rather than neuronal activity. These and other issues will be further discussed in Chapter 8 (section 8.4).

Finally, there is also the question of whether the effects seen here resulted from elevated extracellular 5-HT levels in the amygdala, or from neurodevelopmental changes

due to the absence of the 5-HTT in early life. Indeed, 5-HTTKO mice show several alterations in their amygdala neurons and in other brain structures relevant for fear learning (Li et al., 2003; Wellman et al., 2007; Nietzer et al., 2011; Jennings et al., 2012; see also Chapter 6). To further validate the neuroplasticity theory of 5-HT function being tested in this thesis, a different approach that raises 5-HT levels specifically at the time of conditioning should be conducted in normal mice subjected to the same task to verify whether a similar behavioural phenotype was produced. In the following chapter, this will be achieved with pharmacological blockade of the 5-HTT function in subjects from the mouse strain used to generate the 5-HTTKO mice tested here.

Chapter 6. Effects of pharmacological blockade of 5-HTT function on emotional learning

6.1. Abstract

The experiments described in Chapters 4 and 5 generated evidence of enhanced discriminative aversive learning in 5-HTTKO mice, which could result from the elevated synaptic 5-HT levels of those mice at the time of conditioning. However, an alternative explanation for the enhanced learning observed could be that this resulted from the effects of high levels of 5-HT availability on neural circuits underlying fear learning during the development and early life of these mice. This chapter investigated whether increasing 5-HT levels specifically during conditioning was sufficient to enhance discriminative aversive learning.

C57BL/6J mice were treated systemically with the SSRI citalopram and subjected to the same discriminative aversive learning procedures described in Chapter 4. During Experiment 1, mice were trained to discriminate between a fully predictive aversive cue (CS+ = 100% CS-US contingency) and a fully predictive non-aversive cue (CS– = no US). In Experiment 2, a separate group of mice was trained in the same procedure but the CS+ was replaced with an ambiguous aversive cue that predicted footshock on only 20% of trials (CSa = 20% CS-US contingency). During fear conditioning, half of the mice received citalopram and the other half saline 30 minutes before each session. For the extinction sessions, each group was then split in two, with half receiving citalopram and the other half saline 30 minutes before each session. Thus, in each experiment there were four conditions reflecting treatment during training and extinction respectively (i.e. saline-saline, saline-citalopram, citalopram-saline, and citalopram-citalopram).

In Experiment 1, mice treated with citalopram during conditioning showed enhanced CS+/CS− discrimination compared to the saline-treated group during a fear memory recall (FMR) session. Despite the result obtained with fully predictive cues, citalopram administration during conditioning did not alter CSa/CS− discrimination learning nor fear memory recall in Experiment 2.

The result obtained with the experimental procedure using fully predictive aversive cues indicates that the enhanced fear memory recall exhibited by mice that received citalopram during training likely resulted from increased 5-HT availability at the time of learning. This finding is consistent with the enhanced discriminative learning seen with 5-HTTKO mice in Chapter 4 and provides support for one of the predictions of the neuroplasticity theory of 5-HT function: higher levels of 5-HT availability will facilitate learning of aversive experiences. However, the study using an ambiguous aversive cue failed to provide support for this prediction. Therefore, only partial support has been gathered for the neuroplasticity theory of 5-HT function with an approach of pharmacological blockade of 5-HTT function.

6.2. Introduction

The 5-HTTKO mice used in Chapters 4 and 5 of this thesis exhibit markedly higher extracellular 5-HT levels (and therefore presumably elevated synaptic 5-HT), as measured by both microdialysis and fast cyclic voltammetry (e.g. Mathews et al., 2004; Jennings et al., 2010). It is therefore tempting to speculate that the facilitated aversive learning found in Chapters 4 and 5 is due to the increased 5-HT availability at the time of fear conditioning, magnifying 5-HT signalling at 5-HT receptors. However, when mutations or genetic deletions are present throughout the life-course of animals, secondary changes can occur which might affect the normal function of a given gene or protein (Routtenberg, 1995; Gerlai,

1996; Flint, 2006). These changes may alter the development of neuronal circuits involved in emotional processing (Cryan & Holmes, 2005). This is particularly important to take into account when considering the role of 5-HT in modulating neurodevelopmental processes (Gaspar et al., 2003), which includes maturation of brain systems involved in emotional function (Gross et al., 2002; Ansorge, 2004).

As such, apart from the increased extracellular 5-HT levels (e.g. Jennings et al., 2010; Mathews et al., 2004) and altered 5-HT homeostasis (Kim et al., 2005b), many phenotypic changes are present in the 5-HTTKO mice (Murphy & Lesch, 2008; Kalueff, et al., 2010). Importantly, some of these phenotypic changes could influence fear learning. For example, 5-HTTKO mice show changes in BLA neuronal morphology, such as increased pyramidal neuron spine density (Wellman et al., 2007; Nietzer et al., 2011). This may impact anxiety-like behaviour (Roozendaal et al., 2009), and may be associated with a reduced inhibitory control in the BLA, which can contribute to enhanced emotional processing (Homberg et al., 2016). Additionally, changes in 5-HT receptor expression and function have also been reported (Li, 2006a). For example, 5-HTTKO mice show reduced expression and function of 5-HT_{1A} receptors in the DRN and amygdala (Fabre et al., 2000; Li et al., 2000; Holmes et al., 2003b), which are thought to modulate the expression of fear conditioning (Stiedl et al., 2000; Risbrough et al., 2003; Li et al., 2006b). Moreover, the amygdala of 5-HTTKO mice exhibits an increased density of 5-HT_{2C} receptors (Li et al., 2003) and decreased 5-HT_4 receptor binding (Jennings et al., 2012). This is relevant when testing 5-HTTKO mice because activation of 5-HT_{2C} receptors is thought to increase anxiety and enhance fear learning (Bauer, 2015), and 5-HT_4 receptors have been found to mediate a long-lasting facilitation of synaptic transmission in the amygdala associated with memory for learned fear (Huang & Kandel, 2007).

Subjects	Task/process	Effect/ results	SSRI (route, dose)	Authors/year
Rats	1 pre-training injection → cued fear conditioning	↑ fear acquisition & expression	Citalopram (I.P., 10 mg/kg)	Burghardt et al., 2004
		– fear expression	Citalopram (S.C., 10 mg/kg)	Gravius et al., 2006
		↑ fear acquisition & expression	Fluoxetine (I.P., 10 mg/kg;	Ravinder et al., 2013
	Cued fear conditioning → 1 pre-testing injection	↑ fear expression (both drugs) – extinction learning	Citalopram & Fluoxetine (I.P., 10 mg/kg)	Burghardt et al., 2007
	Cued fear conditioning → 1 pre-testing injection	– fear expression	Citalopram (S.C., 10 mg/kg)	Gravius et al., 2006
	▪ Habituation → cued fear conditioning → 1 pre-testing injection → extinction → extinction recall	↑ fear expression – extinction learning & recall	Fluoxetine (I.P., 10 mg/kg)	Lébron-Milad et al., 2013
	Cued fear conditioning → chronic SSRI treatment → extinction	↓ extinction learning	Citalopram & Fluoxetine (I.P., 10 mg/kg; 21 days)	Burghardt et al., 2013
	▪ Exp.1: a) Cued fear conditioning → 3days → b) chronic SSRI treatment → c) extinction ▪ Exp.2: b) → 3days → a) → c)	– extinction learning	Fluoxetine (I.P., 7mg/kg;)	Deschaux et al., 2011
Mice	▪ Exp.1: a) chronic SSRI treatment → b) cued fear conditioning → c) extinction ▪ Exp.2: b) → a) → c)	↑ extinction learning ↑ loss of fear memory	Fluoxetine (via drinking water, ~10 mg/kg per day)	Karpova et al., 2011
	Cued fear conditioning using a conspecific as a cue	↑ fear expression	NA (possibly Paroxetine, 10mg/kg)	Toth et al., 2012

Table 6.1. Examples of rodent studies investing the effects of SSRI administration on the processing of emotional stimuli, and on emotional learning. I.P., intraperitoneal injection. S.C., subcutaneous. ↑ enhancement. –, no change. ↓ diminution. Exp., Experiment; → , followed by.

Subjects	Task/process	Effect/ results	SSRI (route, dose)	Authors/year
Rats	Contextual fear conditioning (drug-free test)	↑ acquisition	Citalopram (S.C., 10 mg/kg)	Gravius et al., 2006
		↑ acquisition	Escitalopram (S.C., 1mg/kg)	Montezinho et al., 2010
		↓ acquisition (both doses)	Citalopram (S.C., 3 mg/kg, 10 mg/kg)	Inoue et al., 1996
	Contextual fear conditioning (fully counterbalanced design)	↓ acquisition	Citalopram (S.C., 10 mg/kg)	Hashimoto et al., 2009

Table 6.2. Examples of rodent studies investing the effects of acute SSRI administration on contextual fear learning. S.C., subcutaneous. ↑ enhancement. ↓ diminution.

Considering the above-mentioned phenotypic changes, an alternative way to investigate whether increased extra-cellular 5-HT levels, specifically at the time of training, can alter fear learning, is to subject rodents acutely treated with a SSRI administration to fear conditioning (see Table 6.1). SSRIs raise extracellular 5-HT levels (Bel & Artigas, 1992; Nutt, 2002; see Chapter 1, section 1.1.3), and it has been shown in rats, for instance, that an acute systemic administration with SSRI citalopram (10 μmol/kg ≈ 3.25 mg/kg) increases 5-HT levels in the amygdala to 175% of basal levels (Bosker et al., 2001). Using single-cue fear conditioning procedures, researchers have indeed found that rats treated with either citalopram or fluoxetine 60 minutes before the conditioning session show enhanced acquisition and fear memory recall of fear responses (Burghardt et al., 2004; Ravinder et al., 2013). However, other researchers have not found the same results with a single-cue fear conditioning procedure (e.g. Gravius et al., 2006). Regarding the acquisition of contextual fear conditioning, a procedure that increases 5-HT concentration in the amygdala (Zanoveli et al., 2009), contradictory effects have also been reported (see Table 6.2). For instance, two studies reported that the acute effects of the SSRIs citalopram and escitalopram treatment prior to conditioning can lead to an enhanced acquisition of contextual fear conditioning

(Gravius et al., 2006; Montezinho et al., 2010). However, two other studies reported the exact opposite effects with citalopram (Hashimoto et al., 2009; Inoue et al., 1996).

Variation between studies investigating the effects of acute SSRI administration on fear learning may result from the use of different (i) regimens of SSRI treatment, (ii) tests, (iii) stage of learning tested, and from the differences regarding side effects of the various SSRIs used in different studies (Cassel, 2010; Burghardt & Bauer, 2013; further discussed in section 6.7). Overall, these studies with single-cue fear conditioning procedures and contextual fear learning with rats have not yet provided clear support for the neuroplasticity theory of 5-HT function given that enhanced fear learning has not been consistently observed.

Mutant mice are a relevant research tool in the study of the role of a particular molecule in neuronal circuits mediating emotional behaviours particularly when used in conjunction with other tools/techniques to produce converging lines of evidence regarding such role (Holmes & Cryan, 2006). Accordingly, this chapter aimed to investigate whether increasing 5-HT levels shortly before fear learning was sufficient to facilitate discrimination between aversive and non-aversive conditioned stimuli. Two separate cohorts of normal, adult mice from the same background strain as the 5-HTTKO mice used in this thesis were tested on the same discriminative aversive learning procedures used in Chapter 4. To increase 5-HT levels across the whole brain, a 10 mg/kg dose of the SSRI citalopram was administered 30 minutes prior to each conditioning (or extinction) session. In mice, 30 minutes is sufficient time to detect increased extracellular levels of 5-HT following citalopram administration with a similar dose to the one used in this thesis (8 mg/kg, I.P.; David et al., 2003). Additionally, a daily dose of 10 mg/kg is typically used in other mouse studies investigating SSRI effects on fear memory (Burghardt & Bauer, 2013), with greater doses (e.g. 30 versus 10 mg/kg) causing anxiogenic effects (e.g. Mombereau et al., 2010).

Considering the results obtained in this thesis when subjecting 5-HTTKO mice to discriminative aversive learning procedures, and some of the above-mentioned rat studies,

the hypothesis guiding the studies presented in this chapter was that mice treated with citalopram prior to conditioning training would exhibit facilitated discriminative aversive learning compared to saline-treated mice, just as 5-HTTKO mice showed compared to their WT controls. Additionally, citalopram effects on fear extinction learning and freezing to the background context during fear conditioning were also investigated. As in Chapter 4, it was expected that using a discriminative aversive learning procedure instead of a single-cue paradigm could help to clarify some inconsistencies found among different laboratories investigating the effects of acute SSRI treatment on fear learning and fear extinction.

6.3. Methods

6.3.1. Subjects

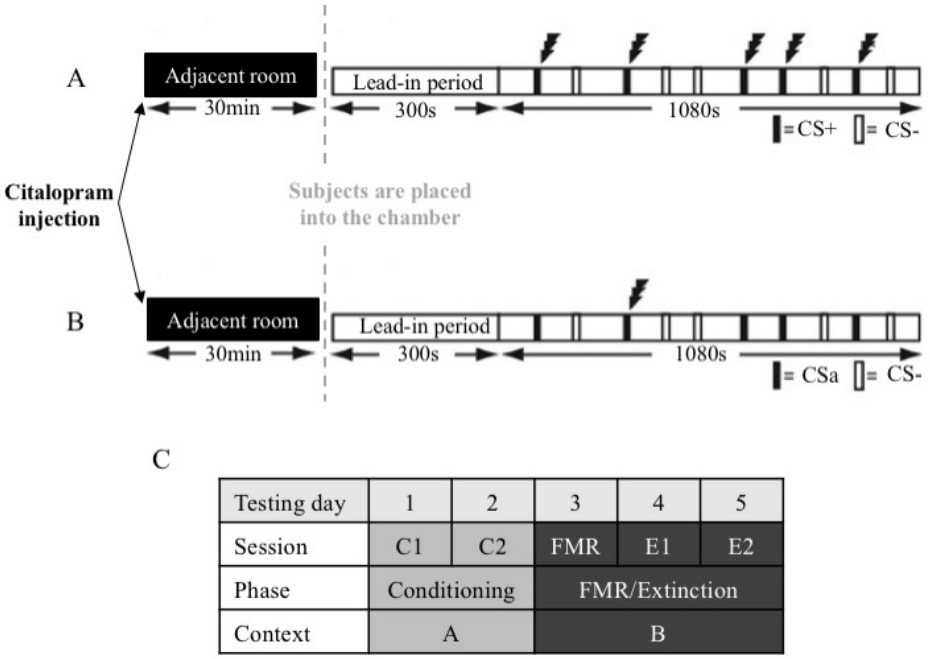
Ninety-Six C57BL/6J female mice were used in each of the two experiments reported in this chapter. They were ordered from Charles River Laboratories (Oxford, U.K.) and allowed to acclimatise to the holding facilities for at least one week before being tested at the age of 7-8 weeks. Handling, tail-marking, weighing and acclimatisation to the temporary holding room located in the laboratory unit were provided during the three days prior to the start of the experiment. Mice were also tail-marked and weighed at least 1h before each experimental session.

6.3.2. Drug administration

A 10mg/kg dose of citalopram hydrobromide ($C_{20}H_{21}FN_2O.HBr$; Tocris Bioscience, Bristol, U.K.) was used in both studies. The vehicle in which citalopram was dissolved was saline (sodium chloride 0.9% w/v, Aqupharm 1, Animalcare, York, U.K.), and the diluted concentration of the injection given was 1mg/ml (e.g. a mouse weighing 20g would receive an injection volume of 0.2ml). The route of administration chosen was the systemic

intraperitoneal (I.P.) route due to the advantage of this involving a simple technique that leads to rapid absorption (Branch, 1991). Injections alternated between the lower left and right quadrant of the abdomen on each day, and 27G needles were used as recommended for mice (see, for instance, Wolfensohn & Lloyd, 2003).

6.3.3. Procedures



C

Testing day	1	2	3	4	5
Session	C1	C2	FMR	E1	E2
Phase	Conditioning		FMR/Extinction		
Context	A		B		

Figure 6.1. A, B) Diagram of the conditioning sessions used in Experiments 1 (A) and 2 (B) of this chapter. The black lightning marks indicate the 0.3mA footshocks. No shocks were given during the fear memory recall (FMR) or extinction sessions. Adapted from McHugh et al. (2014)., under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/3.0/). Licensed Material: McHugh, S., Barkus, C., Huber, A. et al. Journal of Neuroscience 2 July 2014, 34 (27) 9024-9033. https://doi.org/10.1523/JNEUROSCI.4465-13.2014. **C)** 5-day procedure used in both experiments. The 2-day conditioning phase consisted of two conditioning sessions (C1 and C2). This was followed by a 3-day FMR/Extinction phase. Technically, FMR, E1 and E2 were all extinction sessions. These were administered in a context different from that used during the conditioning phase (e.g. context B, if conditioned in context A).

		Conditioning phase (C1-C2)	FMR/Extinction phase (FMR, E1 and E2)
Groups (N=24)	**Sal-Sal**	Saline	Saline
	Sal-Cit		Citalopram
	Cit-Sal	Citalopram	Saline
	Cit-Cit		Citalopram
		Context A	Context B

Table 6.3. Four groups of mice were used in both Experiments 1 and 2, each group differing in what they would receive throughout the 5-day procedure. Half of the 96 mice would either receive saline or citalopram during the conditioning phase. Afterwards, each half would be split in two, with half of the mice receiving saline and the other half citalopram during the FMR/Extinction phase (i.e. a fully counterbalanced design).

At the start of each session, two mice were placed in individual cages and taken to a quiet and dimly-lit room near the experimental room. Following the administration of saline or citalopram (10 mg/kg, I.P.), they were left for 30 min in that room and then placed in the conditioning chambers in the experimental room.

Mice were subjected to the same discriminative fear conditioning procedures described in Chapter 4. They were required to discriminate between an auditory cue (e.g. white noise) paired with an aversive US (a 0.5s-long, 0.3 mA footshock) and another auditory control cue (e.g. a 2900 Hz tone, counterbalanced across mice) which was followed by the absence of the aversive US (fully counterbalanced). Each day mice received five presentations of each cue type, with fear conditioning sessions during the first two days in one context and then extinction sessions in a different context. During fear conditioning sessions one of the cues was paired with footshock (CS+), whereas the other cue (CS–) was not. In Experiment 1, shocks occurred on 100% of CS+ training trials, making this cue a fully predictive aversive cue (see Figure 6.1, diagram A). In Experiment 2, shocks occurred only on 20% of CS+ trials, making this cue a less reliable predictor of footshock, and thus an ambiguous cue (designated CSa; see Figure 6.1, diagram B).

Following the 2-day conditioning phase (conditioning sessions 1 and 2, or C1 and C2, respectively), mice underwent three extinction sessions in the alternative context, with no footshock deliveries following either the CS+ or CSa (see Figure 6.1, C). The first of these three extinction sessions is called the fear memory recall testing session (FMR), whereas the following two sessions are called extinction sessions 1 and 2 (E1 and E2, respectively). Further information about experimental settings, the apparatus, the different contexts used, and how freezing behaviour was measured can be found in Chapters 2 (section 2.3.4) and 4 (sections 4.3.1 and 4.4.1).

During the conditioning phase, half of the mice received citalopram and the other half a vehicle injection (i.e. saline) just before each session (see Table 6.3). For the

FMR/extinction phase, each group was then equally divided, with half receiving citalopram and the other half receiving saline before each session. This fully counterbalanced design was implemented to control for state-dependent learning, an example of contextual stimulus control where the drug provides a contextual (interoceptive) cue (Overton, 1985). Such a situation might lead, for instance, mice conditioned under the influence of citalopram to perform worse in a non-drug state, compared to controls in which there was no state change (e.g. Cit-Cit).

6.3.4. Data analysis

Freezing behaviour was measured, processed and analysed as previously described in Chapters 2 (section 2.3.4). An ANOVA using a general linear model (GLM) was performed on the data, as previously described (see Chapter 2, section 2.6). Given that subjects did not have many opportunities to learn about the meaning of the CSa during the C1 session of Experiment 2, data from this session are shown in the figures but were excluded from the statistical analyses (similarly to what was done in Chapter 4, section 4.4.2).

Prior to the data analyses performed on the conditioning and extinction data, analyses were also conducted on the raw pre-CS freezing responses across the five days. The results of these data analyses can be found in Appendix 3. Figures showing raw freezing during CS periods and freezing levels during pre-CS periods across all trials of Experiments 1 and 2 can be found in Appendices 8-15. Results from data analyses conducted on pre-US activity levels can be found in Appendix 16.

6.4. Experiment 1: Processing of predictable aversive cues

6.4.1. Results

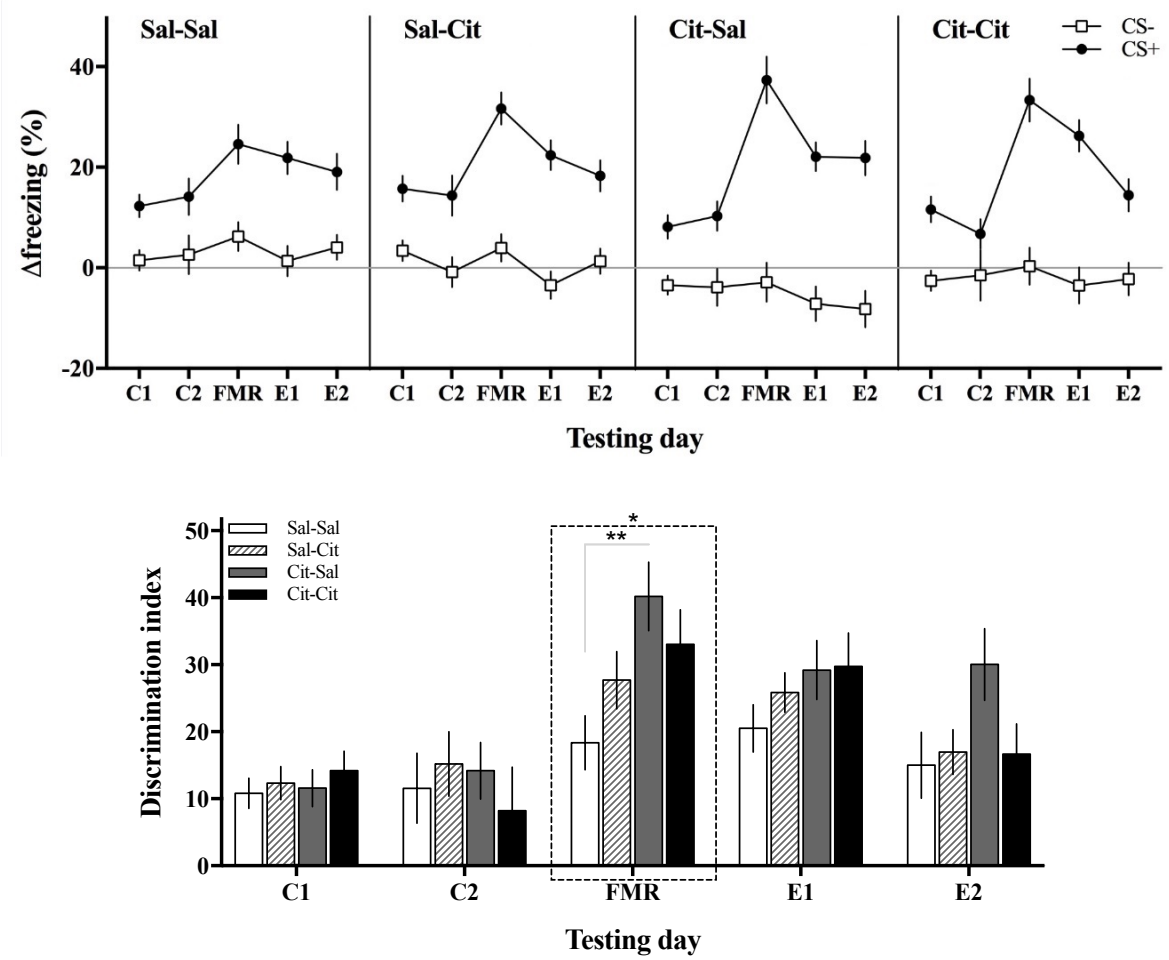


Figure 6.2. Top, Cue-evoked Δfreezing on the conditioning (C1, C2) and extinction sessions (FMR, E1 and E2) for the four groups used in the Experiment 1 (N=96). All groups learned to significantly discriminate between the CS+ and the CS– cues. **Bottom,** Discrimination index (CS+ Δfreezing – CS– Δfreezing) for all groups (N=96) during the 5-day procedure. Mean ± SEM, *p < .05 (main effect of group), **p < 0.01 (pairwise comparison between the Sal-Sal and the Cit-Sal groups).

First, cue-evoked Δfreezing responses were analysed over the 5-day procedure, using separate between-subjects factors for (1) drug treatment during conditioning sessions, and (2) drug treatment during extinction sessions; and using within-subjects factors for day (5 levels: C1, C2, FMR, E1, E2) and CS type (CS+, CS-) (ANOVA model:

Drug(Conditioning)$_2$×Drug(Extinction)$_2$×Day$_5$×CS$_2$×S$_{96}$). This analysis revealed a 3-way interaction between Drug(Conditioning), Drug(Extinction) and CS type ($F(1,92) = 4.1$, $p = .046$). This interaction reflected the fact that citalopram had different consequences on cue-evoked Δfreezing responses during conditioning versus extinction (see Figure 6.2, top).

Citalopram-treated mice exhibit enhanced fear memory recall

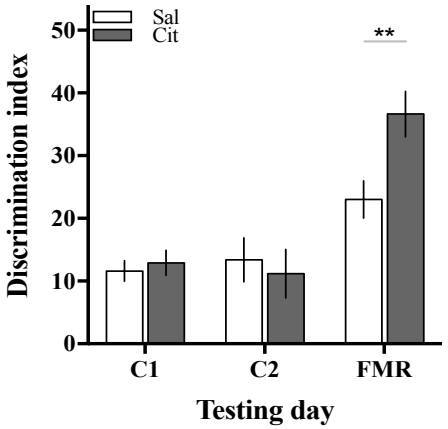


Figure 6.3. Discrimination index (CS+ Δfreezing – CS− Δfreezing) during the conditioning (C1, C2) and FMR sessions for all mice tested in Experiment 1 (N=96). The Sal bar comprises the Sal-Sal and Sal-Cit groups (N=48), whereas the Cit bar comprises the Cit-Sal and Cit-Cit groups (N=48). Mice treated with citalopram during conditioning training exhibited superior cue discrimination when tested on FMR, regardless of whether they received saline or citalopram before that session. Mean ± SEM, $^{**}p < 0.01$ (simple effect of treatment during conditioning on FMR).

Analysis of the discrimination index indicated that citalopram administration did not affect cue discrimination learning during the conditioning training sessions (main effect of day, drug treatment during conditioning or interaction: all Fs < 1; see Figure 6.3). Both citalopram- and saline-treated mice showed cue discrimination during C1 and C2. However, a clear effect of the administration of citalopram during conditioning was seen in the FMR session, regardless of which type of injection (citalopram or saline) was administered just before the FMR session. Specifically, mice treated with citalopram during conditioning sessions exhibited stronger fear memory recall compared to mice that had received saline

during the conditioning sessions (effect of drug treatment during conditioning on FMR: $F(1,92) = 8.5$, $p = .004$; see Figure 6.3).

Comparisons across the four groups on FMR indicated that the only significant group difference occurred between the Sal-Sal and the Cit-Sal groups (main effect of group on FMR: $F(3,92) = 3.9$, $p = .012$; pairwise comparison between Sal-Sal and Cit-Sal: $p = .008$; see Figure 6.2, bottom). That is, the citalopram effect on cue discrimination learning was particularly clear in the subsets of mice that received saline injections immediately before the FMR session. Although no other significant group differences were found, the Cit-Cit group exhibited better discrimination than the Sal-Sal group, and the Cit-Sal group discriminated better than the Sal-Cit group.

Citalopram has bi-directional effects on emotionally-relevant cues

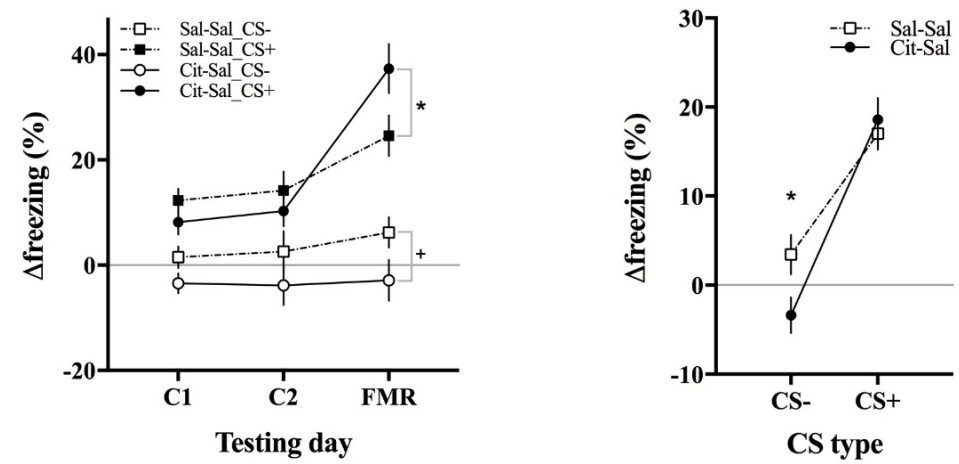


Figure 6.4. Left. Cue-evoked Δfreezing on the first three days of testing for the Sal-Sal and Cit-Sal groups. The CS+ Δfreezing was significantly higher during the FMR session for mice fear conditioned under the effect of citalopram (N=24) compared with those treated only with saline (N=24). An opposite effect was seen for the CS− Δfreezing, where a stronger reduction was observed in citalopram-treated mice. Mean ± SEM, $+p < 0.1$, $*p < 0.05$ (simple effect of group within each level of the CS type, that is, CS+ and CS−). **Right.** Cue-evoked Δfreezing averaged over the first three days of testing for the same groups (N=48). Citalopram-treated mice exhibited reduced freezing to the CS− cue across the three days. Mean ± SEM, $*p < 0.05$ (simple effect of group within each level of the CS type, that is, CS+ and CS−).

Regardless of drug-treatment, mice froze significantly more in the presence of the CS+ than the CS– right from the first conditioning day (C1), and continued to discriminate between the cues during C2 and FMR (main effect of CS: $F(1,46) = 106.9$, $p < .001$; see Figure 6.4, left). Notably, however, the Cit-Sal group exhibited a stronger CS+ Δfreezing response on FMR compared to Sal-Sal mice (simple effect of group within CS+ Δfreezing response: $p = .041$; $Group_2 \times Day_3 \times CS_2$ interaction: $F(2,92) = 4.2$, $p = .018$). Moreover, the Cit-Sal group also froze considerably less during the CS– presentations on that same session compared with Sal-Sal mice, although this did not quite reach statistical significance ($p = .066$). However, it was observed that citalopram-treated mice froze significantly less during the CS– presentation compared to saline-treated mice when CS– responses were pooled over C1, C2, and FMR (simple effect of group within the CS– Δfreezing response averaged over 3 days: $p = .027$; $Group_2 \times CS_2$ interaction: $F(1,46) = 6.0$, $p = .018$; see Figure 6.4, right). Notably, citalopram administration during conditioning led to both increased freezing to the aversive cue and also reduced freezing to the non-aversive cue. There was therefore arguably better learning about both cues in citalopram-treated mice compared to saline-treated mice.

State-dependent learning was not observed

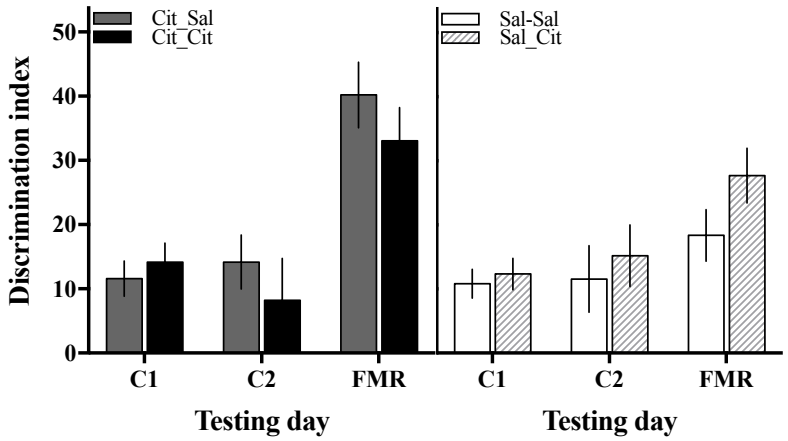


Figure 6.5. Comparison of the discrimination indices (CS+ evoked Δfreezing – CS- evoked Δfreezing) from the Cit-Sal and Cit-Cit groups (N=48), and the Sal-Sal and Cit-Sal groups (N=48) during the first three days of testing. No significant effects of drug administered during FMR were registered. Therefore, state-dependent learning was not observed. Mean ± SEM.

State-dependent learning reflects the observation that memory retrieval is better when the organism experiences the same 'internal' state during the memory retrieval as was experienced during memory encoding. The design of this experiment allowed to investigate state-dependent effects. For example, state-dependent learning during FMR could manifest in the finding that a group receiving the same drug (or vehicle) just before FMR as the one received just before C1 and C2 would exhibit better performance on FMR than a group subjected to a change of drug condition.

Analysis of the discrimination index revealed no significant differences when comparing the Cit-Sal group with the Cit-Cit group (main effect of group: $F < 1$; $Group_2 \times Day_3$ interaction: $F < 1$; simple effect of group on FMR: $p = .331$; (see Figure 6.5). Additionally, no significant differences were seen when comparing the Sal-Sal group with the Sal-Cit group (main effect of group: $F(1,46) = 1.9$, $p = .175$; $Group_2 \times Day_3$ interaction: $F < 1$; simple effect of group on FMR: $p = .118$). This demonstrates that the drug effects observed during FMR were not a result of state-dependent effects.

Citalopram administration does not affect extinction learning

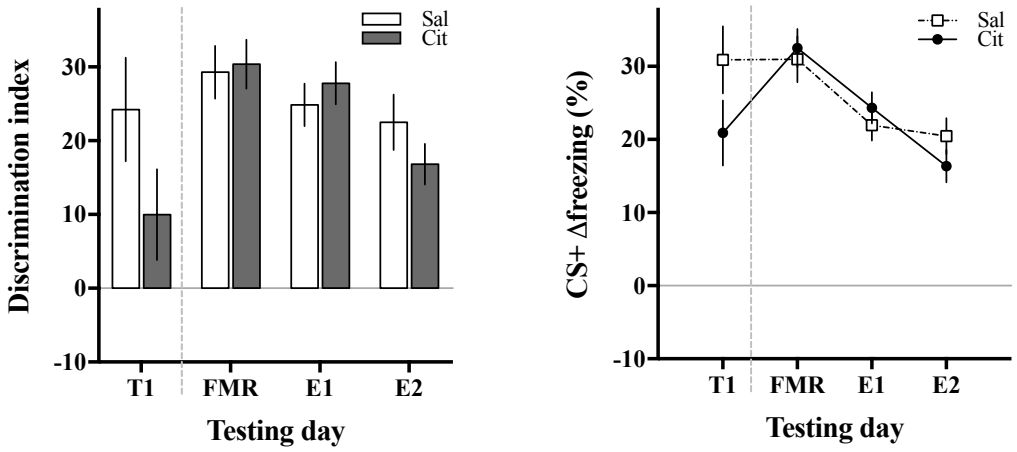


Figure 6.6. Left, Discrimination index (CS+ Δfreezing – CS– Δfreezing) during the extinction sessions (FMR, E1 and E2) for mice treated with either citalopram (N=48) or saline (N=48) during the FMR/extinction phase in Experiment 1. T1 is the first pair of CS+ and CS– presentations (i.e. trial 1) of the FMR session. **Right,** CS+ Δfreezing during T1, FMR, E1 and E2 sessions for the same subjects. These results indicate that there were no significant differences between groups regarding extinction learning. Mean ± SEM.

Analysis of the discrimination index over the FMR/Extinction phase (ANOVA model: Drug(Conditioning)$_2$×Drug(Extinction)$_2$×Day$_3$×S$_{96}$) indicated that both citalopram-treated and saline-treated mice during this phase showed extinction learning (main effect of day: $F(2,184) = 6.9$, $p = .002$; see Figure 6.6, left). No significant differences between the two groups were observed (main effect of drug treatment during the FMR/Extinction phase: $F < 1$; Drug(Extinction)$_2$×Day$_3$ interaction: $F(2,184) = 1.3$, $p = .262$). A similar pattern of results was seen in the analysis of just the CS+ Δfreezing data over the same three extinction sessions (main effect of day: $F(2,184) = 24.2$, $p < .001$; main effect of drug treatment during the FMR/Extinction phase: $F < 1$; Drug(Extinction)$_2$×Day$_3$ interaction: $F(2,184) = 1.6$, $p = .199$; see Figure 6.6, right). In short, effects of citalopram on extinction learning were not observed.

6.5. Experiment 2: Processing of ambiguous aversive cues

6.5.1. Results

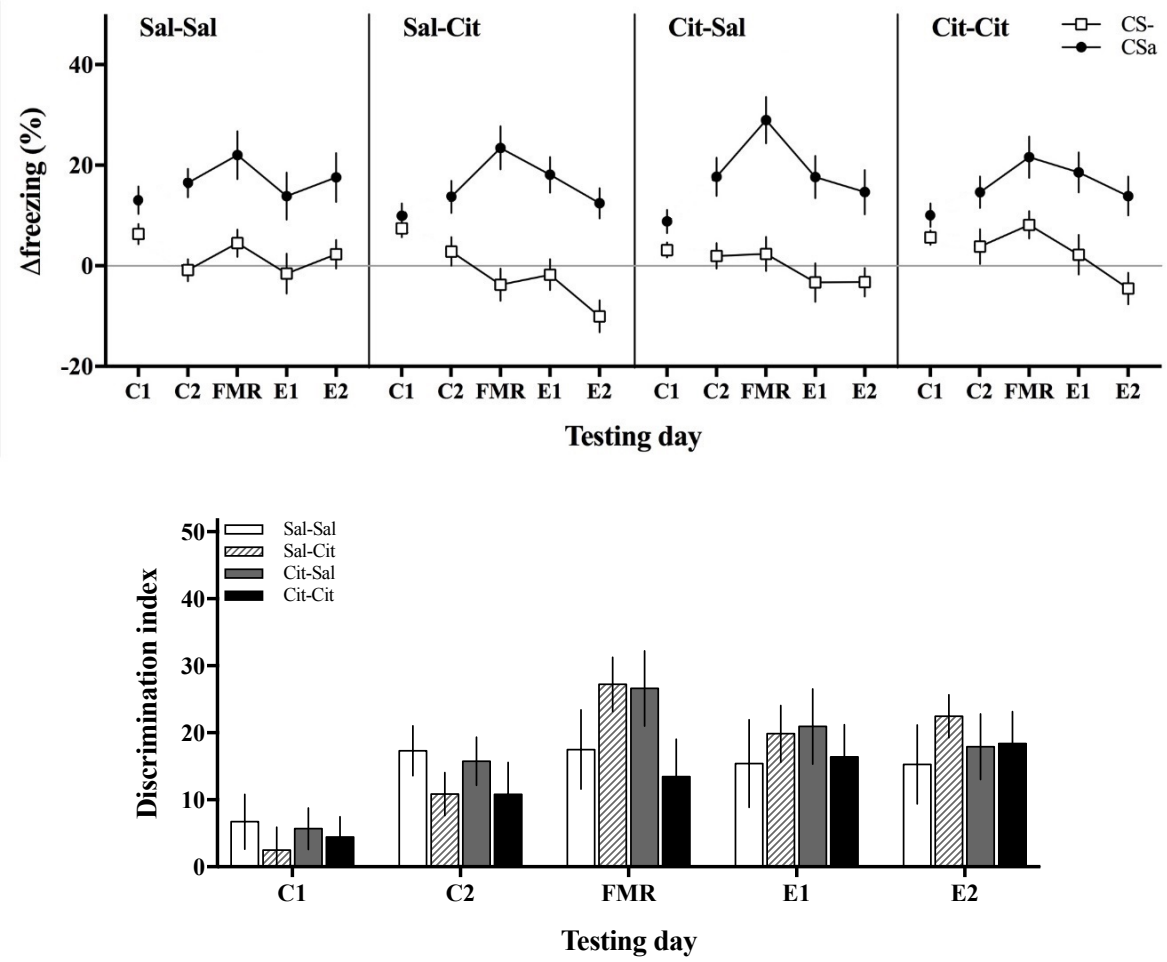


Figure 6.7. Top, Cue-evoked Δfreezing on the conditioning (C1, C2) and extinction sessions (FMR, E1 and E2) for the four groups used in the Experiment 2 (N=96). All groups learned to discriminate between the CS+ and the CS− cues. **Bottom,** Discrimination index (CS+ Δfreezing – CS− Δfreezing) for all groups (N=96) during the 5-day procedure. Mean ± SEM.

In Experiment 2, subjects received only one footshock delivery during C1 (out of five cue presentations), and therefore data from this session are excluded from the statistical analyses but are shown in the graphs. Analyses of cue-evoked Δfreezing (ANOVA model: $Drug(Conditioning)_2 \times Drug(Extinction)_2 \times Day_4 \times CS_2 \times S_{96}$) showed that, contrary to what was observed in Experiment 1, citalopram did not have different effects depending on whether it

was administered during the conditioning phase or the FMR/extinction phase (Drug(Conditioning)$_2$×Drug(Extinction)$_2$×CS$_2$×S$_{96}$: $F(1,92) = 1.5$, $p = .222$; see Figure 6.7). However, considering the results obtained with Experiment 1 and the interest in investigating citalopram effects on acquisition and the FMR/Extinction phase, separate analyses were performed for each phase.

Citalopram-treated mice do not significantly differ from controls on FMR

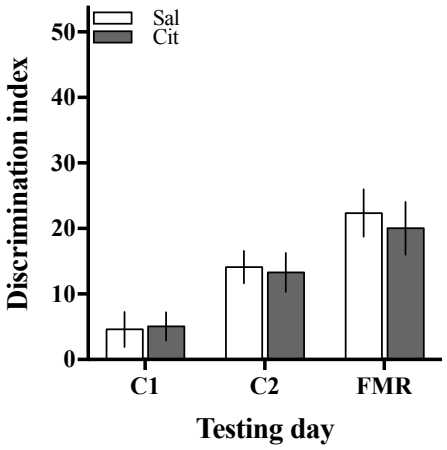


Figure 6.8. Discrimination index (CSa Δfreezing – CS- Δfreezing) during the first three days of testing (C1, C2 and FMR) for all mice tested in Experiment 2. The Sal bar comprises the Sal-Sal and Sal-Cit groups (N=48), whereas the Cit bar comprises the Cit-Sal and Cit-Cit groups (N=48). No significant group differences were seen in any day. Mean ± SEM.

Analysis of the discrimination index revealed that citalopram-treated mice did not differ from saline-treated mice during the conditioning training session C2 ($t(94) = .209$, $p = .835$; see Figure 6.8). There was no effect of drug-treatment during the FMR session (effect of treatment during conditioning on FMR: $F < 1$), even when the four groups were compared to each other (main effect of group on FMR: $F(3,92) = 1.6$, $p = .188$; see Figure 6.7, bottom). In short, citalopram administration during training did not enhance the discrimination of a CSa cue from a CS− cue.

Citalopram did not affect fear extinction

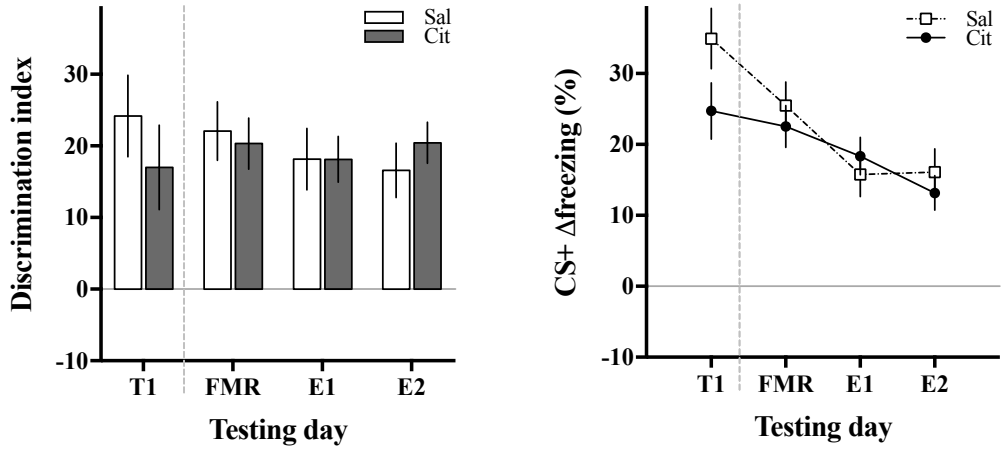


Figure 6.9. Left, Discrimination index (CSa Δfreezing – CS– Δfreezing) during the extinction sessions (FMR, E1 and E2) for mice treated with either citalopram (N=48) or saline (N=48) during the FMR/extinction phase in Experiment 2. T1 is the first pair of CS+ and CS– presentations (i.e. trial 1) of the FMR session. **Right,** CSa Δfreezing during T1, FMR, E1 and E2 sessions for the same subjects. These results indicate that there were no significant differences between groups regarding extinction learning. Mean ± SEM.

Analysis of the CSa Δfreezing over the same three extinction sessions revealed that mice in both groups learned to significantly reduced their response to the CSa ($F(2,184) = 16.3$, $p < .001$). No significant effect of citalopram was observed during the FMR/Extinction phase on the CSa Δfreezing, with both groups extinguishing at a similar rate (main effect of drug treatment during the FMR/Extinction phase: $F < 1$; $\text{Drug(Extinction)}_2 \times \text{Day}_3$ interaction: $F(2,184) = 1.8$, $p = .171$; see Figure 6.9, right). However, examination of the discrimination index over the FMR/Extinction phase failed to show extinction across days in either group (Figure 6.9, left).

6.6. Other citalopram effects

Finally, the effects of citalopram on freezing to the background context were also investigated as a surrogate for a test on contextual fear learning. Additionally, the URs to

the footshock were also investigated to test whether any potential differences in learning reflect a difference in sensory perception to footshocks.

Citalopram administration does not affect freezing to the background context

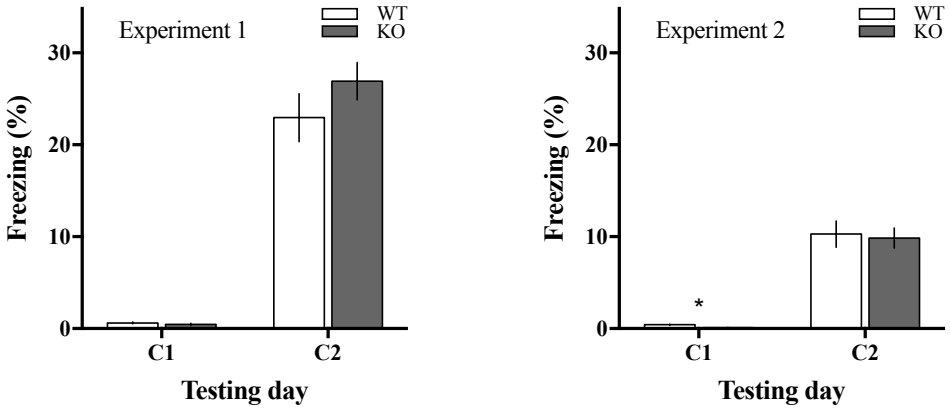


Figure 6.10. Left, Mean levels of freezing behaviour registered during the lead-in period of C1 and C2 for mice treated with either citalopram (N=48) or saline (N=48) during the conditioning phase of Experiment 1. **Right,** Mean levels of freezing behaviour registered during the lead-in period of C1 and C2 for mice treated with either citalopram (N=48) or saline (N=48) during the conditioning phase of Experiment 2. Mean ± SEM. *$p < .05$ (simple effect of drug treatment during conditioning on C1).

In Experiment 1, both groups exhibited a significant increase in freezing to the background context from C1 to C2 (main effect of day: $F(1,94) = 220.6$, $p < .001$; see Figure 6.10, left). The groups did not significantly differ from each other (Drug(Conditioning)$_2$×Day$_2$ interaction: $F(1,94) = 1.6$, $p = .212$).

A similar pattern of results was seen in Experiment 2. Groups did significantly differ on their freezing levels on C1 before shocks were delivered, reflecting a mild hyperactivity in citalopram-treated mice (simple effect of drug treatment during conditioning on C1: 300s: $p < .001$; see Figure 6.10, right). However, both groups exhibited a significant increase in freezing to the background context from C1 to C2 (main effect of day: $F(1,94) = 117.8$, $p < .001$; Drug(Conditioning)$_2$×Day$_2$ interaction: $F < 1$).

Finally, it is clear that a greater number of US presentations leads to stronger conditioning to the background context, with greater freezing during C2 in Experiment 1 compared to Experiment 2 (main effect of experiment on C2: $F(1,190) = 61.9$, $p < .001$). However, the contribution of habituation cannot be ruled out, which would also manifest as an increase in inactivity. To control for habituation, it would be necessary to have control groups subjected to the same conditions apart from the footshock administration.

Citalopram administration does not affect footshock reactivity

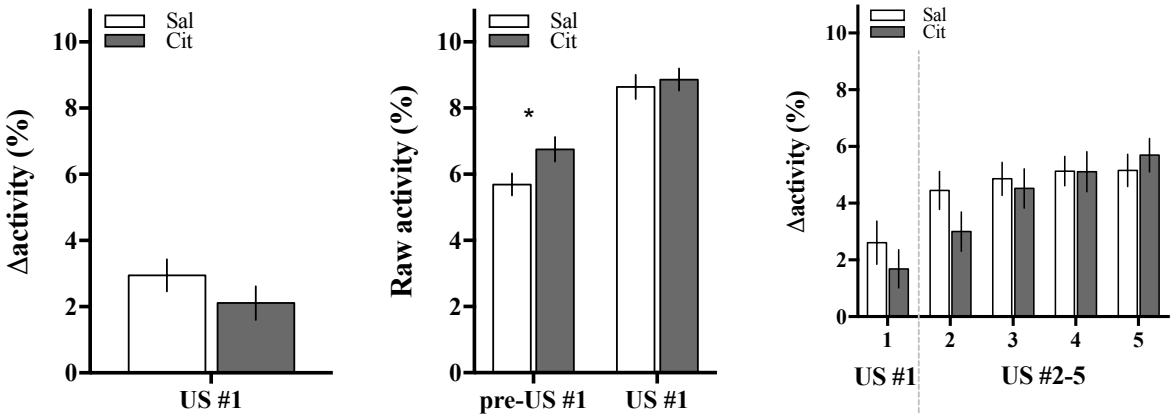


Figure 6.11. Left, Changes in activity levels (Δactivity (%) = % pixels changed during the 5s-long post-US period minus % pixels changed during a 5s-long period before the footshock-predicting CS) for the first footshock delivery (US #1) of C1 administered to citalopram treated-mice (N=96) and saline-treated mice (N=96) in Experiments 1 and 2. **Middle,** Raw activity levels during the pre-US period and the post-US period of the first footshock administered to the same mice. **Right,** Changes in activity levels for the five US deliveries (US #1-5) of C1 administered to the mice used in Experiment 1 only (N=48 for each group). Mean ± SEM. *$p < .05$ (main effect of treatment during conditioning on pre-US activity levels).

Analyses of the UR to footshock were obtained by analysing the Δactivity elicited by the first footshock ("US #1") delivery during the C1 session. The analyses revealed no significant differences between the citalopram-treated mice (N=96) and the saline-treated mice (N=96) (main effect of treatment during conditioning: $F(1,188) = 1.4$, $p = .235$; see Figure 6.11, left). Given that the pre-US activity levels were significantly higher in citalopram-treated mice than in saline-treated mice (main effect of treatment during

conditioning: $F(1,188) = 4.6$, $p = .033$; see Figure 6.11, middle), an additional analysis was performed with the raw activity levels before and after the footshock delivery (ANOVA model: $Drug(Conditioning)_2 \times Phase_{(pre\text{-}US,\ post\text{-}US)}$). This analysis revealed an absence of citalopram effects on the UR to footshock (main effect of phase: $F(1,188) = 51.6$, $p < .001$; $Drug(Conditioning)_2 \times Phase_{(pre\text{-}US,\ US)}$ interaction: $F(1,188) = 1.4$, $p = .235$; simple effect of the treatment during conditioning on the post-US period: $p = .650$; see Figure 6.11, middle).

To investigate if citalopram-treated mice, compared with saline-treated mice, would become more sensitive to the US as the session progressed and subjects received further footshocks, the analyses conducted with the 'US #1' were extended to include also the following four footshock deliveries of C1 in Experiment 1 ('US #2', 'US #3', 'US #4', 'US #5'; see Figure 6.11, right). After a similar UR to the first footshock presentation of C1 ($t(94) = 0.9$, $p = .367$), groups exhibited increased reactivity to footshocks throughout the session (main effect of trial US# 1-5: $F(4,376) = 9.7$, $p < .001$). However, citalopram-treated mice did not significantly differ from saline-treated mice in the development of this increased reactivity ($Group_2 \times Trial_5$ interaction: $F < 1$), with both groups exhibiting similar reactivity levels across the remaining four footshocks of the session (main effect of group during US# 2-5: $F < 1$).

6.7. Discussion

Summary of the results

In this chapter, it was shown that a transient increase of extracellular 5-HT levels during the conditioning training sessions enhances discriminative aversive learning when a fully predictive aversive cue (CS+) is presented together with a non-aversive cue (CS–), but not when an ambiguous aversive cue (CSa) is used instead of a CS+. Mice treated with citalopram prior to the conditioning sessions in Experiment 1 exhibited enhanced learning

in the form of a significantly stronger fear memory recall compared with the saline-treated mice. However, this effect was not found in Experiment 2, with no significant effects of citalopram during learning or recall. Citalopram also did not affect fear extinction learning or conditioned freezing to the background context in either experiment.

Citalopram administered during conditioning facilitates fear memory recall when fully predictive cues are used

Unlike with 5-HTTKO mice, citalopram-treated mice did not exhibit enhanced aversive discriminative learning in the form of faster acquisition compared to their controls. However, citalopram-treated mice did exhibit enhanced fear memory recall. Citalopram effects on URs to the footshocks could not account for the improvement seen. Genotypic differences regarding URs to the auditory cues could not be investigated as there was no measurable UR (see Appendix 20). State-dependent learning was not observed, and differences in pre-CS freezing levels did not significantly influence the results (see Appendices 3, 8-15).

The enhanced learning seen with the procedure using fully predictive cues (Experiment 1) is consistent with the studies of Burghardt and colleagues (2004) and Ravinder and colleagues (2013). Considering the absence of an effect of citalopram on fear learning reported by Gravius and collaborators (2006), it is possible that methodological differences can explain the discrepancy between the different studies. For instance, compared with Burghardt et al. and Ravinder et al., Gravius and colleagues (2006) used different footshock intensities and time intervals between injections and testing.

The result obtained here with a discriminative aversive learning procedure provides support for the claim that a transient administration of citalopram before learning can lead to enhanced fear learning. However, it also challenges some authors' interpretation that the enhancement of fear responses seen is an instance of pharmacologically induced anxiety

(Burghardt et al., 2007, 2004). An exacerbation or potentiation of a conditioned fear response due to symptoms of anxiety might be expected to produce increased stimulus generalisation (or, in an alternative conceptualisation, a failure to inhibit fear in the presence of the CS–), which could impair CS+/ CS– discrimination (Lissek et al., 2005). In contrast, citalopram-treated mice clearly showed enhanced fear discrimination learning in Experiment 1, which indicates that they were dealing with each cue in a distinct way. Indeed, some evidence that citalopram has bi-directional effects on emotionally-relevant cues has been gathered in Experiment 1 supporting this interpretation. Notably, the enhanced learning about both cues in citalopram-treated mice compared to saline-treated controls is also consistent with data obtained with the 5-HTTKO mice in Chapter 4 (e.g. see section 4.3.2).

Considering why citalopram-treated mice did not show faster acquisition of aversive discriminative learning as 5-HTTKO mice did, it is possible that different amounts of extracellular 5-HT availability might be present in each group of mice and that this could have affected how and when mice would show CS+/CS– discrimination. Although the increased extracellular 5-HT availability has been estimated for the 5-HTTKO mice (up to 10-fold, depending on the brain region; Fabre et al., 2000; Jennings et al., 2010; Mathews et al., 2004; Shen et al., 2004), to my knowledge no equivalent information has been gathered regarding an acute citalopram administration in mice. If rat studies can be of any reference for this question, then the increase is certainly lower in citalopram-treated rodents compared to 5-HTTKO mice (an increase between 175% - 538% depending on the brain region; Bel & Artigas, 1992; Bosker et al., 2001).

Additionally, the results of Experiment 1 challenge the claims that citalopram administration during conditioning training can lead to faster fear acquisition. However, these claims were based on comparisons of fear responses during three individual trials of the conditioning training (Burghardt et al., 2004), and in a later report from the same group

it was already possible to see citalopram-treated subjects would match saline-treated controls when two more CS-US pairings were added to the session (Ravinder et al., 2013). Indeed, only in one trial (e.g. the third of the five presented during the conditioning session) it was possible to detect a significant group difference. Therefore, the claimed fast fear acquisition was a rather subtle effect of an acute citalopram administration. The absence of enhanced learning during conditioning in this chapter is thus not totally unexpected when considering the empirical support for the claims of Burghardt et al. and Ravinder et al.

Despite the enhanced learning obtained in Experiment 1, citalopram administration during conditioning did not affect cue discrimination when an ambiguous aversive cue (CSa) was used together with a control cue (CS–) in Experiment 2. This was evident during conditioning training but also during the FMR session. Moreover, no significant changes in cue discrimination levels were seen from C2 to FMR in citalopram-treated mice and saline-treated mice. This result is surprising considering the strong result obtained in Experiment 1 using the same dose and pre-treatment time, which has been also used by other researchers using to investigate SSRI effects on emotionality in C57BL/6J mice (e.g. Jin et al., 2017).

One hypothesis for this result of no changes in learning following citalopram treatment could be that just as different conditioning procedures affected how and when 5-HTTKO mice would should significantly stronger fear discrimination than WT controls (C1 in Experiment1, FMR in Experiment 2; see Chapter 4, sections 4.3.2 and 4.4.2), the same would happen with citalopram-treated mice compared to saline-treated controls. That is, if 5-HTTKO mice showed their stronger cue discrimination at a later stage when an ambiguous aversive cue was used instead of a fully predictive cue, it is possible that citalopram-treated mice would have needed more conditioning training sessions (before a FMR and extinction sessions were administered) to show altered learning.

Citalopram does not affect extinction learning

Citalopram did not affect extinction learning in Experiment 1, as had been expected considering the results obtained with the 5-HTTKO mice in Chapter 4 (see section 4.3.2). It is possible, however, that just as citalopram-treated mice took longer to exhibit their enhanced learning compared to 5-HTTKO mice, they would need more extinction sessions to exhibit altered fear extinction. Regarding Experiment 2, it is not unexpected that altered fear extinction was not observed in citalopram-treated mice given that (i) this was also not observed in 5-HTTKO mice subjected to Experiment 2 of Chapter 4, and (ii) a partially reinforced stimulus usually shows greater resistance to extinction (Wagner et al., 1967). In short, it would be more difficult to see such an effect in Experiment 2 than in Experiment 1.

Burghardt and collaborators have reported that an acute SSRI injection leads to no change in the rate of extinction learning, whereas chronic SSRI treatment between conditioning and extinction training leads to impaired extinction learning (Burghardt et al., 2007, 2013). Considering the former claim, another research group has provided additional support (Lébron-Milad et al., 2013), whereas the latter claim was disputed by other researchers, who either found facilitated extinction (Karpova et al., 2011), or no changes (Deschaux et al., 2011). The present chapter provides no evidence for altered extinction following citalopram. Nevertheless, it is known that fear extinction can be enhanced by means of distinct pharmacological interventions and SSRIs are thought to be an agent capable of such effects (Fitzgerald et al., 2014), although the regimens used for drug treatment and procedures chosen to test its effects play an important role in producing observable effects.

Citalopram did not affect conditioning to the background context

According to some authors, acute SSRI treatment seems to have opposite effects on cued and contextual freezing expression (Homberg, 2012; Burghardt & Bauer, 2013).

Several reasons have been advanced for this situation, particularly the following: cued and contextual fear conditioning tasks recruit different neuronal circuits with different serotonergic inputs (Burghardt et al., 2007); SSRI administration decreases hippocampal activity, and if there are differences in the salience of the contextual cues used in different studies then this could influence the subject's ability to form a representation of the context (Bauer, 2015).

In this chapter, no effects of citalopram were seen regarding freezing to the background context during discriminative aversive conditioning. This result could not help with the dispute summarised in Table 2. As previously mentioned in the discussion of Chapter 4 (section 4.7), investigating conditioned freezing to the background context using the 300s-long lead-in periods of the two conditioning sessions (C1-C2) is a less than satisfactory way of assessing contextual fear learning. It would thus be worth conducting a proper study without any simultaneous cued-fear conditioning, and with a fully counterbalanced design to control for habituation processes. This would be important to do particularly because only one rodent study has used such a design so far (Hashimoto et al., 2009), but also because of the enhanced contextual fear conditioning observed in 5-HTTKO mice during the footshock reactivity test (see Chapter 4, section 4.6.2).

Conclusion

As predicted, mice treated with citalopram, specifically during conditioning, exhibited enhanced discriminative aversive learning. The result obtained with Experiment 1 strengthens the hypothesis, at least in part, that the superior learning previously seen with 5-HTTKO mice is associated with higher levels of 5-HT availability, just as the neuroplasticity theory of 5-HT function predicts. However, this was only seen in a procedure using fully predictive aversive cues, and not with one using an ambiguous aversive cue versus a control cue. The absence of enhanced learning in a procedure using an ambiguous aversive cue also

matches the absence of differences seen on the conditioned freezing to the background context, which can also be conceived of as an ambiguous aversive cue (McHugh et al., 2015). Therefore, overall, pharmacological blockade of 5-HTT function in normal mice only partially supported the hypothesis that the behavioural phenotype seen in 5-HTTKO mice in the experiments reported in Chapter 4 was essentially driven by increased extracellular 5-HT levels at the time of learning. It is thus possible that the considerably longer duration of increased 5-HT availability might prime 5-HTTKO mice to process emotionally relevant stimuli in their environment in a more efficient way than a transient boost of the serotonergic system can achieve. The enhanced learning observed in the 5-HTTKO mice here could result both from higher 5-HT availability at the time of learning and altered neuronal development (Bocchio, et al., 2016).

Finally, it is also possible that citalopram might have affected other phases of memory instead, namely consolidation (Johansen et al., 2011; Burghardt & Bauer, 2013). To verify at what time point (or at what stage of memory) citalopram was having its effects, another discriminative aversive learning experiment could be conducted, for instance, to verify the effects of citalopram when this drug was administered shortly after conditioning training was completed (Johansen et al., 2011).

Chapter 7. Processing of rewarding cues in 5-HTTKO mice

7.1. Abstract

The neuroplasticity theory of 5-HT function predicts that individuals with low 5-HTT expression levels will exhibit increased sensitivity to both aversive and non-aversive events. Chapter 4 provided evidence that 5-HTTKO mice do indeed exhibit increased sensitivity to aversive events, and suggested a similar sensitivity towards safety-signalling cues. This chapter investigated further whether 5-HTTKO mice have altered sensitivity for non-aversive events by studying sensitivity to rewards.

To this end, 5-HTTKO mice and WT controls were trained in a discriminative appetitive learning task (CS+ = reward; CS– = no reward), followed by reversal learning and extinction, using a Pavlovian conditioning procedure similar to the discriminative aversive learning task used in previous chapters. During each session, food-deprived mice were exposed to 15 CS+ and 15 CS– cues, with the CS+ always rewarded with sucrose solution. It was predicted that 5-HTTKO mice would exhibit enhanced discriminative appetitive learning, enhanced reversal learning and facilitated extinction learning compared to WTs.

Results showed that enhanced discriminative appetitive learning was not observed in 5-HTTKO mice. In fact, nearly half of 5-HTTKO mice failed to meet the learning criterion set for CS+/ CS– discrimination by the end of conditioning. This failure was largely confined to male 5-HTTKO mice, with female 5-HTTKO mice matching the performance of female WT controls. Analyses of the performance in those mice that met the learning criterion still revealed no significant genotypic differences during the reversal learning or extinction phases.

In summary, these data do not provide support for the neuroplasticity theory of 5-HT function. However, it is possible that higher anxiety in 5-HTTKO mice might have influenced performance during acquisition, and might, therefore, be a confound in the interpretation of these findings.

7.2. Introduction

Traditionally, the serotonergic system has been strongly associated with adaptive responses to negative events, particularly due to its role in predicting and adapting behaviours in response to aversive events (e.g. Deakin & Graeff, 1991; see Chapter 1). However, in addition to processing aversive events, evidence also implicates the serotonergic system in the processing of positive emotions, particularly rewarding stimuli (Kranz et al., 2010; Hayes & Greenshaw, 2011;).

A key assumption of the neuroplasticity theory of 5-HT function being tested in this thesis is that higher extracellular 5-HT levels increase responsiveness not only to negative environmental experiences, but also to positive experiences (Belsky et al., 2009; Branchi, 2011; Homberg & van den Hove, 2012). Support for this idea has been provided, for instance, by the finding that individuals with putatively higher extracellular 5-HT levels, such as those carrying the 5-HTTLPR *s* allele, benefit more from positive environmental influences than those homozygous for the long allele (Pluess & Belsky, 2013). This has been shown in an experimental setting by Fox and collaborators (2011), who found that participants homozygous for the 5-HTTLPR *s* allele and individuals with two copies of the *l* allele were differently affected by training in the "attention bias modification" (ABM) technique. This technique can induce or modify attentional biases (AB) towards either negative stimuli or positive stimuli depending on the stimuli used. In their study, Fox et al. subjected different groups of participants to either a negative AB or a positive AB by using affective pictures

as stimuli. Homozygotes for the *s* allele developed both stronger negative and positive biases than *l*/*l* homozygotes, depending on which ABM procedure they had been subjected to. This showed that in s/s homozygotes attentional bias is more strongly modified by both positive and negative stimuli, compared to *l*/*l* homozygotes.

Subjects	Task/process	Effect/ results	Authors/year
5-HTTLPR s/s or s/l individuals	Discriminative appetitive conditioning 8s-long visual stimuli only the CS+ was reinforced with erotic pictures	– SCRs to any CS ↑ amygdala activity (contrast CS + > CS-) ↑ amygdala reactivity to the US	Klucken et al., 2013b
5-HTTKO rats	▪ Conditioned place preference cocaine injection (10mg/kg, I.P.) ▪ Extinction of intravenous cocaine self-administration	↑ place preference [♂] ↓ extinction	Homberg et al., 2008
	Pavlovian/Simultaneous conditioning 45 mg sucrose pellet delivered coincidentally with the start of a CS	– conditioning	Nonkes et al., 2012
5-HTTKO mice	Conditioned place preference cocaine injection (10mg/kg, S.C.)	↑ place preference	Sora et al., 1998, 2001
5-HTTOE mice	▪ Reward magnitude discrimination (low reward vs high reward) ▪ Spatial working and reference memory tasks (reward vs no reward)	↓ sensitivity to positive reinforcers [♀] – discrimination learning [♀]	Line et al., 2014

Table 7.1. Selection of rodent studies investigating the effects of different 5-HTT expression levels on appetitive conditioning. ↑, enhanced; ↓, diminished; –, no changes; ♀, only females were used; ♂, only males were used; I.P., intraperitoneal; S.C., subcutaneous.

Other studies in humans support the finding of increased attentional bias for positive stimuli in low 5-HTT-expressing individuals (Roiser et al., 2007; Beevers et al., 2009, 2011; but see Pergamin-Hight et al., 2012). Moreover, at least one study has already subjected individuals with varying 5-HTT expression levels to a discriminative appetitive conditioning procedure, using erotic pictures as the unconditioned stimulus (Klucken et al., 2013b; see

Table 7.1). Although no genotypic differences were detected regarding the behavioural discrimination, measures of the fMRI BOLD signal during the task revealed that the amygdala of *s* allele carriers exhibited significantly increased reactivity to the CS+ as compared to the CS– (i.e. contrast CS+ > CS–), and to the US, compared to the amygdala of *l*/*l* carriers. Other structures relevant to appetitive CS-US learning, such as the ventral striatum and insula, also showed increased haemodynamic responses to the CS+ as compared to the CS– in *s* allele carriers.

Given that the serotonergic system is potentially involved in stimulus-reward learning (Kranz et al., 2010; Hayes & Greenshaw, 2011), one might expect that direct manipulations of this system would interfere with learning in appetitive tasks. Indeed, evidence has been obtained with 5-HTTKO rodents subjected to place conditioning, a procedure involving associative learning in which a conditioned subject chooses to spend more time in a location previously paired with a positive or rewarding stimulus (cocaine) than in an alternative, unrewarded location. In this task, 5-HTTKO rodents exhibit enhanced conditioned place preference compared with WT controls (Sora et al., 1998, 2001; Homberg et al., 2008).

Subjects	Task/process	Effect/ results	Authors/year
s allele carriers	Probabilistic response reversal task tryptophan-depleted participants positive and negative feedback	↑ reversal learning (i.e. fewer errors)	Finger et al., 2007
rh5-HTTLPR SS rhesus macaques	▪ Reversal learning: CS+ reinforced, CS- unreinforced 9 reversals; no correction trials ▪ Instrumental extinction	– discrimination learning ↓ reversal learning ↓ extinction	Izquierdo et al., 2007
rh5-HTTLPR SL rhesus macaques	Reversal learning low reward vs high reward CSs at least 15 reversals	– discrimination learning ↑ reversal learning	Jedema et al., 2010
	Reversal learning 3 stimuli used, only 1 reinforced Polymorphisms in the 3' untranslated region (UTR) of the 5-HTT: the 5-HTT T:G:T 30' UTR haplotype has similar effects to the S allele, whereas the C:C:C haplotype has similar effects to the L allele	SL vs LL: – discrimination learning – reversal learning 5-HTT 3' UTR: – discrimination learning ↑ reversal learning in the T:G:T haplotype	Vallender, et al., 2009
5-HTTKO rats	Visuospatial reversal learning No explicit Pavlovian CSs two nose poke units four reversals were administered Reinforcer: 45-mg food pellet	– acquisition of spatial discrimination – reversal learning in the four reversals [♂♀, inferred]	Homberg et al., 2007b
	Pavlovian reversal learning	– discrimination learning ↑ reversal learning	Schipper et al., 2011
	7 CS+/7CS- per session 8s-long visual-auditory stimuli Reinforcer: 45mg sucrose pellet	– discrimination learning ↑ reversal learning ↑ acquisition of the new CS+ → US association	Nonkes et al., 2011
5-HTTKO mice	Operant-based reversal learning 85% FFW; discrete visual cues Incorrect responding negatively reinforced (timeout + correction trial)	– discrimination learning [♂] ↑ reversal learning [♂]	Brigman et al., 2010b

Table 7.2. Selection of human, monkey and rodent studies investigating the effects of different 5-HTT expression levels on reversal learning (shaded area). ↑, enhanced; ↓, diminished; –, no changes. ♀, only females were used; ♂, only males were used;

However, apart from conditioned place preference, evidence of enhanced learning in other appetitive conditioning tasks (simple or discriminative) has not been found in 5-HTTKO rodents (e.g. Nonkes et al., 2012). The only hint of a possible enhanced learning effect seen in an appetitive conditioning task was provided by Brigman and colleagues (2010b), who reported a trend for the KOs requiring fewer trials to learn a CS+/CS– discrimination during the initial acquisition phase of an operant-based reversal learning task. However, this result was statistically non-significant.

Notwithstanding the absence of significant genotypic differences during the formation of the initial stimulus-reward associations, some studies have reported enhanced reversal learning in low or null 5-HTT-expressing subjects (see Table 7.2). A (discrimination) reversal learning procedure involves having subjects learn an initial CS+/ CS– discrimination (CS+ = reward; CS– = no reward) and then switching the reward association (e.g. the former CS– now becomes the reward-predicting CS+). Subjects show reversal learning by changing their response towards the now more rewarding stimulus. This new learning requires other processes than just the learning of new reward contingencies. Specifically, subjects must (i) detect the change, (ii) be sensitive to negative reinforcement and (iii) avoid the aversive event that is a loss of reward, particularly by (iv) being capable of response inhibition to a previously reward-predicting cue (Roberts, 2006; Brigman et al., 2010a; Brigman et al., 2010b). Importantly, it has been shown that prefrontal cortex 5-HT is necessary for reversal learning (Clarke, et al., 2004).

Enhanced performance of 5-HTTKO rodents has been observed in operant-based reversal learning tasks (Brigman et al., 2010b) and in Pavlovian reversal learning tasks (Nonkes et al., 2011; Schipper et al., 2011), though not in a visuospatial reversal learning task (Homberg et al., 2007b). Notably, some studies have also reported enhanced reversal learning in low 5-HTT-expressing monkeys (Jedema et al., 2010; Vallender et al., 2009),

and in human s-allele carriers tested on a probabilistic response reversal task (Finger et al., 2007). Reversal learning is also a measure of cognitive flexibility, which can be defined as the 'ability to adapt behaviours in response to changes in the environment' (Brigman et al. , 2010a, p. 20). Of note, s-allele carriers perform significantly better than controls homozygous for the long variant in tasks designed to assess cognitive flexibility, such as the Wisconsin Card Sorting Test (Borg et al., 2009).

Considering that few studies have so far investigated how variation in 5-HTT expression levels affect appetitive associative learning in rodents, monkeys and humans, this chapter aimed to subject 5-HTTKO mice for the first time to a cued appetitive (Pavlovian) conditioning procedure (i.e. a non-operant-based task such as the one used by Brigman et al., 2010b). Specifically, a similar discriminative procedure to that used in Chapters 4 and 5 was adapted for this study by replacing the aversive footshock by a rewarding delivery of sucrose solution. The hypothesis guiding this study was that 5-HTTKO mice would demonstrate enhanced learning in a discriminative appetitive learning procedure. In addition, based on the study by Brigman et al. (2010b), reversal learning was also studied. Finally, it was also investigated whether 5-HTTKO mice exhibited altered extinction learning in the appetitive domain.

7.3. Method

Subjects

This experiment used 16 5-HTTKO mice (female: n=8) and 16 WT mice (female: n=8), which were aged between 4-5 months at the start of testing (KOs: M=4.6; WTs: M=5.3). Subjects were group housed with littermates in a temperature-controlled colony room on a 12/12h light/dark cycle (lights on at 7 am) with water always available. During

the two weeks prior to the start of the experiment, they were reduced to, and then maintained at, 90% free-feeding (body) weight (90% FFW) throughout the following period of testing.

Apparatus

Sixteen experimental chambers were used (ENV-307W, Med Associates,), each one with a food magazine (ENV-303M, Med Associates) containing a liquid receptacle into which sucrose solution was delivered via tubing (PHM-122-18, Med Associates; 2609722-1PAK, Sigma-Aldrich) connected to a syringe pump (PHM-100SA-UK, Med Associates) placed outside the sound-attenuating cubicle enclosing each chamber. Besides including a houselight and loudspeaker connected to an audio stimulus generator, chambers contained several other components that were not operational during the sessions (nose poke panels, levers and LED stimulus lights). Syringe pumps accommodated 20 ml syringes carrying sucrose solution made by dissolving 20g of sucrose (16104, Sigma-Aldrich) in 100ml of water. Reinforcement consisted in a delivering 40µl of sucrose solution.

A purpose-built script running in Med-PC IV software controlled the delivery of stimuli and registered the number and duration of head entries in the magazine detected by beam breaks. All sessions were video-recorded with a video camera placed above the chambers.

Pre-training procedure

The experimental subjects and their littermates were first exposed to the sucrose solution in their home cages for three days. Afterwards, they were gradually habituated to drink in the experimental chamber with a 3-day procedure, which also allowed the selection of the most efficient mice at drinking (i.e. the soon-to-be 32 experimental subjects). First, all 5-HTTKO mice and WT controls (i.e. the soon-to-be experimental subjects, plus 2 WTs and

5 KOs) were left in an unclosed experimental chamber with the liquid receptacle filled with sucrose solution for up to 10mins (day 1), or until they started drinking continuously (i.e. over 3s). If a subject did not drink during this period, this step would be repeated up to two times. Next (day 2), all mice were subjected to 20 deliveries of sucrose solution following a variable interval 60-second (VI 60 s) schedule (session duration: 1210s). This would get them used to drink in similar conditions to those used during the experiment, where the sucrose solution would be delivered in total darkness by means of a sucrose pump. The 16 KOs mice and 16 WTs exhibiting the highest levels of sucrose-cup responding during this day were then subjected to 20 deliveries following a variable interval 120-second (VI 120 s) schedule 24h later (day 3; session duration: 2410s). The amount of sucrose solution delivered each time (40 μl) was the same that would be used as a reward during the main task. The range of intertrial interval (ITI) durations used during the VI 120 s was also the same that would be used during conditioning (90-155s; M=120s).

Training procedure

Subjects then received a discriminative Pavlovian conditioning procedure in which an 8-second long auditory cue predicted a reward (CS+) while another auditory cue with the same duration predicted the absence of reward (CS−). The auditory cues were either a white noise or a 3KHz pure tone, and were allocated in a counterbalanced way as the CS+ cue. They differed in loudness level (white noise: 71 dB; tone: 85 dB) because previous mouse studies in the laboratory had found that this leads to similar rates of acquisition learning of CS-US associations in this task (Anna Huber, unpublished).

Every testing session involved the presentation of 15 CS+ and 15 CS− cues, presented in a pseudo-random order that avoided more than two presentations of each cue type in a row (session duration: 3865s). The order of cue presentation changed every day, as

well as the order of the different ITI durations used. However, an interval of 150s was always used at the beginning of every session as a lead-in period to the first cue presentation. Because two orders of presentation of cues were used during the testing (e.g. 'CS+, CS–, CS+' versus 'CS–, CS+, CS–'), care was taken to ensure that all cages in an experimental room would be playing white noise or a tone at the same time, although potentially signalling different consequences for the subjects (e.g. white noise functioning as CS+ in 4 cages, but as a CS– in the other 4).

After a steady-state cue discrimination level was achieved by the experimental subjects (see below), reversal learning was assessed by simply reversing the reinforcement contingencies previously learned. Everything else remained constant throughout the reversal learning phase. After a new stable cue discrimination level was obtained, extinction sessions were administered in the same experimental chamber by the omission of the reward after the presentations of the CS+ that had been reinforced during the reversal learning phase. Mice were tested every day.

Learning was assessed by means of conditioned sucrose-cup responding, which was recorded as the number and duration of cue-evoked head entries into the magazine for each CS (e.g. mean duration of head entries during the CS period minus mean duration of head entries during the 8s-long pre-CS period). Sucrose-cup responding during a reinforcement period was also recorded. This period was the 5s following the end of CS+ presentation, which included the 1s-long delivery of the reward and the next 4s.

Data analysis

To assess cue discrimination learning (and extinction), acquisition curves were plotted daily. Training continued until the strength of the conditioned response (CR) elicited by the CS+ reached a steady state, and the response strength elicited by the CS– dropped to,

and remained at, zero. For clarity, a discrimination index incorporating pre-CS baseline periods (i.e. CS+ evoked responses minus CS– evoked responses) was calculated for each day of testing and used for most of the figures and the statistical analyses reported in this chapter. For conciseness, only the results obtained with the duration measures are reported in this chapter. However, results produced with head entries data provided a similar picture.

Two criteria were used for establishing steady-state learning in the experimental subjects: one for asymptotic group learning and another for individual learning. A group would be considered to have acquired stable cue discrimination (or stable extinction) when (i) visual inspection of the plotted discrimination index data revealed a stable phase (i.e. if the learning curve reached a plateau), and (ii) no statistically significant differences were detected among the averaged discrimination indexes obtained during the last three sessions for each group. After the asymptotic group learning criterion was met for both groups, an individual learning criterion was also used to select the subjects that would be included in the data analyses of the following phase (i.e. the 'discriminators'). This was done to ensure that: (i) the assessment of the reversal learning was not influenced by subjects that had not learned well the initial CS+/CS– discrimination during the conditioning phase; and (ii) that good discriminators were not overtrained. The criterion used for selecting discriminators consisted of ensuring that the number of CS+ evoked head entries exhibited by a given subject was significantly greater than those evoked by the CS–, in two out of the three sessions used to establish the asymptotic group learning. All mice received the exact same number of training sessions in each training phase of the experiment.

Two-way between-subjects ANOVAs, repeated-measures ANOVAs, and Mann-Whitney *U* tests were conducted as described in Chapter 2 (section 2.8). Data from the VI 120 s were subjected to a logarithm ($\log_{10}$) transformation, but neither a logarithmic or square root transformation could solve the assumptions problem in the data obtained with

the VI 60 s. A factor of sex was included in the ANOVAs presented in the conditioning phase (ANOVA model: $Genotype_2 \times Sex_2 \times Day_{20}$) but not for the following phases (reversal and extinction learning) given that only two male KOs met the learning criterion set for the acquisition phase. Graphs show means and standard errors of the mean (SEM). Non-parametric data are plotted in refined boxplots, which show not only the medians and inter-quartile ranges (IQRs) but also outliers, which were defined as data values exceeding the IQRs by 1.5 times.

7.4. Results

7.4.1. Pre-training

5-HTTKO mice exhibited reduced sucrose-cup responding during pre-training compared to WT controls

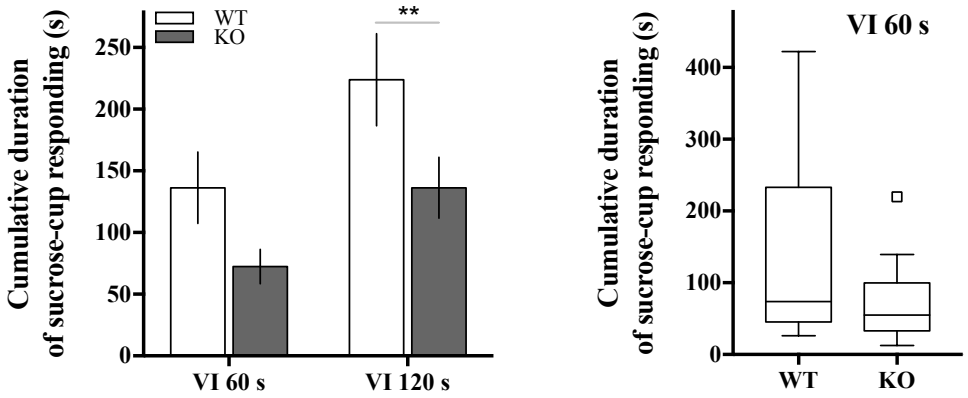


Figure 7.1. Left, Cumulative duration of sucrose-cup responding (s) during each of the Variable-Interval (VI) schedules administered during the pre-training phase to the 5-HTTKO (N=16) and WT (N=16) mice. Mean ± SEM. **Right,** Refined boxplots with the data obtained during the VI 60-second schedule (white square: outlier exceeding the IQR 1.5 times).

During VI pre-training, 5-HTTKO mice exhibited less sucrose-cup responding than WT controls (see Figure 7.1, left). Although this was non-significant during the VI 60 s

schedule (U = 85.0, p = .105; WTs Median = 73.8; KOs Median = 54.9; see Figure 7.1, right), a significant genotypic difference was detected during the VI 120 s schedule (main effect of genotype: $F(1,28) = 13.4$, $p = .001$), with 5-HTTKO mice responding less than WT mice. Female subjects exhibited higher levels of sucrose-cup responding in both genotypes (main effect of sex: $F(1,28) = 76.7$, $p < .001$), but with no interaction between genotype and sex ($F(1,28) = 1.2$, $p = .272$).

7.4.2. Discriminative appetitive conditioning

Male 5-HTTKO mice exhibited poorer discrimination than male WT mice

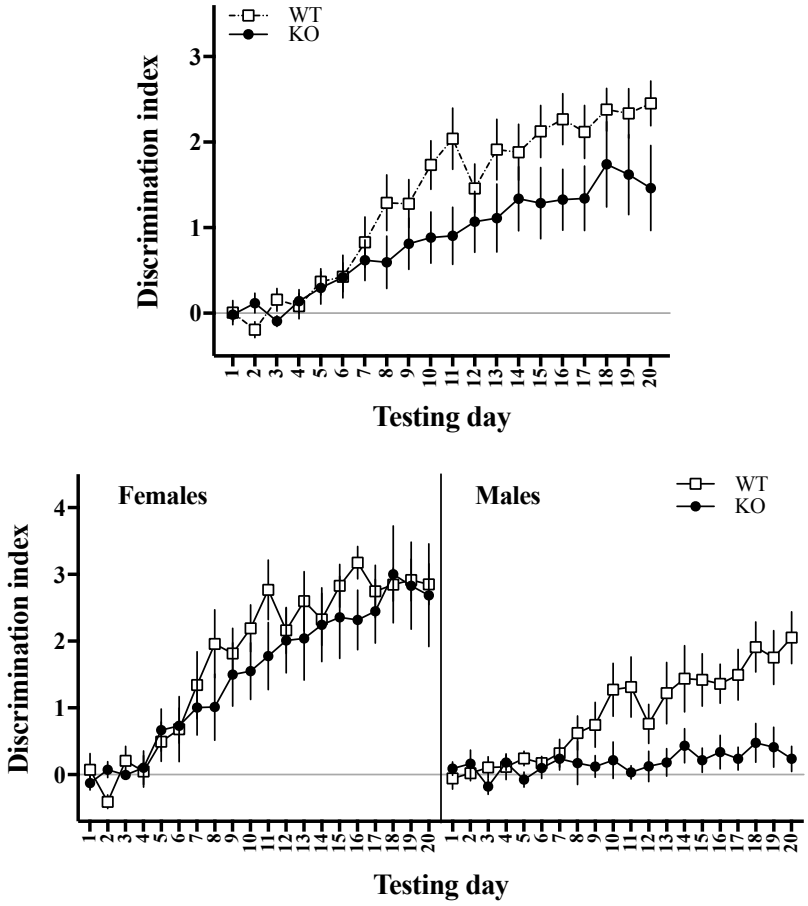


Figure 7.2. Top, Discrimination index (duration of the CS+ evoked sucrose-cup responding minus the duration of CS- evoked sucrose-cup responding) for each of the testing days administered to the 5-HTTKO (N=16) and WT (N=16) mice during the conditioning phase. **Bottom,** Discrimination index exhibited separately by the females (8 WTs, 8 KOs) and males (8 WTs, 8 KOs) in each group during the same days. Both genotypes acquired cue discrimination, though the male 5-HTTKO mice exhibited poorer discrimination than male WT mice. This significantly decreased the overall discrimination levels of the 5-HTTKO mice compared to WT controls. Mean ± SEM.

Analysis of sucrose-cup responding revealed that both genotypes acquired the CS+/CS– discrimination (main effect of day: $F(19,532) = 34.3$, $p < .001$), but 5-HTTKO mice exhibited significantly worse discrimination levels than the WT mice during acquisition ($Genotype_2 \times Day_{20}$ interaction: $F(19,532) = 2.6$, $p = .024$; see Figure 7.2, top). Further analyses revealed worse discrimination in males versus females (main effect of sex:

$F(1,28) = 18.1$, $p < .001$; see Figure 7.2, bottom). Although no statistically significant interactions with genotype were detected, probably due to underpowered analyses (i.e. only 8 subjects for each sex in a group), inspection of the data revealed that male 5-HTTKO mice were not only considerably worse than female KOs, but also worse than male WT mice. Indeed, male KOs seem to have learned nothing at all. When a separate ANOVA was conducted with only male data, a significant genotypic difference was found (main effect of genotype: $F(1,14) = 8.4$, $p = .012$; $Genotype_2 \times Day_{20}$ interaction: $F(19, 266) = 4.0$, $p < .001$). In contrast, when performing this analysis with females, no significant genotypic differences were found (main effect of genotype: $F < 1$; $Genotype_2 \times Day_{20}$ interaction: $F < 1$).

Additional analyses focused on the 5-second reinforcement period after the cues revealed that male 5-HTTKO mice drunk significantly less during these periods on most of the testing days compared with their female 5-HTTKO counterparts, but also compared with male WTs ($Genotype_2 \times Sex_2 \times Day_{20}$ interaction: $F(19,532) = 3.4$, $p = .013$). Visual inspection of the recorded videos revealed that, compared with all the other subjects being tested, male 5-HTTKO mice preferred to drink during the inter-trial intervals or, as training progressed, immediately after a sucrose delivery (see Appendix 21). Note that males were heavier than females for both genotypes but, importantly, no significant genotypic differences were found for body weight during testing (90% FFW for male KOs: Mean = 30.3g; female KOs: 21.4g; male WTs: 29.8g; female WTs: 20.3g; main effect of sex: $F(1,28) = 60.6$, $p < .001$; main effect of genotype and $Genotype_2 \times Sex_2$ interaction: both Fs < 1).

At the end of the conditioning phase (i.e. 20 sessions), 15 WTs (7 males, 8 females) and 9 KOs (2 males, 7 females) met the individual learning criterion set for the acquisition of cue discrimination. In short, 5-HTTKO mice were worse than WT mice in acquiring a CS+/ CS– discrimination, but this was driven mainly by the performance of male KOs.

7.4.3. Reversal learning

Groups acquired reversal learning at a similar rate

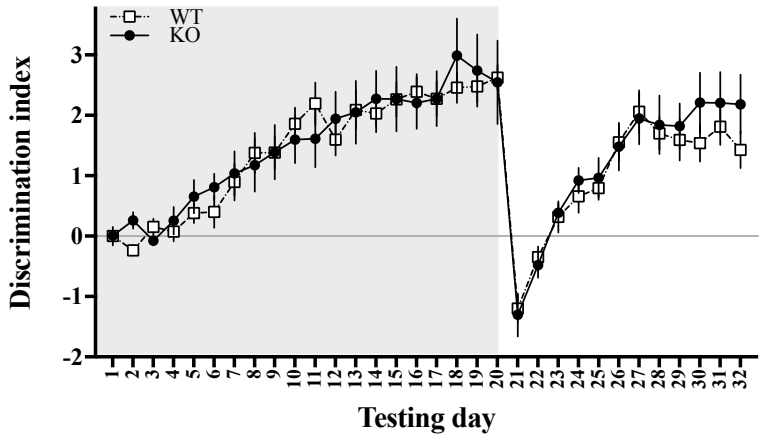


Figure 7.3. Discrimination index (duration of the CS+ evoked sucrose-cup responding minus the duration of CS- evoked sucrose-cup responding) for each of the conditioning (grey-shaded area) and reversal learning (non-shaded area) testing days administered to the 5-HTTKO (N=9) and WT (N=15) mice that met the discrimination learning criterion by the end of the conditioning training. The shaded area thus includes the same data presented in Figure 7.2 but for a reduced number of subjects. Both groups acquired reversal learning and no significant genotypic differences were detected regarding the rate of reversal. Mean ± SEM.

Subjects from each genotype that managed to meet the learning criterion set for the acquisition phase showed virtually no differences in the learning curves during that phase ($Genotype_2 \times Day_{20}$ interaction: $F < 1$; see Figure 7.3, grey-shaded area). In addition, no significant genotypic differences were seen during the reversal learning phase (non-shaded area in Figure 7.3). Both groups learned the new associations at the same rate, matching the level of cue discrimination in each of the twelve sessions administered during this phase (main effect of day: $F(11,242) = 28.7$, $p < .001$; main effect of genotype: $F < 1$; $Genotype_2 \times Day_{12}$ interaction: $F < 1$). Two WT mice (1 male and 1 female) failed to meet the individual learning criterion set for the acquisition of cue discrimination by the end of the 12 sessions of reversal training.

7.4.4. Extinction learning

5-HTTKO and WT mice exhibited a similar rate of extinction learning

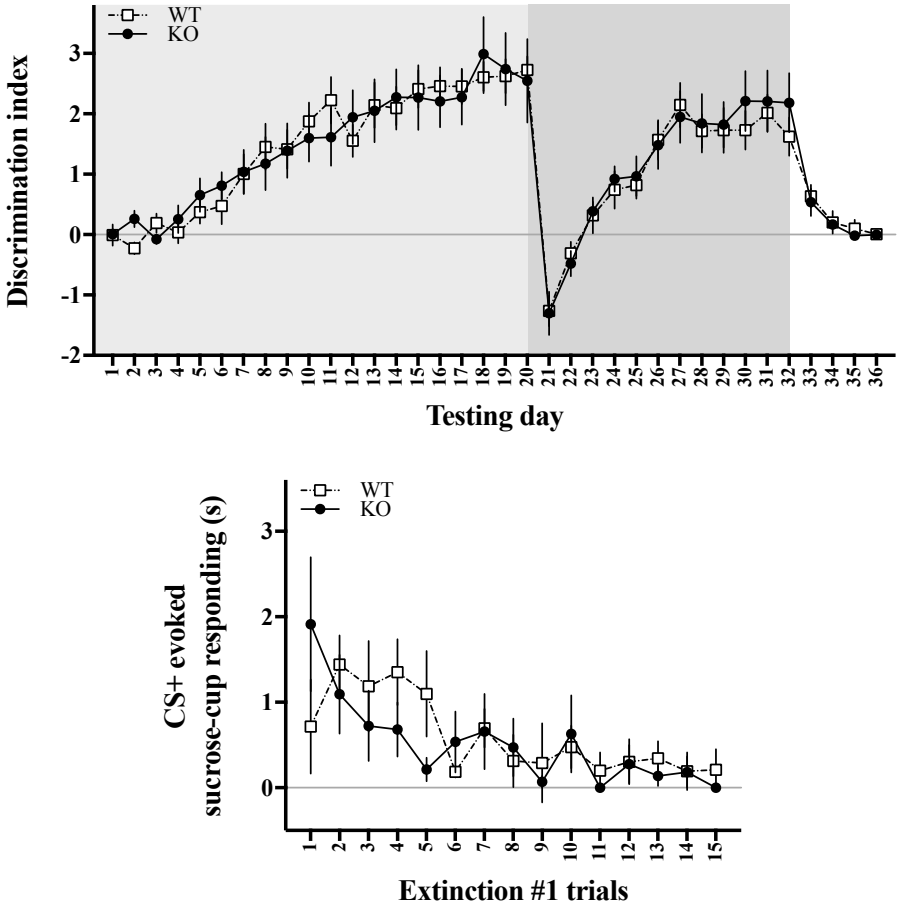


Figure 7.4. Top, Discrimination index (duration of the CS+ evoked sucrose-cup responding minus the duration of CS- evoked sucrose-cup responding) for each of the conditioning (light-grey-shaded area), reversal learning (dark-grey-shaded area) and extinction (non-shaded area) testing days administered to the 5-HTTKO (N=9) and WT (N=13) mice that met the learning criterion by the end of the reversal learning phase. The shaded area thus includes the same data presented in Figure 7.3 but for a reduced number of subjects. **Bottom,** CS+ evoked sucrose-cup responding (i.e. CS+ minus pre-CS+) for the same subjects during the 15 CS+ trials of extinction day 1. Genotypes showed equivalent levels of within-session extinction learning during the first extinction training session, and did not significantly differ across all four days of extinction training. Mean ± SEM.

The 13 WTs (6 males, 7 females) and 9 KOs (2 males, 7 females) that met the learning criterion from the reversal learning phase exhibited the same levels of cue discrimination throughout each session of that phase ($Genotype_2 \times Day_{12}$ interaction: $F < 1$;

see Figure 7.4, dark-grey-shaded area). Subsequently, both genotypes effectively extinguished the CS+/ CS– discrimination acquired during the reversal learning phase (main effect of day: $F(3,60) = 7.0$, $p < .001$), with no differences in the extinction learning rate ($Genotype_2 \times Day_4$ interaction: $F < 1$). Investigation of the extinction of the response towards the CS+ cue only during the first session of extinction also revealed no significant genotypic differences (main effect of genotype: $F < 1$), with genotypes showing equivalent extinction learning rates for the CS+ evoked sucrose-cup responding (main effect of trial: $F(14,280) = 3.5$, $p = .005$; $Genotype_2 \times Trial_{15}$ interaction: $F(14,280) = 1.2$, $p = .327$).

7.4.5. Other analyses

WTs exhibited greater pre-CS levels of sucrose-cup responding than KOs

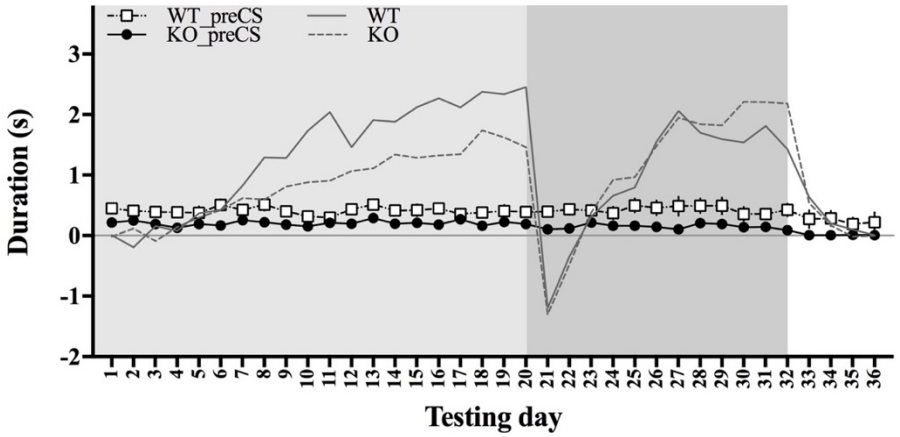


Figure 7.5. Average duration (30 trials) of the pre-CS levels of sucrose-cup responding during the conditioning (16 WTs, 16 KOs; light-grey-shaded area), reversal learning (15 WTs, 9 KOs; dark-grey-shaded area) and extinction (13 WTs, 9 KOs; non-shaded area) phases. The grey curves depict the discrimination index to the cues themselves exhibited by each genotype on the respective phase (as shown in Figure 7.2, 7.3 and 7.4). Mean ± SEM.

Throughout the experiment, WT mice were generally more interested in the sucrose solution (i.e. reward) than 5-HTTKO mice. For example, WT mice exhibited more sucrose-cup responding during the pre-CS periods compared with 5-HTTKO mice (see Figure 7.5).

During conditioning, a significant genotypic difference was found (main effect of genotype: $F(1,28) = 12.6$, $p < .001$; $Genotype_2 \times Sessions_{20}$ interaction: $F < 1$). In the reversal learning phase, WT mice also exhibited significantly more sucrose-cup responding than 5-HTTKO mice during the baseline pre-CS periods (main effect of genotype: $F(1,22) = 4.5$, $p = .046$; $Genotype_2 \times Sessions_{12}$ interaction: $F < 1$). However, some caution should be taken in interpreting the statistics obtained with these measures given that the assumptions of normality and homogeneity of variance were not met for all sessions, mostly due to the presence of many zeros in the data. Transforming the data could not solve this problem.

7.5. Discussion

Summary of the results

This chapter aimed to investigate if 5-HTTKO mice exhibited altered discriminative appetitive learning, reversal learning and extinction in an appetitive Pavlovian conditioning procedure similar to the aversive task used in previous chapters. Although the first analyses pointed towards a significantly poorer acquisition of discriminative appetitive learning in 5-HTTKO mice compared with WT controls, this seems to have been driven mostly by the performance of male KOs. In fact, no significant genotypic differences regarding cue discrimination were detected when only female subjects from each genotype were compared. It is possible that the male KOs' performance was due to a lack of motivation, as reflected in lower pre-CS cup responding compared with WT controls and during pre-training. Subsequently, the 5-HTTKO mice and WT controls that acquired the CS+/ CS– discrimination successfully during the conditioning phase exhibited a similar rate of reversal learning, with no significant genotypic differences being detected at end of this phase. Both

groups also successfully extinguished the CS+/ CS− discrimination acquired during the reversal learning at the same rate.

No enhanced discriminative appetitive learning was observed in 5-HTTKO mice

Prima facie, the results here reported suggest no facilitation, and even an impairment, in 5-HTTKO mice during appetitive discrimination learning and reversal. However, it is possible that the learning impairment seen during the conditioning phase reflects a more general performance impairment on this task in the 5-HTTKO mice. Indeed, significant genotypic differences in sucrose-cup responding were present during the pre-training VI schedules. 5-HTTKO mice drank significantly less than WT controls in a situation where no CSs were presented and subjects could drink *ad libitum*. Moreover, 5-HTTKO mice showed significantly reduced sucrose-cup responding during the pre-CS periods compared with WT mice throughout acquisition and reversal. Taken together, these two results suggest that 5-HTTKO mice were less interested or motivated to drink the sucrose solution than WT mice in the experimental chamber.

Consistent with this likely reduction in motivation in the KOs, a pilot study conducted in the laboratory using an appetitive maze procedure with food-deprived 5-HTTKO and WT mice had to be terminated because the KOs did not drink during the pre-training phase of the maze task. Specifically, 5-HTTKO mice drank significantly less than WT mice (Fisher's exact test: $p < .001$) during short exposures with two to three littermates on a T-maze with rewards available at both goal arms (food wells topped with diluted, sweetened, condensed milk). This occurred despite all subjects drinking the milk in their home cages during the previous days. This result together with the results obtained with the hyponeophagia tests described in Chapter 3 (see section 3.4) argue that 5-HTTKO mice show increased anxiety-like behaviours when given opportunities to consume desirable foods in novel environments after food deprivation. Although testing mice inside

conditioning chambers in total darkness should in theory be less anxiogenic than the previous maze situation or during the hyponeophagia test, particularly after several days of training, it is nevertheless possible that the reduction seen in motivation to drink the sucrose solution in the current experiment can also result from increased anxiety-like behaviours in 5-HTTKO mice.

Considering why male 5-HTTKO mice were markedly worse than the female KOs, it is worth mentioning one study conducted by Joeyen-Waldorf and colleagues (2009). These researchers subjected male and female 5-HTTKO mice of different ages to several anxiety and stress tests, and found an interaction between sex and 5-HTT expression. The male KOs exhibited high baseline emotionality but normal rates of increase, whereas female KOs exhibited the opposite pattern; that is, the females showed normal baseline emotionality but a greater vulnerability to develop high emotionality states with ageing. In their study, males exhibit higher levels of anxiety between the ages of 3 to 6 months, whereas female KOs showed it at later stages (6-12 months). Considering that the 5-HTTKO mice used in this chapter were 4.6 months old on average (males: 4.9; females: 4.3), it is possible that, in agreement with these authors' claim, male KOs could exhibit more anxiety-like behaviours in a relatively anxiogenic environment compared with same-aged female KOs. However, support for this hypothesis warrants further investigation.

Brigman and collaborators (Brigman et al., 2010b) reported a statistically non-significant trend in their results which indicated that 5-HTTKO mice require fewer trials than WTs to acquire a CS+/ CS– discrimination with an appetitive procedure. However, no similar suggestion of enhanced discriminative appetitive learning was found here. It is possible that methodological differences may explain the discrepancy between studies, particularly the use of very different tasks. For example, Brigman et al. used an operant-based visual discrimination learning task that required nose-poking to a touch-screen whereas a purely Pavlovian discrimination procedure was used in this chapter. Brigman et

al. also made incorrect responding more than just non-reinforced (i.e. costlier). Accordingly, their CS− did not just signal absence of the reward but would also lead to a timeout period in total darkness, followed by a correction trial. The contrast between positive reinforcement and a time-out punishment may be necessary to detect enhanced discriminative appetitive learning in 5-HTTKO mice.

In addition, another explanation for the absence of significant genotypic differences during conditioning training could be that subjects with low 5-HTT expression levels do not need to attend to a reward-predicting CS if they can directly approach a reward location whenever they want. In the procedure used here, there was no need to track the CSs for cost-efficient behavioural responding because the availability of the reward did not depend on the CS. Conversely, in the study conducted by Brigman and collaborators (2010b), the visual stimuli would remain on the screen until a response was made. As Nonkes and colleagues (2014) have suggested for 5-HTTKO rats, the following might be true for 5-HTTKO mice: "the environment sensitive 5-HTT−/− rats only acquire tight CS−US associations when the CS is the only cue available signalling the availability of the US" (p. 272).

In summary, no enhanced discriminative appetitive learning was seen in 5-HTTKO mice relative to WT controls in this procedure. This absence of significant alterations in the acquisition of the CS+/ CS− discrimination replicates observations reported by other research groups testing either monkeys or rodents with low 5-HTT expression levels (Homberg et al., 2007b; Izquierdo et al., 2007; Jedema et al., 2010; Nonkes, et al., 2011; Vallender et al., 2009).

No improved reversal learning was observed in 5-HTTKO mice

Following the absence of significant genotypic differences during the acquisition of the CS+/ CS− discrimination during the conditioning phase, no significant genotypic

differences were again seen during the reversal learning phase. That is, both genotypes required the same number of trials to achieve a stable learning of a new CS+/CS– discrimination.

This result contrasts with that of Brigman and collaborators (2010b), who found enhanced reversal learning in 5-HTTKO mice, which made significantly fewer errors than WT controls to achieve the reversal criterion. Notably, in the Brigman et al. study, KOs needed more trials on average to meet the new learning criteria (~600) compared with those needed for the initial CS+/CS– discrimination (456.6 ± 157). This is the opposite of what was observed in this chapter, where KOs were faster to acquire the reversal criterion compared to the acquisition of the initial CS+/CS– discrimination (12 sessions versus 20 sessions, respectively). As previously mentioned regarding the acquisition of the initial CS+/CS– discrimination, methodological differences might explain the different results obtained here compared with those provided by Brigman and colleagues (2010b). As these authors suggested in their report, the enhanced learning seen in KOs could have been driven by several processes apart from the learning of a new CS-US contingency, including the heightened sensitivity to the negative reinforcement used in their experiment. Brigman et al. predicted that if the latter process was the main reason responsible for the enhanced learning observed, then a new experiment where incorrect responding was not negatively reinforced would not produce improved reversal learning in the 5-HTTKO mice. Although the present study did not use an operant-based task, the absence of any time-out punishment following the CS– might have nevertheless contributed to the absence of any increased reversal learning in the KOs. Therefore, the contrast between positive reinforcement and a time-out punishment might not only have played a crucial role in the trend result obtained by Brigman et al. during the acquisition of the CS+/CS– discrimination, but also when the CSs were reversed.

Apart from these two studies with 5-HTTKO mice, different results have also been obtained with 5-HTTKO rats when different methods were used in the investigation of reversal learning. Accordingly, whereas a study using a Pavlovian reversal learning procedure has led to a learning enhancement (Nonkes et al., 2011), another using a spatial reversal learning task testing four consecutive reversals has not found any significant genotypic differences in any of the reversals (Homberg et al., 2007b). Results have also differed when different methods were used to test reversal learning with rhesus macaques carrying the long–short variant (LS) genotype of the rh5-HTTLPR polymorphism compared with controls homozygous for the long variant (LL) (Vallender et al., 2009; Jedema et al., 2010).

In summary, differences in the methods might explain why improved reversal learning was not found in the current experiment. Other researchers have already pointed out (e.g. Brigman et al., 2010b) that studying the effects of the genetic deletion of the 5-HTT on reversal learning seems to depend upon interactions with factors such as task specifics and environmental factors. Indeed, evidence collected with different species and tasks so far seems to point towards a subtle effect that can only be detected in specific conditions.

No altered extinction learning was observed in 5-HTTKO mice

Evidence for facilitated appetitive extinction learning was also not verified in the current experiment. This result might be related to the absence of genotypic differences during reversal learning, given that in both situations a previously reinforced CS+ has now two meanings in the same context and is thus considered an ambiguous cue (Roberts, 2006). That is, in the same way that 5-HTTKO mice did not differ from WT controls in learning a new meaning for the previously reinforced CS+ (i.e. it no longer predicts reward in that context) during the reversal learning phase, the same happened during the learning of the new meaning of the CS+ during the extinction phase (i.e. it also does not predict reward).

Conclusion

The experiments in this chapter found no evidence for enhanced appetitive discrimination learning, or reversal learning, or extinction learning in 5-HTTKO mice. However, these results do not rule out the possibility that variation of 5-HTT expression levels might produce subtle effects, limited to only some aspects of reward information processing. In support of this hypothesis, 5-HTTOE mice show a deficit in reward processing, in particular an insensitivity to reward magnitude. Specifically, they showed no difficulties in tasks where they can choose between options that are associated with either reward or no reward, but are impaired in tasks involving relative reward discriminations, where two options are associated with rewards of different sizes (i.e. low reward vs high reward; Line et al., 2014).

Chapter 8. General discussion

8.1. Overview

The experiments reported in this thesis aimed to clarify the role of the 5-HTT on emotional behaviour. This objective was achieved by manipulating 5-HTT function in mice in two ways (genetic deletion and pharmacological blockade) and using approach–avoidance conflict tests, social assays and discriminative learning tasks with negative and positive emotional stimuli. In this chapter, the main results are summarised, and their implications for the neuroplasticity theory of 5-HT function are discussed. Evaluation of the thesis and suggestions for future work are also addressed.

8.2. Summary of results

The effects of genetic deletion of 5-HTT function on emotional behaviour

Increased anxiety-like behaviours were observed in each of the approach–avoidance conflict tests used (EPM, HPN-1 and HPN-2), which means the high-anxiety phenotype typically seen in 5-HTTKO mice was replicated, and extended, here. 5-HTTKO mice exhibited reduced social interaction when forced to interact with a conspecific in the reciprocal social interaction test, but showed equivalent levels of social investigation to the WT controls during the less anxiogenic sociability phase of the three-chamber test. The KOs also showed increased preference to investigate a novel mouse stimulus over a familiar mouse in the three-chamber test, suggesting enhanced social recognition memory.

5-HTTKO mice undergoing a discriminative aversive conditioning task exhibited enhanced cue discrimination compared with WT controls, both when fully predictive aversive cues (CS+) or ambiguous aversive cues (CSa) were used versus a control cue (CS–). When fully predictive aversive cues were used, the enhanced discrimination learning seen

in the KOs was paralleled by larger BLA haemodynamic responses to the footshock, the CS+ and, to a lesser extent, the CS–. Moreover, throughout conditioning training, the CS+ came to evoke stronger theta-frequency oscillations than the CS– in both genotypes, but this change developed faster in the KOs than WT mice. Importantly, 5-HTTKO mice exhibited not only increased sensitivity/reactivity to the repeated presentations of an aversive stimulus (i.e. footshock) and the threat predicting CS+ cue, but also increased sensitivity towards the positive, safety-signalling CS– presentations. Additionally, other forms of altered conditioned learning were observed in 5-HTTKO mice, namely contextual fear conditioning and fear extinction learning. Regarding the processing of rewarding conditioned cues, no evidence for enhanced appetitive discrimination learning, reversal learning or appetitive extinction learning was found in the 5-HTTKO mice.

The effects of 5-HTT pharmacological blockade on emotional learning

Normal C57BL/6J mice treated with a SSRI 30 mins before each fear discrimination learning session exhibited enhanced discrimination in the form of stronger fear memory recall compared with saline-treated mice. This was only seen in the task using fully predictive aversive cues.

8.3. Implications of the thesis

Few experimental studies have so far provided direct evidence for the neuroplasticity theory of 5-HT function (e.g. Branchi et al., 2013; Kästner et al., 2015). This thesis provides empirical support for two main predictions of this theory on the effects of increased 5-HT availability: increased sensitivity to positive and negative emotional stimuli, and enhanced aversive learning. However, although evidence for enhanced sensitivity for positive stimuli was obtained (e.g. a safety-signalling cue), enhanced appetitive learning in subjects with

increased 5-HT availability was not found. Additionally, it was also observed that there are instances where increased 5-HT availability does not affect emotionality per se, but the testing environment may influence the expression of an anxiety-like phenotype (e.g. reduced social investigation). Furthermore, increased sensitivity to social information in the environment was observed in subjects with higher extracellular 5-HT levels. In summary, although evidence for one of its predictions was not found, the overall outcome of this thesis is considerable support for the neuroplasticity theory of 5-HT function.

5-HTT function and emotional learning

In this thesis, the testing of a mouse model with increased levels of 5-HT availability (i.e. the 5-HTTKO mice; e.g. Jennings et al., 2010) has revealed that they exhibit improved learning on three different forms of associative learning: discriminative aversive learning, contextual fear conditioning and fear extinction learning. Moreover, these mice showed increased sensitivity to negative and positive stimuli in their learning environment.

Altered sensitivity to negative emotional stimuli in the KOs was seen as increased reactivity to the presentation of successive footshocks, which was shown behaviourally (i.e. stronger changes in activity levels) and with stronger BLA reactivity to these USs during the first training session of a discriminative aversive learning task. Additionally, compared with WT controls, 5-HTTKO mice froze more to a context that was the sole predictor of footshock (i.e. enhanced contextual fear learning) and exhibited larger BLA haemodynamic responses to a fully predictive aversive cue (CS+). The KOs also exhibited a faster development of stronger CS+ evoked theta compared to WT controls. Additionally, during a task using a partially-reinforced aversive cue, they required fewer pairings of CS with a footshock than WT controls to show a fear memory during a recall session.

Regarding the altered sensitivity to positive stimuli, 5-HTTKO mice showed *reduced freezing* (i.e. compared to the pre-CS period) during the presentation of a safety-signalling

cue (CS–) to that exhibited during the presentation of an aversive cue (i.e. freezing to the CS+). One interpretation of this would be inhibition of fear or 'relief' (Bouton, 2007), and is in line with claims that the CS– can work as a safety cue (see Chapter 4, section 4.7; see also Genud-Gabai et al., 2013). Moreover, 5-HTTKO mice also exhibited larger BLA haemodynamic responses to the CS– than WT mice, although to a lesser extent (i.e. non-significantly) than those seen with CS+ cues. Furthermore, the KOs exhibited facilitated fear extinction learning by showing less freezing to a CS+ after two extinction sessions compared with WT controls. During extinction training, the CS+ can also be considered a safety-signalling cue (Likhtik & Paz, 2015), which means that, arguably, two instances of enhanced learning about positive, safety signals were seen with the KOs. Notably, the BLA is also involved in fear extinction learning (Herry et al., 2006; Lee & Kim, 1998) and augmented functioning of this brain area could perhaps facilitate this form of learning.

The above-mentioned instances of enhanced sensitivity to negative and positive emotional stimuli likely contributed to the enhanced fear discrimination learning seen in the 5-HTTKO mice. Increased sensitivity to the footshock during the first conditioning session may have made these mice pay more attention to both CSs, which led to bidirectional effects on the processing of emotionally-relevant cues: fear in the presence of the CS+ and relief in the presence of the CS–. This differential response to each cue helped the 5-HTTKO mice to exhibit significantly stronger cue discrimination early in conditioning training compared with WT controls. Together, these processes provide support for a prediction of the neuroplasticity theory of 5-HT function that increased sensitivity to emotionally-relevant events can facilitate emotional learning. This claim is further strengthened by the demonstration that increasing 5-HT availability at the time of conditioning training in normal mice also leads to enhanced discriminative aversive learning (see Chapter 6, section 6.4.1).

Importantly, the enhanced BLA responsivity found in 5-HTTKO mice during the presentation of aversive cues provides support for long-standing predictions that these mice should exhibit augmented amygdala function (e.g. Hariri & Holmes, 2006). Moreover, this result is in line with other findings showing increased functional activation of the amygdala in 5-HTTKO mice (Narayanan et al., 2011; Pang et al., 2011; Kolter et al., 2016), and in individuals with low 5-HTT levels (Rhodes et al., 2007; see also Klucken et al., 2015). Together, these results support the view that increased amygdala function in individuals with low 5-HTT levels (and thereby with increased 5-HT availability) can lead to hypervigilance (Homberg & Lesch, 2011) and deeper processing of emotional stimuli (Homberg et al., 2016). In addition, the stronger CS+ evoked theta-frequency oscillations seen in the KOs early in conditioning training strengthens claims that 5-HT can modulate theta oscillations in the BLA during fear conditioning, with higher 5-HT availability associated with stronger theta power in the BLA (Barkus et al., 2014; Bocchio et al., 2016).

Mechanisms underlying the altered emotional responding seen in the KOs

Considering a biological mechanism underlying the altered emotional responding seen in 5-HTTKO mice in this thesis, it is worth considering one theorised by Hariri and Holmes in 2006 (Hariri & Holmes, 2006) addressing a (then) putative altered amygdala activity in 5-HTTKO mice. These authors suggested that these mice would exhibit amygdala "hyper-excitability" because of the combined effects of the following circumstances: i) the downregulation of 5-HT_{1A} autoreceptors would lead to a decrease in inhibitory control of 5-HT firing and release; ii) the downregulation of 5-HT_{1A} receptors would decrease the inhibitory effects of DRN 5-HT neurons on postsynaptic neurons; iii) the upregulation of the postsynaptic 5-HT_{2C} receptors would lead to a further increase of excitatory effects.

Another biological mechanism addressing changes in emotional responding in 5-HTTKO mice can be inferred from a hypothesis advanced by Bocchio and colleagues

(Bocchio et al., 2015, 2016) regarding one of the roles of 5-HT in the BLA during fear memory acquisition and expression. These authors suggest that one way in which 5-HT can modulate fear learning is by modulating fear-driven BLA theta oscillations via the recruitment of parvalbumin-expressing (PV) interneurons (INs), which can synchronize principal neurons (PNs) (Courtin et al., 2014; Amilhon et al., 2015). It is known that 5-HT can regulate the inhibition of BA PNs via excitation of PV INs, which can shape PN spike precision and synchrony and control fear learning (Woodruff & Sah, 2007; Wolff et al., 2014). Importantly, the recruitment of BA PV INs during fear memory retrieval (measured by c-Fos expression) is reduced in 5-HTTOE mice (Bocchio et al., 2015). According to Bocchio and colleagues, this reduced excitation of PV INs in 5-HTTOE mice can i) contribute to the already observed reduction of theta rhythms in the BLA of these mice (Barkus et al., 2014), ii) affect/impair the encoding of the CS and US by the PNs, and iii) likely affect the BLA output to the CeA (i.e. the processing of fear responses). Considering that 5-HTTKO mice exhibit enhanced theta synchronisation between the BLA and the mPFC (Narayanan et al., 2011), these authors propose that increased 5-HT levels might positively correlate with BLA theta power and theta synchrony between the BLA, mPFC and the HPC. Additionally, Bocchio and colleagues (2016) also suggest that 5-HT may also modulate synaptic plasticity of excitatory synapses to BA PN (e.g. by facilitating long-term potentiation induction from axons in the external capsule). This mechanism could also be affected by alteration of 5-HT levels in the amygdala, for instance.

However, it should be noted that neurodevelopmental changes might still play a role in the altered theta rhythmicity observed in 5-HTTKO and 5-HTTOE mice. For instance, the abnormal 5-HT-evoked depolarisation of PV INs in the 5-HTTOE mice seems to result from reduced functionally of 5-HT_{2A} receptors (Bocchio et al., 2015). This is relevant given that administration of 5-HT_{2A} receptor agonists in mice have been found to i) enhance consolidation of cued and context fear conditioning memories, ii) facilitate extinction

learning of delay and trace fear conditioning (Zhang et al., 2013), iii) and produce anxiety-like behaviours when injected directly into the BLA (Campbell & Merchant, 2003; de Mello Cruz et al., 2005). Moreover, 5-HT_{2A} knockout mice show a decrease in anxiety-like behaviour (although no changes in a single-cue fear conditioning procedure were observed; Weisstaub et al., 2006). Taking this into account, it could be thought that the reduced theta rhythmicity in 5-HTTOE mice can result from aberrant 5-HT_{2A} receptor signalling (Bocchio et al., 2016). Considering that 5-HTTKO mice and 5-HTTOE have been found to exhibit opposite alterations in other aspects of 5-HT_{2A} receptor function (Jennings et al., 2008; Qu et al., 2005), it cannot be ruled out that compensatory changes in 5-HT_{2A} receptor function may play a role in the altered theta oscillations in 5-HTTKO mice reported in this thesis and, consequently, in the behavioural phenotypes reported.

5-HTT function and the processing of conditioned rewarding cues

Contrary to what might be predicted by the neuroplasticity theory of 5-HT function, 5-HTTKO mice did not exhibit enhanced appetitive discrimination learning. Although it is possible that higher anxiety in the 5-HTTKO mice might have been a confound in the study reported in Chapter 7 (see section 7.5), it is worthwhile reasoning about what could produce such a result.

Considering the above-mentioned hypothesis that elevating 5-HT levels results in superior aversive learning via modulation of BLA function, the absence of genotypic differences in the acquisition of the appetitive CS+/CS− discrimination in the task used in Chapter 7 might not be so unexpected. Indeed, it has been shown that BLA lesions in rodents do not affect acquisition of conditioned responses in simple and discriminative appetitive Pavlovian conditioning tasks (Hatfield et al., 1996; Killcross, 2000; Blundell et al., 2001; Parkinson et al., 2000). However, the BLA may be necessary for instrumental responses to appetitively motivated CSs (Ambroggi et al. , 2008; but see Schoenbaum et al., 2003), which

could explain the trend in the data reported by Brigman and colleagues (2010) of a faster initial CS+/CS− discrimination acquisition in the 5-HTTKO mice compared with WT controls, because their study required an explicit instrumental response (nose poking the correct stimulus on a screen).

Regarding reversal learning, it is still unclear what role the BLA plays in this form of learning (Schoenbaum et al., 2003; Izquierdo & Murray, 2007). Nevertheless, Wassum and Izquierdo (2015) have suggested that the BLA might be, at least, less involved in reversal learning tasks like the one used in this thesis (i.e. where only one type of reward is provided), but may be necessary for tasks where two very different outcomes must be compared (e.g. positive versus negative reward; see Schoenbaum et al., 2003). Accordingly, it is possible that altered functioning of the BLA might have influenced the 5-HTTKO's mice performance in the task used by Brigman and collaborators (2010), but not in the task used here (i.e. which did not use a reward versus time-out punishment was the one used by Brigman et al.).

It is also possible that increased 5-HT availability might have affected the responding to the CS+ in 5-HTTKO mice in the appetitive task. Researchers have shown that increasing extracellular 5-HT levels with an acute administration of d-fenfluramine (a 5-HT releaser) can attenuate responding to a stimulus previously paired with reward (Fletcher, 1995). Conversely, 5,7-DHT lesions of both the DRN and MRN neurons in the same task lead to enhanced responding (Fletcher et al., 1999). Additionally, chronic administration of the SSRI fluoxetine has been found to facilitate responding in a similar task (Sasaki-Adams & Kelley, 2001). Considering these results together, McBride (2010) has suggested that the difference between the results of the drug studies could rely on the fact that d-fenfluramine releases 5-HT at all 5-HT synapses, whereas the SSRI has its strongest effects at the "active" 5-HT synapses. As McBride (2010) put it, "although some parts of the 5-HT system may be actively involved in a positive manner on CR, activation of the majority of 5-HT synapses

may produce negative effects and counter any positive actions of 5-HT" (p.406). It could be that a similar situation is occurring with the 5-HTTKO mice in this discriminative appetitive task used in Chapter 7, where increased 5-HT availability in some brain areas might potentially counteract facilitated reward-related behaviour promoted by other brain areas.

Despite the above-mentioned explanations about why no enhanced learning in the KOs was seen in Chapter 7, it is still worthwhile investigating further whether these mice exhibit altered appetitive conditioning. For instance, it has been shown that the BLA is involved in reward-seeking behaviours (e.g. Stuber et al., 2011), in responses to reward-predictive cues (e.g. Shabel & Janak, 2009) and representation of appetitive USs (Gore et al., 2015). Moreover, cue-reward learning is accompanied by synaptic plasticity changes in the LA early in training (Tye et al., 2008). Therefore, if 5-HTTKO mice have enhanced BLA responsivity to emotionally relevant cues in general, and not only to aversive and safety-signalling cues, there is still a chance of detecting alterations in the BLA function of these mice during appetitive conditioning procedures.

Furthermore, as previously said in Chapter 5 (section 5.5), some authors consider learned safety signals as rewarding, albeit non-appetitive (Likhtik & Paz, 2015; see also Rogan et al., 2005). Importantly, Sangha and colleagues (2013) have shown that a subset of BLA neurons that respond to the CS– also fire to a CS predicting reward. If the 5-HTTKO mice have increased sensitivity to safety-signalling cues, as exhibited in their behaviour and BLA responsivity, it is also possible that they might exhibit increased sensitivity to appetitive rewarding cues, at least under some circumstances.

In addition, besides the examples mentioned in Chapter 7, there is evidence that the serotonergic system, in addition to processing aversive events, is important for the processing of rewarding stimuli (Kranz et al., 2010; Hayes & Greenshaw, 2011). For example, Cohen and colleagues (2015) have shown that optogenetically-identified DRN serotonergic neurons can be phasically excited by reward-predicting cues, and have their

tonic activity increased during blocks of several reward trials. Additionally, Liu and collaborators (2014) have shown that DRN neurons increase activity to a reward-predicting cue (A+) during a Go/No-go olfactory discrimination task, but not to the reward-negative cue (B–), and they require 5-HT to do this. Liu and collaborators (2014) also showed that optogenetic stimulation of DRN neurons can guide the learning of sensory discrimination during acquisition (A+/B–), switch (to a novel pair of cues: C+/D–), and reversal learning (D+/C–). Therefore, serotonergic neurons unequivocally signal information about a reward.

In summary, the processing of conditioned rewarding cues was not enhanced in 5-HTTKO mice as might have been predicted by the neuroplasticity theory of 5-HT function. However, there is strong evidence that both the serotonergic system and the BLA are involved in the processing of rewarding cues. Therefore, it can be assumed that, although no enhanced appetitive discrimination learning was observed in the KOs with the methods used in Chapter 7, it might still be possible to observe it with a different approach. After all, it is worthwhile remembering that 5-HTTKO mice do show enhanced associative learning with reward in other tasks. This is the case with (cocaine) conditioned place preference (Sora et al., 1998, 2001), which is a form of associative learning dependent on the BLA function (Everitt et al., 1991).

5-HTT function and social investigation

This thesis also extended the investigation of the high-anxiety phenotype in 5-HTTKO mice to the investigation of social anxiety. Notably, the reduced social interaction seen in the KOs during the reciprocal social interaction test is consistent with the higher levels of social anxiety commonly seen in 5-HTTLPR *s* allele carriers, compared to *l*/*l* carriers (Way & Taylor, 2011; Stoltenberg et al., 2013). Additionally, the increased social novelty preference seen in the 5-HTTKO mice that underwent the three-chamber test also

supports the claim that 5-HT contributes to pro-social behaviour (Kiser et al., 2012). Considering that the subjects tested in this thesis experienced the same environmental conditions during early life (i.e. no more nor less stressful conditions across different cohorts), it is reasonable to assume that it were differences in the two social assays and the different anxiogenic levels they could elicit that led to observation of different social investigation levels by the 5-HTTKO mice in each assay. This also indicates that alterations of the serotonergic system alone do not impair social interaction.

The enhanced social recognition observed here in 5-HTTKO mice indicates that another type of memory is affected in 5-HTTKO mice besides that observed with conditioned aversive cues in Chapters 4 (e.g. Experiment 2) and 5 (e.g. on C2). Moreover, it provides support for a prediction of the neuroplasticity theory of 5-HT function that higher 5-HT availability increases sensitivity to environmental social influences (Belsky & Pluess, 2009; Branchi, 2011; Kiser et al., 2012; see also Way & Taylor, 2010, for a review focused on the effects of the 5-HTTLPR on differential sensitivity to social experiences). It is possible that an increased preference for social novelty reflects an instance of increased sensitivity to a novel social aspect in the testing environment. In other words, perhaps the 5-HTTKO mice were more interested in what was novel in the environment because it was a new social stimulus, but possibly no genotypic differences would be seen if another type of stimulus had been used (e.g. an inanimate object).

The increased neophobia seen in 5-HTTKO mice in the novel object test (i.e. less exploration when a novel object is introduced in a familiar arena; Kalueff et al., 2007a) suggests indeed that the KOs might be particularly interested in investigating new social stimuli in a test of recognition memory. Perhaps testing 5-HTTKO mice in a novel object recognition test (NOR) could clarify this, but to my knowledge no study has yet reported such investigation. 5-HTTKO rats, however, do not exhibit any alterations in this test (Olivier et al.., 2008a), and although one study has reported improved memory function in

5-HTT +/– mice compared to WT controls in the NOR test (Van den Hove et al., 2011), this was only observed with one of the delay intervals used (i.e. 3h, but not with 2 or 4h). Therefore, the question of whether the enhanced recognition memory in 5-HTTKO mice is specific to social stimuli still needs further investigation.

Importantly, some researchers have advanced that an altered amygdala function (or "hyper-plasticity" as they put it) in the 5-HTTKO mice could lead to increased sensitivity to social stimuli (Kiser et al., 2012). This is consistent with the enhanced BLA responsivity seen in Chapter 5 of this thesis. Additionally, altered BLA function might explain the differences seen between the reciprocal social interaction test and the three-chamber test.

Although the role of the amygdala in ethologically-based tests for anxiety like-behaviour is still a matter of debate (Gonzalez et al., 1996; Treit & Menard, 1997; McHugh et al., 2004), optogenetic activation of BLA projections to the ventral hippocampus (vHPC) in mice being tested in the EPM and HPN test leads to an increase in anxiety-like behaviours (Felix-Ortiz et al., 2013). Similarly, activation of BLA-vHPC projections during the sociability phase of the three-chamber test and a resident-juvenile intruder procedure has been shown to reduce social behaviours in mice (Felix-Ortiz & Tye, 2014). Notably, optogenetic inhibition of the same pathway significantly increased social interaction in the latter task. There is, thus, a crucial role for the BLA-vHPC pathway in modulating both social interaction and anxiety-like behaviours. It is possible that this pathway might have been differently activated in the reciprocal social interaction test and the three-chamber test reported in Chapter 3, and that an altered BLA function in a more anxiogenic environment could explain the differences in behaviours seen in the KOs.

Implications for theories of 5-HT function

Considering that the 5-HTTKO mice did not show enhanced discriminative appetitive learning in Chapter 7, and that half of them failed to even show CS+ evoked

responding (see Appendix 21), it is worthwhile considering what other explanations for the role of 5-HT could be suitable for the distinct results obtained with aversive versus appetitive learning tasks.

As previously said, the serotonergic system has been traditionally considered as being involved in aversive learning (e.g. the Deakin/Graeff hypothesis; Chapter 1, section 1.1.3). In addition to this, some authors consider that 5-HT is involved in a "functional opponency" to dopamine (DA): whereas DA is associated with better-than-expected outcomes (e.g. reward delivery) and their effects on learning, 5-HT is associated with worse-than-expected outcomes (e.g. punishment) and their effects on inhibition and fear learning (Boureau & Dayan, 2011). 5-HT would be important in situations involving "actions that should be avoided to prevent the loss of otherwise expected rewards" (Boureau & Dayan, 2011, p. 79). However, the finding that single 5-HT neurons can be activated both by reward-predictive cues and punishments strongly undermined the claims of this 'opponency model' (Cohen et al., 2015; Dayan & Huys, 2015). Additionally, the tasks used in this thesis do not allow direct 'testing' of this hypothesis (e.g. there are no worse than expected rewards in any experiment, for instance).

An alternative hypothesis to those mentioned above that could potentially explain the performance of male 5-HTTKO mice during the discriminative appetitive learning task is one suggesting that 5-HT is involved in waiting for reward (Miyazaki et al., 2012; Miyazaki et al., 2014). For instance, Miyazaki and colleagues (2012) conducted an experiment with rats where they would inhibit DRN 5-HT neurons during a sequential food-water navigation task with variable delayed rewards by the local application of a 5-HT_{1A} receptor agonist. They showed that such inhibition affected performance during the long (7–11 s) delayed reward condition, but not in the short (2 s) delayed reward condition. Considering their findings, they postulated a role for central 5-HT "in 'waiting to obtain reward' and behavioural withholding to acquire future rewards" (Miyazaki et al. 2012, p. 10456; see also

Miyazaki et al., 2014, for a follow-up study with optogenetically activated DRN 5-HT neurons). Notably, the 5-HTTKO mice that did not show any cue discrimination during the discriminative appetitive conditioning phase were becoming more efficient at eating immediately at the end of the 8-second long presentation of the CS+ (see Appendix 21). Perhaps, the male KOs were just more primed to wait for the reward withholding any action than the male WTs.

A third idea for the role of 5-HT could be that it has a general effect on perception and attention to emotional material (Merens et al., 2007; Hensler, 2010) as suggested by studies showing that increasing 5-HT availability increases attention to both negative and positive emotional stimuli. For instance, healthy volunteers subjected to a single administration of a SSRI can have the recognition of, and attention to, positive and negative emotional stimuli facilitated. This can be seen as increases in the recognition of fearful facial expressions (Browning et al., 2007) or detection of a higher number of facial expressions of fear and happiness with reduced response times (Harmer et al., 2003). Additionally, acute tryptophan loading has been shown i) to enhance the perception of fearful and happy facial expressions in healthy volunteers versus a placebo condition; Attenburrow et al., 2003), and ii) to improve immediate vigilant attention in the letter cancellation task (versus a tryptophan depletion condition; (Luciana et al., 2001). In addition to this, 5-HTTLPR *s* allele carriers have been found to perform better than *l*/*l* carriers on attention tests (e.g. affective Go/No-go test; Roiser et al., 2007). Finally, certain alterations in 5-HT neurotransmission in mouse models (5-HT_3 receptor overexpressing in the forebrain of mice) have been shown to lead to enhanced attention in a latent inhibition paradigm (Harrell & Allan, 2003).

However, it could happen that individuals with increased 5-HT levels are more sensitive to the salience of negative stimuli compared with that of positive stimuli (Homberg & Lesch, 2011). Consistent with this hypothesis, *s* allele carriers have been found to be more sensitive to negative environmental cues than positive ones in some situations (Dannlowski

et al., 2010; Pergamin-Hight et al., 2012). Considering the results of this thesis, 5-HTTKO mice could perhaps be paying more attention to the cues in an aversive context, probably due to their anxiety-like phenotype (Gray & McNaughton, 2000), which thus led to better learning in this context compared with the relatively less aversive context used in Chapter 7.

Finally, it should be noted that it may be that there is no simple theory about a general role for 5-HT that could explain its effects both in aversive and appetitive learning (Hayes & Greenshaw, 2011; Dayan & Huys, 2015).

8.4. Strengths and limitations of the thesis

Strengths of the thesis

This thesis confirmed the importance of running different assays when investigating behavioural phenotypes in genetically modified animals (Holmes & Cryan, 2006; Crawley, 2008). Particularly, providing a complete assessment of the anxiety profile in the 5-HTTKO mice, by using two social assays and several common approach-avoidance conflict tests, was crucial to elucidate the role of anxiety in the reduced social interaction seen in the KOs during the reciprocal social interaction test. Additionally, the observation of enhanced social novelty preference in 5-HTTKO mice showed that other forms of altered discrimination acquisition can be studied in these mice besides those relying on discriminative conditioning tasks.

Testing 5-HTTKO mice for the first time in a discriminative aversive learning task, instead of a single-cue procedure, proved to be a fruitful strategy to detect significant differences in emotional learning between the KOs and the WT controls. Moreover, it is a task that provides some protection against other competing accounts of the associative learning mechanisms that single cue tasks have more difficulties in controlling for (e.g. sensitization; Kamprath & Wotjak, 2004; Lissek et al., 2005). The enhanced learning result

obtained here further stresses the importance of continuing to use discrimination learning in the study of altered emotional learning in 5-HTTKO mice. Particularly, because it was clear that these mice show increased sensitivity not only to negative events (e.g. footshocks; CS+ or CSa presentations) but also to positive, safety-signalling ones (e.g. CS– presentations).

Combining TO_2 amperometry with LFPs recordings is also a strength of this thesis because they represent an indirect (TO_2) and direct (LFPs) measure of brain activity, and both revealed altered BLA function in the KOs compared with the WT mice. Convergent results obtained with these different measurements strengthened the interpretation that was obtained separately with each one: 5-HTTKO mice show increased BLA responsivity to an aversive cue compared to WT controls, particularly during the early stages of fear discrimination learning (e.g. the second day of conditioning training).

A further strength of the thesis is the use of different methods (i.e. genetic deletion of the 5-HTT and pharmacological blockade of 5-HTT function, with citalopram) to investigate the hypothesis that increasing 5-HT levels results in superior aversive learning. This 'combination' approach has been used by other studies to better understand the behavioural phenotypes of 5-HTTKO mice (e.g. anxiety-like phenotype; Ansorge, 2004).

Limitations of the thesis

One limitation of this thesis is that only 5-HTTKO mice and their WT controls were used, and no experiment made use of 5-HTT +/– mice. For some researchers, these mice closely resemble 5-HTTLPR *s* allele carriers regarding 5-HTT expression levels and function (Murphy et al., 2001; Murphy & Lesch, 2008), and would thus be a more relevant mouse model of these individuals given that they are a less confounded model of 5-HTT dysfunction for behavioural and pharmacological studies (Kalueff et al., 2007c).

Another limitation of this thesis was that mice treated with citalopram during conditioning training did not exhibit enhanced discrimination learning when tested in a task

using ambiguous aversive cues (Chapter 6, Experiment 2), but only when fully predictive aversive cues were used (Experiment 1). This result indicates that increased extracellular 5-HT levels at the time of conditioning training might not be the sole factor determining the enhanced discrimination learning seen in 5-HTTKO mice undergoing discriminative aversive learning tasks. Accordingly, it is likely that neurodevelopmental effects resulting from the lack of 5-HTT expression still play a role in the more efficient way in which the KOs to process emotionally relevant stimuli. However, it should also be noted that acute administration of an SSRI is not equivalent to a lifetime loss of the 5-HTT.

Another limitation of this thesis concerns the results regarding the altered BLA haemodynamic responses reported here. As described in Chapter 5 (section 5.3.5), the analysis of the data obtained with TO_2 amperometry involved obtaining a local baseline immediately before each cue presentation to detect relative changes in the local concentration of TO_2. Despite this, and the fact that specific cue-evoked changes developed throughout the conditioning training, there is the possibility that other effects of 5-HT on the cardiovascular system could have affected the altered haemodynamic responses recorded in the 5-HTTKO mice. Serotonin affects neurovascular coupling and vascular reactivity, and this should be considered when interpreting BOLD-like signal dynamics (Mohammad-Zadeh et al., 2008; Rauch et al., 2008; Martin, 2014). Moreover, genetic manipulations of the 5-HTT are thought to modulate the cardiovascular system (Ni & Watts, 2006). Although studies with 5-HTTKO mice have shown normal haemodynamic parameters (e.g. heart rate; haematocrit), these animals exhibit alterations such as an absence of blood 5-HT (Eddahibi et al., 2000; Chen et al., 2001b), which is usually involved in the regulation of blood flow (Cohen et al., 1996). Therefore, the impact of a possible altered neurovascular coupling cannot be completely ruled out when investigating altered haemodynamic responses in 5-HTTKO mice (Pang et al., 2011; Barkus et al., 2014), particularly when these mice exhibit increased BLA responses to both CS+ and CS– cues.

Lastly, another limitation of this thesis concerns the origin of the theta oscillations measured in Chapter 5. There is strong evidence that LFPs can be intrinsically generated within the BLA (Pape & Paré, 2010; Bocchio et al., 2017; see Appendix 22), even though they can also be modulated by synaptic inputs from other areas. However, it is not possible with the technique used in this thesis to be sure of the origin of the changes in theta power evoked by the presentation of the CS+. To clarify this issue, it would be necessary to perform multiple single unit and LFP recordings simultaneously from the BLA and other areas from which passive volume conduction could probably occur, and then proceed to individual inactivation of those candidate areas (i.e. one at a time) to verify whether BLA theta oscillations would still arise (as suggested in Barkus et al., 2014).

8.5. Suggestions for future work

It would be important that the combination of the reciprocal social interaction test with the three-chamber test should be repeated using 5-HTTKO mice generated with other background strain. This is important given that phenotypic abnormalities can sometimes result from effects of background genes located at loci distinct from the null allele (Gerlai, 1996), including the anxiety-like phenotype in 5-HTTKO mice (Holmes et al., 2003a). Furthermore, it is crucial to verify whether the enhanced recognition memory in these mice only works for social cues, or whether a similar enhancement could also be seen in the novel object recognition task.

Additionally, contextual fear learning should be investigated further in a 2-day experiment solely focused on this form of learning. This study should control for the contribution of habituation processes (i.e. effects of exposure to the box which would also manifest as increases in immobility) by using four groups in a fully counterbalanced design (Wahlsten, 2011). That is, both the 5-HTTKO mice and WT mice would be split into two

groups each, where one group would be shocked on day 1 only but the other would never be shocked. Differences in activity levels from day 1 to day 2 in the controls would likely reflect habituation.

To formally test if the CS– can act as a safety signal, a new discriminative aversive learning task could be administered to a new cohort of 5-HTTKO mice. In this new task, the fear memory recall session would be followed, for instance, by a retardation-of-acquisition test to test for a conditioned inhibitor (Bouton, 2007). In this test, the CS– would be paired with footshock, and if the freezing response developed slowly (i.e. if it was 'retarded'), this could be taken as further evidence that the CS– evokes the opposite response to that evoked by the CS+ (e.g. relief instead of fear). This test should also control for latent inhibition effects by having control groups exposed to the CSs but not to the footshocks.

Regarding the discriminative appetitive learning task used in this thesis, some improvements could be made to increase the responding levels of 5-HTTKO mice. This could be achieved, for instance, by making the sucrose solution only temporarily available (i.e. during a short reinforcement period). Alternatively, a rule could be set only to reward those trials where at least one head entry was made during the 8-second long presentation of the CS+, thus making the procedure instrumental. Furthermore, considering that the significant genotypic differences observed in the discriminative aversive learning tasks were obtained mostly because they relied on a bidirectional response system (Bouton, 2007), it would be worthwhile to devise an equivalent system to be used in the study of discriminative appetitive learning with 5-HTTKO mice. In other words, it could help to have a task where the conditioned responses could go either above or below a baseline level when a CS+ or a CS– were presented, respectively.

Future studies with 5-HTTKO mice should also investigate the occurrence of an altered function of the bed nucleus of the stria terminalis (BNST), which is part of the

extended amygdala. This structure is thought to have an important role in mediating anxiety (Tovote et al., 2015), a modulatory role in generating stimulus-specific conditioned fear responses, and a role in mediating the acute fear-enhancing effects of SSRIs (Ravinder et al., 2013; Marcinkiewcz et al., 2016). Additionally, it has been shown that optogenetic stimulation of serotonergic DRN-BNST pathway can significantly increase both cued and contextual fear recall in mice, as well as enhance anxiety-like behaviour in approach-avoidance tests (Marcinkiewcz et al., 2016).

Finally, future work also should include optogenetic manipulations of 5-HT in WT mice (i.e. the controls for 5-HTTKO mice). For instance, considering the issue of the role neurodevelopmental effects in the behavioural phenotype of 5-HTTKO mice, it would be important to control 'tagged' DRN 5-HT axons input into the BLA with high spatial and temporal selectivity (Bocchio et al., 2016). Doing this, for instance, precisely at the time of discriminative fear conditioning training could perhaps not only demonstrate the necessity of 5-HT for the enhanced learning seen in the 5-HTTKO mice, but possibly its sufficiency. Additionally, combining the optogenetic approach with field potential recordings in the BLA during discriminative fear learning could also elucidate whether manipulating BLA 5-HT affects theta oscillations. This could be achieved, for instance, by inhibiting 5-HT release in the BLA during fear conditioning and observe whether theta oscillations would still occur (possibly volume conducted to the amygdala from structures like the hippocampus; See Appendix 22).

Future directions for testing the neuroplasticity theory of 5-HT function

This thesis has shown that it is not only the sensitivity to emotional information that is altered in the 5-HTTKO mice but also the sensitivity to social information. Particularly, KOs exhibited enhanced social recognition, which some authors consider to be a form of short-term memory (Thor & Holloway, 1982; Ferguson et al., 2002; Winslow, 2003). It

would be worthwhile investigating whether long-term memory for social recognition is also altered in 5-HTTKO mice. Additionally, this could be complemented with research on whether 5-HTTKO mice exhibit altered short-term memory for fear discrimination learning (e.g. at different stages of memory consolidation; Slipczuk et al., 2009). The results from discriminative aversive learning task using ambiguous cues suggest that the 5-HTTKO mice retain cue discrimination better than WT control after a few CS-US pairings. However, it is less clear at what stages of memory consolidation a possible enhanced memory could be formed.

In addition, a study focused on the relationship between increased social recognition performance and discrimination learning during discriminative aversive learning tasks would also be worthwhile. Indeed, preliminary results obtained with mice that underwent both the three-chamber test and, later, the discriminative aversive learning task used in Chapter 5 indicated that those 5-HTTKO mice exhibiting stronger social novelty preference later showed greater levels of CS+/CS– discrimination during the first day of conditioning (see Appendix 23). No equivalent significant relationship between these two measures was seen in the WT controls. Therefore, it is relevant to confirm that enhanced sensitivity to different environmental influences can be seen in the same KO subjects undergoing different tasks (see Homberg & Lesch, 2011). Experimentally, the same cohort of mice could be, for instance, tested in the three-chamber test, then in a discriminative appetite task, and finally in a discriminative aversive task.

Finally, further aversive learning tasks using ambiguous aversive cues (see Chapter 4) could be conducted with 5-HTTKO mice to advance research on the cognitive mechanisms mediating a differential susceptibility to the environment resulting from ‘plasticity genes’ such as the 5-HTT gene. One of those mechanisms was advanced by Fox and Beevers (2016; see also Fox et al., 2011): cognitive processing biases. To put it simply, the interaction between the 5-HTTLPR and early negative or positive environmental events

are thought to lead to the development of "toxic" or "enhancing" cognitive biases, respectively. These biases can then mediate affective outcomes, namely psychopathology or positive affect resiliency. Notably, it has been shown that 5-HTTOE mice, compared with WT controls, exhibit a reduced negative cognitive bias by interpreting an ambiguous aversive cue (i.e. a CS partially reinforced with footshock) as being more similar to a non-aversive CS− than to an aversive CS+ (McHugh et al., 2015). It would be worthwhile to investigate whether 5-HTTKO mice can exhibit both increased negative and positive cognitive biases for stimuli with uncertain outcomes depending on the learning environment.

8.6. Conclusion

This thesis investigated the role of 5-HTT function on emotional behaviour by testing the neuroplasticity theory of 5-HT function, which predicts that increased 5-HT levels can affect sensitivity to the environment and emotional learning via enhanced neuronal plasticity. Accordingly, the experiments reported here have shown that mice with a null expression of the 5-HTT (and thereby with increased 5-HT availability) exhibit altered sensitivity towards emotionally-relevant negative and positive events and towards social influences. Moreover, increased 5-HT levels resulted in superior aversive learning via augmented BLA function. However, the nature of the stimuli used in the experiments reported here was crucial for the observation of such facilitated learning, given that learning about reward-predicting cues was not facilitated in 5-HTTKO mice.

Overall, the results obtained in this thesis provide direct evidence for the neuroplasticity theory of 5-HT function at the behavioural and neurophysiological level. However, further studies are warranted to clarify the boundaries of this theory.

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Appendix 1

Generating of 5-HTTKO mice

The first step in creating a knockout mouse involves incorporating a genetic targeting construct (in this case, a non-functional sequence of DNA inserted in a vehicle) into the specific coding region of the gene known to disrupt its function. This is achieved by making use of a natural phenomenon occurring during meiosis called recombination, in which homologous DNA regions (i.e. with strong sequence similarity) can be exchanged between equivalent chromosomes. Genetic engineering techniques allow researchers to artificially induce such a process and replace an endogenous gene with an altered, non-functional version of that gene by flanking the latter with regions containing sequences of DNA homologous to those surrounding the former (Carter & Shieh, 2010; Flint et al., 2010). These regions of homology are what ensures targeting specificity to a particular locus in the genome, which is usually later verified by testing for the presence of selectable markers (e.g. a drug-resistant gene) also included in the construct.

Following this first step, the altered DNA is delivered into mouse embryonic stem (ES) cells, capable of differentiating into all types of cells. These are then injected into the inner cell mass of a blastocyst, also made of ES cells. After inserting the blastocyst into the uterus of a pseudopregnant female mouse, all those ES cells will develop into a chimeric mouse made of tissues originated from both modified and unmodified ES cells. Given that blastocysts are usually obtained from one strain and the ES cells are derived from another strain, ensuring that the strains differ, for instance, in fur colour helps to visually determine which offspring are chimeric (they will exhibit patches of both colours). Mating chimeric mice carrying the mutation in germline cells with WT mice (+/+) from the strain providing the blastocyst, will lead to some offspring mice expressing the altered gene, but also the endogenous gene (i.e. gene targeting only hits one chromosome). These heterozygote mice

(+/–) are then bred with each other to produce a full knockout mouse (–/–) under a Mendelian inheritance rule, that is, with a genotypic ratio of 2:1:1 for heterozygote, WT and knockout (KO) mice, respectively.

Methodological considerations on the use of genetically modified mice

When KO mice are being generated, the offspring generated by mating chimeric mice with mice from an inbred strain (e.g. C57BL/6J) are heterozygous at all loci that are allelic between those strains, and not only at the null mutant allele (Flint, 2006). This means that the first generation of KO mice will be homozygous for several genes of the strain that provided the ES cells used to generate null mutations (e.g. one of the 129 inbred strains), across different chromosomes. Importantly, this will happen in genes flanking the altered gene.

To deal with this problem, a commonly used strategy is backcrossing heterozygous mice for the mutation to one parental inbred strain for at least 10 generations to purge the genome of all variants and make it 99% identical to the recipient strain (Crusio et al., 2009; Flint, 2006). This strategy ensures reduced within-genotype variability and also provides an appropriate behavioural baseline, considering how well studied some inbred strains are (Holmes & Cryan, 2006). However, although the purge affects most of the chromosome containing the altered gene, it is very difficult to completely eliminate the effects of flanking gene regions (Bailey et al., 2006; Crusio et al., 2009; Wahlsten, 2011). Genes in this region can potentially interact with those of the altered gene and, alternatively, the latter can affect the expressing pattern of neighbouring genes (Flint, 2006). This can confound the interpretation of what is causing the phenotype. This problem is known as the 'flanking allele problem' (Gerlai, 1996) and although it may happen that such effects do not affect the phenotype of interest, the problem cannot be ignored.

Importantly, there are also other problems of genetic background effects due to complex and epistatic interactions between the mutation and genes located in regions outside the flanking region (Gerlai, 1996; Holmes, 2001; Flint et al., 2010). For instance, a given mutation might lead to divergent effects when backcrossed onto different strains (e.g. Holmes et al., 2003).

In addition, there are also concerns about a constitutive mutation of a gene, where transcription is prevented throughout development and adulthood. Secondary developmental consequences of knocking out a gene can occur, leading to either compensation for the effect of the mutation and masking of the gene's normal function, or, conversely, being the actual cause of the observed phenotype (Routtenberg, 1995; Holmes, 2001; Cryan & Holmes, 2005; Flint, 2006; Carter & Shieh, 2010).

Despite these limitations, some steps can be taken to tackle them. A considerably long backcrossing procedure into a well-known inbred strain is always desirable, ideally with the parental strain that provided the ES cells for target mutation. However, sometimes this is not convenient, as is the case with mice from the 129 strain family, which are particularly susceptible to a number of diseases and locomotor abnormalities (Gerlai, 1996; Holmes & Cryan, 2006). In this case, it is then preferable to choose a behaviourally and physiologically well-studied inbred strain that does not exhibit extreme traits that could mask the outcome of the manipulation, and to which littermate WT mice can be compared if necessary (Bailey et al., 2006; Crawley, 2008). If the mutation is implemented in several genetic strains and the phenotype is evident in most of them, this strengthens the hypothesis that the mutation is necessary to generate a phenotype.

Finally, considering behavioural phenotyping, it is important to assess general health, sensory abilities, and motor functions in order to avoid false positive phenotypes, as well as conducting several behaviour tasks to avoid false negative phenotypes (Crawley, 2008; Phillips et al., 2002).

fMRI methodology

In a typical fMRI task, a subtraction methodology is used to isolate a specific sensory, motor or cognitive process. This involves scanning and contrasting two sets of brains: either the brain of a subject in different states, such as those elicited by an experimental task and a control task; or the brains of subjects belonging to different experimental groups (who can also be subjected to an experimental and control task in certain experiments). In the case of the same subjects experiencing different conditions, the experimental and control task make similar (sensory, motor and cognitive) demands on the brain apart from the process of interest. Then, the brain activation elicited by the control condition is subtracted to the one elicited by the experimental condition and any differences in the brain images obtained can then be attributed to the process of interest (Logothetis, 2008).

Depending on the aims of the study, the method of subtraction can be combined with several strategies of presentation of stimuli, among which are the block designs, the event-related designs or a mixture of the two (Carter & Shieh, 2010). The block designs consist of alternating the presentation of each category of stimuli being tested every 1-2 minutes, leading to maximal signal intensity in active brain regions. They are useful for producing (statistical parametric) activation brain maps from the registered haemodynamic changes. Alternatively, event-related designs can be used to measure the degree of signal intensity to each type of stimulus, with the advantage of analysing the course of haemodynamic responses with a temporal resolution of seconds (Carter & Shieh, 2010; Logothetis, 2008). This is achieved with high-speed fMRI methods and can be analysed by plotting the BOLD signal response (% of the change in image intensity) against time (s).

The shape of a typical curve is affected by several factors, but a typical pattern of a positive BOLD signal would be an initial negative dip (undetectable at lower layers of the cortex) followed by a positive rise, reaching its peak a few seconds after the stimulation

started (Passingham & Rowe, 2016). This reflects an initial consumption of O2 followed by the overcompensating increase in O_2 by the increased CBF, with the positive phase lasting for a varying number seconds depending on the nature of the stimuli (e.g. phasic versus continuous) (Logothetis & Wandell, 2004). The amplitude of the BOLD signal can sometimes indicate processing, as is the case in some learning experiments. For instance, O2 demands can be higher during the initials stages of learning which can reflect, for instance, increased attention levels. However, these can decrease as learning improves, reflecting a reduced demand for attention and/or good associative learning (Passingham & Rowe, 2016).

fMRI has several limitations besides not measuring neuronal activity directly. First, it is not possible to use it in a free environment, thus the subject must remain still to avoid producing artefacts in the BOLD signal. Second, the deoxyhaemoglobin content is dependent on other factors besides the $CMRO_2$ such as, for instance, the local basal state of the brain region under investigation. Third, the size and shape of the BOLD responses vary across areas, and its association with $CMRO_2$ and/or neuronal activity is not the same in all brain regions. Finally, the underlying neuronal activity prompting the BOLD response can differ within the same area depending on the circumstances (Logothetis, 2008; Small & Heeger, 2013; Passingham & Rowe, 2016). Therefore, a better understanding of the neural mechanisms underlying the BOLD signal via electrophysiological studies is needed, particularly those involving *in vivo* experimental animal research (Martin, 2014).

Notwithstanding these limitations, the fMRI technique also has several advantages and it has had an enormous, unparalleled influence. These advantages include the possibility of imaging simultaneously over the whole brain in a non-invasive way and a relative high spatiotemporal resolution compared with other brain imaging techniques (Carter & Shieh, 2010). Moreover, it can additionally be used to study functional connectivity between two brain areas through the detection of positive covariance in slow fluctuations in the BOLD signal measured in those two areas (Passingham & Rowe, 2016). Therefore, it still is one of

the best tools to investigate brain function, though a multimodal approach would always be needed for a complete understanding.

Appendix 2

Correlations from the reciprocal social interaction test

Correlations between two variables were investigated using the Person's correlating coefficient (r). No significant correlations were seen between weight differences and either the number of events or total duration of events for each genotype.

WT mice:

Correlation between weight difference and number of social events per pair:

r = -.31, p = .241;

Correlation between weight difference and duration of social events per pair:

r = -.23, p = .385

5-HTTKO mice:

Correlation between weight difference and number of social events per pair:

r = .32, p = .267;

Correlation between weight difference and duration of social events per pair:

r = .23, p = .436

Raw data and DR results obtained in the proximity areas and full chambers of the three-chamber test by tracking of the head movement of experimental subjects

		Sociability phase (Proximity areas)					
		Entries			Duration		
		Raw data		DR	Raw data		DR
		Empty	Social		Empty	Social	
300s	WTs	23.0 (2.4)	29.6 (3.1)	**0.56 (.03)**	50.6 (4.2)	112.4 (8.2)	**0.68 (0.02)**
	KOs	16.4 (2.4)	23.9 (2.8)	**0.59 (.05)**	37.2 (4.5)	91.3 (9.4)	**0.68 (0.05)**
600s	WTs	41.0 (3.5)	51.2 (5.7)	**0.54 (.03)**	94.5 (6.9)	189.4 (16)	**0.65 (0.03)**
	KOs	29.6 (3.1)	42.4 (4.3)	**0.57 (.04)**	67.5 (6.7)	152.5 (15)	**0.68 (0.03)**

		Sociability phase (Chambers)					
		Entries			Duration		
		Raw data		DR	Raw data		DR
		Empty	Social		Empty	Social	
600s	WTs	9.1 (0.9)	9.3 (0.8)	**0.51 (.01)**	208.5 (13.7)	313.3 (18.0)	**0.59 (.03)**
	KOs	6.5 (0.9)	7.1 (0.8)	**0.54 (.03)**	210.0 (22.6)	298.5 (24.4)	**0.58 (.04)**

		Social novelty phase (Proximity areas)					
		Entries			Duration		
		Raw data		DR	Raw data		DR
		Familiar	Novel		Familiar	Novel	
300s	WTs	14.5 (1.7)	23.0 (4.3)	**0.58 (.04)**	39.4 (5.1)	82.5 (9.0)	**0.64 (.04)**
	KOs	8.8 (1.3)	20.1 (2.3)	**0.70 (.03)**	26.8 (4.9)	70.9 (7.5)	**0.73 (.04)**
600s	WTs	28.2 (3.2)	45.2 (6.6)	**0.58 (.03)**	77.4 (9.5)	139.5 14.0)	**0.62 (.04)**
	KOs	19.9 (2.3)	35.9 (3.1)	**0.65 (.03)**	57.0 (7.4)	120.9 12.4)	**0.67 (0.3)**

Note: The values are means and standard errors of the mean (SEM; bracketed).

Exposure to social stimulus during the sociability phase of the three-chamber test

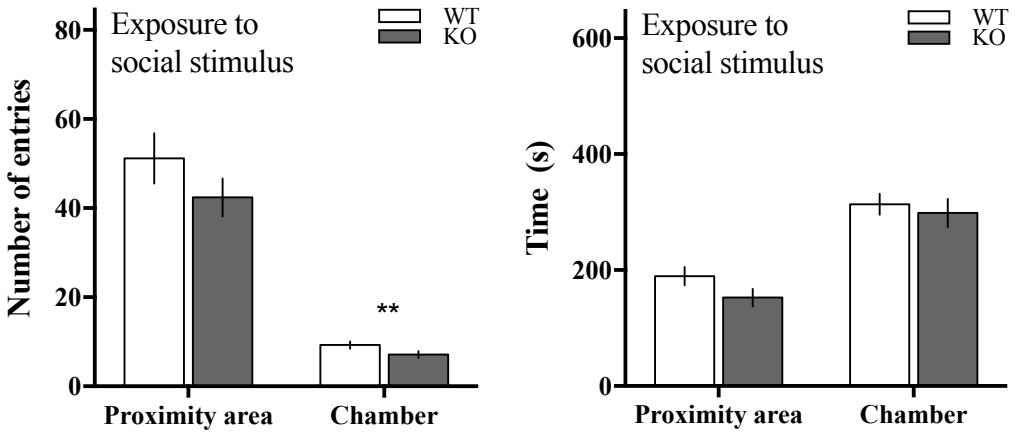


Figure A2.1. Left, Absolute number of head entries into the proximity areas and full chambers of the three-chamber test during the 600-second long sociability phase for 5-HTTKO (N=22) and WT (N=22). **Right,** Absolute time spent with the head in the proximity areas and full chambers of the three-chamber during the same period for the same subjects. Overall, genotypes did no significantly differ regarding the amount of exposure to the social stimulus. However, WT mice made significantly more head entries into the chamber than the KOs. Mean ± SEM. $^{**}p < .01$ (main effect of genotype on the exposure to the social stimulus during the 600s-long sociability phase).

Number of entries:

Proximity area – $F(1,38) = 1.4$, $p = .252$

Chamber – $F(1,38) = 8.0$, $p = .007$ Note: This result does not support the hypothesis that the performance of 5-HTTKO mice during the preference for social novelty phase reflected greater levels of exposure to the social stimulus during the sociability phase.

Time:

Proximity area – $F(1,38) = 3.1$, $p = .084$

Chamber - $F < 1$

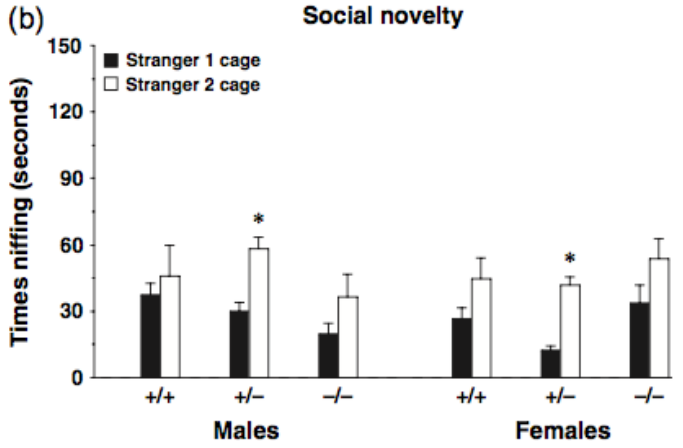


Figure 5: Time spent sniffing each cage by *Slc6a4* mice during the tests for (a) sociability and (b) preference for social novelty. During the test for sociability, all groups had a significant preference for the cage containing stranger 1. Data shown are mean + SEM for each group. *$P < 0.05$, within-group comparison, stranger 1 side different from empty cage side (a) or stranger 2 side (b).

Figure 5.b. from Moy et al., 2009. Reprinted with permission from John Wiley and Sons under the terms of the Creative Commons Attribution (CC BY) License (https://creativecommons.org/licenses/by/4.0). Licensed Material: Moy, S. S., Nadler, J. J., Young, N. B. et al. (2009), Social approach in genetically engineered mouse lines relevant to autism. Genes, Brain and Behavior, 8: 129-142. https://doi.org/10.1111/j.1601-183X.2008.00452.x

Appendix 3

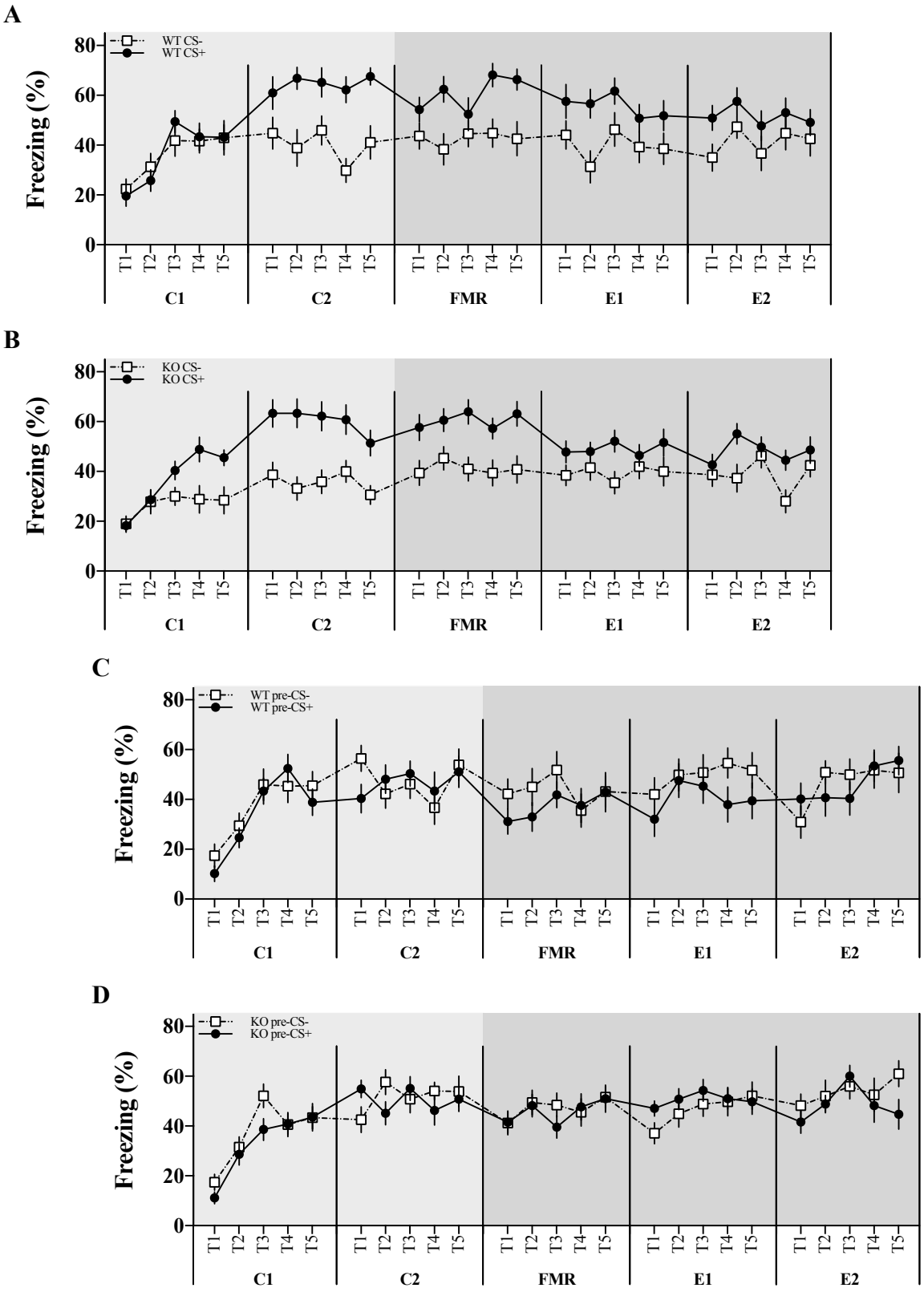
Figure	Results of the statistical tests	Appendix with raw data
4.2, left	No overall genotypic differences were observed during the 5-day procedure (main effect of genotype: $F(1,33) = 1.5$, $p = .232$; $Genoype_2 \times Day_5$ interaction: $F<1$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,33) = 1.3$, $p = .261$; $Genotype_2 \times Day_5 \times pre\text{-}CS_2$ interaction: $F(4,132) = 2.2$, $p = .063$).	4
4.2, right	The result obtained with the discrimination index cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype during C1-FMR: $F(1,33) = 1.3$, $p = .268$; interactions between genotype, day and pre-CS type: $Fs < 1$).	
4.3	Freezing levels during the pre-CS periods (pre-CS+ and pre-CS–) did not significantly differ between groups (main effect of genotype: $F <1$; $Genotype_2 \times Trial_5$ interaction: $F(4,132) = 1.0$, $p =.409$; $Genotype_2 \times pre\text{-}CS_2$ and $Genotype_2 \times Trial_5 \times pre\text{-}CS_2$ interactions: Fs < 1), particularly during the pre-CS– periods ($p = .954$).	
4.4, left	The results obtained with the discrimination index cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype during FMR-E2: $F(1,33) = 1.9$, $p = .179$; $Genotype_2 \times Day_3$ interaction: $F < 1$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,33) = 2.5$, $p = .121$; $Genotype_2 \times Day_3 \times pre\text{-}CS_2$ interaction: $F(2,66) = 3.1$, $p = .054$).	
4.4, right	This result cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype: $F(1,33) = 4.1$, $p = .052$; $F(2,66) = 1.4$, $p = .247$).	
4.6, left	Freezing levels during the pre-CS periods (pre-CSa and pre-CS–) did not significantly differ between groups (main effect of genotype: $F <1$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,44) = 1.6$, $p = .207$; and $Genotype_2 \times Day_4 \times pre\text{-}CS_2$ interaction: $F < 1$). A significant interaction between genotype and day was detected ($F(3,132) = 3.0$, $p = .034$, but no statistically significant genotypic difference was detected on any of the four days included in the analysis.	5
4.6, right	This result cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype during C2-FMR: $F < 1$; $Genotype_2 \times Day_2$ interaction: $F(1,44) = 2.1$, $p = .156$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,44) = 1.2$, $p = .285$; $Genotype_2 \times Day_2 \times pre\text{-}CS_2$ interaction: $F(2,66) = 1.8$, $p = .185$).	
4.7, left	The above-mentioned results cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype and $Genotype_2 \times Day_3 \times pre\text{-}CS_2$ interaction during FMR-E2: $Fs < 1$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,44) = 2.5$, $p = .123$). A significant interaction between genotype and day was registered ($Genotype_2 \times Day_3$ interaction: $F(2,88) = 5.3$, $p = .007$), but pairwise comparisons in each of the days of testing did not reveal any significant genotypic difference regarding pre-CS freezing levels.	
4.7, right	This result cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype: $F < 1$). A significant interaction between genotype and day was detected ($F(2,88) = 4.4$, $p = .014$) but no statistically significant genotypic difference was detected on any of the three days included in the analysis.	

Figure	Results of the statistical tests	Appendix with raw data
5.5, left	No significant differences were detected regarding the freezing responses before the 'to-be-allocated' CS types (main effect of genotype and $Genotype_2 \times preCS_2$ interaction: $p < 1$).	7
5.6, top left and top right	This result cannot be explained by genotypic differences regarding pre-CS freezing levels (main effect of genotype during C1-FMR: $F < 1$; $Genotype_2 \times Day_2$ interaction: $F < 1$; $Genotype_2 \times pre\text{-}CS_2$ interaction: $F(1,57) = 1.4$, $p = .241$; $Genotype_2 \times Day_2 \times pre\text{-}CS_2$ interaction: $F(3,171) = 1.3$, $p = .286$).	
5.6, bottom right	A main effect of genotype across the four days was detected, with the WT freezing significantly more than the KOs during the pre-CS periods ($F(1,25) = 6.0$, $p = .022$). However, no significant interaction between genotype, day and pre-CS type was detected (all $F < 1$).	
6.2, top and bottom	No significant group differences regarding pre-CS freezing levels were registered during FMR among the four groups (main effect of group and interaction between group and pre-CS type: all $Fs < 1$).	8 & 9
6.3	No significant group differences regarding pre-CS freezing levels were registered during this 3-day period (main effect of drug treatment during conditioning: $F(1,94) = 2.5$, $p = .118$; $Drug(Conditioning)_2 \times Day_3$ interaction: $F(2,188) = 1.7$, $p = .184$; $Drug(Conditioning)_2 \times preCS_2$ interaction: $F < 1$; $Drug(Conditioning)_2 \times Day_3 \times preCS_2$ interaction: $F < 1$; simple effect of drug treatment on FMR: $p = .778$).	10
6.4, left and right	No significant group differences regarding pre-CS freezing levels were registered during this 3-day period (main effect of group: $F < 1$; $Group_2 \times Day_3$ interaction: $F < 1$; $Group_2 \times preCS_2$ interaction: $F(1,46) = 2.0$, $p = .162$; $Group_2 \times Day_3 \times preCS_2$ interaction: $F(2,92) = 2.1$, $p = .130$).	8
6.5	Sal-Sal vs. Sal-Cit: This result cannot be explained by group differences regarding pre-CS freezing levels (main effect of group: $F < 1$; $Group_2 \times Day_3$ interaction: $F < 1$; $Group_4 \times Day_3 \times preCS_2$ interaction: $F(2,92) = 1.8$, $p = .178$). A significant interaction was detected between day and group across the three days ($F(1,46) = 4.4$, $p = .041$), with the Sal-Cit group freezing significantly more before the pre-CS– periods than the pre-CS+ periods ($p = .020$). However, no similar significant difference was seen in any of the days individually for this group (C1: $p = .130$; C2: $p = .081$; FMR: $p = .278$). Cit-Sal vs. Cit-Cit: This result cannot be explained by group differences regarding pre-CS freezing levels (main effect of group: $F(1,46) = 1.8$, $p = .181$; $Group_2 \times Day_3$ interaction: $F(2,92) = 1.2$, $p = .306$; $Group_4 \times CS_2$ interaction: $F(1,46) = 1.3$, $p = .252$; $Group_4 \times Day_3 \times preCS_2$ interaction: $F < 1$).	8 & 9
6.6, left	No significant group differences regarding pre-CS freezing levels were registered during this 3-day period (main effect of group: $F < 1$; $Group_2 \times Day_3$ interaction: $F(2,188) = 1.2$, $p = .307$; $Group_2 \times preCS_2$ interaction: $F < 1$; $Group_2 \times Day_3 \times preCS_2$ interaction: $F(2,188) = 1.1$, $p = .322$).	11
6.6, right	No significant group differences regarding pre-CS freezing levels were registered during this 3-day period (main effect of group and interaction between group and day: $Fs < 1$);	

Figure	Results of the statistical tests	Appendix with raw data
6.7, top and bottom	No significant group differences regarding pre-CS freezing levels were registered during FMR among the four groups (main effect of group: $Fs < 1$; $Group_4 \times preCS_2$ interaction: $F(3,92) = 1.4$, $p = .238$).	12,13
6.8	A significant main effect of genotype across the two days was registered ($F(1,94) = 7.3$, $p = .008$), with the saline-treated mice exhibiting significantly more freezing during the pre-CS periods compared to citalopram-treated mice. However, no significant interactions between group, day and pre-CS type were detected ($Drug(Conditioning)_2 \times Day_3$ interaction: $F(1,94) = 3.9$, $p = .051$; $Drug(Conditioning)_2 \times preCS_2$ interaction: $F(1,94) = 1.5$, $p = .217$; $Drug(Conditioning)_2 \times Day_3 \times preCS_2$ interaction: $F < 1$; simple effect of drug treatment on FMR: $p = .176$).	14
6.9, left	This result cannot be explained by group differences regarding pre-CS freezing levels (main effect of group: $F(1,94) = 1.8$, $p = .187$; $Group_2 \times Day_3$ interaction: $F(2,188) = 2.4$, $p = .091$; $Group_2 \times preCS_2$ interaction: $F < 1$; $Group_4 \times Day_3 \times preCS_2$ interaction: $F < 1$).	15
6.9, right	No significant group differences regarding pre-CS freezing levels were registered during this 3-day period (main effect of group: $F(1,94) = 1.8$, $p = .186$; $Group_2 \times Day_3$ interaction: $F(2,188) = 1.8$, $p = .164$).	

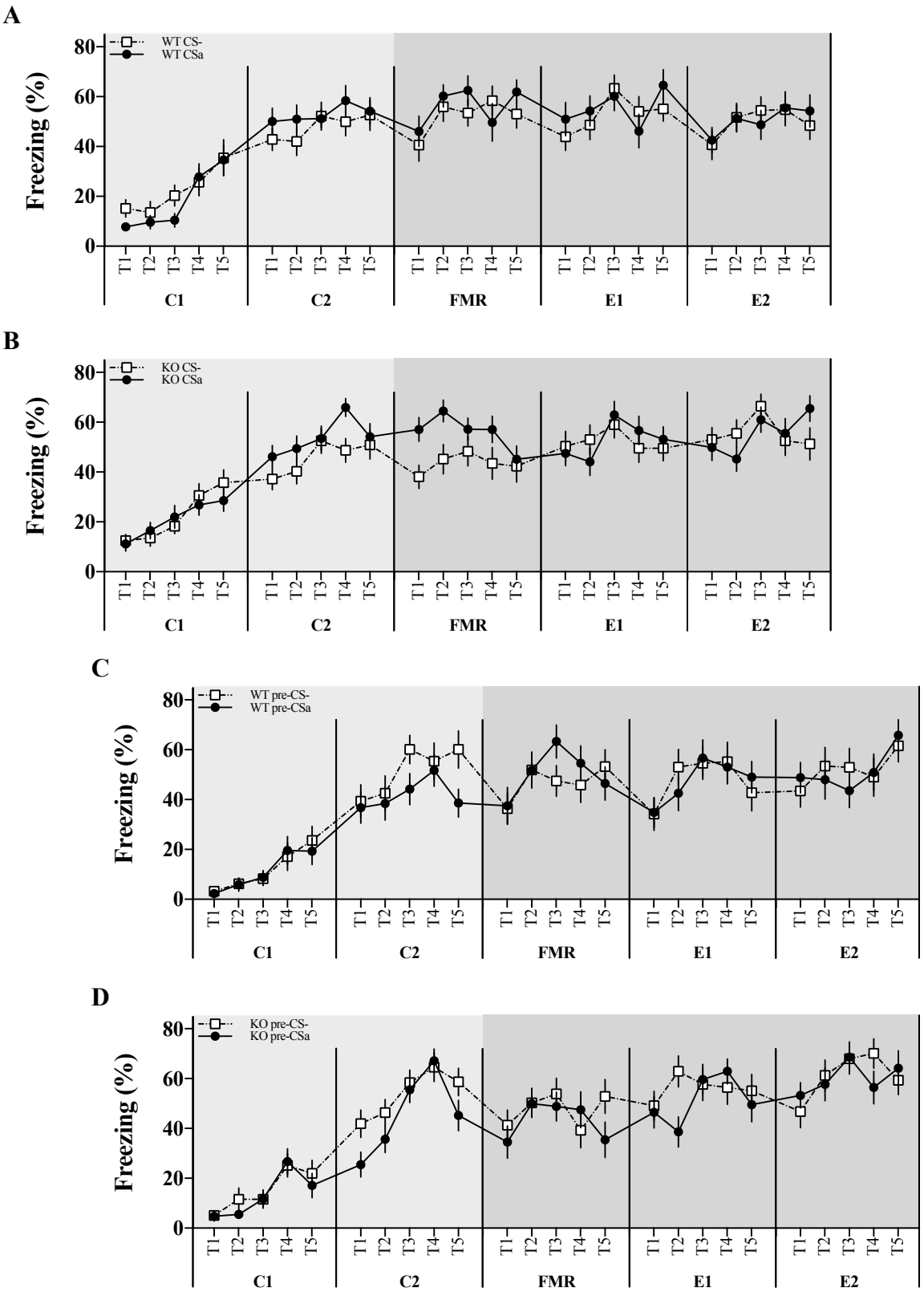
Appendix 4

Raw freezing levels during the CS and pre-CS periods of Experiment 1 of Chapter 4



Appendix 5

Raw freezing levels during the CS and pre-CS periods of Experiment 2 of Chapter 4



Appendix 6

Information regarding sample sizes, sex and age of subjects used in the analysis of UR to the first footshock delivery ('US #1') and footshock reactivity during the first fear conditioning session ('US #1-5').

Cohort	Chapter / Experiment	Genotype	mean age (months)	N size
1	Ch. 4 - Experiment 1	WT	5.2	19 (9 females)
		KO	6.5	19 (9 females)
2	Ch. 4 - Experiment 2	WT	4.2	23 (5 females)
		KO	3.4	23 (12 females)
3	Ch. 5	WT	6.9	31 (16 females)
		KO	6.8	29 (14 females)
4	Pilot study 1	WT	4.4	13 (5 females)
		KO	4.3	13 (5 females)
5	Pilot study 2	WT	4.6	12 (6 females)
		KO	4.6	12 (6 females)
Data analyses of footshock #1		WT	5.1	98 (41 females)
		KO	5.1	96 (46 females)
Data analyses of footshocks #1-5		WT	5.3	75 (36 females)
		KO	5.6	73 (34 females)

Note: Two 5-HTTKO mice and one WT mice were excluded from the analyses of Experiment 1 of Chapter 4. This was due to two mice being exposed to a defective auditory cue during C1, and another mouse jumping through the hole in the ceiling of the conditioning chamber after the delivery of the first shock on C2, thus avoiding all the remaining CS-US pairings of the session. However, these mice were included in the analyses of unconditioned responses to the footshocks (and to the auditory cues; see next page).

No observable unconditioned responses to the auditory cues were detected

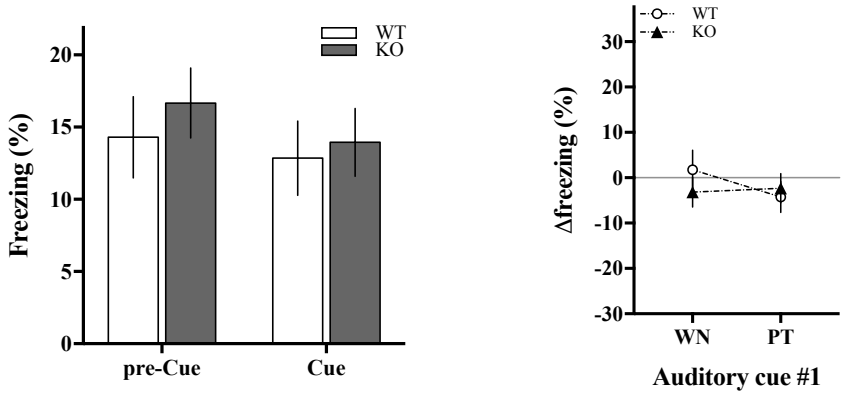


Figure A6.1. Left, Unconditioned freezing response (UR) to the first auditory cue (AC) presented to all 5-HTTKO (N=96) and WT (N=98) mice fear conditioned in this thesis. Freezing during the 5 s prior to the first AC was compared with freezing during the 5 s immediately after the AC onset. **Right,** Δfreezing (cue − preCue) evoked when the first AC presented was either a white noise (WN) or pure tone (PT) for the same mice. Mean ± SEM.

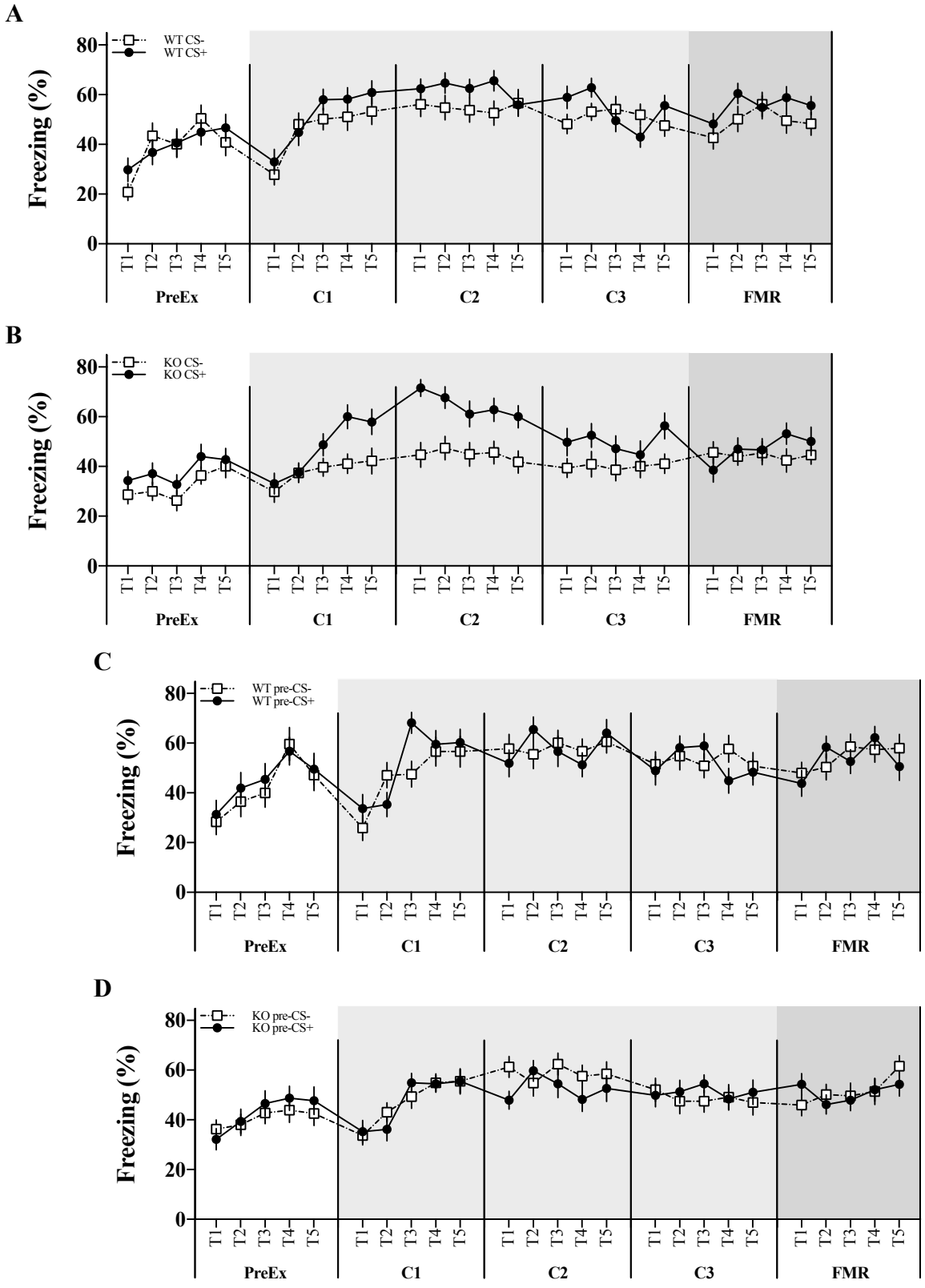
To investigate whether the effects found in Experiments 1 and 2 could result from altered sensitivity to the auditory cues (e.g. the auditory cues being more salient to the KOs than WTs), the UR to the auditory cues was investigated. The procedure used followed a similar approach to that conducted by Line and colleagues (2014). Accordingly, analyses of the freezing response during presentation of the very first auditory cue presented were focused in two phases: the 5s-long phase immediately after the first auditory cue onset ("cue phase"), and the 5s-long phase preceding the onset of that auditory cue ("pre-Cue" phase). All mice fear conditioned in this thesis were included in the analyses (KOs: N=96; WTs: N=98).

Analyses of the freezing responses revealed no observable responses to the first auditory cue presented (main effect of phase $F < 1$), both in 5-HTTKO mice and their WT controls (main effect of genotype and $Genotype_2 \times Phase_2$ interaction: Fs < 1; see Figure A6.1, left). An additional two-way between-subjects ANOVA, with genotype and auditory cue as factors, used on the data provided by 96 5-HTTKO mice (white noise: n=44) and 98 WT mice (white noise: n=46) indicated that both auditory cues do not seem to evoke an UR in

5-HTTKO mice and WT mice (main effect of auditory cue, genotype and interaction: *F*s < 1; see A6.1, right).

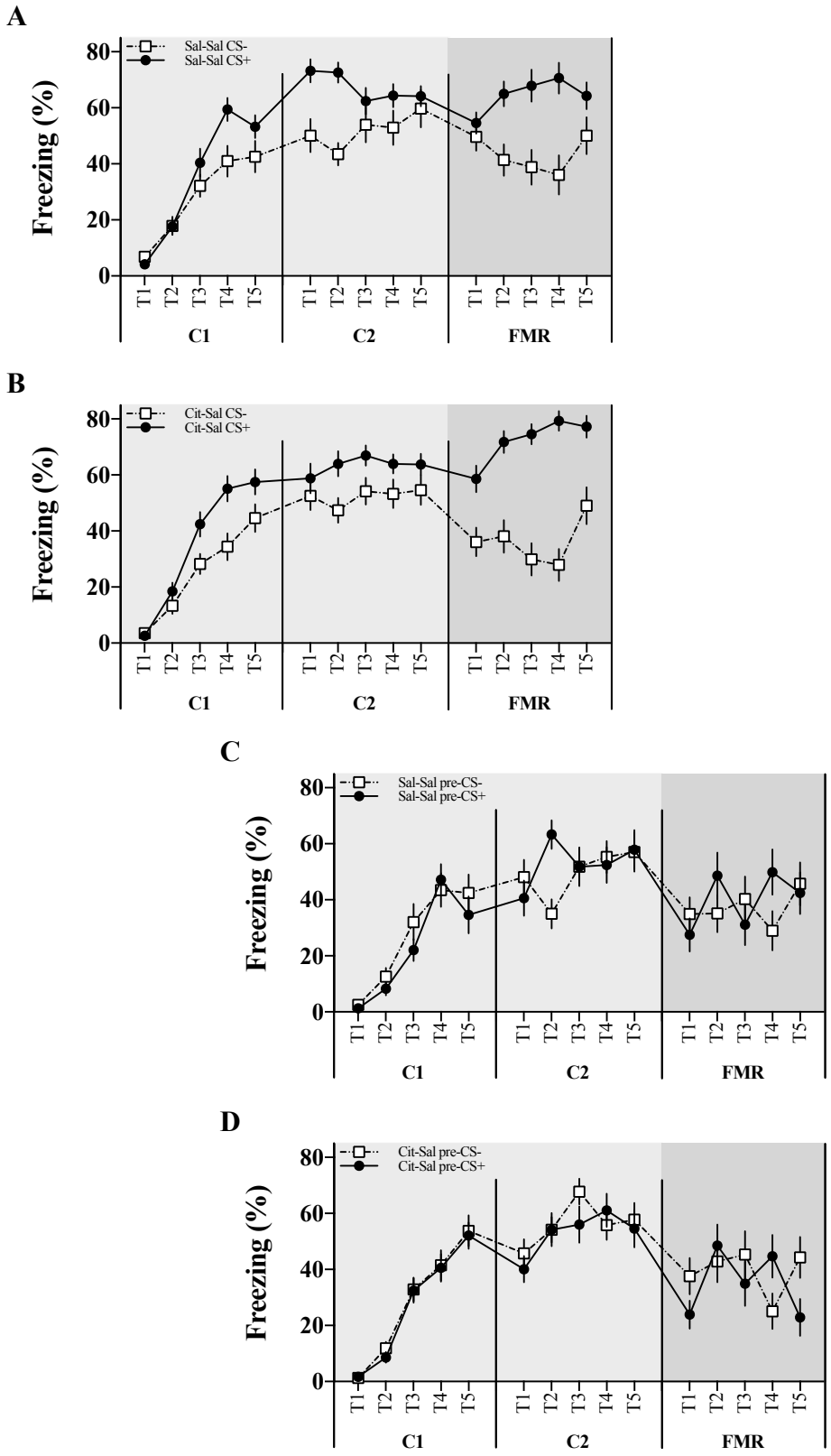
Appendix 7

Raw freezing levels during the CS and pre-CS periods of the experiment of Chapter 5



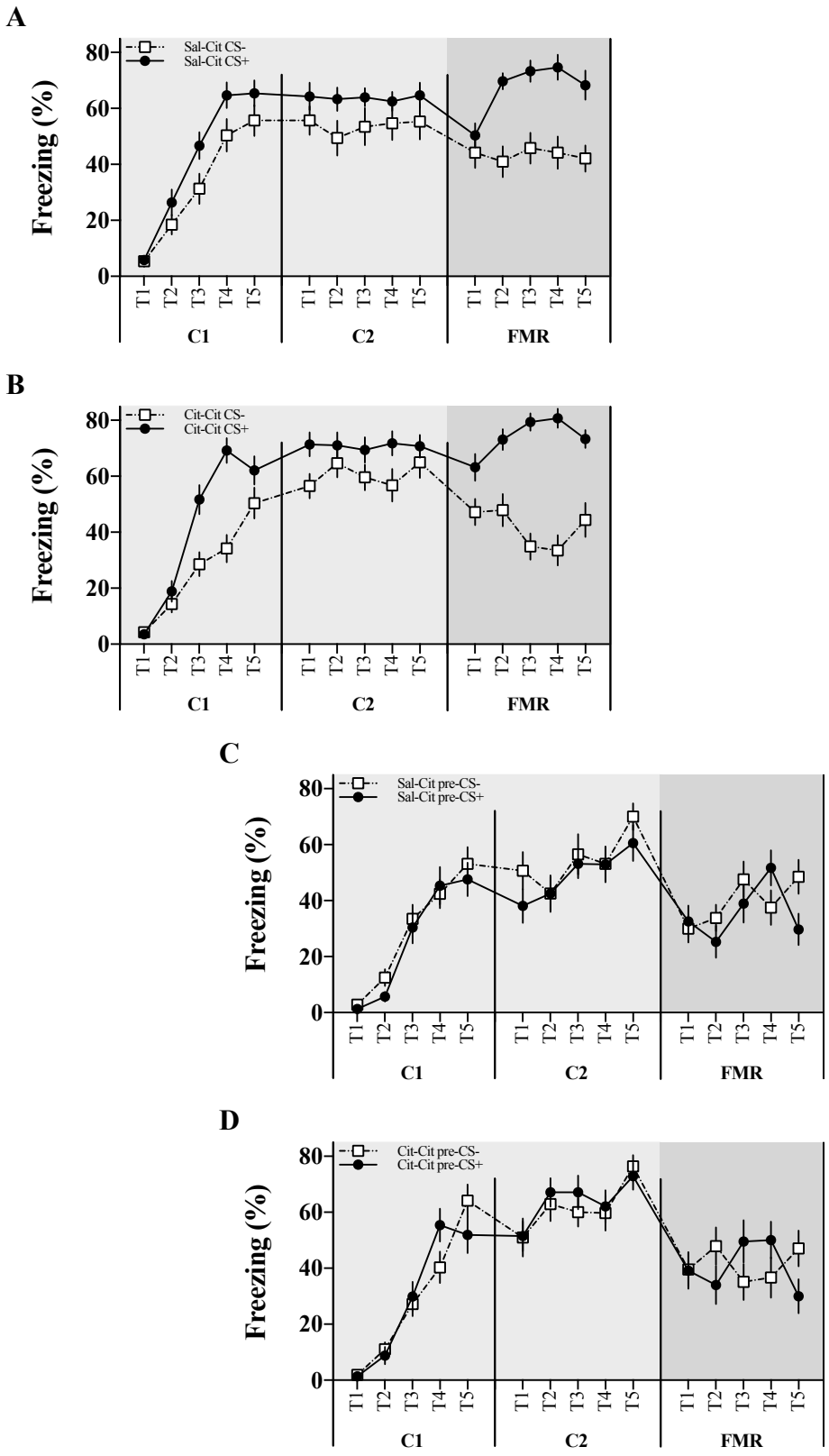
Appendix 8

Raw freezing levels during the CS and pre-CS periods of Experiment 1 of Chapter 6



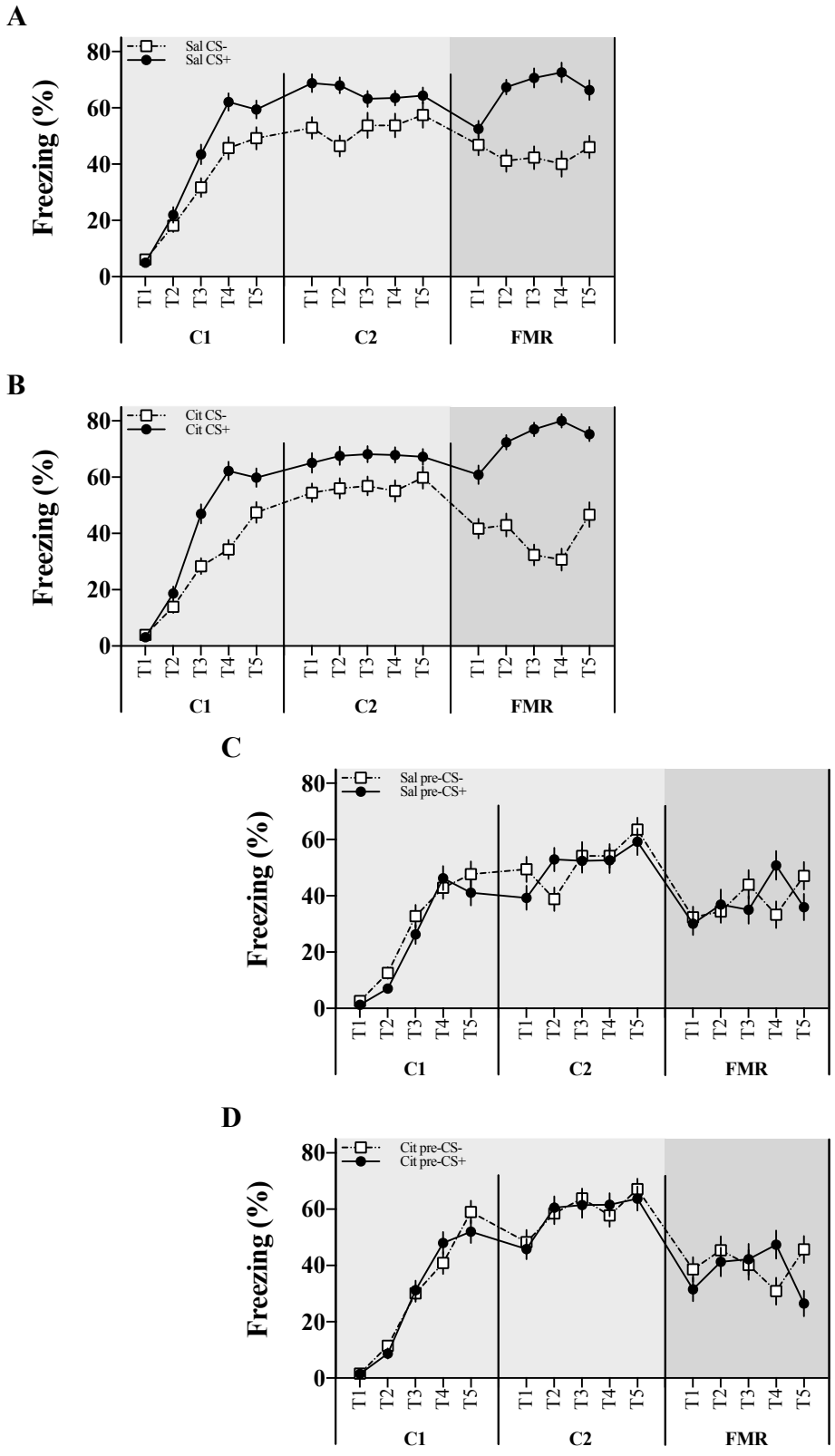
Appendix 9

Raw freezing levels during the CS and pre-CS periods of Experiment 1 of Chapter 6



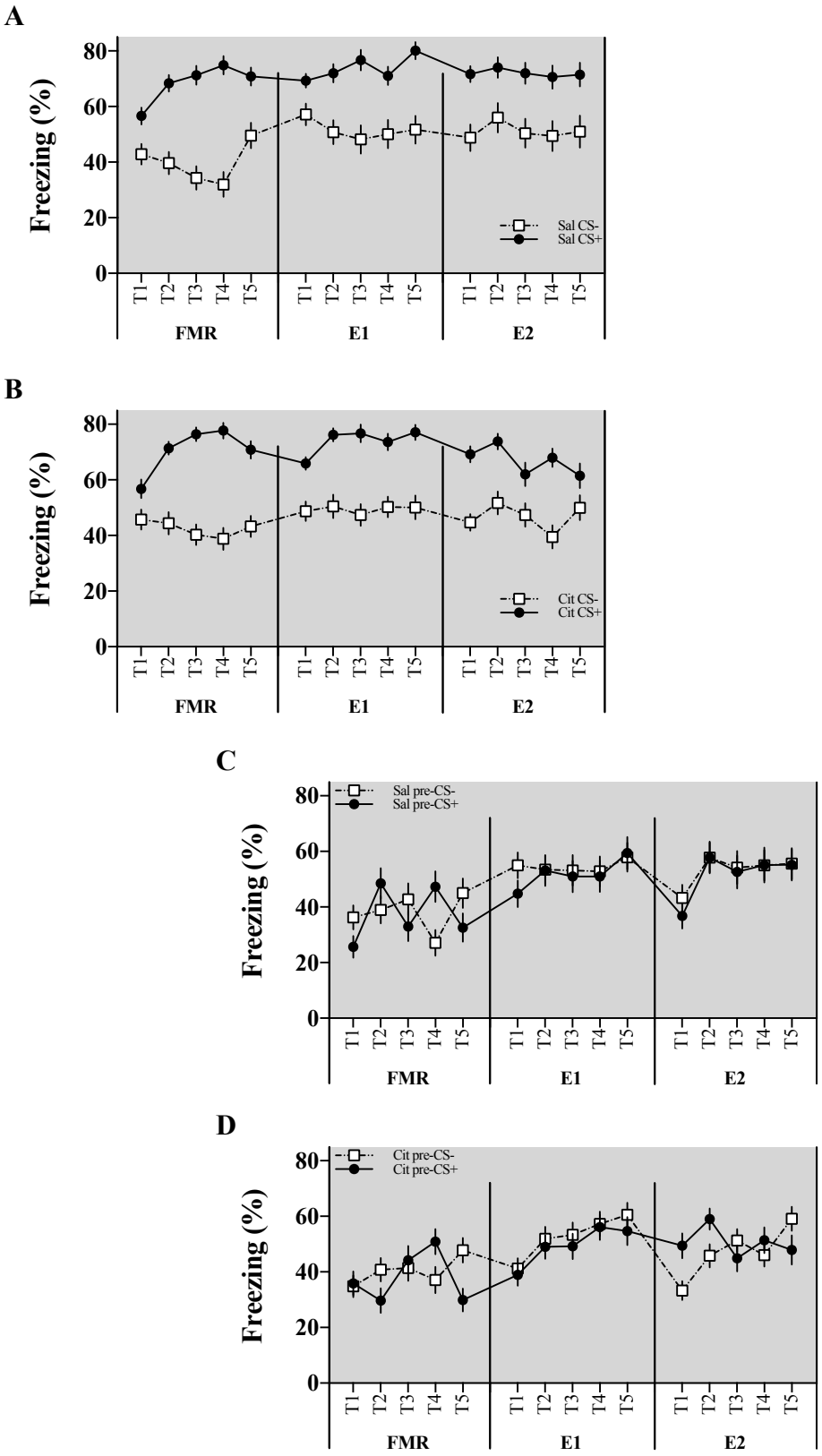
Appendix 10

Raw freezing levels during the CS and pre-CS periods of Experiment 1 of Chapter 6



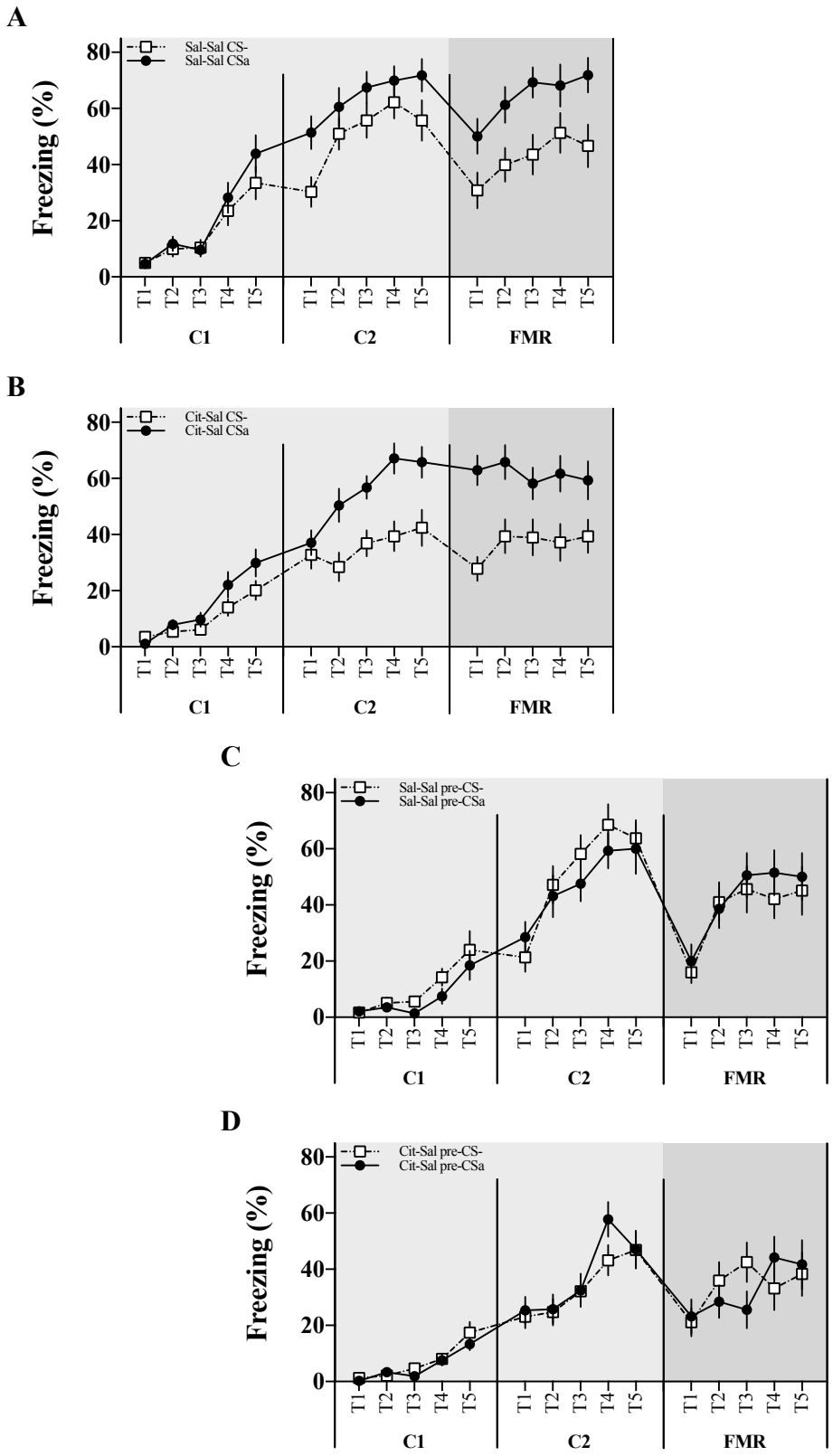
Appendix 11

Raw freezing levels during the CS and pre-CS periods of Experiment 1 of Chapter 6



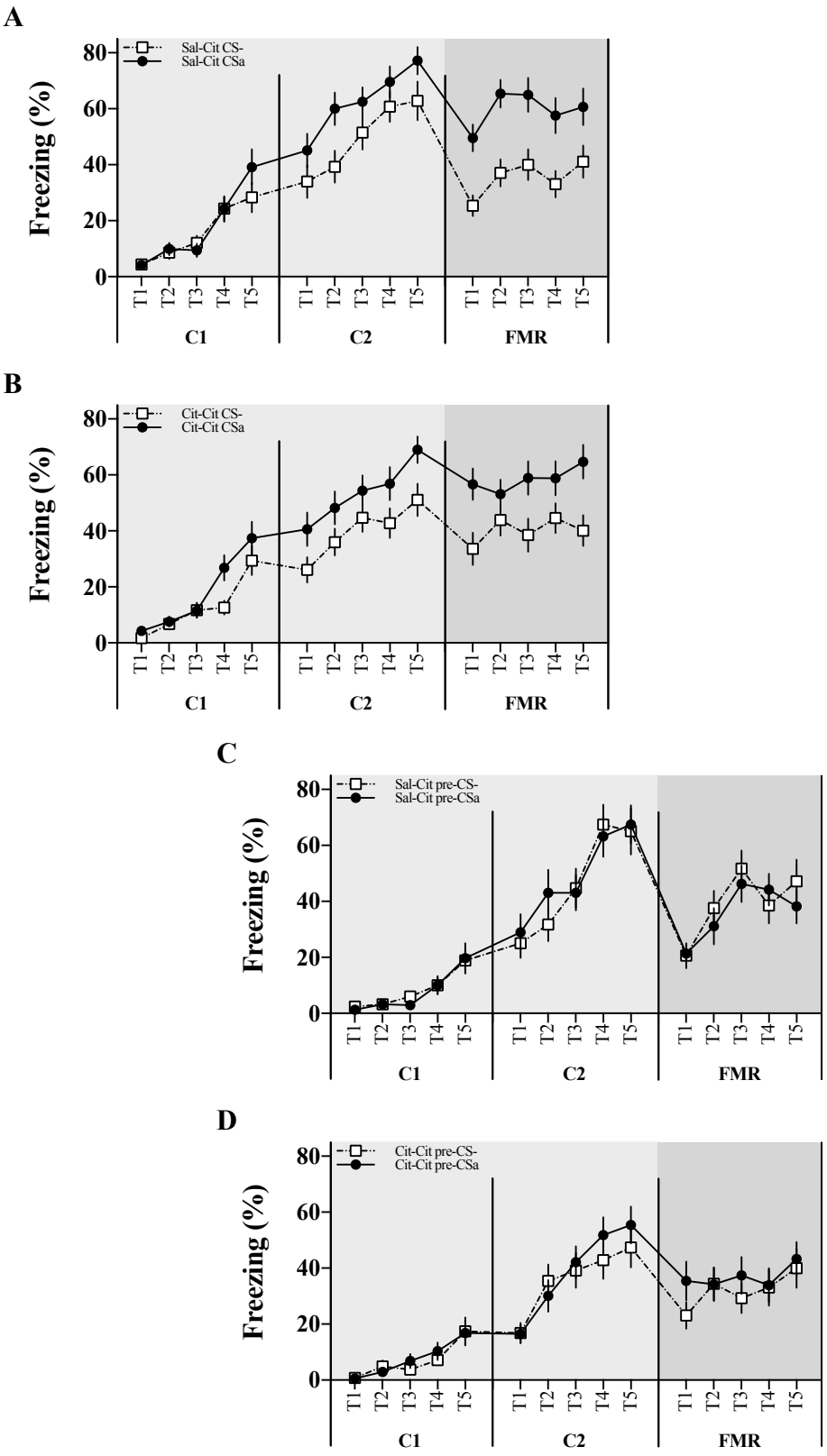
Appendix 12

Raw freezing levels during the CS and pre-CS periods of Experiment 2 of Chapter 6



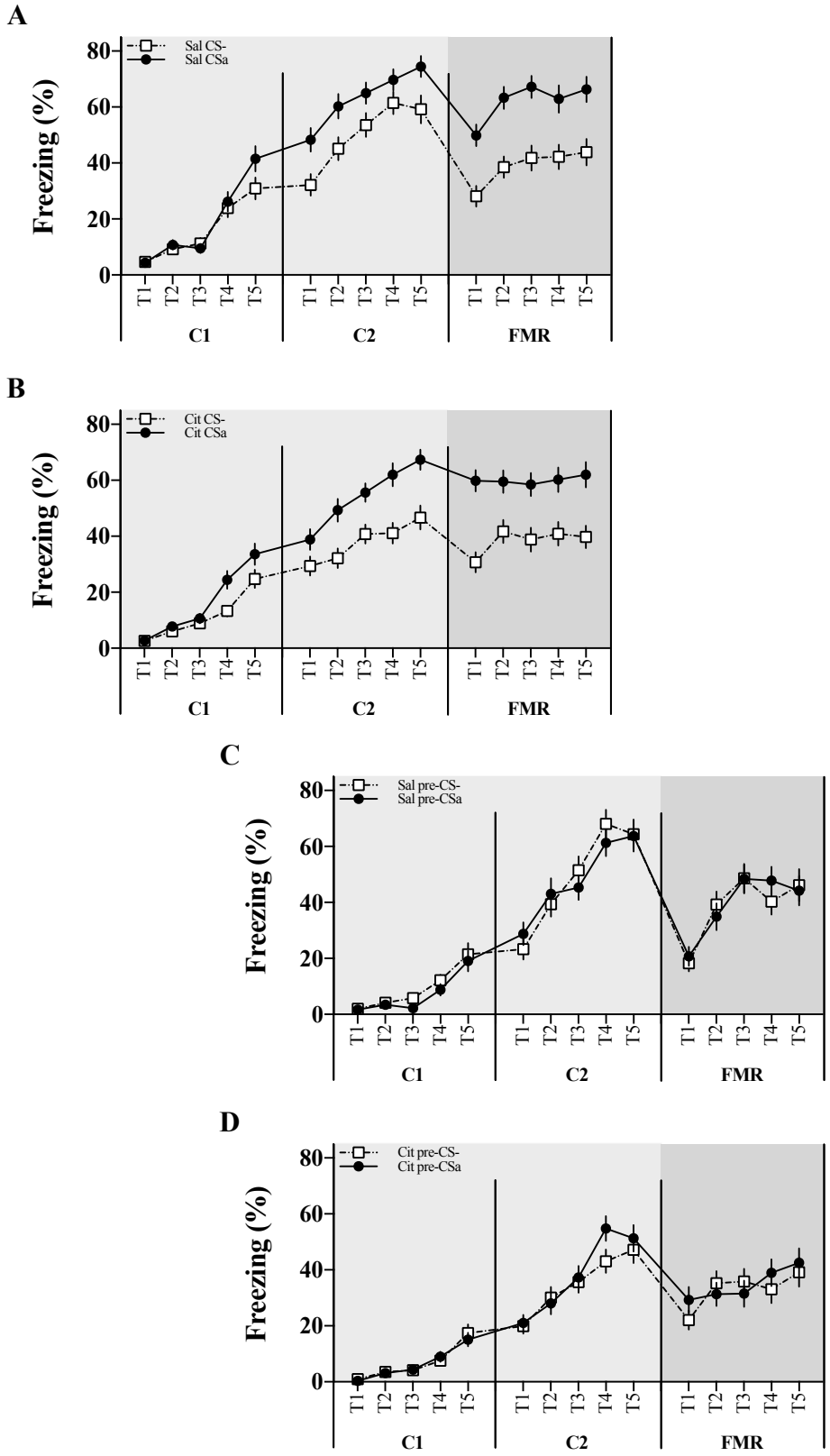
Appendix 13

Raw freezing levels during the CS and pre-CS periods of Experiment 2 of Chapter 6



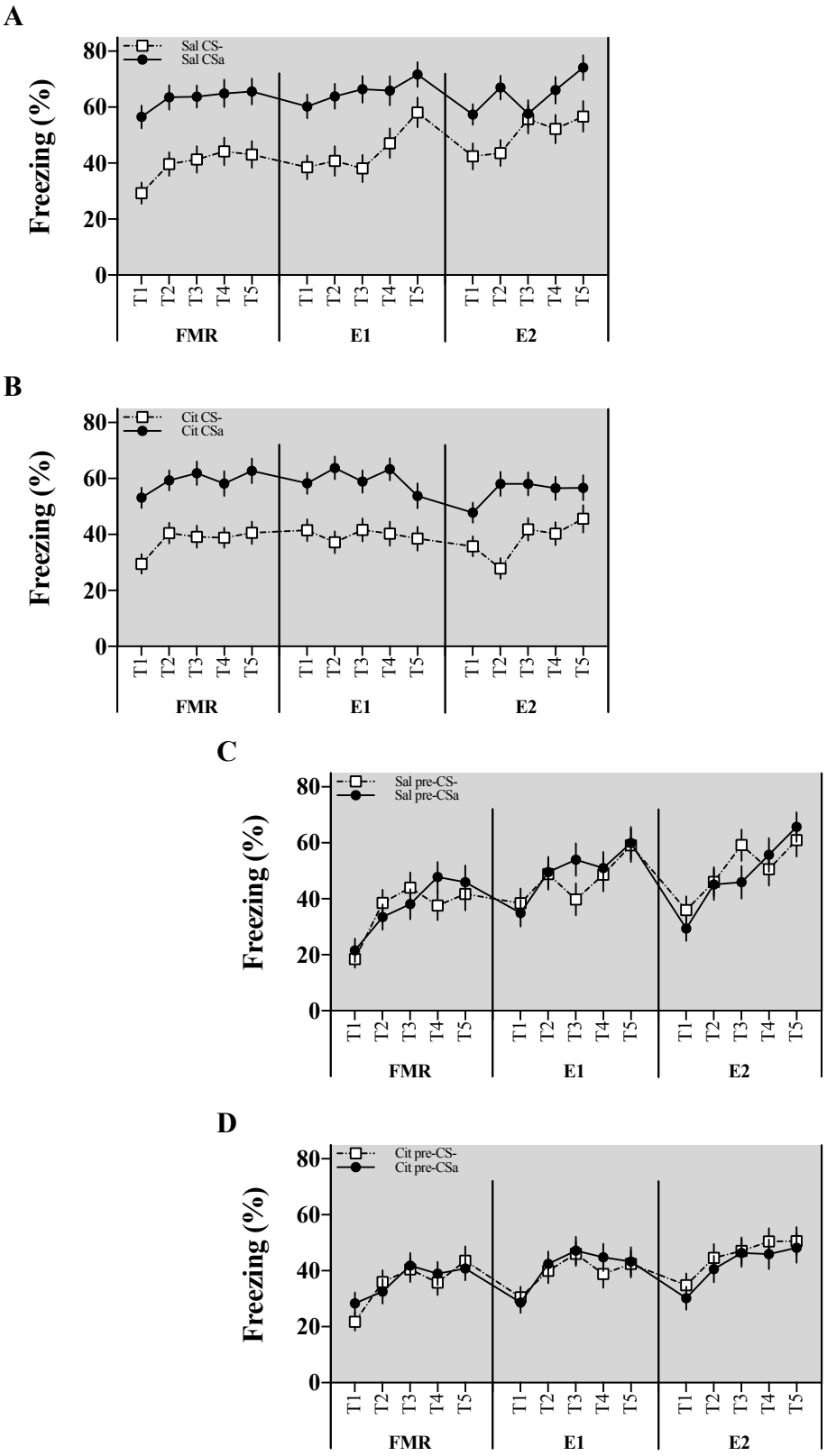
Appendix 14

Raw freezing levels during the CS and pre-CS periods of Experiment 2 of Chapter 6



Appendix 15

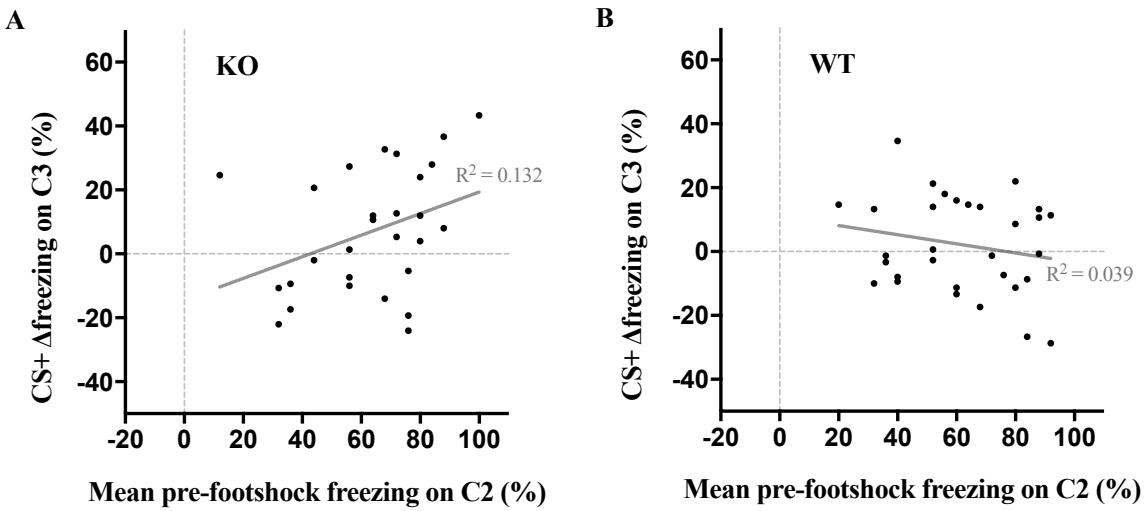
Raw freezing levels during the CS and pre-CS periods of Experiment 2 of Chapter 6



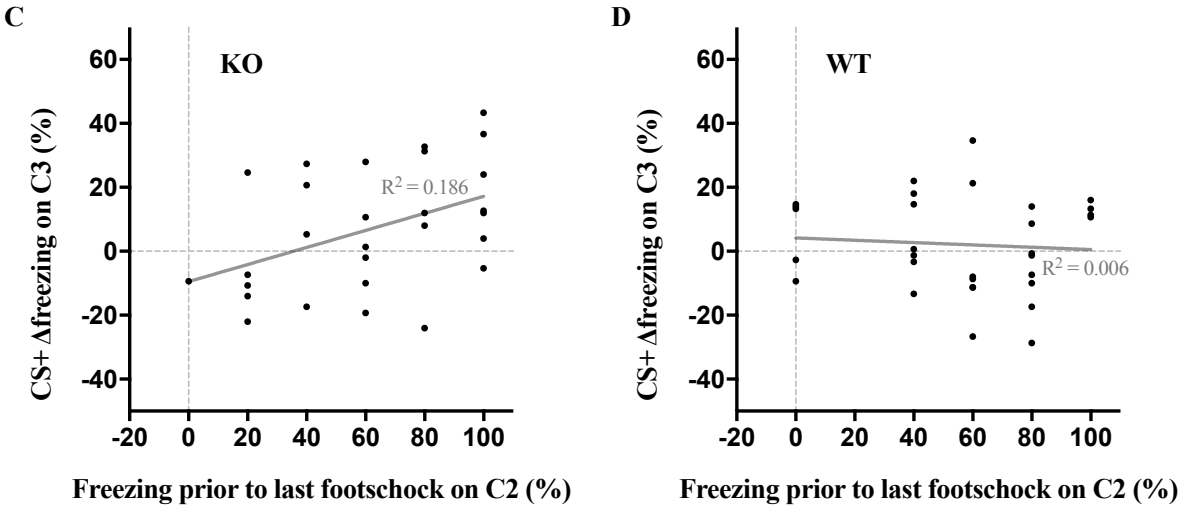
Appendix 16

Figure	Results of the statistical tests
4.10, top	These results were not affected by differences regarding baseline levels (main effect of genotype: F(1,13) = 1.3, p = .283; $Genotype_2 \times pre\text{-}shock_5$ interaction: $F < 1$).
4.11, left	This result cannot be explained by differences in pre-US activity levels (main effect of genotype: $F < 1$).
4.11, right	These results cannot be explained by differences in pre-US activity levels (main effect of genotype during US# 1-5 and US# 2-5: Fs < 1; $Genotype_2 \times Trial_5$ interaction during US# 1-5: $F < 1$; $Genotype_2 \times Trial_5$ interaction during US# 2-5: $F(3,420) = 1.1$, $p = .332$).
6.11, left	Main effect of treatment during conditioning: $F(1,188) = 4.6$, $p = .033$
6.11, right	These results cannot be explained by group differences on pre-US activity levels (main effect of treatment during conditioning during US# 1-5: $F(1,94) = 3.6$, p = .059; main effect of treatment during conditioning during US# 2-5: $F(1,94) = 2.2$, p = .142; Drug(Conditioning)2×Trial5 interaction: F(4, 376) = 1.4, p = .238; Drug(Conditioning)2×Trial4 interaction: F(3,282) = 1.4, p = .237).
6.11, right	This result cannot be explained by differences in pre-cue freezing levels (main effect of treatment during conditioning: $F < 1$; $Drug(Conditioning)_2 \times preCue_2$ interaction: $F(1,184) = 3.8$, $p = .054$).

Appendix 17



A regression analyses on the KO data (see Figure A) indicated that the mean average freezing shown during the 5s up to a footshock delivery on C2 accounted for 13.2% of the variance of the CS+ Δfreezing on C3, which was almost significantly greater than zero ($F(1,26) = 3.9$, $p = .058$). Accordingly, a statistical trend was detected in the positive correlation between the two variables (standardised β coefficient: .363; $t(26) = 2.0$, $p = .058$). To sum up, greater freezing just before the footshock deliveries on C2 practically predicted greater response to the CS+ on C3 in the KOs. Data from the WT mice are shown for comparison (see Figure B).



A regression analyses on the KO data (see Figure C) indicated that the freezing shown during the 5s up to the last footshock delivery on C2 accounted for 18.6% of the variance of the CS+ Δfreezing on C3, which was significantly greater than zero ($F(1,26) = 5.6$, $p = .022$). Accordingly, a significant positive correlation between the two variables was detected (standardised β coefficient: .432; $t(26) = 2.4$, $p = .022$). To sum up, greater freezing just before the last footshock deliveries on C2 predicted greater response to the CS+ on C3 in the KOs. Data from the WT mice are shown for comparison (see Figure D).

Appendix 18

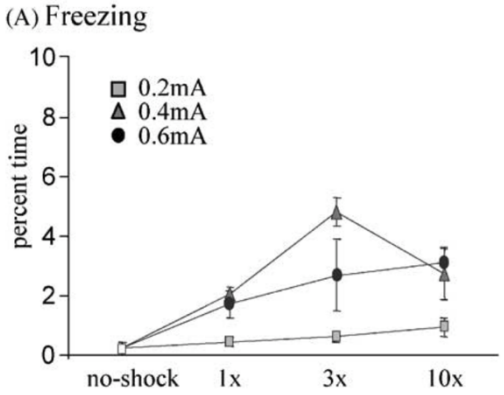


Figure 2.A from Laxmi et al., 2003. According to the authors, this figure shows the following: "Auditory cued fear memory. (A) Freezing behaviour during fear memory retrieval generally increased with the number of CS+/US pairings and with US intensity applied during training. A maximum of conditioned freezing was achieved after three pairings with an US of 0.4mA." (Laxmi et al., 2003, p. 91). Source: Laxmi et al. (2003). Reprinted from Behavioural Brain Research, Vol. 145, T. Laxmi, Oliver Stork, Hans-Christian Pape, Generalisation of conditioned fear and its behavioural expression in mice, pp. 89-98 (10 pages), Copyright (2003), with permission from Elsevier.

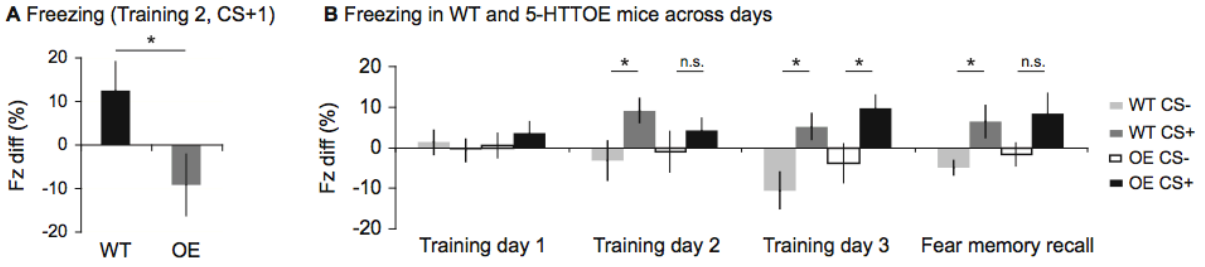


Figure 3. Cue-evoked freezing behavior in wild-type (WT) and 5-hydroxytryptamine transporter (5-HTT) overexpressing (OE) mice during discriminative fear conditioning. **(A)** WTs froze more than 5-HTTOE mice during the first conditioned stimulus (CS)+ presentation on Training Day 2. **(B)** WT mice discriminated between the CS+ and CS− on Training Days 2 and 3 and the fear memory recall day, but discrimination was seen in 5-HTTOE mice on Training Day 3 only, with a trend on the fear memory recall day. Each bar represents the mean freezing difference score (Fz diff) ± 1 SEM. $*p < .05$; $**p = .07$. n.s., not significant.

Figure 3 from Barkus et al., 2014. Reprinted under the terms of the Creative Commons Attribution License (CC BY) (https://creativecommons.org/licenses/by/4.0). Licensed Material: Barkus, C., Line, S. J., Huber, A. et al. (2014). Variation in serotonin transporter expression modulates fear-evoked hemodynamic responses and theta-frequency neuronal oscillations in the amygdala. Biological psychiatry, 75(11), 901-908. https://doi.org/10.1016/j.biopsych.2013.09.003

Appendix 19

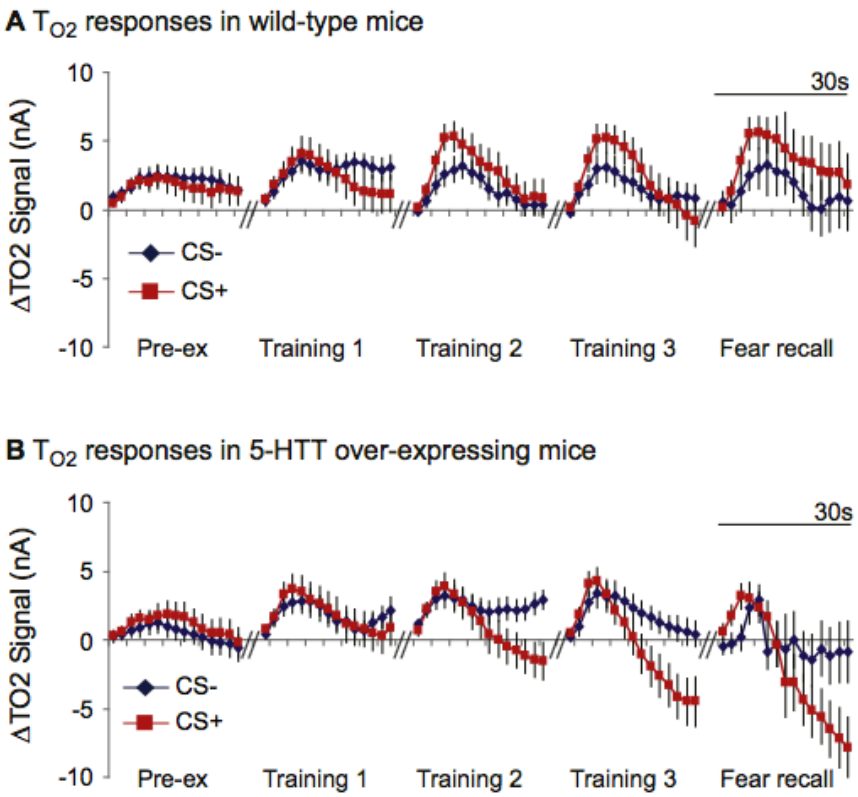


Figure 2 from Barkus et al., 2014. According to the authors, this figure shows the following: "Cue-evoked tissue oxygen (T_{O2}) signals in wild-type (WT) and 5-hydroxytryptamine transporter (5-HTT) overexpressing (OE) mice during discriminative fear conditioning for the pre-exposure (Pre-ex) day, the 3 days of training, and the first trial of fear memory recall. **(A)** In WT mice, conditioned stimulus (CS) evoked responses (red) increased relative to CS– evoked responses (blue) over training. **(B)** In 5-HTTOE mice, CS+ evoked responses were not higher than CS– evoked responses and had a pronounced "negative tail" by Training Day 3." (Barkus et al., p .904). Reprinted under the terms of the Creative Commons Attribution License (CC BY) (https://creativecommons.org/licenses/by/4.0). Licensed Material: Barkus, C., Line, S. J., Huber, A. et al. (2014). Variation in serotonin transporter expression modulates fear-evoked hemodynamic responses and theta-frequency neuronal oscillations in the amygdala. Biological psychiatry, 75(11), 901-908. https://doi.org/10.1016/j.biopsych.2013.09.003

Appendix 20

No observable unconditioned freezing responses to the auditory cues were detected both in citalopram-treated mice and in their controls

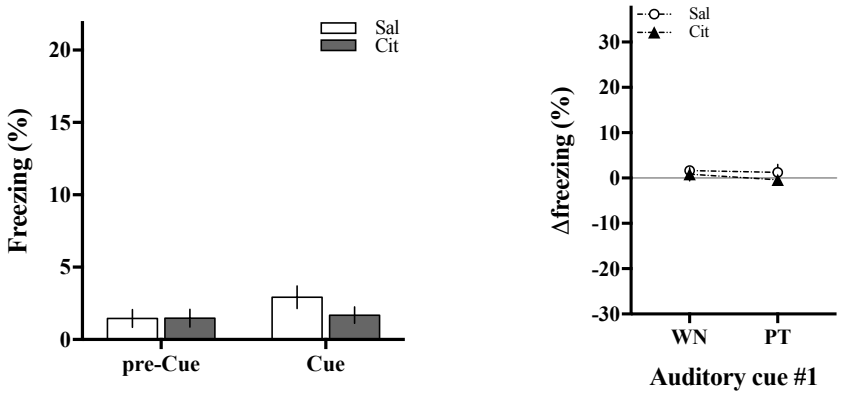


Figure A20.1. Left, Unconditioned freezing response (UR) to the first auditory cue (AC) presentation of the citalopram-treated mice (N=96) and saline treated mice (N=96) used in Experiments 1 and 2 (i.e. combined data). Freezing during the 5s prior to the first AC was compared with freezing during the 5s immediately after the AC onset. **Right,** Δfreezing (cue – preCue) evoked when the first AC presented was either a white noise (WN) or pure tone (PT) for the same mice. Mean ± SEM.

To investigate whether any of the effects found in Experiments 1 and 2 could result from altered responses to the auditory cues allocated to the CS (e.g. citalopram treatment making the cues more salient), the UR to the auditory cues was investigated. The procedure used followed a similar approach to that conducted by Line and colleagues (2014). Accordingly, analyses of the freezing response during presentation of the very first auditory cue presented were focused in two phases: the 5s-long phase immediately after the first auditory cue onset ("cue phase"), and the 5s-long phase preceding the onset of that auditory cue ("pre-Cue" phase). All mice tested in Chapter 6 were included in the analyses.

Analyses of the freezing responses revealed no observable responses to the first auditory cue presented (main effect of phase $F(1,188) = 1.7$, $p = .196$) in both groups (main

effect of treatment during conditioning and Drug(Conditioning)$_2$×Phase$_2$ interaction: Fs < 1; see Figure A20.1, left). A two-way between-subjects ANOVA, with treatment during conditioning and auditory cue as factors, using the data provided by 96 citalopram-treated mice (white noise: n=44) and 98 saline-treated mice (white noise: n=46) indicated that neither of the auditory cues seems to evoke an UR (main effect of auditory cue, treatment during conditioning and interaction: Fs < 1; see Figure A20.1, right).

Appendix 21

Supplemental results for Chapter 7.

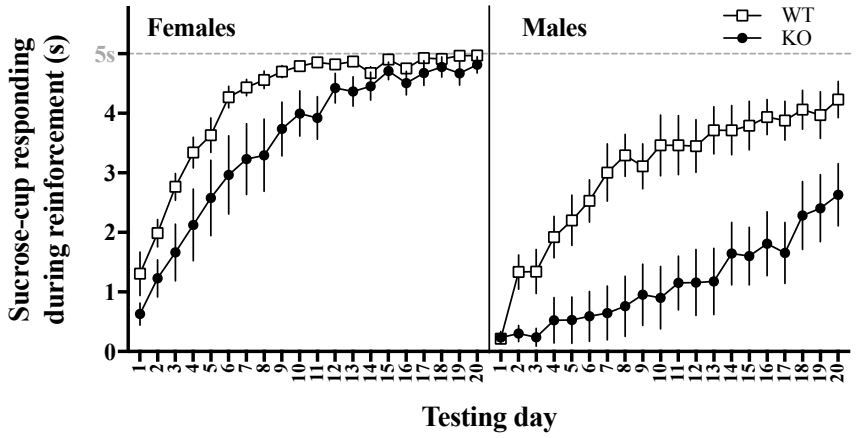


Figure A21.1. Duration of the sucrose-cup responding during the 5s-long reinforcement period for each of the testing days administered to the 5-HTTKO (N=16) and WT (N=16) mice during the conditioning phase. Mean ± SEM.*5*

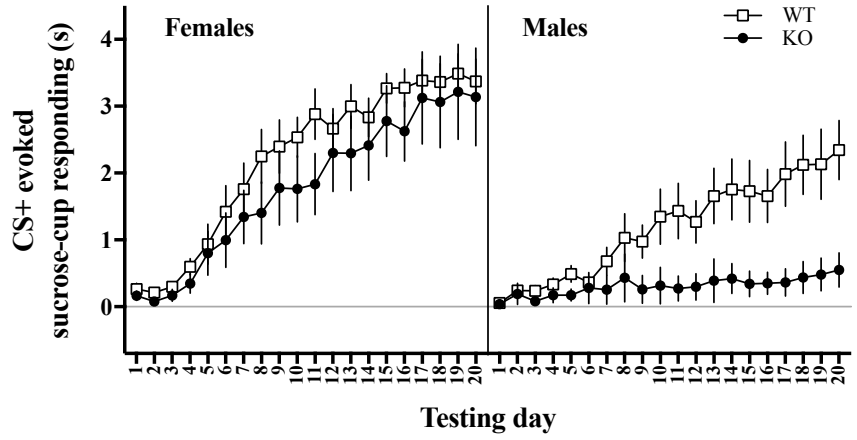


Figure A21.2. Mean CS+ evoked sucrose-cup responding for each of the testing days administered to the 5-HTTKO (N=16) and WT (N=16) mice during the conditioning phase. Mean ± SEM.

Appendix 22

Are amygdala theta oscillations locally generated?

Intrinsic properties of the membrane of amygdala neurons make it apt to exhibit voltage-dependent resonance and (self-sustained) oscillations at the theta frequency (Paré et al., 1995; Pape & Driesang, 1998; Pape et al., 1998). Two types of intrinsic theta oscillations (subthreshold and suprathreshold to spike generation), each one produced by the periodic interplay of two opposing sets of membrane currents, functionally interact to adjust the action potential output of the neuron at the theta frequency range (Pape et al., 2005). These mechanisms, together with synaptic inputs from other brain areas connected to the amygdala such as the hippocampus and mPFC, are assumed to promote population activity in theta frequency rhythm (Paré et al., 2002; Pape & Paré, 2010).

However, some researchers doubt that theta activity can be locally generated by the amygdala, and consider that the properties of these neurons would just facilitate their entrainment to theta frequency oscillations (Buzsáki, 2002; Amzica & Lopes da Silva, 2010). Considering that there is also controversy about how local is the phenomena that LFPs usually measure (Reed et al., 2015), some researchers would likely consider that the LFPs measured in the above-mentioned studies consist of a mixture of local potentials with potentials from other structures that are volume conducted to the amygdala (e.g. Kajikawa & Schroeder, 2011). Moreover, it is unclear if the amygdala cytoarchitecture allows for a geometrical arrangement of neurons that allows them to generate theta oscillations (i.e., its neurons are not organised in a laminar array, which typically leads to strong electric dipoles that do not cancel each other; Berens et al., 2010).

Nevertheless, Paré and Pape (2010) strongly claim that amygdala theta activity recorded as LFPs in the amygdala are unlikely to reflect volume conduction from other brain structures, such as the hippocampus, and draw attention to some aspects of their findings to

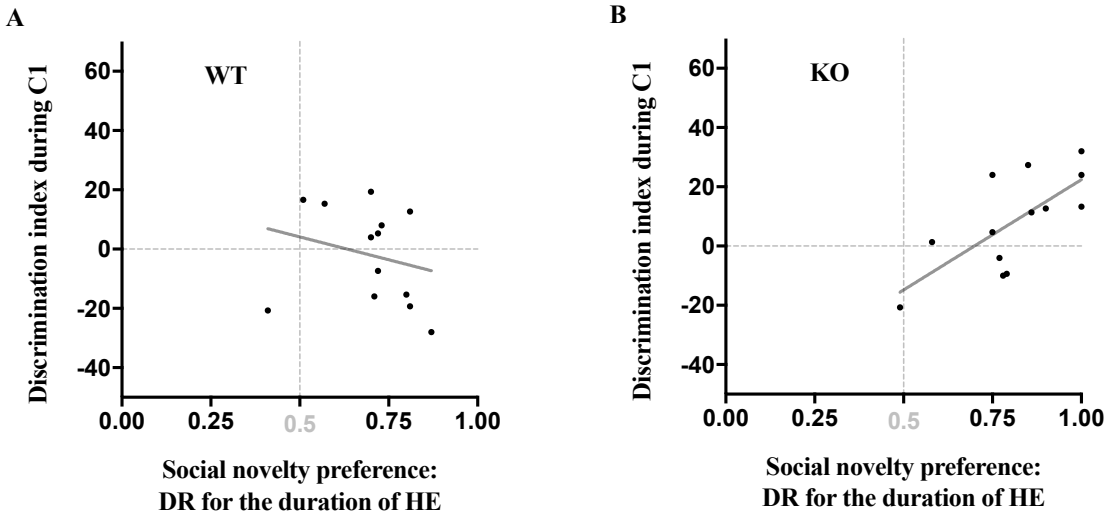
support that position. For instance, in the study conducted by Seidenbecher and colleagues (2003), strong theta correlation between LA-CA1 accompanied CS+ evoked freezing but not risk-assessment behaviour of controls, and strong theta activity was seen in CA1 during exploratory behaviour but not in the LA. Later, Lesting et al. (2011) observed that the degree of theta coupling between CA1, LA and mPFC varied throughout testing, increasing during FMR, declining during extinction, and partly rebounding during extinction recall (i.e. the CA1-LA coupling remained low in this stage). Additionally, Popa and colleagues (2010) registered a weak correlation between the amplitude of theta oscillations in the BLA and hippocampus during paradoxical sleep after conditioning. Together, these observations indicate that the coupling between brain regions with the amygdala varies with different states of a fear memory and, particularly, that theta synchronisation between the hippocampus and the amygdala is not always present.

Additionally, simultaneously recorded LFPs and unit activity in the LA and BLA shows rhythmic theta-related unit activity at these sites (i.e. the firing probability fluctuates with the oscillations in LFPs), which suggests that phase-locked synaptic activity contributes to the generation of local theta LFP in the amygdala (Popa et al., 2010; Lesting et al., 2011). Therefore, even if theta is not entirely generated in the amygdala, its neurons are clearly influenced by oscillations at this frequency, which makes them likely contributors to the local generation of that oscillation (Logothetis, 2008; Buzsáki et al., 2012; Frederick et al., 2015).

Appendix 23

Correlations between social recognition performance and discriminative aversive learning

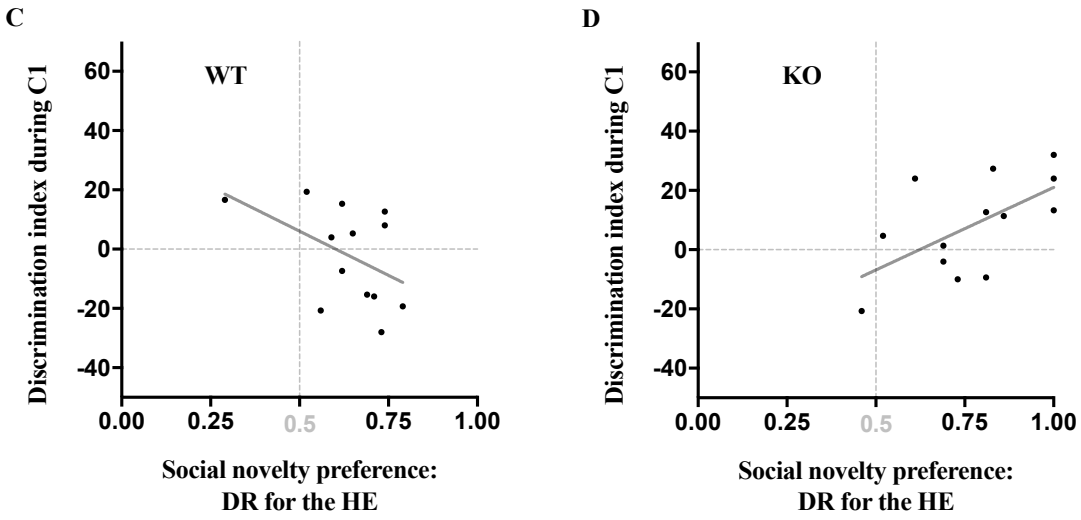
Correlations between two variables were investigated using the Person's correlating coefficient (r). Non-parametric data were analysed with a Spearman's correlation coefficient (r_s) instead. Results obtained with mice that underwent both the three-chamber test and, later, the discriminative aversive learning task used in Chapter 5 indicated that those 5-HTTKO mice exhibiting stronger social novelty preference later showed greater levels of CS+/CS– discrimination during the first day of conditioning. No equivalent significant relationship between these two measures was seen in the WT controls.



WT: r_s = -.36, p = .225

KOs: r_s = .63, p = .021

r = .71, p = .007



WT: $r = -.47$, $p = .102$

KOs: $r = .6$, $p = .029$

Appendix 24

Figure	Results of the statistical tests
3.1, top left	Latency to enter the centre: No significant main effect of sex or interaction between genotype and sex were detected (F's < 1). Latency to enter an open arm: No significant main effect of sex (F(1,78) = 3.2, p = .078) or interaction between genotype and sex (F < 1) were detected.
3.1, bottom left	Proportion of entries in the open arms: No significant main effect of sex (F(1,78) = 2.7, p = .106) or interaction between genotype and sex (F < 1) were detected. Proportion of time in the open arms: No significant main effect of sex (F(1,78) = 1.3, p = .259) or interaction between genotype and sex (F < 1) were detected.
3.2, left	No significant main effect of sex (F(1,78) = 2.1, p = .144) or interaction between genotype and sex (F(1,78) = 1.1, p = .300) were detected.
3.2, right	A significant main effect of sex on total distance travelled was detected (F(1,78) = 9.3, p = .003; females > males) but not significant interaction between genotype and sex was obtained (F < 1).
3.3, top	Latency to first contact with the milk (N=50): No significant main effect of sex or interaction between genotype and sex were detected (F's < 1). Latency to start drinking (N=50): A significant main effect of sex on latency to start drinking was detected (F(1,46) = 4.5, p = .039; females < males) but not significant interaction between genotype and sex was obtained (F < 1). Latency to start drinking (N=45): A significant main effect of sex on latency to start drinking was detected (F(1,41) = 10.8, p = .002; females < males) but not significant interaction between genotype and sex was obtained (F < 1). Weight difference: A significant main effect of sex was detected (F(1,46) = 12.0, p = .001) as well was a significant interaction between genotype and sex (F(1,46) = 5.0, p = .029). Female WT mice were heavier than male WT mice and female KO mice.
3.3, bottom	Latency to first contact with the pellets (N=39): A significant main effect of sex on latency to start drinking was detected (F(1,35) = 4.3, p = .046; females < males) but not significant interaction between genotype and sex was obtained (F(1,35) = 1.2, p = .287). Latency to first contact with the pellets (N=33): No significant main effect of sex (F(1,29) = 2.1, p = .155) or interaction between genotype and sex were detected (F(1,29) = 1.2, p = .276). Latency to start eating (N=39): No significant main effect of sex (F(1,35) = 3.3, p = .077) or interaction between genotype and sex were detected (F's < 1). Weight difference: A significant main effect of sex was detected (F(1,35) = 6.4, p = .016) as well was a significant interaction between genotype and sex (F(1,35) = 9.3, p = .004). Female WT mice were heavier than male WT mice and female KO mice.

3.5	Distance travelled: A significant main effect of sex was detected ($F(1,26) = 5.3$, $p = .030$) but no significant interaction between genotype and sex ($F(1,26) = 1.7$, $p = .208$), or between genotype, sex and session ($F < 1$) were obtained. Time immobile: No significant main effect of sex ($F(1,26) = 1.2$, $p = .286$) or interaction between genotype, sex and habituation session ($F < 1$) were detected. However, a significant interaction between genotype and sex was detected ($F(1,26) = 6.3$, $p = .018$). Male KO mice spent significantly more time immobile than female KO mice and male WT mice over the 4 habituation sessions. This pattern of results was kept on day 4. [This is consistent with what was seen in Chapter 7: male KO mice exhibited more anxiety in the apparatus than female KO and male WT mice, even after several exposures to the apparatus.]
3.6	Total number of social interaction events per pair: No significant main effect of sex or interaction between genotype and sex were detected (F's < 1). Total duration of social interaction events per pair: No significant main effect of sex ($F(1,26) = 3.2$, $p = .084$) or interaction between genotype and sex were detected ($F < 1$). Weight difference: No significant main effect of sex or interaction between genotype and sex were detected (F's < 1). $Genotype_2 \times Sex_2 \times$'$WeightDifferencesGroup'_3$ interaction for the number of events: all F's < 1, apart from the interaction between genotype and sex ($F(1,18) = 3.8$, $p = .067$). $Genotype_2 \times Sex_2 \times$'$WeightDifferencesGroup'_3$ interaction for the duration of events: all F's < 1, apart from the interaction between genotype and sex ($F(1,18) = 1.4$, $p = .247$).
3.7	Distance travelled: No statistically significant results were obtained regarding main effect of sex ($F(1,31) = 2.3$, $p = .142$), interaction between genotype and sex ($F(1,31) = 1.6$, $p = .218$), and interaction between genotype, sex and session ($F(3,93) = 1.2$, $p = .331$). Time immobile: No statistically significant results were obtained regarding main effect of sex, interaction between genotype and sex, and interaction between genotype, sex and session (all F's < 1).
3.8, left	Head entries during the sociability phase: No statistically significant results were obtained regarding main effect of sex ($F < 1$), interaction between genotype and sex ($F < 1$), and interaction between genotype, sex and cohort ($F(2,32) = 1.2$, $p = .304$). Duration of head entries during the sociability phase: No statistically significant results were obtained regarding main effect of sex ($F(1,32) = 1.1$, $p = .296$), interaction between genotype and sex ($F < 1$), and interaction between genotype, sex and cohort ($F(2,32) = 3.1$, $p = .057$).
3.8, right	Head entries during the preference for social novelty phase: No statistically significant results were obtained regarding main effect of sex, interaction between genotype and sex, and interaction between genotype, sex and cohort (all F's < 1). Duration of head entries during the preference for social novelty phase:

	No statistically significant results were obtained regarding main effect of sex ($F < 1$), interaction between genotype and sex ($F(1,32) = 2.1$, $p = .157$), and interaction between genotype, sex and cohort ($F < 1$).
4.2, left	No statistically significant results were obtained regarding main effect of sex ($F < 1$), interaction between genotype and sex ($F < 1$), and interaction between genotype, sex, day and CS ($F(4,124) = 1.3$, $p = .284$).
4.2, right	No statistically significant results were obtained regarding main effect of sex (F (1,31) = 1.9, $p = .176$), interaction between genotype and sex ($F < 1$), and interaction between genotype, sex, day and CS ($F(4,124) = 1.3$, $p = .284$).
4.3	No statistically significant results were obtained regarding main effect of sex (F (1,31) = 3.3, $p = .080$), interaction between genotype and sex ($F(1,31) = 2.6$, $p = .115$), and interaction between genotype, sex and CS ($F(1,31) = 1.4$, $p = .245$), and interaction between genotype, sex, trial and CS ($F < 1$).
4.4	CS+ Δfreezing during T1: No statistically significant results were obtained regarding main effect of sex and the interaction between genotype and sex (F's < 1). Discrimination index during T1: No statistically significant results were obtained regarding main effect of sex and the interaction between genotype and sex (F's < 1). CS+ Δfreezing during FMR, E1 and E2: No statistically significant results were obtained regarding main effect of sex, the interaction between genotype and sex, and the interaction between genotype, sex and session (F's < 1). Discrimination index during FMR, E1 and E2: No statistically significant results were obtained regarding main effect of sex ($F < 1$), the interaction between genotype and sex ($F < 1$), and the interaction between genotype, sex and session ($F(2,62) = 1.3$, $p = .273$).
4.6	Discrimination index during C2 and FMR: No statistically significant results were obtained regarding main effect of sex, interaction between genotype and sex, and interaction between genotype, sex and session (F's < 1). Cue-evoked Δfreezing during C2 and FMR: No statistically significant results were obtained regarding main effect of sex (F (1,42) = 1.0, $p = .30$), interaction between genotype and sex ($F < 1$), and interaction between genotype, sex and CS ($F < 1$), and interaction between genotype, sex, session and CS ($F < 1$).
4.7	CSa Δfreezing during T1: No statistically significant results were obtained regarding main effect of sex ($F(1,42) = 1.5$, $p = .227$ and the interaction between genotype and sex ($F < 1$). Discrimination index during T1: No statistically significant results were obtained regarding main effect of sex ($F(1,42) = 1.1$, $p = .304$ and the interaction between genotype and sex ($F < 1$). Discrimination index during FMR, E1 and E2: No statistically significant results were obtained regarding main effect of sex ($F(1,21) = 1.3$, $p = .267$) or session and sex interaction ($F < 1$).

	CSa Δfreezing during FMR, E1 and E2: No statistically significant results were obtained regarding main effect of sex or session and sex interaction (*F*'s < 1).
4.10, top	A significant main effect of sex was detected ($F(1,11) = 5.6$, $p = .037$) but not significant interaction between genotype and sex ($F < 1$) or between intensity and sex ($F < 1$). However, a significant interaction between genotype, sex and intensity was obtained ($F(4,44) = 3.0$, $p = .027$. WT males showed significantly lower reactivity for the first two intensities, whereas male KOs showed significantly lower reactivity for the highest intensity than female KOs.
4.10, bottom	No statistically significant results were obtained regarding the main effect of sex ($F < 1$), the interaction between genotype and sex ($F(1,11) = 1.2$, $p = .305$), interaction between genotype, sex and intensity ($F(4,44) = 1.7$, $p = .136$), and the interaction between genotype, sex, intensity and bin ($F(16,176) = 1.0$, $p = .427$).
4.11, left	No statistically significant results were obtained regarding the main effect of sex ($F < 1$) and the interaction between genotype and sex ($F < 1$). The same was true when using a 5s-long local baseline period immediately before the bin containing the footshock delivery (*F*'s < 1)
4.11, right	$Genotype_2 \times Trial_4$: No statistically significant results were obtained regarding the main effect of sex, the interaction between genotype and sex, and interaction between genotype, sex and trial (*F*'s < 1). The same was true when using a 5s-long local baseline period immediately before the bin containing the footshock delivery (*F*'s < 1). $Genotype_2 \times Trial_5$: No statistically significant results were obtained regarding the main effect of sex, the interaction between genotype and sex, and interaction between genotype, sex and trial (*F*'s < 1). The same was true when using a 5s-long local baseline period immediately before the bin containing the footshock delivery (*F*'s < 1). Main effect of genotype on trial 1 only (N=148): No statistically significant results were obtained regarding the main effect of sex and the interaction between genotype and sex (F's < 1). The same was true when using a 5s-long local baseline period immediately before the bin containing the footshock delivery (*F*'s < 1).
4.14	$Genotype_2 \times Sex_2 \times Bin_{30}$: No statistically significant result were obtained regarding the main effect of sex ($F(1,11) = 1.6$, $p = .227$), the interaction between genotype and sex ($F(1,11) = 3.8$, $p = .078$) and the interaction between genotype, sex and bin ($F < 1$). $Genotype_2 \times Sex_2 \times Bin_{18}$: No statistically significant result were obtained regarding the main effect of sex ($F(1,11) = 3.8$, $p = .079$), the interaction between genotype and sex ($F(1,11) = 4.0$, $p = .071$) and the interaction between genotype, sex and bin ($F < 1$). C1 (180s): No statistically significant result were obtained regarding the main effect of sex ($F(1,11) = 1.7$, $p = .225$) and the interaction between genotype and sex ($F < 1$). C1 (300s): No statistically significant result were obtained regarding the main effect of sex ($F(1,11) = 2.0$, $p = .189$) and the interaction between genotype and sex ($F < 1$).

4.15, left	No statistically significant result was obtained regarding the main effect of sex, the interaction between genotype and sex, and the interaction between genotype, sex and session (F's < 1).
4.15, right	No statistically significant result was obtained regarding the main effect of sex ($F(1,42) = 1.0$, $p = .318$), the interaction between genotype and sex ($F(1,42) = 1.4 = .240$), and the interaction between genotype, sex and session ($F(1,42) = 2.7$, $p = .110$). Main effect of experiment on C2: No significant main effect of sex or interaction between experiment and sex were seen on C2.
5.5, left	$Genotype_2 \times CueAllocation_2 \times AuditoryCue_2$: No statistically significant result was obtained regarding the main effect of sex ($F(1,51) = 1.9$, $p = .176$) and the interaction between genotype, sex and cue allocation ($F < 1$). However, a significant interaction between genotype and sex was obtained ($F(1,51) = 5.9$, $p = .019$. Female WT mice froze significantly less than male WTs, and these male WTs froze significantly more than male KOs. No statistically significant interaction was obtained regarding the interaction between genotype, sex and auditory cue ($F < 1$), and the interaction between genotype, sex, cue allocation and auditory cue ($F < 1$). $Genotype_2 \times$ 'to-be-allocated CS'$_2$: No statistically significant result was obtained regarding the main effect of sex ($F(1,55) = 1.3$, $p = .262$) and the interaction between genotype, sex and to-be-allocated CS ($F < 1$). However, a significant interaction between genotype and sex was obtained ($F(1,55) = 6.7$, $p = .012$). However, a significant interaction between genotype and sex was obtained ($F(1,51) = 5.9$, $p = .019$. Female WT mice froze significantly less than male WTs, and these male WTs froze significantly more than male KOs
5.5. middle	$Genotype_2 \times AuditoryCue_2 \times Bin_{15}$: No statistically significant results were obtained regarding the main effect of sex($F < 1$), the interaction of genotype and sex ($F < 1$), and the interaction between genotype, sex auditory cue, and bin ($F(14,266) = 1.5$, $p = .100$). However, a significant interaction between genotype, auditory cue and sex was obtained ($F(1,19) = 5.4$, $p = .032$). Male KO mice exhibit significantly greater mean TO_2 signals than male WT mice ($p = .037$). $Genotype_2 \times$'to-be-allocated CS'$_2 \times Bin_{15}$: No statistically significant result was obtained regarding the main effect of sex, the interaction between genotype and sex, the interaction between genotype, sex and to-be-allocated CS, and the interaction between genotype, sex, to-be-allocated CS and bin (F's < 1).
5.5., right	$Genotype_2 \times AuditoryCue_2$: No statistically significant result was obtained regarding the main effect of sex ($F(1,16) = 3.6$, $p = .076$), the interaction between genotype and sex ($F(1,16) = 1.1$, $p = .298$), and the interaction between genotype, sex and auditory cue ($F(1,16) = 1.5$, $p = .239$). $Genotype_2 \times$'to-be-allocated CS'$_2$: No statistically significant result was obtained regarding the main effect of sex ($F(1,16) = 3.6$, $p = .076$), the interaction between genotype and sex ($F(1,16) = 1.1$, $p = .298$), and the interaction between genotype, sex and to-be-allocated CS ($F < 1$).
5.6, top	No statistically significant result was obtained regarding the main effect of sex ($F(1,55) = 9.4$, $p = .003$), the interaction between genotype and

	sex ($F < 1$), the interaction between genotype, sex and day ($F < 1$), the interaction between genotype, sex and CS type ($F < 1$), and the interaction between genotype, sex, session and CS type ($F(3,165) = 1.5$, $p = .215$). Discrimination index: No statistically significant result was obtained regarding the main effect of sex ($F < 1$), the interaction between genotype and sex ($F < 1$), and the interaction between genotype, sex and day ($F(3,165) = 1.5$, $p = .215$).
5.6, bottom left	No statistically significant result was obtained regarding the main effect of sex, the interaction between genotype and sex, and the interaction between genotype, sex and session (F's < 1).
5.6, bottom right	No statistically significant result was obtained regarding the main effect of sex, the interaction between genotype and sex, and the interaction between genotype, sex and day (F's < 1).
5.7	No statistically significant result was obtained regarding the main effect of sex ($F(1,19) = 3.4$, $p = .079$), the interaction between genotype and sex ($F(1,19) = 2.1$, $p = .168$), the interaction between genotype, sex and session ($F(3,57) = 2.1$, $p = .117$), the interaction between genotype, sex and stimuli ($F < 1$), the interaction between genotype, sex, session and stimuli ($F(6,114) = 1.1$, $p = .392$) and the interaction between genotype, sex, session, stimuli and bin ($F(84, 1596) = 1.4$, $p = .235$).
5.8	$Genotype_2 \times Day_3 \times Bin_{15}$ interaction: No statistically significant result was obtained regarding the main effect of sex ($F(1,19) = 2.6$, $p = .122$), the interaction between genotype and sex ($F(1,19) = 3.2$, $p = .088$), the interaction between genotype, sex and session ($F < 1$), the interaction between genotype, sex, session and bin ($F(28,532) = 1.9$, $p = .143$). $Genotype_2 \times Bin_{15}$ interaction: No statistically significant result was obtained regarding the main effect of sex, the interaction between genotype and sex, the interaction between genotype, sex and bin (F's < 1).
5.9	No statistically significant result was obtained regarding the main effect of sex ($F(1,16) = 1.5$, $p = .243$), the interaction between genotype and sex ($F < 1$), the interaction between genotype, sex and session ($F < 1$), the interaction between genotype, sex, session and CS type ($F(3,48) = 1.9$, $p = .134$).

Appendix 25

The aversive CS+ evoked a shift from delta-dominant to theta-dominant neuronal oscillations that was stronger in the 5-HTTKOs than in WT mice.

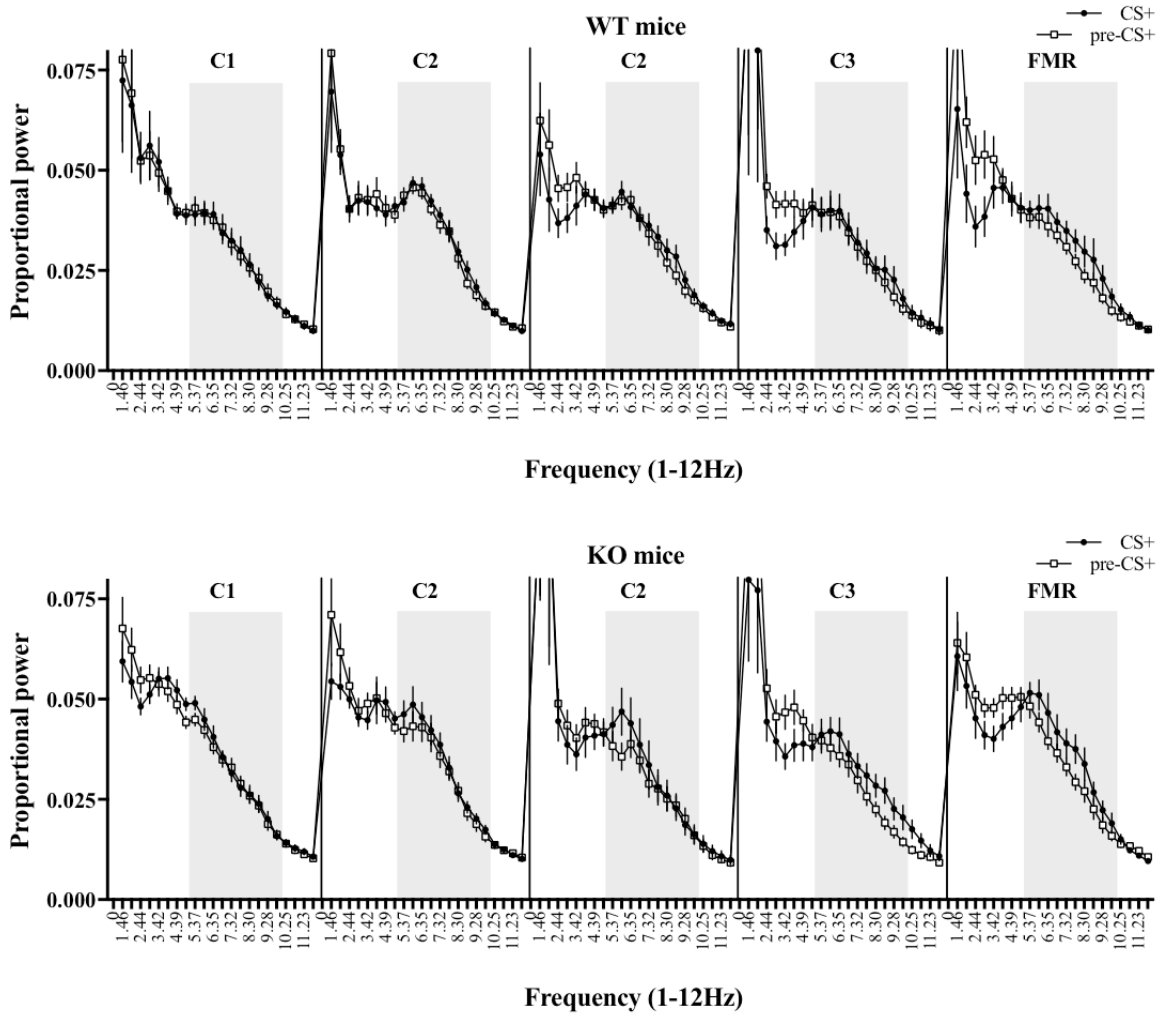


Figure A25.1. Power spectra (expressed as a proportion of the total spectral power between 1-45Hz) for the 30s-long pre-CS+ (white squares) and CS+ (black circles) periods for each of the testing days administered to the 5-HTTKO (N=10) and WT (N=10) mice during the conditioning and FMR phases. The grey-shaded are delimits the theta range (5-10Hz). Mean ± SEM.

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