An Investigation into the Interaction of Dopamine and Glutamate in Behaviours Relevant to Schizophrenia



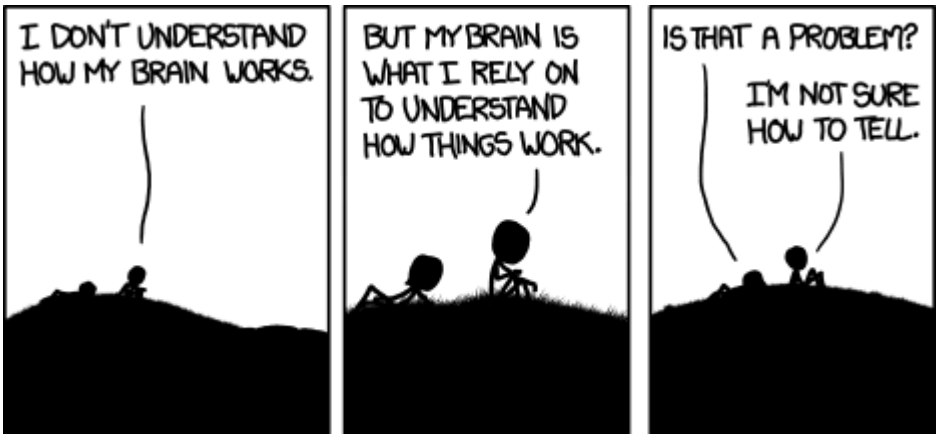
Thomas Börner

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Wolfson College, Department of Experimental Psychology

University of Oxford

Thank you Linda



Randall Munroe, xkcd.com

An Investigation into the Interaction of Dopamine and Glutamate in Behaviours Relevant to Schizophrenia

Aberrant dopaminergic activity is widely believed to underlie at least some of the symptoms seen in schizophrenia. This belief is partly based on all currently used antipsychotics antagonising the dopaminergic D2 receptor. However, there is less agreement on what causes this aberrant dopamine activity. One candidate is a glutamatergic change upstream of the dopamine system. I investigated this glutamate theory of schizophrenia by examining the effect of glutamatergic manipulations on the dopamine system during behaviours relevant to schizophrenia in rodents.

One of these manipulations consisted of systemic injections of the putative antipsychotic LY354740, a group II metabotropic glutamate receptor agonist. This revealed that the effect of LY354740 on the dopamine system was apparently dependent on the internal state of the animal. Even though it is proposed as a putative antipsychotic, LY354740 could actually enhance phasic dopamine responses to reward in the nucleus accumbens core as measured by fast-scan cyclic voltammetry (FCV). Furthermore, at least under some circumstances, LY354740 appeared to exacerbate behavioural effects of amphetamine.

The second manipulation of the glutamate system consisted of a knockout of the GluA1 glutamatergic AMPA-receptor subunit in mice. These GluA1 KO mice displayed deficits in short-term memory and hyperactivity which may reflect an aberrant salience phenotype which could be relevant to schizophrenia. The group II metabotropic glutamate receptor agonist, LY354740, could ameliorate the hyperactivity but failed to rescue the cognitive, short-term memory deficits in a test of spatial working memory.

The phenotype of GluA1 KO mice was further investigated using FCV in tasks relevant to schizophrenia. These experiments showed that GluA1 KO mice exhibited a hyperdopaminergic phenotype. They had increased phasic dopamine responses to neutral stimuli which were slower to habituate. They also exhibited increased dopamine signals to reward delivery in an instrumental conditioning task. In a simple Pavlovian conditioning task, however, they did not show increased phasic dopamine responses.

In summary, two very different glutamatergic interventions relevant to schizophrenia could, under certain circumstances, increase phasic dopamine responses in tasks relevant to schizophrenia. This gives additional evidence for a role of glutamate in schizophrenia.

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Chapter 1: General Introduction

Investigating the brain confronts the researcher with an immense complexity. One way to subdivide the connections made by billions of neurons (Lent et al., 2012) is to classify them by the way signals are transferred from one cell to the next. These connections can be classified by the physical identity of the signalling molecule used, the neurotransmitter. This thesis will investigate the role of two of these neurotransmitters, glutamate and dopamine, with particular emphasis on their potential interactions relevant to schizophrenia.

Mutations at the locus of genes coding for glutamate and dopamine receptor proteins were found to be associated with schizophrenia in a genome-wide association study (Ripke et al., 2014). This thesis will explore the role of two of these glutamatergic receptor proteins associated with schizophrenia in behaviours relevant to schizophrenia. These are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA1 and the metabotropic glutamate receptor 3 (mGluR3). mGluR3 is a member of the group II mGluRs, which have been used as a target in antipsychotic drug development.

Firstly, I will introduce key aspects of schizophrenia. Secondly, the role of dopamine in brain function and behaviour will be discussed. Finally, the role of glutamate as a neurotransmitter in the brain will be described with a special focus on GluA1-containing AMPA receptors and the group II metabotropic glutamate receptors.

Schizophrenia

Schizophrenia is a neurodevelopmental disorder marked by five partly overlapping symptom categories (delusions, hallucinations, disorganized speech, disorganized behaviour, negative symptoms) as defined in the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM 5) (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., 2013). The disorder is usually first diagnosed between the late teens and early thirties with men having an earlier onset and worse prognosis than women (McGrath et al., 2008, van Os and Kapur, 2009). Before delusions and hallucinations lead to the diagnosis of schizophrenia, evidence suggests the existence of a prodromal phase during which negative symptoms and a set of cognitive deficits are developing (Lencz et al., 2006). These deficits include problems with learning and memory and attentional abnormalities. After the first psychotic episode the disorder presents with an unexplained heterogeneity in terms of symptom expression, prognosis and progression. However, the most common is a relapsing/remitting pattern with the patient needing at least some form of support in daily living. Life time prevalence of schizophrenia is approximately 0.3-0.7% across the world (McGrath et al., 2008, van Os and Kapur, 2009).

The two most salient symptom categories are the positive symptoms of delusions and hallucinations. Delusions are counterfactual beliefs that persist in the presence of contradictory evidence. Hallucinations are perception-like experiences that are subjectively sensed by the patient even though there is no

associated stimulus (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., 2013).

However, as the former name of schizophrenia, “dementia praecox” (Angst, 2002), suggests, this disorder encompasses a more complex symptomatology encompassing cognitive deficits. Patients often present with disorganized thought, which is normally inferred from their speech. This speech can consist of a sequence of loose associations rather than a logical progression of thought. This makes the patient tangentially move away from the topic of conversation or in extreme cases, become completely incoherent (Mueser and McGurk, 2004, van Os and Kapur, 2009).

Motor behaviour can be equally disorganized and abnormal. Problems with goal-directed behaviour can extend from abnormal repetitive behaviours to catatonia, an extreme loss of motor function (Manschreck et al., 1982).

The category of negative symptoms distinguishes schizophrenia from other psychotic disorders (American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., 2013). As the term “negative” suggests it consists of a set of functional losses; a loss of motivation (avolition), diminished speech output (alogia), a loss of hedonic pleasure (anhedonia), and a lack of interest in social interaction.

Treatment

These symptoms differentially respond to currently available pharmacological treatments. Currently, antipsychotics broadly fall into two groups. Typical or first generation antipsychotics act as antagonists on the dopaminergic D2 receptor (Seeman and Lee, 1975). Their extrapyramidal side effects like muscle rigidity and tremor, due to the lack of specificity at the level of the dopamine pathways, led to the development of atypical or second generation antipsychotics. These drugs have a more diffuse binding profile, depending on the specific drug, showing affinity to a variety of dopaminergic, serotonergic, and cholinergic receptors. But all currently used antipsychotics show at least some affinity to the D2 receptor (Kapur and Mamo, 2003). And all of these drugs are effective in ameliorating positive symptoms but show little efficacy in treating the negative or cognitive symptoms of schizophrenia (Kirkpatrick et al., 2006). As the mode of action of antipsychotic medications suggests, dopamine plays an important role in the pathology of schizophrenia. Therefore, the role of dopamine in the central nervous system in general, and schizophrenia in particular, will be discussed next.

What does dopamine do?

To identify the role of a single molecule in a system as complex as the human brain, it is useful to split up the investigation based on more specific questions.

1. How does the molecule dopamine interact with other molecules in the brain to affect cells?
2. In what way is dopamine involved in generating adaptive behaviour?

In order to answer the first question, this thesis will explain how the body synthesises and metabolises dopamine, and then detail its role in neural signalling. Next, this thesis will illustrate the anatomy of the dopamine system, focusing on the midbrain. For the remainder of this thesis the term “dopamine system” will denote the group of cells which metabolises dopamine and uses it for neuronal signalling. To address the second question, this thesis will present different theories to explain the role of dopamine in adaptive behaviour.

Dopamine at the Molecular and Cellular Level

Dopamine, being a catecholamine, is synthesized from tyrosine. Tyrosine is hydroxylised by tyrosine hydroxylase into the precursor L-DOPA. L-DOPA in turn is decarboxylated by Aromatic L-amino acid decarboxylase (AADC) into dopamine which is then packaged into vesicles by VMAT2 (Kandel et al. (2013)).

Dopamine functions as a neurotransmitter, a signalling molecule that enables communication from a neuron to another cell (Carlsson et al., 1957, Carlsson, 1993, Benes, 2001). This signal is transmitted upon depolarization of the presynaptic cell sending the information. More specifically, if an action potential reaches the area in which dopamine vesicles are present, dopamine will be

released via fusion of the vesicle with the cellular membrane (Kandel et al., 2013).

Once in the extracellular space, dopamine shows affinity to a number of G-protein coupled receptors (GPCRs), a class of membrane-bound proteins that activate intracellular signalling cascades. Dopamine receptors are categorised into two groups: the family of D_1-like receptors, containing D_1 and D_5 receptors, and the D_2-like family, containing D_2, D_3 and D_4 receptors (Beaulieu and Gainetdinov, 2011). D1-like receptors are coupled to the G-protein $G_{s\alpha}$ which increases intracellular cyclic adenosine monophosphate (cAMP) levels via activation of adenyl cyclase, making the neuron more excitable when activated. D2-like receptors on the other hand, are coupled to the g-protein $G_{i\alpha}$ which decreases cAMP levels, making the cell less excitable (Beaulieu and Gainetdinov, 2011).

Only a very small subset of all neurons synthesize dopamine (~20 000–30 000 in mice and 400 000–600 000 in humans (Bjorklund and Dunnett, 2007)) and these neurons are therefore classified as dopaminergic. However, this category contains a considerable anatomical, biochemical and physiological diversity.

Dopaminergic neurons can be identified using histofluorescence, a method in which formaldehyde vapour is used to convert dopamine into isoquinoline. Isoquinoline emits a yellow-green fluorescence that can then be detected (Falck et al., 1962). Using this technique, dopaminergic neurons were identified in brain regions as diverse as the retina, the olfactory bulb, the hypothalamus, and the

midbrain (Fig. 1.1) (Dahlstrom and Fuxe, 1964, Bjorklund and Dunnett, 2007). However, a dopaminergic neuron might not necessarily only release dopamine; it may release other neurotransmitters such as GABA (Tritsch et al., 2012), or be coupled to other neurons via gap junctions(Grace and Bunney, 1983, Vandecasteele et al., 2005). For the purposes of this thesis the focus will be on the properties of dopaminergic neurons in the midbrain.

These dopaminergic neurons in the midbrain can be anatomically subdivided into two major groups of adjacent cell populations, the substantia nigra and the ventral tegmental area (VTA). In rodents the dopaminergic neurons of the substantia nigra pars compacta (SNc) project to the dorsal striatum via the nigrostriatal pathway (Fig. 1.1). The SNc contains neurons that in vitro show slow conduction velocity, low firing rates, long duration action potentials, and pacemaker firing (Margolis et al., 2006). However, in vivo they show burst firing (Roeper, 2013), suggesting that this is a physiological pattern arising from network activity rather than properties inherent to the neurons themselves.

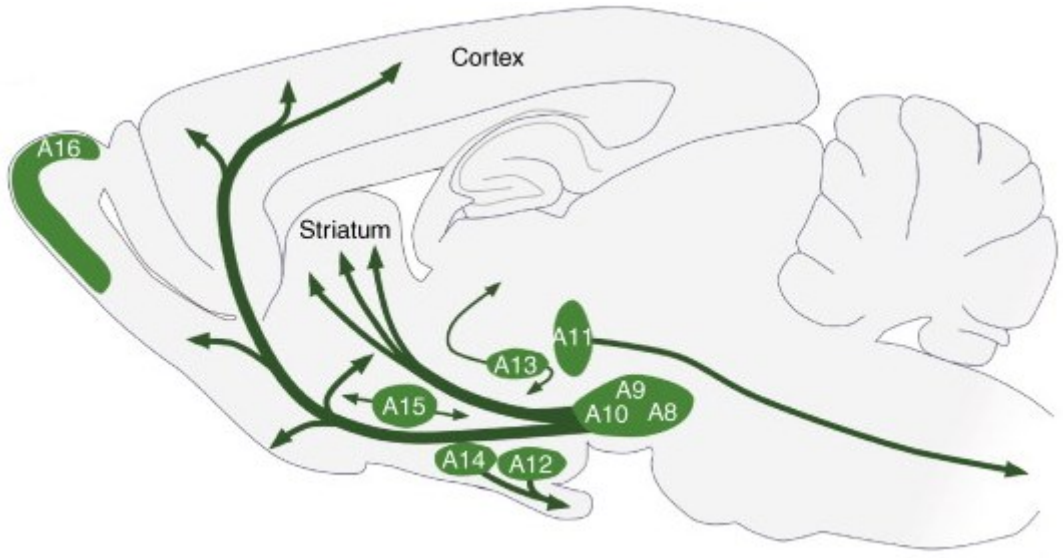


Figure 1.1: Location of dopamine neurons in a sagittal slice of an adult rodent brain and their projections. The nomenclature of the cell groups was introduced by Dahlström and Fuxe (1964). The locus A10 of dopamine neurons corresponds to dopaminergic neurons in the VTA and A9 corresponds to dopaminergic cells in the SNc. Taken from Bjorklund and Dunnett (2007)

Medial to the SNc lies the VTA which has major projections to the ventral striatum (including the nucleus accumbens) via the mesolimbic pathway, and to the frontal cortex via the mesocortical pathway (Menegas et al., 2015). Additional projection areas include the cingulate gyrus, the hippocampus, and the amygdala. In contrast to the SNc, dopaminergic neurons in the VTA cannot be identified as being dopaminergic based on their firing pattern (Ungless and Grace, 2012).

The Role of Dopamine in Adaptive Behaviours

The function of the dopamine system at a systemic level will be discussed next. To clarify this role foraging will be used as an example. Every animal faces the same problem: How to acquire food to fulfil its caloric needs? Obviously there is a high evolutionary pressure on this process. An organism that cannot find food

without becoming food itself will not be able to transmit its genes. To understand different potential roles of dopamine, it is instructive to first look at this process of foraging for a food source.

Hunger, or the knowledge that it will soon be hungry, motivates an animal to explore the environment. This movement will lead to a change in the sensory input it sees, smells, hears, and touches. From this flood of stimuli the animal has to recognize input that is associated with food in order to find a food source. To make use of these stimuli they have to be inherently attractive (hard coded by evolution) or need to have become associated with food. In any case, the stimulus has to have a value that differentiates it from the plethora of stimuli the animal perceives at the same time.

In order to exploit the food source (potentially) associated with a stimulus, the animal has to adapt its behaviour. The animal has to process the stimulus, make a decision, and implement this decision. A mouse, for example, would approach the smell of fruit. Based on whether the decision to approach the smell of fruit is advantageous or detrimental (i.e. leads to food or not) the animal would update its future strategies accordingly. Dopamine has long been thought to play a central role in this process. However, different theories have been proposed as to exactly what this role is.

The Anhedonia Hypothesis

In the 1950s, Olds and Milner conducted a series of experiments in which rats had the opportunity to press levers to receive intracerebral electrical stimulation (Olds and Milner, 1954). Areas where electrical stimulation reinforced lever pressing were said to be “pleasure centres” (Olds, 1956). Wise and colleagues showed that these areas were colocalized with the locus of dopaminergic cells in the midbrain (Corbett and Wise, 1979), and that the rewarding effect of this electrical stimulation could be weakened by the systemic injection of dopamine receptor antagonists (Fouriezos and Wise, 1976).

This led to the formulation of the anhedonia hypothesis: As dopamine is normally released in response to rewarding stimuli, and can be accompanied by a subjective experience of pleasure, it is therefore thought to code the motivational arousal needed for goal directed behaviour (Wise, 1982, 2008). In the above mentioned example, dopamine would be released when the mouse finds the fruit and would motivate it to search out the smell of fruit the next time the smell is encountered.

The Reward Prediction Hypothesis

Undoubtedly, dopamine plays an important role in reward processing, but today’s dominant theory attributes a different role to dopamine function. In a seminal experiment Schultz et al. (1997) presented a monkey with a reward and measured the activity of VTA neurons. As predicted by the anhedonia

hypothesis, the neurons fired when the reward was presented. However, when a cue was presented before the reward, neurons behaved in a surprising way after the association was acquired. In apparent contradiction to the anhedonia hypothesis, the cells were active when the cue that predicted reward was presented but not when the reward itself was given. Furthermore, if the conditioned cue was presented but the predicted reward was absent, the firing of the VTA neurons actually decreased at the time point during which the reward was predicted to occur.

This pattern fits a concept used in the field of reinforcement learning. In reinforcement learning the optimization of a function leads an agent to perform actions in a defined environment so as to maximize reward, just as in the foraging example described above. If a reward is already fully predicted, as can be the case after a conditioned cue presentation, the behaviour does not have to change. But if there is a discrepancy between prediction and reality, for example if a reward comes without being predicted or a predicted reward is absent, the behavioural output has to be adapted. For this to happen, the difference between prediction and reality, the so called reward prediction error (RPE), has to be determined (Montague et al., 1996). As Schultz et al. (1997) have shown, dopamine neurons increase their activity when more reward than predicted is present and are less active if less reward than predicted is present, suggesting that dopamine potentially fills the role of the RPE. In terms of our foraging example, the dopamine system would be active if the mouse is consuming the fruit. Over multiple presentations, the smell would become predictive of the fruit

and the dopamine system would be active when the mouse starts to smell the fruit.

At this point it is important to note that these two hypotheses discussed so far, the anhedonia hypothesis and the RPE hypothesis of dopaminergic actions, are not necessarily mutually exclusive. Dopamine instead appears to contribute simultaneously to both motivation and learning. Hamid et al. (2016) measured dopamine levels in the NAc in rats performing a complex choice task. They found that dopamine codes a single decision variable over multiple time scales, the available reward to be gained for the effort invested. This combines the reward prediction error hypothesis (reward) with the motivational account (effort) of dopamine function. In addition, dopaminergic cells in the SNc play an important role in the initiation and control of movement as is evident by its central role in Parkinson's Disease (Kalia and Lang, 2015). However, discussion of this topic would be beyond the scope of this thesis. Instead this thesis will concentrate on the nucleus accumbens core. If not stated otherwise "dopamine levels", "dopaminergic changes", etc. denote changes in dopamine in the nucleus accumbens core, a structure identified as part of the "reward circuit" (Haber and Knutson, 2010), which receives its dopaminergic input preferentially from the VTA as opposed to from the SNc.

The Incentive Salience Hypothesis

A third hypothesis of dopamine's role is related to both of the above discussed ideas. According to the incentive salience hypothesis, dopamine's role is to turn

neutral stimuli into stimuli that are capable of capturing attention by inducing motivational “wanting” (Berridge, 2007), making the animal approach the stimulus. This explains how a neuronal signal coding motivation can occur at reward presentation at the beginning of training and then move to the cue after the association is acquired. Additionally, it addresses some data inconsistent with the RPE theory of dopamine.

If dopamine plays a causal role in learning, a mouse without dopamine should not be able to process rewards. As described above tyrosine hydroxylase is necessary to synthesise dopamine. Therefore, tyrosine hydroxylase knockout mouse should not prefer inherently rewarding sucrose solution over water since its reward processing is putatively abolished. However, tyrosine hydroxylase knockout mice do prefer sucrose over water (Cannon and Palmiter, 2003), putting the central exclusive role of dopamine in reward processing in doubt. Dopamine activity could instead be the result of a learning mechanism somewhere else in the brain. Dopamine could instead give salience to a previously neutral stimulus. What used to be a cold piece of information is now a desirable “wanted” stimulus that attracts the agent (Berridge, 2007). In our example, the dopamine system would be activated when the mouse smells fruit which makes the mouse approach the smell and thereby find the piece of fruit.

Dopamine and Schizophrenia

The effect of known antipsychotics on the dopamine system was the first evidence for a role of dopamine in schizophrenia (Carlsson and Lindqvist, 1963).

Later, Seeman and Lee (1975) showed that the inhibitory effect an antipsychotic had on a receptor, later to be identified as the dopamine D2 receptor, was highly correlated to the clinical dose of that antipsychotic. Hence, the dopamine hypothesis of antipsychotic action was born; the idea that an antipsychotic's clinical effect emerged from its reaction with the dopamine system. This was supplemented by the finding that a drug which exacerbates psychotic symptoms, amphetamine, also acts on the dopamine system (Heikkila et al., 1975, Lieberman et al., 1987, Heal et al., 2013). Hence not only antipsychotic action could be explained by dopaminergic mechanisms, but it became feasible to think of schizophrenia as a disorder of a hyperactive dopamine system.

This rather unspecific theory was elaborated on by Davis et al. (1991). If increased dopamine in the whole central nervous system was to underlie schizophrenia, one would expect increased dopamine metabolites in the cerebrospinal fluid, which wasn't the case (Post et al., 1975). Davis et al. (1991) and Weinberger (1987) instead reconceptualised the dopamine hypothesis by proposing hyperdopaminergia in the striatum as the cause of positive symptoms and hypodopaminergia in the prefrontal cortex as the cause for the negative symptoms seen in schizophrenia. Striatal hyperdopaminergia has been found in schizophrenia patients using positron emission tomography (Fusar-Poli and Meyer-Lindenberg, 2013). With this method striatal hyperdopaminergia has also been found to be present in the prodromal phase of schizophrenia (Howes et al., 2009) thereby preceding the development of psychosis, and suggesting a causal role. Cortical hypodopaminergia has been found in drug naïve schizophrenia patients (Abi-Dargham et al., 2012) showing that it not only is present in patients,

but is not simply an effect of antipsychotic medication. This dominant theory of the biology of schizophrenia, even though highly successful in the literature, has one major gap. It fails to connect the biology and chemistry with the subjective feelings of the patient. It is hard to imagine how a change in the concentration of a chemical should lead to a profound loss of connection to reality.

To bridge this gap, Howes and Kapur (2009) proposed that changes upstream of the dopamine system as varied as genetic predispositions, perinatal changes, and environmental factors lead to an alteration in presynaptic dopaminergic control. The resulting aberrant dopamine system is underlying psychotic symptoms. The connection between aberrant dopamine and psychosis is made via the concept of aberrant salience. As discussed earlier, dopamine plays a role in assigning value to stimuli. If the dopamine system is active inappropriately, salience may be attributed to unimportant stimuli or representations. Howes and Kapur (2009) argue that delusions are an attempt of cognition to explain this aberrant salience attribution.

However, Howes and Kapur (2009) did not state that changes to the dopamine system are the cause of the symptoms seen in schizophrenia. Instead aberrant dopamine is hypothesised to be the final common pathway through which upstream changes affect the organism. One of the most promising candidates for this upstream change is an alteration in the glutamate system (Javitt and Zukin, 1991, Moghaddam and Javitt, 2012). Therefore, the workings of the glutamate system will be discussed in the following sections with an emphasis on the constructs and manipulations used in this thesis.

Glutamate

Glutamate acts as the major excitatory neurotransmitter in the central nervous system. It therefore plays an essential part in most processes in the mammalian brain. To understand processes involving glutamatergic signal transduction it is informative to examine the role of its different receptors. These can be subdivided into fast channel-forming ionotropic receptors and slow G-protein coupled metabotropic receptors.

Ionotropic Glutamate Receptors

The ionotropic class, in turn, can be subdivided based on their pharmacology into N-Methyl-D-aspartic acid (NMDA), kainic acid (kainate) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, named after synthetic compounds acting as competitive agonists at the respective subgroups (Watkins and Jane, 2006). A fourth subgroup the delta receptors are included into the ionotropic glutamate receptor family based on sequence homology. However, proteins of this family do not bind glutamate (Lomeli et al., 1993).

NMDAR

The channel-forming NMDA receptor is made up of two ubiquitously expressed obligatory GluN1 subunits and two identical GluN2 subunits from a group of four possible distinct subtypes (GluN2A-D). In addition to these diheteromers, triheteromeric NMDA receptors have been found (e.g. GluN1/GluN2A/GluN2B),

but their properties are largely unknown and evidence suggests that they are in the vast minority (Kohr, 2006).

NMDA receptors are expressed at high levels throughout the brain (Monyer et al., 1992) in astrocytes (Lalo et al., 2006), oligodendrocytes (Karadottir and Attwell, 2006) and neurons (Kohr, 2006). In neurons NMDA receptors are typically expressed postsynaptically (Paoletti et al., 2013), but have been reported presynaptically (Tzingounis and Nicoll, 2004) and extrasynaptically (Steigerwald et al., 2000).

Activation (i.e. channel opening) of the NMDA receptor is dependent on binding of glycine (or D-serine) and glutamate, and depolarization of the neuron on which membrane the receptor is located (Paoletti et al., 2013). This voltage dependency arises due to a Mg^{2+} ion inside the channel blocking ions passing through the channel at the normal resting potential of neurons (-70mV)(Johnson and Ascher, 1990). When the cell is depolarized to (e.g. -40mV) the Mg^{2+} block is removed, allowing the flow of Na^{+} and small amounts of Ca^{2+} ions into the neuron and K^{+} ions out of the neuron.

Since NMDA receptors are mainly located in the postsynapse, they modulate their activity based on the membrane potential of the postsynapse. In addition activity of the channel is dependent on glycine and glutamate binding. Glutamate is released into the synapse by depolarization of the presynapse. Therefore, NMDA receptors can work as coincidence detectors of pre- and postsynaptic activity. Only if both are depolarized is the NMDA receptor active. This enables

the NMDA receptors to play an important role in synaptic plasticity, the change of synaptic strength over time (Paoletti et al., 2013). This is a process of self-organisation in which neural connections change, for example, due to neural activity patterns.

Hebb (1949) formulated a set of basic rules to explain this (back then hypothetical) process of self-organisation. Today, his model is often summed up by the maxim “neurons wire together if they fire together”(Lowel and Singer, 1992). If pre- and postsynaptic neurons are active at the same time, the synapse connecting the two will increase in strength.

Bliss and Lomo (1973) found a persistent increase in synaptic efficiency in the hippocampus after the presynapse was stimulated with a high-frequency sequence of individual stimulations (tetanic stimulation). This long-term potentiation (LTP) is defined as an increase of synaptic strength at least 30 minutes after initiation. However, visual inspection of the change in synaptic strength (Fig. 1.2) shows that synaptic facilitation actually consists of two components, an early rapidly decaying component and the long-term potentiation itself.

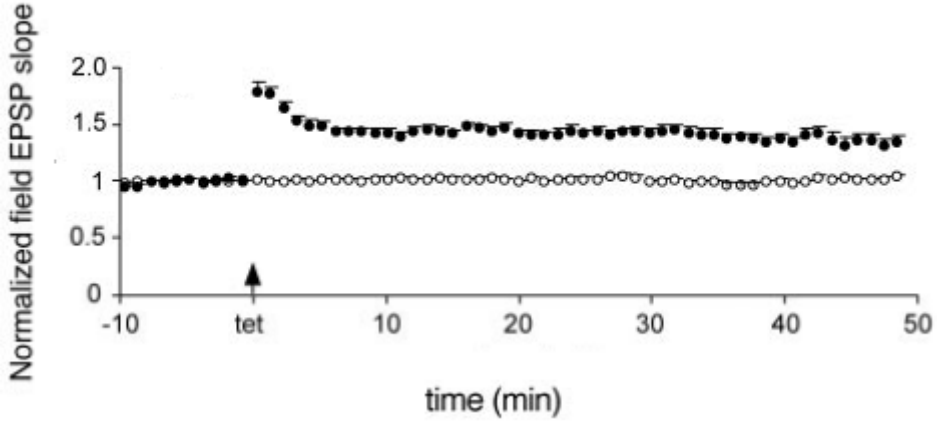


Figure 1.2: Time course of long term potentiation (LTP) of synaptic strength in a neural pathway in which LTP was induced in an in vitro hippocampal slice preparation of the CA1 subregion by a tetanic stimulus at t=0 (black) vs controls (white, a non-tetanized pathway). Modified from Zamanillo et al. (1999)

NMDA receptors play an important role in the induction of LTP. Collingridge et al. (1983) showed that when APV is introduced onto a slice preparation, LTP induced by tetanic stimulation is abolished in the hippocampus. NMDA receptors have also been suggested to play an important role in learning and memory (Morris et al., 1986, Tsien et al., 1996, Martin et al., 2000). A knockout mouse that lacks NMDA receptors in the CA1 principal cells of the hippocampus, for example, shows impaired spatial reference memory (Tsien et al. (1996), but see Bannerman et al. (2012)). It is important however, to note that NDMA receptor-dependent hippocampal LTP does not necessarily underlie spatial memory processes, as some spatial memory processes function in the absence of hippocampal LTP (Bannerman et al., 2014).

AMPA Receptors

AMPA receptors are composed of combinations of four subunits, GluA1-4, coded for by the Gria1-4 genes. Each of these subunits consists of an extracellular terminal domain, an extracellular ligand-binding domain, a transmembrane domain with three transmembrane segments and an intracellular C-terminal tail. These subunits can combine in 7 different configurations of hetero- and homotetramers, forming a channel pore permeable to K^+ and Na^+, and Ca^{2+} if the receptor is lacking the GluA2 subunit. If the receptor contains the GluA2 subunit it is impermeable to Ca^{2+} ions (Fleming and England, 2010).

AMPA receptors mediate fast synaptic transmission (Riedel et al., 1999) and mediate the expression of long-term potentiation. As discussed above, NMDA receptors are needed to induce LTP. The continued expression of synaptic facilitation however is dependent on the addition of AMPA receptor to the postsynaptic membrane (Malinow and Malenka, 2002, Kessels and Malinow, 2009). In order to be inserted in the membrane, AMPA receptors have to be trafficked there. This trafficking is controlled by proteins that bind to the c-terminal end of the subunits. Therefore the identity of the C-terminal end determines how these receptors are trafficked. GluA1 has a long intracellular c-terminal tail. GluA3 has a short c-terminal tail and, GluA2 and GluA4 can have a long or short c-terminal tail dependent on the splice variant. Subunits with a long c-terminal tail are inserted into the postsynaptic membrane to increase synaptic strength in an activity-dependent manner (Shi et al., 2001). Short c-terminal

tailed subunits on the other hand continuously cycle in and out of the membrane without affecting connection strength (Zhu et al., 2000).

As their role in synaptic plasticity might suggest AMPA receptors play a role in learning and memory. This has been investigated by local drug infusion into different brain areas. For example, infusions of the AMPA/kainate antagonist LY326325 into the hippocampus reduced basal fast excitatory transmission by 90% and induced deficits in spatial memory as measured in the Morris water maze (Riedel et al., 1999). In the Morris water maze task rodents are repeatedly placed into the pool at various locations inside a water tank. To escape this water tank they have to reach a hidden platform the position of which stays fixed. Performance in this task is measured by the time taken and path swum (distance) until the platform is localized. This task is often referred to as a spatial reference memory task since extramaze cues have to be associated with the location of the platform over many trials and the solution (i.e. the correct spatial location) stays constant (Sanderson et al. 2010). Furthermore, avoidance learning is impaired when another AMPAR/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) is infused into the entorhinal cortex, hippocampus or the amygdala (Bianchin et al., 1993, Quillfeldt et al., 1994). Even though these drugs are selective to non-NMDA ionotropic glutamate receptors, they, in effect, silence the brain region they are introduced into as is shown by the large reduction of basal fast excitatory transmission (Riedel et al., 1999). This complicates the interpretation of these experiments with respect to their relevance for schizophrenia. It is unlikely that AMPAergic signalling is completely

absent or even absent in certain brain regions in schizophrenia patients. Such a change would inactivate the whole region or even the whole brain.

One potential experimental strategy would be to target receptor subunits more specifically. Hence, to investigate the role of AMPA receptors further, this thesis will focus on the GluA1 subunit. This receptor subunit is mainly expressed in the forebrain and the cerebellum (Martin et al., 1993). In the neocortex, while still being expressed in layer V pyramidal cells, it is preferentially expressed in interneurons. In the hippocampus, GluA1 is strongly expressed throughout the Ammon's horn and the dentate gyrus. In the midbrain it is expressed in the dopamine rich, ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) among other regions. In the SNc it has been shown to be expressed specifically in dopaminergic neurons (Martin et al., 1993).

No AMPA receptor subunit specific compounds are available. Still, a big literature on the role of GluA1 in physiology and behaviour has accumulated. This literature is largely based on the use of a GluA1 knockout mouse.

The GluA1 knockout mouse

To create this GluA1 knockout (KO) mouse Zamanillo et al. (1999) utilized homologous recombination and transient expression of Cre recombinase in embryonic stem cells to delete exon 11 (the region of the Gria1 gene coding the first two transmembrane segments) of the GluA1 protein.

The resulting constitutive GluA1 null mutant does not express the GluA1 protein anywhere in its brain, at any point during its life-time. Still, it develops normally, has a normal life expectancy, and does not show any difference in weight, gross motor behaviour, or brain structure. Indeed, the GluA1KO mouse displays a behaviour that is generally indistinguishable from wildtype littermates in a home cage environment (Zamanillo et al., 1999). Concentrating, their electrophysiological investigations on the hippocampus Zamanillo et al. (1999) made three major findings in the GluA1 KO mouse:

1. Field excitatory postsynaptic potentials (fEPSP) in GluA1 KO mice were unchanged as measured by extracellular recordings. This suggested that basal synaptic transmission was unchanged.
2. LTP in the Schaffer collateral-CA1 pyramidal cell synapse in the hippocampus was absent when assessed with the classical tetanus stimulation.
3. Spatial learning was normal in GluA1 KO mice as assessed in the Morris water maze.

However, multiple studies have since elaborated on all three findings. For example, basal synaptic transmission was found to be reduced in GluA1 KO mice. The same presynaptic depolarization led to a reduced EPSP in GluA1 mice when Zamanillo et al. (1999)'s experiment was repeated (Jensen et al., 2003, Romberg et al., 2009).

Continued investigation of the synaptic plasticity phenotype reproduced the lack of LTP in the hippocampus of adult GluA1 KO mice in response to tetanic stimulation (Jensen et al., 2003, Frey et al., 2009, Romberg et al., 2009). However, investigation of the Schaffer collateral-CA1 synapse with other stimulation paradigms revealed a more complex picture. When both the pre- and postsynapse were stimulated with bursts (100Hz) of stimulation at 5 Hz (paired theta-burst stimulation), LTP was detected at the Schaffer collateral-CA1 synapse in GluA1 KO mice (Hoffman et al., 2002, Frey et al., 2009, Romberg et al., 2009). Furthermore this potentiation showed a peculiar pattern. As discussed above, potentiation seen upon electrical stimulation usually consists of an early rapidly decaying part and the long-lasting stable part. GluA1 KO mice lacked the early rapidly decaying component but displayed the long-lasting stable component. Thus, GluA1 appear to lack a form of short-term plasticity (see also Erickson et al. (2010)).

Finally, when memory processes were further investigated in GluA1KO mice, a more complex picture emerged. Reisel et al. (2002) reproduced the intact performance of GluA1 KO mice in the Morris water maze. Even though they were indistinguishable from controls on this test of spatial reference memory, GluA1 KO mice were impaired on a test of spatial working memory, namely rewarded alternation on a T-shaped maze (Reisel et al., 2002). Here correct responding is dependent on recall of unique information from a single sample trial. During testing, each trial consisted of two runs; a sample and a choice run. In the sample run the animal was placed into the start arm facing the investigator, and forced (by blocking off one of the arms) to turn left or right into one of the goal

arms to gain a food reward. After the animal consumed the reward it was removed from the maze and both arms were opened (i.e. the block/door was removed). After an intra-trial interval (e.g. 5-10 seconds) the animal was placed in the start arm of the maze, facing the investigator, and allowed to make a free choice between the left and right goal arms. Animals were rewarded for entering the arm that they had not previously visited, *i.e.* for alternating or displaying a win-shift behavioural response. On this test GluA1 KO mice were impaired; alternating at chance levels, suggesting that they had compromised spatial working memory.

This specific impairment in spatial working memory with intact spatial reference memory can further be elucidated using Olton's radial maze (Sanderson et al., 2010). With the radial maze both types of memories can be investigated in the same task at the same time and in the same animal. The radial maze consisted of a central platform from which 6 arms radiated out equidistantly. Over many trials three of these arms (the same arm on every trial) were baited. The remaining arms were never baited. The mice had to learn to discriminate between the baited and unbaited arms. This is described by Olton as the spatial reference memory component of the task. The animals have to use extramaze cues to locate the food rewards which are in constant location throughout the experiment. As would be predicted from their performance in the Morris water maze, GluA1 KO mice were indistinguishable from wildtypes in this spatial reference memory component of the radial arm maze (Schmitt et al., 2003). GluA1 KO mice were as unlikely to enter an arm that is never baited as were wildtypes. If the food rewards are not replaced within a trial, the animal has to

remember which arms they have visited in the present trial and avoid re-entering those arms. This means displaying win-shift behaviour, similar to the behaviour measured by rewarded alternation on a T-maze. Therefore, the number of arm entries into (previously) baited arms during the radial arms maze is a measure of spatial working memory. GluA1 KO mice, which displayed normal reference memory, were impaired in this spatial working memory component of the task (Schmitt et al., 2003).

Further studies suggested that the spatial working memory deficit in GluA1 KO mice is due to deficits in short-term habituation. Sanderson et al. (2007) tested knockouts and controls in a non-rewarded single trial spontaneous alternation task. Mice were placed on a Y-shaped maze with one of the three arms being blocked (sample run). Mice could explore the two open arms and no rewards were given. Then the mouse was removed, the third arm was opened and the animal was placed in the maze again (choice run). Wildtype mice now preferentially entered the previously blocked arm compared to the previously visited arm even though there was no previous training and there no rewards were given. This suggests that wildtype mice alternate spontaneously showing novelty preference. GluA1 KO mice, however, showed no novelty preference. This suggests that they did not habituate to the previously visited arm, perceiving it as novel.

Interestingly, long-term habituation actually seems to be facilitated in GluA1 KO mice. Sanderson et al. (2009) tested wildtypes and GluA1 KO mice on a spatial novelty preference Y-maze task in which mice were repeatedly placed at the end

of an arm of a Y-shaped maze with only one other arm being open. The interval between these exposure trials was either short (1min) or long (24h). After completion of the exposure sessions another short (1min) or long (24h) interval (pre-test) was imposed. In the following novelty preference test the third arm was now opened. Relying upon the subject's ability to remember extramaze cues to determine which arm was new, the animal was inclined to spend more time in the unfamiliar arm due to habituation to the two known previously experienced arms. Considering this, the time spent in the new arm was used as a measurement for the subject's spatial memory. GluA1 KO mice were impaired compared to wildtypes if the interval between exposure trials was short, suggesting an impairment of short-term spatial habituation. If the intervals were long on the other hand, GluA1 KO mice spent more time in the novel arm than controls, suggesting increased long-term habituation and long-term memory.

This dissociation between long term and short-term habituation can be explained in terms of Wagner's sometimes opponent process theory of animal learning (Wagner, 1981, Sanderson et al, 2010). Wagner described short-term and long-term habituation as two independent processes. Short-term habituation is a non-associative process to recently presented stimuli. In the one trial Y-maze task, this means that a rodent could habituate to the open arm during the sample run. And since rodents have a tendency to explore and forage they will choose the novel arm during the choice run over the recently visited (i.e. habituated) arm. In GluA1 KO mice this non-associative process is impaired. They do not habituate and therefore do not prefer the novel arm over the previously visited arm. Long-term habituation, however, according to Wagner (1981), is associative relying on

the retrieval of associative memories by a presented stimulus. This process is intact in GluA1 KO mice as can be seen by their preference for the novel arm of the Y-maze when training sessions have a long intertrial interval between them.

The impairment of short-term habituation in GluA1 KO mice is not restricted to the spatial domain. For example, GluA1 KO mice also showed impaired habituation in a novel object recognition task (Sanderson et al., 2011a). This task again consisted of two phases. In the exposure phase of this task mice were placed into an open field environment with two similar objects and were allowed to explore these items. After a two minute interval the mice were again placed in the open field for a test phase. In this test phase, one of the objects was switched with a novel one, the other with a duplicate of the now familiar object. Wildtype mice habituated to the "known" object and therefore spent more time exploring the novel object. GluA1 mice, however, showed no preference for the novel object.

Finally, Sanderson et al. (2011b) found evidence of short-term sensitization as opposed to short-term habituation in GluA1 KO mice in the habituation of the orienting response to neutral stimuli (HORNS) task (Fig. 1.3). The task was built on rodent's natural tendency to orient towards novel visual stimuli, a behaviour known to habituate after repeated presentation of the same stimulus (Hall, 1985). Mice were trained to nose-poke in a food magazine to gain sucrose rewards. If the mice oriented to a stimulus rather than continued to nose-poke, there would be a reduction in the duration of "head entries" into the food magazine. Hence suppression of head entry can be used as a surrogate measure of orienting. To

assess stimulus-specific short-term habituation pairs of lights were presented for 10s each, independent of reward delivery. Each trial consisted of a pair of lights with an interval of 30s between them. The second stimulus in this trial was either the same as the first (condition SAME), or different from the first (condition DIFFERENT). If a mouse habituated to the first stimulus it would show weaker head entry suppression to the familiar stimulus (SAME) than to novel stimulus (DIFFERENT). Wildtype mice oriented more in the DIFFERENT than in the SAME condition, thereby giving evidence for stimulus-specific habituation of the orienting response to neutral stimuli. GluA1 KO mice, however, showed stronger orienting in the SAME than in the DIFFERENT condition, thereby showing sensitization to neutral stimuli.

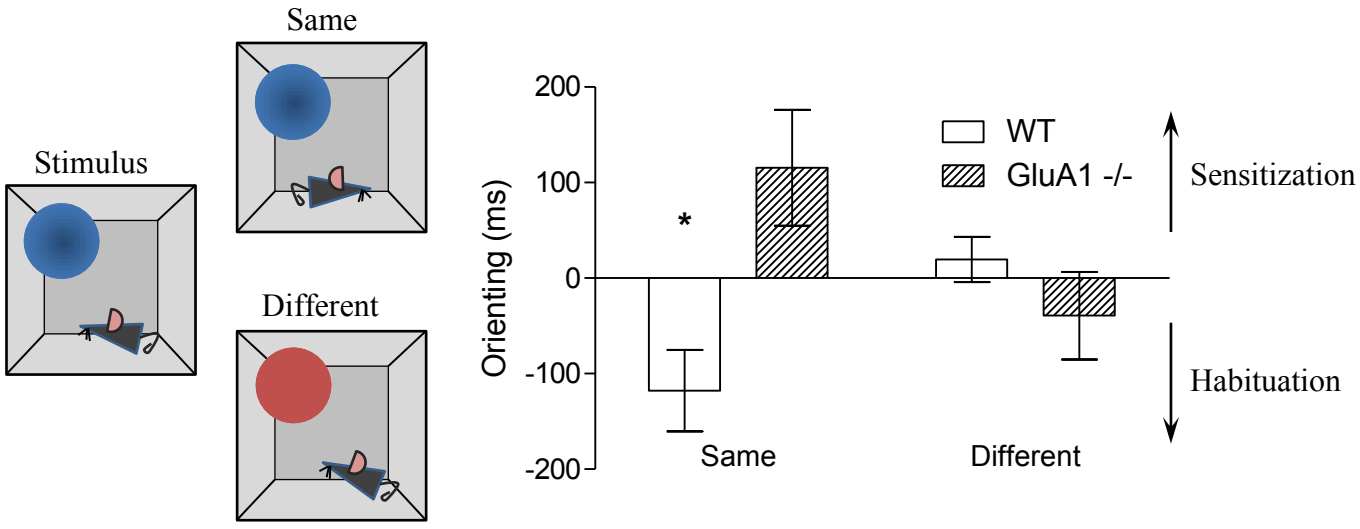


Figure 1.3: Habituation of the orienting response to neutral stimuli (HORNS) task: Mice were presented with pairs of stimuli in which the 2nd stimulus was either the same as the first stimulus or different from it. Wildtype mice oriented less if the second stimulus was the same as the first stimulus. GluA1 knockout mice displayed increased orienting (a measure of attention) to a recently presented light stimulus. * p < .05 (Taken from Barkus et al. (2014))

So far we have conceptualized the behaviour of the GluA1 KO mouse in terms of memory processes. However, the HORNS task can also be viewed from the angle of attention. From this angle the orienting response is a read out of the salience attributed to a stimulus, just like eye movement is in humans, albeit much less sophisticated on a methodological level. This enables a connection to the previously discussed theory of aberrant salience. However, it has to be mentioned that the salience measured in this task is most likely guided by the stimulus, whereas salience as understood by Berridge (2007) is guided by association of the stimulus with aversive or rewarding stimuli (for further discussion see chapter 8). Still, GluA1 KO mice have a knockout of a gene with relevance to schizophrenia (Ripke et al., 2014) and display aberrant salience attribution. If dopamine is the final common pathway in which upstream changes relevant to aberrant salience are reflected, GluA1 KO mice would be expected to show increased dopamine activity in the striatum. Baseline dopamine levels in GluA1 KO mice, however, are indistinguishable from controls (Wiedholz et al., 2008). Dopamine clearance in the nucleus accumbens on the other hand is slower in the nucleus accumbens (Wiedholz et al., 2008). However, this measurement was made in anaesthetized animals. This makes it impossible to investigate behavioural correlates of this change.

Metabotropic Glutamate Receptors

All mGluRs have 7 transmembrane segments that are linked to a large Venus fly trap domain containing the glutamate binding site via a cysteine rich domain. They share this structure with the $GABA_B$ receptor. And just like the $GABA_B$

receptor, mGluRs form dimers. These dimers can either be homodimers or heterodimers of mGluRs coupled with G-proteins (Nicoletti et al., 2011).

The 8 known metabotropic glutamate receptors (mGluRs) can be subdivided into groups based on their sequence homology. Group I receptors consist of mGluR1 and mGluR5, and are coupled to Gq/G11 which raises PLC levels and is therefore excitatory. Group II (mGluR2, 3) and group III (mGluR4, 6, 7, 8) receptors are both coupled to Gi/G0 that reduce adenyl cyclase activity and are therefore inhibitory (Niswender and Conn, 2010). Since this thesis is interested in the group II mGluRs the following discussion will concentrate on this subgroup.

Group II mGluRs are diffusively expressed throughout the brain. mGluR3 is located on the pre- and the postsynapse as well as in glial cells. mGluR2 is expressed in neurons, preferentially presynaptically in the preterminal zone, on the axon just outside the terminal bouton. They can therefore be activated by excess glutamate spilling out of the synapse (Scanziani et al., 1997). This enables them to act as an inhibitory feedback loop to decrease further glutamate release (i.e. they are inhibitory autoreceptors). This is illustrated by evidence that activating these receptors reduces glutamate levels (Battaglia, 1997 and Xi, 2002, Moghaddam and Adams 1998).

The literature investigating the influence of pharmacological interventions targeting group II mGluRs is complex. This is not surprising, as group II mGluRs modulate the major excitatory neurotransmitter glutamate. Glutamate would be expected to be involved in most behaviour. Furthermore, Riedel et al (1999)

showed that blocking the major ionotropic glutamate receptor subtype AMPAR in an area in effect shuts down the brain region in question. The effects of group II mGluR agonist and antagonists are not as profound since metabotropic receptors are modulating neural activity rather than driving it. Still, they affect a wide variety of behaviours (Swanson et al., 2005, Niswender and Conn, 2010, Nicoletti et al., 2011), discussion of which would go beyond the scope of this thesis. Therefore, only the effect of the group II mGluR agonist used in this thesis (LY354740) will be discussed in chapter 3.

The role of group II metabotropic glutamate receptors has also been examined using double knockout mice for both mGluR2 and mGluR3 receptors (Lyon et al., 2011). These mGluR2/3 double knockout mice were impaired on appetitively motivated spatial memory tasks, regardless of whether the task tested spatial reference or spatial working memory. However, mGluR2/3 double knockout mice acquired the Morris water maze, an aversively motivated spatial reference memory task at a rate indistinguishable from wildtype controls. Lyon et al. (2011) hypothesised that the memory deficit is absent in the aversively motivated task because higher arousal levels rescue performance in the double knockouts. To test this hypothesis, mice were exposed to injection stress to increase arousal before being tested in an appetitively motivated spatial working memory task, the rewarded alternation T-maze. This injection stress impaired alternation performance in wildtypes but increased performance in mGluR2/3 double knockout mice. This was taken to suggest an altered arousal–cognition relationship in mGluR2/3 double knockout mice and thereby a role of group II mGluR at the interface of cognition and arousal.

Glutamate and Schizophrenia

Even though the dopamine theory of schizophrenia is the most widely accepted framework, there is little agreement about what causes the changes in dopamine seen in schizophrenia patients in the first place. One potential causal factor could be a primary change in the glutamate system.

The idea that glutamate plays a role in schizophrenia developed from the finding that healthy subjects developed psychosis-like symptoms when given NMDA receptor antagonists like ketamine, MK-801, or phencyclidine (PCP) (Javitt and Zukin, 1991, Moghaddam and Javitt, 2012). Interestingly, the effect of ketamine seems to more closely mimic the spectrum of symptoms of schizophrenia than amphetamine, especially with respect to the negative and cognitive symptoms. This led to the idea that the effect of ketamine on the brain (i.e. NMDAR hypofunction) might more closely resemble the underlying pathology in schizophrenia patients.

It has been hypothesized that the behavioural effects of NMDA-receptor antagonists like Ketamine, MK-801, and PCP arise from their effect on inhibitory interneurons in the prefrontal cortex (Seamans, 2008). Interneurons are less polarized than pyramidal cells. Hence, they are more likely to have a membrane potential in a range allowing the NMDA-receptor to be active. Presumably, a higher percentage of them will therefore be active at any one time. Hence, blocking NMDA receptors on interneurons will have a bigger effect than in pyramidal cells. The inhibitory interneurons will get less excitatory input due to

the NMDA receptors being blocked by their antagonists. Thereby inhibition in the network is removed and neural processing in the network changed (Moghaddam et al., 1997). Additionally, PCP also increases striatal dopamine (Moghaddam & Adams, 1998), showing that the dopamine and glutamate accounts of schizophrenia could be complementary.

In recent years an additional set of intriguing evidence is beginning to emerge. Anti-NMDA encephalitis is an inflammation of the brain during which the body develops antibodies to the NMDA receptor (Dalmau et al., 2008). The first symptoms of this disorder show a remarkable similarity with schizophrenia. Additionally NMDAR antibodies have been found in some first episode schizophrenia patients (Zandi et al., 2011, Deakin et al., 2014). This suggests that NMDARs are linked to psychosis in a pathophysiological process in humans. Another stream of evidence comes from studies of post mortem brain tissue. NMDA receptor levels appear to be increased in the cortex and putamen of schizophrenia patients (Ishimaru et al., 1994, Aparicio-Legarza et al., 1998). However, in a recent review Hu et al. (2015) found no conclusive evidence for an up- or downregulation of glutamate receptors in the post mortem brains of schizophrenia patients.

NMDA receptors are not the only proteins associated with glutamate signalling that play a role in schizophrenia. This is best illustrated by the results of a recent genome-wide association study (GWAS). In GWAS, occurrences of mutation in single base pairs, single nucleotide polymorphisms (SNPs), are compared in patient populations and controls. Ripke et al. (2014) found that changes in the

gene coding for the D2 receptor and changes in genes involved in glutamatergic neurotransmission are associated with schizophrenia. Interestingly, SNPs around the coding region of three separate glutamate receptors were associated with schizophrenia: These were the NMDA receptor subunit GluN2A, the AMPA receptor subunit GluA1, and the group II metabotropic glutamate receptor mGluR3.This showed that the abnormality in glutamate neurotransmission in schizophrenia extends beyond the NMDA receptors and may provide clues as to the nature of the glutamate dysfunction, the psychological processes affected and the potential neurotransmitters involved.

Indeed, interest in the role of GluA1 in schizophrenia predates Ripke et al. (2014) or the generation of a GluA1 knockout mouse by Zamanillo et al. (1999). Post mortem studies found GluA1 mRNA and immunoreactivity to be reduced in the hippocampus of schizophrenia patients (Harrison et al., 1991, Eastwood et al., 1997). This raises the question of how these hippocampal changes could be brought together with the aberrant salience phenotype seen in GluA1 KO mice. A theory proposed by Lisman and Grace (2005) may explain how salience attribution and novelty might be controlled by the hippocampus. They propose hippocampal-striatal-basal ganglia loops that regulate attention paid to stimuli. In this framework, novelty is detected in the hippocampus which compares current sensory input to memories. These cells can then modulate dopaminergic function through a polysynaptic connection to the dopaminergic neurons in the VTA.

Group II metabotropic glutamate receptors have also attracted interest for their potential role in schizophrenia. SNPs in the gene coding for mGluR3 have been

associated with schizophrenia (Egan et al., 2004). However, recent investigations of mGluR3 levels in post mortem brains, found no change in mGluR3 immunoreactivity in schizophrenia (Garcia-Bea et al., 2016). The most extensive research on group II mGluRs has been conducted using drugs targeting this receptor group. These have been proposed as treatments for disorders as diverse as stroke, epilepsy, drug abuse, schizophrenia, depression, anxiety disorders, neurodegeneration, and pain disorders (Imre, 2007, Nicoletti et al., 2011). This thesis is interested in the interaction of dopamine and glutamate in schizophrenia. Therefore, the following discussion will be restricted to this disorder.

This thesis will use the group II mGluR selective agonist, (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) (Monn et al., 1997). Moghaddam and Adams (1998) found that LY354740 ameliorates deficits seen in rewarded alternation on a T-maze induced by the NMDA receptor antagonist PCP. This result suggested that LY354740 can reverse cognitive deficits induced by pharmacological glutamate manipulations relevant to schizophrenia. These encouraging findings led to clinical trials of group II mGluR agonists for schizophrenia. Results of these trials for the agonist LY2140023 (a prodrug of LY404039 (Rorick-Kehn et al., 2007a, Rorick-Kehn et al., 2007b)) initially showed promising results (Patil et al., 2007). More recently, however, LY2140023 did not show significant improvements compared to placebo controls in the primary outcome measure of a stage II clinical trial (Downing et al., 2014). However, exploratory analysis of this clinical trial showed clinical efficacy of the agonist in early disease states (Kinon et al., 2015). This suggests that more

preclinical research in the effect of group II mGluR agonists is necessary to understand their effect on schizophrenia-related behaviours.

Aims of this thesis

This thesis will investigate the role of these neurotransmitters, glutamate and dopamine, in schizophrenia-related behaviours using genetic and pharmacological interventions.

As described above, multiple experiments on the effect of glutamatergic manipulations on dopamine and behaviours relevant to schizophrenia have been conducted. The group II metabotropic receptor agonist LY354740, for example, has been shown to ameliorate PCP-induced hyperlocomotion and working memory deficits (Moghaddam and Adams, 1998), suggesting that LY354740 might ameliorate cognitive deficits seen in schizophrenia. The first aim of this thesis is to test whether this finding can be generalised to other manipulations reminiscent of the symptomatology seen in schizophrenia. For this purpose working memory and locomotor activity will be tested in amphetamine-injected rats (chapter 3) and GluA1 KO mice (chapter 5). LY35740 will be assessed to see if it ameliorates the behavioural phenotype seen with these experimental manipulations

Chapter 3 will assess the effect of LY354740 on the dopamine system by investigating its influence on amphetamine-induced behavioural changes. As its second aim this thesis will examine the effect of LY354740 on dopamine in the

nucleus accumbens directly (chapter 4). To do so a method called fast-scan cyclic voltammetry (FCV) will be used in LY354740 injected, behaving rats. This method enables me to assess phasic dopamine changes with high temporal resolution. Furthermore, neural activity in the nucleus accumbens will also be assessed using oxygen amperometry, a measure assessing tissue oxygen levels. Tissue oxygen levels are dependent on neural activity and are reminiscent of the physiological variable measured in fMRI (i.e. they provide a haemodynamic signal analogous to the BOLD signal).

Thirdly, this thesis will assess whether the GluA1 KO mouse shows a phenotype of aberrant dopamine and aberrant salience as defined by Kapur (2003). To do so, a test of short-term habituation is validated using an established psychotomimetic drug MK-801. This task is then applied in GluA1 KO mice while phasic dopamine is recorded (chapter 6). And finally, the role of any potential change in the dopamine system in GluA1 KO mice will be investigated in the context of Pavlovian conditioning (chapter 7). However, before these questions can be addressed an introduction to several methods used in this thesis is given in chapter 2.

Chapter 2: Methodological Introduction

This chapter will introduce two behavioural assays. The first one is rewarded alternation on a T-maze used to assess short-term memory. The second one is the locomotor activity test which measures activity levels in rodents. Finally, fast-scan cyclic voltammetry (FCV) will be introduced, an electrochemical method to measure phasic dopamine changes.

T-maze

This thesis will use rewarded alternation on a T-maze to assess short-term memory processes in rodents (Deacon and Rawlins, 2006). This task has also been described as a spatial working memory task (but see Sanderson et al. 2008) and as a win-shift memory task.

Equipment

The T-maze consisted of a start arm making up the base of the T (mice: 10 X 47cm, rats: 10 X 80 cm) and two identical goal arms with food wells (mice: 10 X 35cm, rats: 10 X 60 cm). The mouse maze had 20cm high walls, and the entire maze was painted black. This maze's goal arms could be blocked by doors that could be closed at the entrance to the goal arms. The rat maze was elevated 50 cm above the floor, had no walls and entrance to its goal arms could be prevented by a wooden block.

Acclimatisation

Animals were placed on a restricted diet limiting them to 85% - 90% of their free-feeding weight. Access to drinking water was not limited. Animals were placed on the T-maze in home cage groups for acclimatisation. The food wells in the goal arms were filled with condensed milk (the reward) for mice and 40mg sucrose pellets for rats. After several days of group acclimatisation the animals were placed on the maze individually. This was continued until all animals were moving freely and readily consumed food rewards at the end of the arms.

Testing

During rewarded alternation testing, each trial consisted of two runs; a sample and a choice run. In the sample run the animal was placed into the start arm facing the investigator, and forced (by blocking off one of the arms) to turn left or right into one of the goal arms to gain a food reward. After the animal consumed the reward it was removed from the maze and both arms were opened (i.e. the block/door was removed). After an intra-trial interval the animal was placed back in the start arm of the maze, facing the investigator, and allowed to make a free choice between the left and right goal arms. Animals were rewarded for entering the arm that they had not previously visited, *i.e.* for alternating. Therefore an animal that is preferentially entering the novel arm in the choice run displays a memory of previously having been in the other arm during the sample run. 50% correct entries constitute chance performance.

In the sample runs, animals were forced left or right in a pseudorandom manner such that there was an equal number of left and right sample runs overall, and the animal was not forced in the same direction on more than two trials consecutively. During the sample run the time taken from the animal being released to the animal sampling the reward was measured (sample latency). In the choice run the time taken for the animal to choose an arm to enter (defined as all four paws entering into an arm) was also measured (choice latency). An animal was given 120s to complete a run. If the run was not completed in this time, the trial was discarded.

Locomotor Activity Test

In order to screen compounds for an antipsychotic effect locomotor activity tests are widely used (Pratt et al., 2012). Psychomimetic drugs like amphetamine or MK-801 induce psychosis-like symptoms in humans are known also to induce hyperactivity. Therefore, a compound's ability to ameliorate drug-induced hyperactivity is seen as at least preliminary evidence for antipsychotic actions.

If not otherwise specified spontaneous locomotor activity was tested in clear plastic cages (26cm x 16cm x 17cm), containing clean sawdust. Activity levels were assessed by an array of infrared photobeam sensors that covered the x and y axes of the cages (San Diego Instruments). Animals were first habituated to the novel environment, then dosed with the compound of interest, and directly placed back into the cages for a test phase. Experiments were conducted in a light room.

Fast-Scan Cyclic Voltammetry

So far we have been discussing how to measure behaviours. But this thesis is interested in linking psychological to biological processes. Since both schizophrenia and salience attribution are known to involve dopamine (Kapur, 2003, Berridge, 2007), a method for measuring this neurotransmitter as directly as possible is needed.

Salience is tied to a given stimulus. We need to therefore be able to measure stimulus-evoked dopamine release in an event related manner. Classical methods with a low temporal resolution like microdialysis (Wang et al., 2008) may not capture the underlying biological change. However, fast-scan cyclic voltammetry (FCV) allows detecting extracellular dopamine with a high temporal and spatial resolution.

Chemistry

Voltammetry is an electrochemical method based on the property of certain molecules in solution to lose or gain electrons; that is to oxidize or reduce at certain applied potentials which are specific to the given molecule.

At least two electrodes, a working and a reference electrode, are placed into a solution or tissue containing the analyte and a voltage is applied between the two electrodes. Voltammetry is the study of how manipulating this voltage changes

the current arising from the oxidization and reduction of the electrolyte (Clark et al., 2002, Wightman, 2006, Michael and Borland, 2007).

In this thesis the potential between a carbon fibre working electrode and an Ag/AgCl reference electrode is held at a negative potential (-0.4V), which is too low to oxidize dopamine. Once every 0.1s a triangular waveform rising linearly from -0.4V to 1.3V and returning back to -0.4 is applied to the working electrode with a speed of 400V/s. When the voltage between working and reference electrode is moving towards 1.3V, the positive working electrode oxidizes dopamine into dopamine-*o*-quinone around its surface and thereby creates an electrochemical current that peaks at ~+0.6V. On the falling slope of the triangular waveform dopamine-*o*-quinone is reduced back to dopamine (peak reaction at -0.2V). The kinetics of the oxidation and reduction, combined with the diffusion flux (dopamine diffusion to the electrode) lead to an applied voltage to current relationship that is unique to catecholamines (the "cyclic voltammogram"). However, as all the work in this thesis involves recordings made in the nucleus accumbens (NAc) core where there is a large dopamine projection but little if any noradrenaline, this cyclic voltammogram can be considered a signature of dopamine. Studies of TH-knockout mice (where noradrenaline is reintroduced) but which lack dopamine have shown that local electrical stimulation of neurotransmitter release in the NAc core produces no changes in current, consistent with this idea of a dopamine-mediated response (Hnasko et al., 2006). The peak amplitude of the current is linearly proportional to the dopamine concentration around the working electrode.

However, this faradaic current is not the only current measured. There is also simple charge transfer between the two electrodes, called the capacitive current. This capacitive current and additional sources of noise mask the faradaic current dopamine produces. Therefore, a waveform recorded before the event that triggered the dopamine release, typically an average of ten waveforms centred 0.5s before the trigger event, has to be subtracted from waveforms containing a potential dopamine signal. Due to this baselining, FCV can only measure relative changes of dopamine in response to an experimental manipulation.

Electrode Construction

Working electrodes were constructed according to Clark et al. (2010). A 7µm carbon fibre (Goodfellow; Grade 34-700, C 005722, 7µm) was placed in a 8mm long polyamide-coated, fused silica capillary (outer diameter: 90µm; inner diameter 20µm; polyimide coating 12µm). On one end the capillary was sealed with Devcon two-component epoxy leaving 150-200 µm of the carbon fibre exposed. On the other end of the capillary a silver pin was attached to the carbon fibre with silver epoxy ensuring electrical conductivity.

The Ag/AgCl reference electrode was constructed out of a coated silver wire. After the coating was removed the wire was attached to a gold pin (Mill-Max, USA) using silver epoxy and an insulating layer of non-conductive two-component epoxy leaving ~0.2cm of wire exposed. This wire was then bathed in sodium hypochlorite solution for 36-48 hours and the emerging crystals removed.

Implantation

Electrode implantation was performed as described in Syed et al. (2016). Animals were anesthetized using isoflurane (Vet-tech Solutions, Cheshire, UK) (induction 3% in O2, 1-1.5% during surgery). Once anesthetized glucosaline, meloxicam (Metacam, Boehringer Ingelheim; 5mg/kg) and buprenorphine (0.08mg/kg, Vertergesic, Alstoe Animal Health, UK) were given subcutaneously and the scalp shaved. After the animal was placed into a stereotactic frame (Kopf Instruments, Model Clark Electromedical, Reading, UK), the scalp was cleaned with hibiscrub, Bupivacain was applied locally (2mg/kg, Marcain), and the skull was exposed with an incision along the midline. A dental drill was used to drill holes for stainless steel anchoring screws (M1.0 X 2 slot machine screws), and the working electrode(s) and reference electrode, after which animals were implanted with carbon fibre electrodes unilaterally or bilaterally into the nucleus accumbens core (rats: AP: +1.4, ML: ± 1.3, DV: −6.8 mice: AP: +1.4, ML: ± 1.0, DV: −3.8). The reference electrode was implanted into the right hemisphere anterior to lambda. To attach these electrodes to the skull Kemdent Simplex Rapid dental cement (Associated Dental Products Ltd) was applied. The pins of the electrodes were connected to a 2-pin or 3-pin (the latter when two working electrodes were implanted) plastic pedestal (Plastics One, Roanoke, VA, USA) for mice and a 6-pin headstage connector for rats. The Connector was secured in place using dental cement, resulting in a headcap that covered the top of the skull. For rats, an additional headpost was added (the same type of plastic pedestal used for the mouse headstage connector) to help stabilise the headstage during recording.

After surgery animals received 0.9% saline to replace fluid lost during surgery. Buprenorphine and meloxicam were given to provide analgesia after surgery. Animals were monitored daily for seven days and saline and analgesic were administered for at least two days after surgery and then subsequently when deemed appropriate. After surgery all animals were housed singly. Food restriction started at least two weeks after surgery and FCV recording commenced at least four weeks after surgery.

Validation

As previously reported (Gan et al., 2010), dopamine's faradaic current can be distinguished from other molecules based on its waveform with the exception of noradrenaline which is only present at low levels in the nucleus accumbens core. To validate whether dopamine can be detected for a given recording session, voltammograms were baselined and filtered (with a 2 kHz 4^{th} order Butterworth low-pass filter) using Tarheel software. The voltammogram was then linearly regressed against a template voltammogram known to reflect dopamine oxidation and reduction, acquired by electrical stimulation of the dopamine midbrain and adjacent medial forebrain bundle. This tract includes the dopaminergic input from the ventral tegmental area to the nucleus accumbens. Therefore, electrically stimulating this tract induces dopamine release in the NAc. If this standard explains 75% or more of the variance of the extracted voltammogram ($R^2 \geq 0.75$), the tested session was included into the analysis. The dopamine cyclic voltammogram exhibits a lower correlation coefficient with cyclic voltammograms from almost all other analytes (Heien et al., 2003)

Analysis

So far we have discussed that the size of the oxidation peak of dopamine linearly increases with the dopamine concentration around the working electrode. We are only interested in dopamine. So, theoretically, in a flow cell for instance, we could simply measure the peak height of each waveform and thereby determine the dopamine concentration at a given time point. However, *in vivo* data are more complex and cannot be captured with such a univariate method as there are many different analytes that can cause faradaic changes in recorded current, as well as non-chemical noise. The most well-characterised nuisance analyte when performing *in vivo* FCV in the striatum comes from changes in pH value related to neural activity. Therefore a multivariate technique to extract the dopamine signal from the raw data is needed (Heien et al., 2004, Keithley et al., 2009).

To do so, first, different magnitudes of dopamine and pH changes were elicited by applying different intensities of electrical stimulation to the medial forebrain bundle. From these data, a set of (assumed) ideal dopamine and pH waveforms were extracted using principle component regression (Kramer., 1998, Keithley et al., 2009), from which a model can be constructed of expected signal given predicted changes in dopamine and pH. This model not only predicts the dopamine and pH content of the waveform but also divides the variance of the training data into two parts. One part of the variance is explained by the model, the rest is the error term.

This enables me to accomplish two tasks:

- to determine the change in current attributable to changes in dopamine levels.
- to decide whether the model can be applied to the data. If the model explains enough variance it is said to explain the data and therefore to predict accurately the dopamine content of the signal. This is achieved by investigating the squares of the residuals between the model and the data, just as one would for a simple linear regression model. However, since the residuals are not normally distributed, some adaptations have to be made according to Jackson and Mudholkar (1979) and Jensen and Solomon (1972).

If the model does not sufficiently account for the variance in the recorded signal, the model is rejected at that time point meaning that the dopamine current predicted by the model cannot be used. Finally a 0.5s moving average is applied to the data over time for smoothing.

Data Processing Software and Statistics

FCV data were recorded and analysed using software written in Labview (National Instruments). Parts of the data pre-processing and organisation were programmed in custom-written Matlab (Mathworks) scrips and in Visual Basic for Applications (VBA) in an Excel Environment (Microsoft).

All statistical analyses in this thesis were conducted using IBM SPSS Statistics 20 (IBM Corp). Statistical significance was defined as $\alpha = 0.05$. Multiple comparisons were corrected where necessary using Sidak-test. The test statistics (t-value) for each pairwise comparison is reported uncorrected. Only the p-value is reported in its corrected form.

Histology

At the end of recording animals were deeply anesthetized with pentobarbital (200mg/kg), microlesions were made at the working electrode locations via current stimulation, and the animal was transcardially perfused with saline followed by 10% formalin solution. Brains were sectioned in 40 µm coronal slices using a cryostat and were stained with Cresyl violet enabling the identification of the electrode location.

Animal Licenses

All procedures reported in this thesis were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and associated Home Office guidelines.

Chapter 3: The Effect of LY354740 on Amphetamine-induced Behaviours

Introduction

As described in the general introduction (chapter 1), group II mGluRs have been investigated since the late 1990's as a potential target for schizophrenia (Monn et al., 1997, Schoepp et al., 1997) due to their role in glutamate and dopamine modulation and agonist have been used in clinical trials (Patil et al., 2007).

The Group II mGluR agonist LY354740

The first compound published as part of a research program conducted by Eli Lilly was LY354740. It is a highly selective agonist of group II receptors which mainly acts on inhibitory autoreceptors to reduce glutamate release. LY354740 binds widely throughout the brain, but binds particularly strongly in the neocortex and hippocampal formation (Higgins et al., 2004).

At a functional level, LY354740 reduces glutamate release induced by the NMDA receptor antagonist PCP (Moghaddam and Adams, 1998) and the voltage-gated Na^+ channel opener veratridine (Battaglia et al., 1997), without affecting baseline glutamate levels. However, extracellular glutamate levels are notoriously hard to evaluate since glutamate is both a neurotransmitter and an important part of cellular metabolism.

Data on the effects of LY354740 on dopamine levels are more reliable, and tell a similar story. For example, the drug does not affect baseline dopamine levels as measured by microdialysis, but reduces PCP and amphetamine-induced dopamine release in rodents although the effect on PCP was no statistically significant (Moghaddam & Adams, 1998; Pehrson & Moghaddam, 2010). This effect on dopamine is especially interesting considering that the result of direct manipulation of dopamine neurons via VTA stimulation or by L-DOPA injection is not changed by LY354740 (Pehrson and Moghaddam, 2010). This suggests that LY3547470 does not directly affect dopaminergic neurons but rather changes the activity of neurons upstream of the VTA.

At a behavioural level, LY354740 has been found to act as an anxiolytic in a standard test of anxiety, the elevated plus maze (Monn et al., 1997, Helton et al., 1998, Linden et al., 2005a, Linden et al., 2005b, Schlumberger et al., 2009). This test exploits the conflict between a rodent's innate preference to stay in the enclosed sections and its tendency to explore the environment. Two of the four arms in a plus-shaped maze are surrounded by walls. The two remaining arms are open, constituting a potentially more dangerous area for a rodent since it is easily visible and accessible to potential predators. The rodent is placed in the more secure closed arm and is given time to explore the open and closed arms of the maze. The less anxious a rodent is the more it will explore the open arms. LY354740 increased this exploration.

Further evidence consistent an anxiolytic effect of LY354740 comes from electrophysiological measurements in the hippocampus. Gray (1982 and 2000)

discusses how a reduction in theta oscillations in the hippocampus may be used as a biomarker for an anxiolytic effect of a compound. LY354740 induced such a reduction in hippocampal theta oscillations (Siok et al., 2012).

However, as discussed in the introduction interest in this class of drugs is mostly derived from their ability to ameliorate PCP-induced working memory deficits in rats, as measured by rewarded alternation on the T-maze (Moghaddam and Adams, 1998, Blot et al., 2015). This effect in a potential schizophrenia model was later reproduced in humans using ketamine instead of PCP (Krystal et al., 2005). However, even though Moghaddam & Adams (1998) reported to use a dose of 10mg/kg LY354740 that does not by itself induce memory deficits when given alone, later studies found working memory deficits in rats treated at the same dose of LY354740 (Aultman and Moghaddam, 2001, Higgins et al., 2004, Schlumberger et al., 2009). This suggests that LY354740 has a bidirectional effect. The agonist impairs memory on its own, but ameliorates the effect of NMDA-antagonists.

LY354750 has been shown to ameliorate hyperactivity in a locomotor activity test induced by drugs used to model aspects of schizophrenia, like PCP (Moghaddam and Adams, 1998, Cartmell et al., 1999) and amphetamine (Pehrson and Moghaddam, 2010). However, LY354740 fails to ameliorate amphetamine-induced hyperactivity in the studies of Cartmell et al. (1999) and Schlumberger et al. (2009). Still, both show a numerical reduction of activity even if they fail to reach statistical significance, which might indicate that the negative results may be due to a lack of power. This seems especially likely, since

another group II mGluR agonist LY379268 has been shown to reduce amphetamine-induced locomotor activity in the same study (Cartmell et al., 1999).

Investigating the effect of LY354740 on baseline locomotor activity is problematic, however. Since most studies include a 60 minute pre-injection period during which the animal habituates to the environment, baseline activity levels are often quite low by the time the drug is injected. Hence, due to a potential floor effect only some studies find a reduction of basal locomotor activity by LY354740 (Klodzinska et al., 1999, Higgins et al., 2004). Other studies fail to see such effects (Imre et al., 2006, Schlumberger et al., 2009).

The Effect of Amphetamine on Dopamine and Activity Levels

Amphetamine (AMPH) is a widely used stimulant which manipulates a large range of behaviours through its influence on monoaminergic neurotransmitters such as dopamine, noradrenaline and serotonin. Monoamines are cleared from the synapse by being transported into the presynaptic terminal via monoamine reuptake transporters. Structural similarities of amphetamine with monoamines make it a competitive substrate for these transporters i.e. the transporters move amphetamine into the neuron as they would normally transport monoamines (Heal et al., 2013). Once inside the cell, amphetamine blocks uptake of cytosolic monoamines into the vesicle due to its affinity for the protein responsible for this process VMAT2 (Teng et al., 1998). This leads to an increase of the presynaptic cytosolic monoamine levels. The outcome of this is a reversal of the activity of

the monoamine reuptake transporters. Instead of clearing the synapse they now release monoamines into the synapse, thereby increasing extracellular synaptic dopamine concentrations (Robertson et al., 2009).

Amphetamine exists as two enantiomeric mirror images, d-amphetamine and l-amphetamine, with d-amphetamine releasing approximately 4 times more dopamine at the same dose than l-amphetamine (Heikkila et al. (1975). Since this thesis is interested in the role of dopamine in brain function, d-amphetamine will be used in these experiments.

So far the effect of amphetamine has been discussed at a molecular level. However, a drug like amphetamine directly manipulates only a small subset of synapses. The effect of this manipulation depends on the state the rest of the system is in. These non-pharmacological factors can be summarized by the terms “set” and “setting”, describing the internal state (set) and the external physical environment (setting) of the subject, respectively, when the psychoactive substance is given (Zinberg, 1984). In rats, for example, it has been shown that brain areas activated by amphetamine can be modulated by the environmental context (Badiani et al., 1998). When amphetamine was given in the home cage or in a novel environment, the increase in extracellular dopamine was similar in both groups as measured by microdialysis. However, the neuronal activation of the striatum and especially the nucleus accumbens was higher in the novel environment as measured by c-Fos staining. As an example of a potential role of set (i.e. the internal state of the animal) in the generation of behavioural effects of amphetamine, the example of food restriction is especially

interesting. Stuber et al. (2002) found an increase of the amphetamine effect on locomotor activity in food restricted rats without an increase of dopamine release in the ventral striatum. These findings suggest that the effect of amphetamine on the organism can differ depending on the set and setting.

D-amphetamine is known to induce hyperactivity (Cartmell et al., 1999). This effect is mediated at least partly by d-amphetamine's effect on the dopamine system (Rolinski and Scheel-Krüger, 1973, Koob et al., 1981). However, at higher doses amphetamine-induced hyperactivity can be reduced due to the occurrence of stereotypy, including repetitive movements such as sniffing, licking, or gnawing (Segal, 1975, McNamara et al., 2006). These two aspects are mediated by different parts of the striatum. Amphetamine-induced hyperactivity is dependent on the nucleus accumbens, whereas stereotypy is dependent on the dorsal striatum (Kelly et al., 1975).

Manipulations of the dopamine system, like systemic amphetamine injection, also influence working memory. Dopamine levels in the prefrontal cortex of primates have been shown to display an inverted-U shape relationship to working memory performance levels in delayed responding tasks (Castner et al., 2004). Small increases in dopamine levels increase working memory performance whereas larger increases worsen performance. The effect of amphetamine on working memory tests like rewarded alternation on a T-maze in rodents shows a similar dose-response pattern (Shoblock et al., 2003). Low doses increase performance and high doses impair performance. However,

when high doses of amphetamine are given, only reductions in performance are seen (Aultman and Moghaddam, 2001).

Aims

This chapter will investigate the interaction of LY354740 with amphetamine. LY354740 has been shown to successfully counteract the effects of PCP (Moghaddam & Adams, 1998) in a rewarded alternation T-maze task and ameliorate PCP-induced hyperactivity. Group II mGluR agonists have also been shown to attenuate amphetamine-induced locomotor activity (Cartmell et al., 2000). This chapter will first investigate the effect of LY354740 on rewarded alternation on a T-maze followed by the interaction of amphetamine and LY354740 in this task. Specifically, I will investigate whether LY354740 can ameliorate the amphetamine-induced deficit in T-maze rewarded alternation in the same way as it can ameliorate the PCP-induced T-maze rewarded alternation deficit. This is important in the context of group II mGluR agonist being putative antipsychotics. As putative antipsychotics they would be expected to rescue performance deficits induced via a drug relevant to a primary dopaminergic dysfunction. It has been argued that glutamate might underlie the dopaminergic dysfunction in schizophrenia, but Ripke et al. (2014) have shown how variable the genetic background of schizophrenia is. A putative antipsychotic like LY354740 would be expected to ideally be effective independent of the underlying causes. Finally, LY354740 is expected to show effectivity in multiple assays relevant to schizophrenia. Therefore, I will test the ability of LY354740 to ameliorate amphetamine-induced hyperactivity.

Experiments

Experiment 3a: The Group II metabotropic Receptor Agonist LY354740 Impaired Spatial Short-Term/Working Memory

Introduction

Aultman & Moghaddam (2001) showed that 10mg/kg LY354740 impaired spatial working memory in a T-maze rewarded alternation task. This chapter is interested in the interaction between LY354740 and d-amphetamine (AMPH). To understand the interaction of these two drugs it is helpful to know first their effects when given separately. Therefore, the LY354740 rewarded alternation experiment by Aultman & Moghaddam (2001) was first repeated.

Aultman & Moghaddam (2001) tested two doses of LY354740 (1mg/kg and 10mg/kg) at three intra-trial delays (minimal, 10s, and 40s). They did not find an impairment of alternation compared to the controls at a minimal delay, but found an impairment by 10mg/kg LY354740 at a 10s delay, and an impairment by 1mg/kg and 10mg/kg at a delay of 40s. In the following study the same drug dosing regimens were used, but only a minimal or 40s delay was imposed between sample and choice runs.

Methods

A rewarded alternation T-maze task with a within-subjects design, using 28 male Sprague Dawley rats (Harlan Laboratories) was conducted as described in

Chapter 2. This cohort and all the following cohorts were housed in groups of four. Rats were injected intraperitoneally (i.p.) at a dose of 1mg/kg or 10mg/kg, 45-50 min before behavioural testing. Two control conditions consisted of a no injection (no inj) control and a distilled water (veh) injected condition (1ml/kg).

Each session of behavioural testing consisted of 20 trials of rewarded alternation with an intertrial interval of 20s. A minimal and a longer intratrial delay were imposed between sample and choice runs. During the minimal intratrial delay the rat was placed on the maze as soon as possible. During the longer intratrial delay the rat was placed in a holding cage for 40s reproducing Aultman & Moghaddam's (2001) experimental design. Rats received equal numbers of trials with each delay in each session.

Alternation levels were determined by calculating the percentage of trials in which the animal alternated for each delay (minimal vs 40s). Latencies for each rat were calculated by taking the median time to complete the sample and the choice run respectively for each delay (minimal, 40s). Drug-injected animals were often highly lethargic on the first trial, needing encouragement by gentle handling to move down the maze. On subsequent trials, their latencies greatly reduced, resulting in a heavily skewed distribution. Since no transformation could correct this skewness the median was chosen over the mean as a measure of the latency of a single animal in this and the following experiment.

LY354740 (LY) was formulated at a concentration of 10 mg/ml and dissolved in distilled water. A 5 molar solution of NaOH was added dropwise to help the

compound dissolve and to give a solution of pH 7. From this, a diluted 1mg/ml solution was prepared. LY354740 was administered intraperitoneally (i.p.) at a volume of 1ml/kg.

Results

A 10mg/kg dose of systemic LY354740 decreased rewarded alternation performance (Fig. 3.1) and increased latencies in both the sample and choice runs. Furthermore, introducing a delay of 40s between the sample and choice run decreased alternation performance (% choice accuracy) and decreased latencies in the choice run in all conditions.

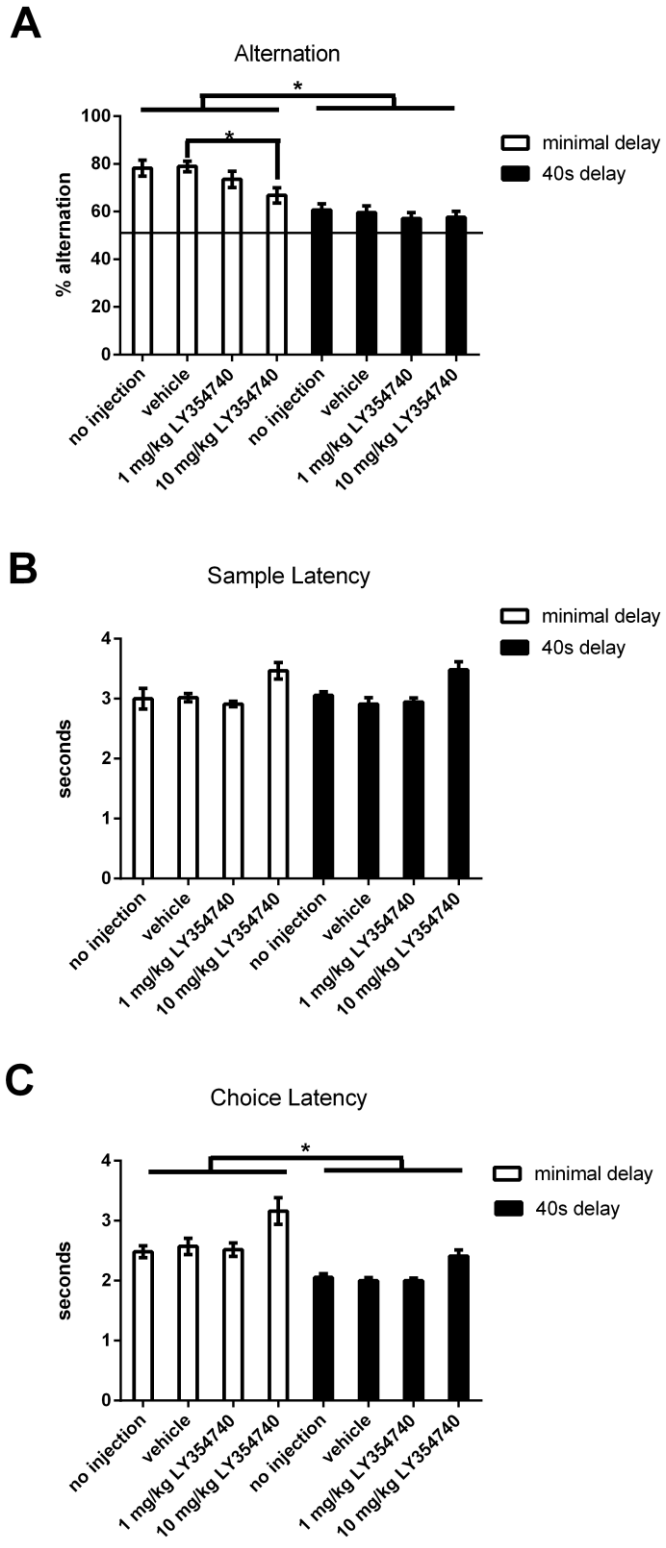


Figure 3.1: Test of the effect of LY354740 (1mg/kg & 10mg/kg) on rewarded alternation with a no injection and a vehicle control. (A) Alternation performance, the horizontal line denotes alternation at chance level (50% correct). (B) Latency to collect reward during the sample run. (C) Latency to enter the goal arm during the choice run (n=28). Data points represent mean ± SEM. * p < .05

Choice Accuracy

The higher dose of LY354740 produced a mild impairment in rewarded alternation performance. A repeated-measures ANOVA with drug (no injection, vehicle (veh), 1mg/kg and 10mg/kg LY354740) and delay (0s, 40s) as within-subjects factors revealed a significant effect of drug ($F(3,81) = 2.987$, $p = .036$) and delay ($F(1,27) = 71.665$, $p<.001$) on alternation performance (Fig. 3.1A). There was no significant drug*delay interaction ($F(3,81) = 1.696$, $p = .174$). Furthermore, investigation of the significant main effect of drug using post-hoc comparisons of the drug groups showed that none of the individual pairwise comparisons were significant despite the significant overall main effect of drug condition.

Visual inspection of the data revealed that alternation performance at the 40s delay was not influenced by LY354740, likely due to a floor effect (i.e. the poor performance of the controls; all conditions <60%). Therefore, the alternation scores at minimal delays were analysed separately and this also revealed a significant effect of drug ($F(3,81) = 4.095$, $p = .009$).

Post-hoc tests on the main effect of drug at minimal delay, with Sidak correction for multiple comparisons, revealed that the 10mg/kg treated rats alternated significantly less than the vehicle injected group ($t(27) = 3.559$, $p = .009$), and the comparison with the no injection control group approached significance ($t(27) = 2.780$, $p = .060$). The 1mg/kg injected group, on the other hand, did not differ

from either control group (no injection – 1mg/kg: t(27) = 1.314, p = .731; veh – 1mg/kg: t(27) = 1.385, p = .690)).

Sample Run Latencies

The higher dose of LY354740 led to animals running more slowly on the maze during the sample run. A repeated-measures ANOVA with drug and delay as within-subjects factors revealed a significant effect of drug ($F(3,81) = 9.413$, $p<.001$) but not delay($F(1,27) = 0.000$, $p> .999$) on latencies in the sample run (Fig. 3.1B). The lack of an effect of delay is not surprising since the training run is taking place before the delay is imposed. There was no significant drug*delay interaction ($F(3,81) = 0.682$, $p = .566$).

Post-hoc tests on the effect of drug with Sidak correction for multiple comparisons revealed that treatment with 10mg/kg slowed the animals compared to all three other conditions, which had no significant differences between them (noInj-10mg/kg: t(27) = 4.502, p = .001; Veh-10mg/kg: t(27) = 3.652, p = .007; 1mg/kg-10mg/kg: t(27) = 4.040, p = .002).

Choice Run Latencies

As during the sample, choice run latencies were increased by the higher dose of LY354740. A repeated-measures ANOVA with drug and delay as within-subjects factors revealed a significant effect of drug ($F(3,81) = 10.297$ $p<.001$) and delay ($F(1,27) = 50.023$ $p<.001$) on latencies in the choice run (Fig. 3.1C). The main

effect of delay reflected the fact that animals completed their choice runs faster after the 40 sec delay. There was no significant drug*delay interaction ($F(3,81) = 1.224$ $p = .306$).

Post-hoc tests on the effect of drug, with Sidak correction for multiple comparisons, revealed that treatment with 10mg/kg LY354740 again slowed the animals compared to all other conditions, which had no significant differences between them (noInj-10mg/kg: $t(27) = 4.346$, $p = .001$; Veh-10mg/kg: $t(27) = 3.384$, $p = .013$; 1mg/kg-10mg/kg: $t(27) = 3.237$, $p = .019$).

Discussion

LY354740 impaired rewarded alternation on the T-maze, replicating Aultman & Moghaddam (2001). In the current experiment, 10mg/kg LY354740 impaired alternation performance at a minimal delay whereas Aultman & Moghaddam (2001) only saw an effect at 10s and 40s delay. Though different at face value, inspection of the alternation performance of the vehicle control groups suggests that performance at the 10s delay in Aultman & Moghaddam (2001) (~80%) is quite well matched to the alternation performance at minimal delay (78.93%) in this experiment. Thus, when the controls are alternating at approximately 80% correct choices, then LY354740 produces a consistent impairment in short-term memory performance.

Additionally, this experiment showed an increase in latencies in the 10mg/kg but not the 1mg/kg condition. Together with the alternation performance results, this

suggests that a dose of 10mg/kg LY354740 produced a robust and reliable phenotype. Therefore a dose of 10mg/kg was used in the following experiments.

Experiment 3b: LY354740 Exacerbated a D-Amphetamine-Induced Increase in Latencies in the Rewarded Alternation T-Maze Task

Introduction

Aultman & Moghaddam (2001) showed that 2.5mg/kg amphetamine impairs rewarded alternation on a T-maze similarly to PCP in Moghaddam & Adams (1998). The following experiment investigated whether the dose of 10mg/kg LY354740 that ameliorated the effect of PCP (Moghaddam and Adams, 1998) on rewarded alternation can also rescue the impairment induced by amphetamine (AMPH).

Methods

The same cohort of rats tested on the T-maze in experiment 3a was used in this rewarded alternation experiment with amphetamine. Rats received two consecutive i.p. injections 45-50 min before behavioural testing. They received either veh/veh, veh/AMPH (2.5mg/kg), or LY354740 (10mg/kg)/AMPH (2.5mg/kg) in a within-subjects design. Due to the low alternation levels with the 40s delay condition in experiment 3a, the following rewarded alternation T-maze experiment was conducted with only a minimal intratrial delay. One rat had to be excluded because it failed to run on the maze in the LY354740/AMPH condition.

LY354740 was prepared as described in experiment 3a. D-amphetamine was formulated at a concentration of 2.5 mg/ml (salt weight) and dissolved in distilled water to give a solution of pH 7.

Results

Somewhat surprisingly given previous findings of Aultman and Moghaddam (2001), systemic amphetamine (2.5mg/kg) alone had no significant effect on choice accuracy during rewarded alternation (Fig. 3.2), but increased latencies in both the sample and choice runs. Combined LY354740 (10mg/kg)/AMPH treatment reduced alternation and increased latencies in both runs compared to the vehicle controls.

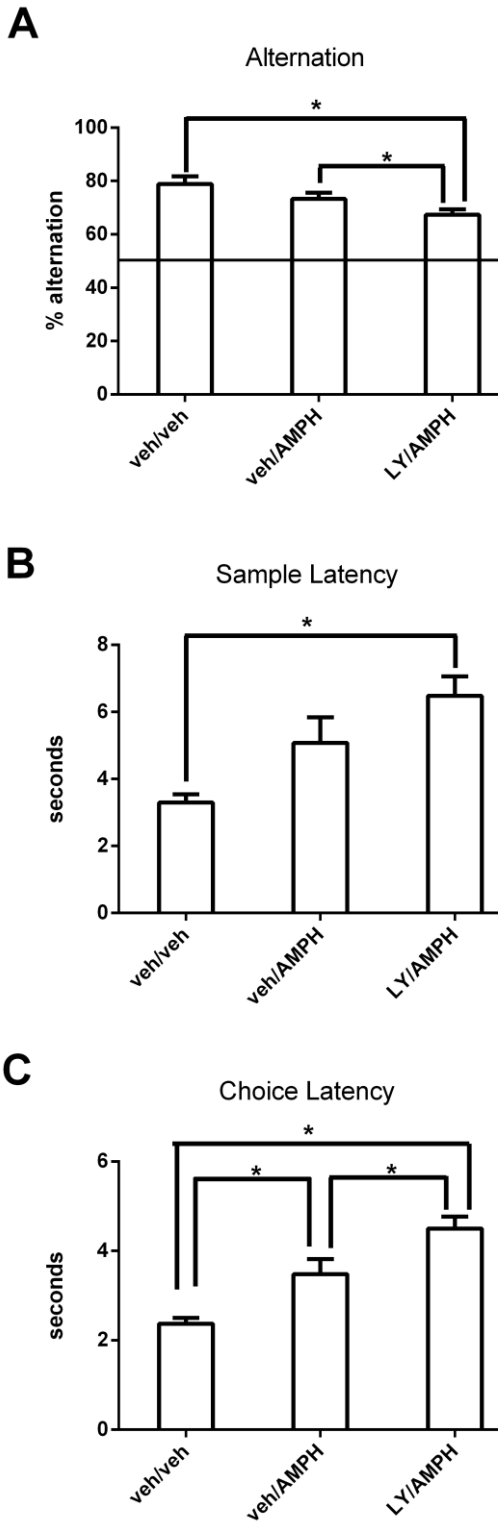


Figure 3.2: Test of a combination design of LY354740 (10mg/kg) and amphetamine (2.5mg/kg) on a rewarded alternation T-maze task. (A) Alternation performance (% correct choices), the horizontal line denotes alternation at chance levels (at 50%). (B) Latency to collect reward during the sample run. (C) Latency to enter the goal arm during the choice run (n=27). Data points represent mean ± SEM. * p < .05

Alternation

An ANOVA with drug condition as a within-subjects factor revealed a main effect of drug on alternation performance ($F(2,52) = 8.140$, $p = .001$) (Fig. 3.2A). Post-hoc tests on the effect of drug with Sidak correction for multiple comparisons revealed that the combined LY354740-amphetamine condition was significantly impaired compared to both remaining conditions (veh/veh - LY35740/AMPH: $t(26) = 4.039$, $p = .001$; veh/AMPH - LY354740/AMPH: $t(26) = 2.842$, $p = .027$), which didn't differ from each other (veh/veh - veh/AMPH: $t(26) = 1.629$, $p = .308$).

Sample Run Latencies

An ANOVA with drug as a within-subjects factor revealed a main effect of drug on latencies in the sample run ($F(2,52) = 8.830$ $p < .001$) (Fig. 3.2B). Post-hoc tests on the effect of drug with Sidak correction for multiple comparisons revealed that rats in the LY354740/AMPH condition were slower than in the veh/veh control condition. The amphetamine on its own had a tendency to increase latencies in the sample run compared to the vehicle control but this didn't quite reach significance (veh/veh - LY354740/AMPH: $t(26) = 5.273$, $p < .001$; veh/AMPH - LY354740/AMPH: $t(26) = 1.536$, $p = .357$; veh/veh - veh/AMPH: $t(26) = 2.450$, $p = .063$).

Choice Run Latencies

A repeated-measures ANOVA with drug as a within-subjects factor revealed a significant effect on latencies in the choice run ($F(2,52) = 21.172$ $p<0.001$)(Fig. 3.2C). Post-hoc tests on the effect of drug with Sidak correction for multiple comparisons revealed that all three conditions were significantly different from each other (veh/veh - LY354740/AMPH: $t(26) = 7.423$, $p <.001$; veh/AMPH - LY354740/AMPH: $t(26) = 3.005$, $p =.017$; veh/veh - veh/AMPH: $t(26) = 3.153$, $p = .012$). Amphetamine on its own increased latencies compared to the vehicle condition. Amphetamine and LY354740 further increased latencies compared to amphetamine alone. Thus, LY354740 exacerbated the effect of amphetamine on running latencies.

Discussion

Surprisingly, this experiment did not find that amphetamine impaired working memory performance on the T-maze. However, the combination of LY354740/AMPH was found to impair alternation performance. This deficit was similar to the deficit seen when LY354740 was given in experiment 3a. Amphetamine by itself and the combination of LY354740/AMPH, both increased latencies on the T-maze with the LY354740/AMPH condition being significantly slower than amphetamine alone in the choice run. These data suggest that LY354740 did not rescue the amphetamine phenotype. In fact, if anything, LY354740 potentially exacerbated or compounded the amphetamine effect as could be seen in the choice run latencies.

Experiment 3c: LY354740 Failed to Ameliorate D-Amphetamine-Induced Hyperactivity in Food Restricted Rats

Introduction

Experiment 3b gave initial indications of a counter-intuitive potential exacerbation of the amphetamine effect by LY354740 at least in terms of running latencies on the T-maze. To test this potential exacerbation further, the effect of LY354740 on amphetamine-induced hyperactivity was investigated further. Previously, Cartmell et al. (1999) found a reduction of amphetamine-induced hyperactivity at an amphetamine dose of 3mg/kg by LY379268, a closely related group II mGluR agonist, structurally similar to LY354740, and a numerical amelioration of amphetamine-induced hyperactivity by LY354740 itself that just failed to reach significance. Therefore, based on the literature, a reduction of amphetamine-induced locomotor hyperactivity was expected in these animals.

Methods

To test the effect of LY354740 on baseline activity and its interactions with AMPH, a locomotor activity experiment in a novel home cage-like environment was performed by measuring beam breaks (See chapter 2c) in the same food restricted cohort previously used in experiments 3a and 3b.

In order to habituate the rats to the novel environment, activity was measured for one hour before injection. Every animal then received two i.p. injections, which consisted either of (i) veh/veh, (ii) veh/LY354740 (10mg/kg), (iii) veh/AMPH

(2.5mg/kg), or (iv) LY354740 (10mg/kg)/AMPH (2.5mg/kg). Drugs were prepared as described in experiments 3a and 3b. These injections were performed almost simultaneously rather than with the 30min interval as reported in the literature (Cartmell et al., 1999) in order to resemble the drug dosing schedule used in the T-maze experiments (Experiments 3a+b). Immediately after injection, rats were placed back in the locomotor cages and their activity was measured for two hours. Behaviour was also observed and scored manually, and no signs of stereotypy were found which might confound locomotor activity measures.

This thesis is interested in the interaction of the dopamine and the glutamate system. These systems are manipulated with d-amphetamine and LY354740 respectively in the present chapter. In particular, I wished to ask the specific question, whether LY354740 would ameliorate or exacerbate the amphetamine-induced locomotor hyperactivity phenotype. Hence, an a priori interest in the comparison of veh/AMPH and LY354740/AMPH exists. Therefore, this comparison will be investigated by simple main effects even if the associated interaction from the main ANOVA is not significant for this and the following experiments.

Results

As expected, amphetamine (2.5mg/kg) injections induced hyperactivity. However, exploratory analysis showed that, somewhat surprisingly, LY354740 appeared to increase amphetamine-induced hyperactivity (Fig. 3.3), which

disagrees with published results that group II mGluR agonists inhibit or reduce amphetamine-induced locomotor hyperactivity (Cartmell et al., 1999, Kim and Vezina, 2002, Galici et al., 2005, Rorick-Kehn et al., 2007b, Woolley et al., 2008, Pehrson and Moghaddam, 2010).

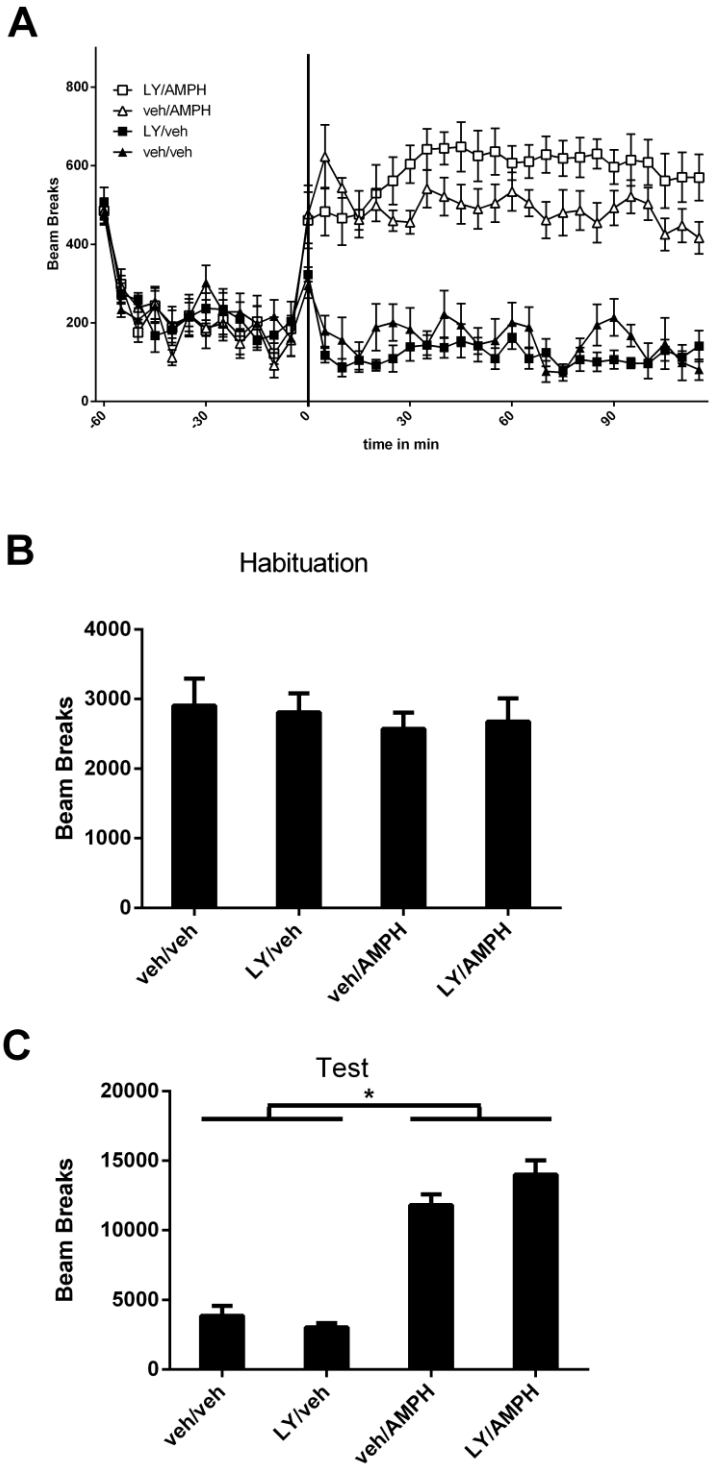


Figure 3.3: Locomotor activity test (LMA) demonstrating the interaction of LY354740(LY) and d-amphetamine (AMPH) in food restricted rats. (A) Beam breaks during the LMA experiment in 5 min bins. The vertical line at time=0 min denotes the time of dosing. (B) Total number of beam breaks during the pre-injection 60 min habituation period. (C) Total number of beam breaks during the 120 min post-injection test period (all n=28, n=7 per group). Data points represent mean ± SEM. * p < .05

A three-way ANOVA with time as a within-subjects factor and amphetamine and LY354740 as between-subjects factors over the one hour habituation phase was conducted. During the habituation phase, before any injections took place, there were no differences in the would-be amphetamine ($F(1,24) = 0.555$, $p = 464$) and the would-be LY354740 ($F(1,24) < 0.001$, $p = .990$) injected condition compared to the control groups, and the interaction of amphetamine and LY354740 would-be groups was not significant ($F(1,24) = 0.101$, $p = 753$) (Fig. 3.3B).

A three-way ANOVA with time as within and, amphetamine and LY354740 as between-subjects factors over the two hour test period revealed significant amphetamine-induced hyperactivity ($F(1,24) = 159.812$, $p < .001$), but no overall effect of LY354740 ($F(1,24 = 0.838$, $p = .369$)(Fig. 3.3C). Since

(i) the interaction of AMPH*LY354740 ($F(1,24) = 4.128$, $p = .053$) and the interaction of AMPH*LY354740*time ($F(23,552) = 1.514$, $p = .060$) were close to significance,

(ii) there was an a priori interest (see methods above),

(iii) visual inspection of the data suggested an increase of the amphetamine response by LY354740, further analyses of LY354740 in amphetamine-injected rats were conducted (i.e. the veh/AMPH and LY/AMPH groups were compared directly).

This revealed a significant effect of time ($F(23,276) = 1.770$, $p = .018$) but not LY354740 ($F(1,12) = 2.969$, $p = .111$). Importantly the time by LY354740

interaction was again significant ($F(23,276) = 2.199$, $p = .002$), and so further exploratory analyses were conducted. Visual inspection of the data suggested that there was a significant difference between the veh/AMPH and the LY354740/AMPH group in the second hour of testing. This was confirmed by a main effect of drug ($F(1,12) = 5.576$, $p = .035$) in a two-way ANOVA with time (60-120 min) as within-subjects factor and drug (veh/AMPH vs LY/AMPH) as between-subjects factor.

Discussion

These results showed that LY354740 did not reduce amphetamine-induced hyperactivity, and unexpectedly, showed a tendency to exacerbate hyperactivity. Interestingly, LY354740 did not increase or decrease activity on its own when compared to the vehicle controls. This suggests that the increase in activity seen in the LY354740+AMPH group was due to an interaction between both drugs and a modulation of the amphetamine effect.

It is not clear why in this experiment I saw an exacerbation of the amphetamine-induced locomotor response, whereas in previous studies this effect was ameliorated (Cartmell et al., 1999). However, in addition to being food restricted for five months as part of experiments 3a and 3b, this cohort differed from rats normally used in such experiments in several ways. They were extensively handled, and expected a food reward as part of being handled since they previously took part in an appetitively motivated experiment in the same suite of

testing rooms. Furthermore, they had also received multiple injections previously (two amphetamine, three LY354740, and four vehicle injections).

Experiment 3d: LY354740 Ameliorated D-Amphetamine-Induced Hyperactivity in ad lib fed Rats

Introduction

Therefore, one explanation for the surprising results in experiment 3c might be the prior history of this cohort. Rats were used in behavioural experiments previously, had been injected with drugs, and remained food restricted during locomotor testing. To test whether it is possible to replicate the published amelioration of the amphetamine effect by LY354740 in our setup at a stimulant dose of 2.5mg/kg, a second locomotor experiment was conducted on experimentally naïve Sprague-Dawley rats with ad libitum access to food, thereby more closely resembling the conditions in the literature (Cartmell et al., 1999).

Methods

A novel cohort of 36 naïve male Sprague Dawley rats (Harlan Laboratories) with ad lib access to food was tested in the same locomotor activity testing paradigm used in the previous experiment (experiment 3c). Again behaviour was scored manually by direct observation and no signs of stereotypy were found. Drugs were prepared as described in experiment 3a and 3b.

Results

The use of naïve rats showed that amphetamine-induced hyperactivity was indeed ameliorated by LY354740 (Fig. 3.4a). This reproduced the results of Cartmell et al. (1999), but was in contrast to my previous experiment (experiment 3c) where LY354740 had a tendency to exacerbate amphetamine-induced hyperactivity.

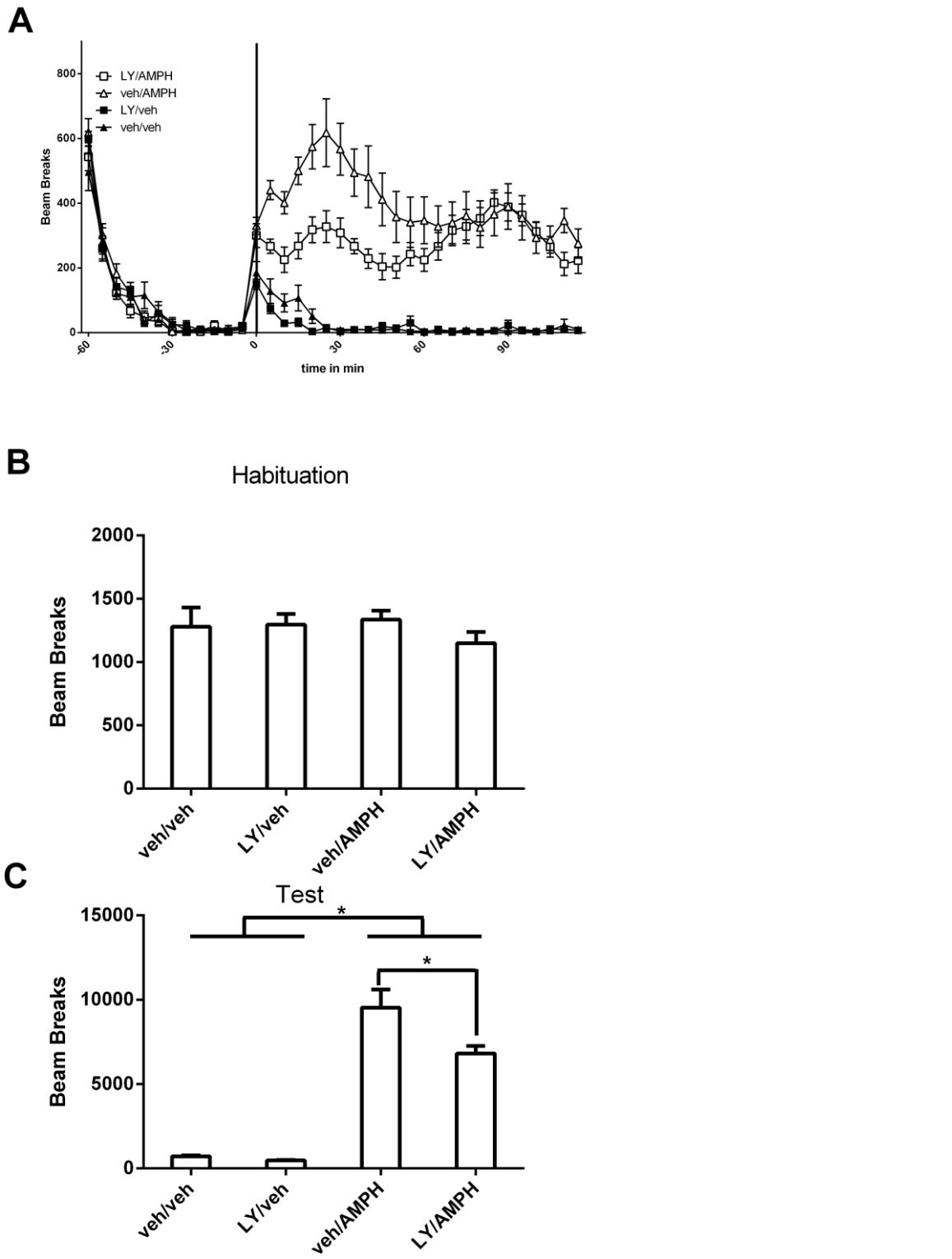


Figure 3.4: Locomotor activity test (LMA) demonstrating the interaction of LY354740(LY) and d-amphetamine (AMPH) in ad lib fed rats. (A) Beam breaks during the LMA test in 5 min bins. The vertical line at t=0min denotes the time of dosing. (B) Total number of beam breaks during the pre-injection 60 min habituation period. (C) Total number of beam breaks during the 120 min post-injection test period (all n = 36, n=9 per group). Data points represent mean ± SEM. * p < .05

During the habituation phase before drug injection, none of the groups that would later receive different drug combinations differed from each other (AMPH: $F(1,32) = 0.189$, $p = .667$; LY354740: $F(1,32) = 0.665$, $p = .421$; interaction of amphetamine and LY354740: $F(1,32) = 0.976$, $p = 331$; Fig. 3.4B).

A three-way ANOVA with time as within and amphetamine and LY354740 as between-subjects factors over the 2 hour test period revealed a significant amphetamine-induced hyperactivity ($F(1,32) = 169.741$, $p < .001$) a significant decrease of activity by LY354740 ($F(1,32) = 6.491$, $p = .016$) and a significant AMPH*LY354740 interaction ($F(1,32) = 4.515$, $p = .041$)(Fig. 3.4C). All effects and interactions including time were highly significant ($F(23,736) > 2.5$, $p < .001$).

A further analysis (two-way repeated-measures ANOVA) of the effect of LY354740 and time in amphetamine injected rats was conducted (i.e. comparing veh/AMPH vs LY354740/AMPH). This revealed a significant reduction of amphetamine-induced hyperactivity by LY354740 ($F(1,16) = 6.491$, $p = .016$).

Discussion

LY354740 ameliorated amphetamine-induced hyperactivity, replicating this widely published property of group II mGluR agonists (Cartmell et al., 1999, Kim and Vezina, 2002, Galici et al., 2005, Rorick-Kehn et al., 2007b, Woolley et al., 2008, Pehrson and Moghaddam, 2010). Moreover, the strong effect of LY354740 seen in this cohort is not only different from experiment 3c but also different from the weak, non-significant reduction of amphetamine-induced hyperactivity seen in Cartmell et al. (1999) specifically with LY354740. Another group II mGluR

agonist seems to have stronger effects on amphetamine-induced hyperactivity (Cartmell et al., 1999).

Experiment 3e: Food Restriction Increased Amphetamine-Induced Hyperactivity

Introduction

One potential explanation for the effect of food restriction in experiment 3e is a changed absorption, distribution, metabolism, or excretion (ADME) profile of d-amphetamine in food restricted rats compared to ad lib rats. Intraperitoneally injected substances largely get absorbed by the visceral peritoneum which drains into the hepatic portal system which, in turn, conducts to the liver (Lukas et al., 1971). Food restriction is likely to change the activity and metabolic properties of organs in this pathway. Potentially, uptake of amphetamine in the food restricted state could be higher. This could push rats into a higher effective dose range in which amphetamine induces subtle stereotypies which would actually reduce observed hyperactivity but were not detectable by visual inspection. LY354740 in this case could reduce the stereotypies by reducing the effective dopamine concentration and thereby increase the observed locomotor hyperactivity.

Veterinarians and industrial ADME specialists were consulted on the issue of changes in metabolism in food restricted rats (personal communication). To the author's knowledge, no experiment on the effect of food restriction on amphetamine metabolism after i.p. injection has been conducted previously. However, Stuber et al. (2002) found an increase of amphetamine-induced

hyperactivity in food restricted rats without an increase of dopamine release in the ventral striatum.

To test whether the effect of food restriction on the amphetamine locomotor activity effect can be reproduced, a dose-response locomotor activity experiment (vehicle, 1mg/kg, 2.5mg/kg or 5mg/kg d-amphetamine) was conducted in food restricted and ad lib fed rats at the Lilly Research Centre (Windlesham, UK) (experiment 3e). Simultaneously, blood level measures were taken from a distinct group of rats. These results are reported in experiment 3f.

Methods

Locomotor activity was measured in two different rooms in complete darkness with 16 clear Perspex boxes each (40x40x30cm) based on infrared fields. Four boxes were placed on each field, which were monitored using overhead infrared cameras. The cameras fed into a Quad compressor unit which, in turn, fed to a PC running the image analysis application Ethovision (Noldus). Ethovision digitized the path made by animals and used this to calculate distance travelled. Rats were habituated to the Perspex box for one hour, dosed, and tested for 2 hours.

90 male Sprague Dawley rats (Charles River) were food restricted or ad lib fed. Food restricted rats were fed in their home cages while in their holding rooms. During the experiment rats were injected with vehicle, 1mg/kg, 2.5mg/kg or 5mg/kg d-amphetamine (See Table 3.1 for group sizes). D-amphetamine was prepared as described in experiment 3f. The change in rat supplier was

necessary due to an infection in Harlan Laboratories breeding facility at the time of the experiment. The sample size for this experiment was large because two separate testing rooms were used, a factor known to increase variance in the sample for studies of locomotor activity. Due to equipment malfunction the last 5 minutes of the testing phase could not be recorded. Hence the testing phase is only 1 hour 55 minutes long.

	Vehicle	1mg/kg	2.5mg/kg	5mg/kg
ad lib	9	12	12	12
Food restricted	9	12	12	12

Table 3.1: Group sizes of experiment 3e.

Results

Rats displayed an inverted-U shape response profile to amphetamine in terms of locomotor activity, independently of feeding state. Food restriction reduced activity during the habituation phase, but increased amphetamine-induced hyperactivity during the test phase (Fig. 3.5A).

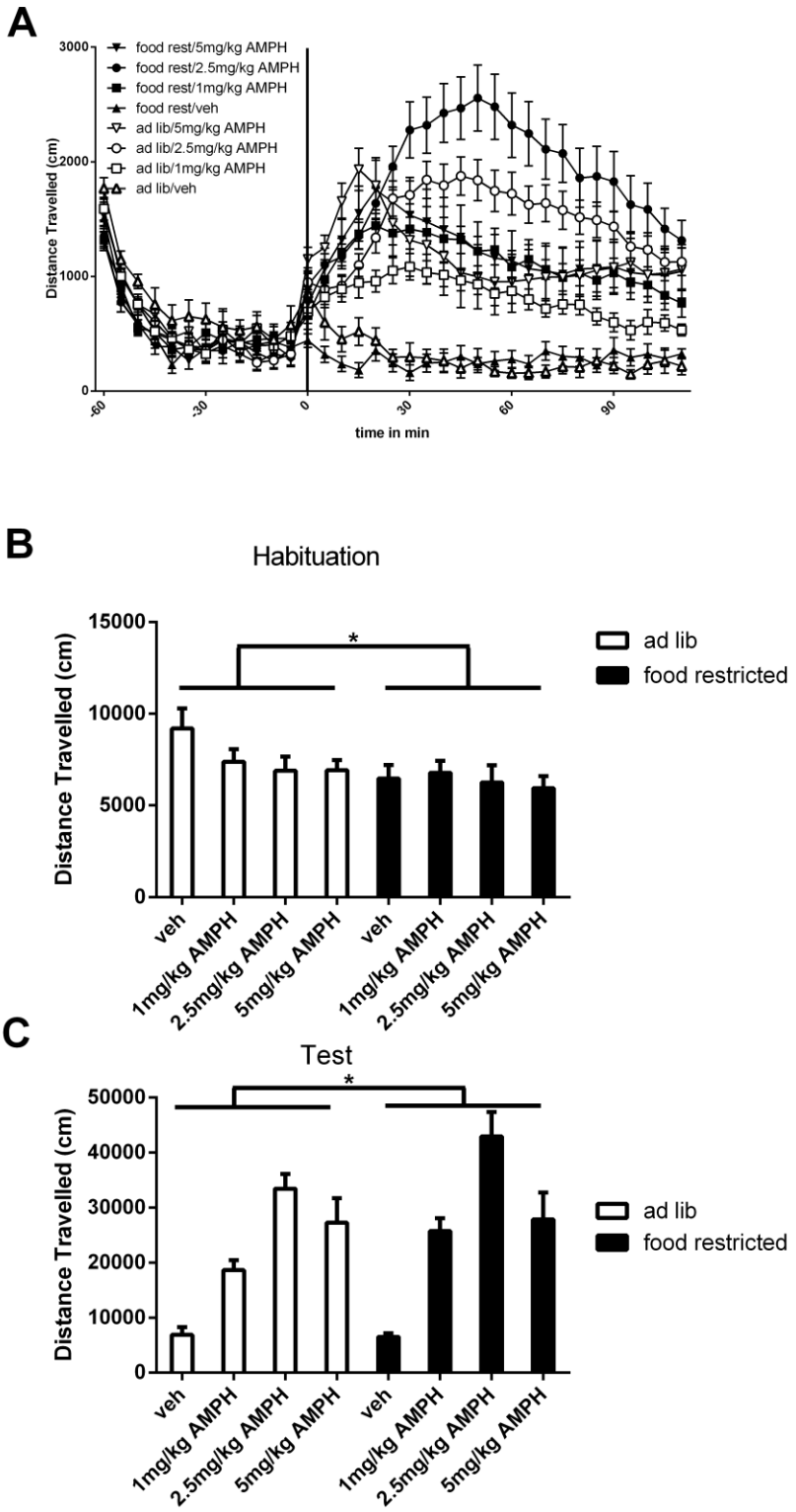


Figure 3.5: Locomotor activity test (LMA) demonstrating the effect of d-amphetamine (AMPH) in ad lib fed rats and food restricted rats. (A) Beam breaks during the LMA test in 5 min bins. The vertical line at t=0min denotes the time of dosing. (B) Total number of beam breaks during the pre-injection 60 min habituation period. (C) Total number of beam breaks during the 120 min post-injection test period (n=90, see table 3.1). Data points represent mean ± SEM. * p < .05

Surprisingly, food restricted rats were less active in the habituation period ($F(1,74) = 9.517$, $p = .003$) as was revealed by a four-way ANOVA with feeding, would-be drug group, and room as between-subjects factors and time as the within-subjects factor. Time ($F(11,814) = 161.877$, $p < .001$), room ($F(1,74) = 100.084$, $p < .001$), and the interaction of time and feeding state ($F(11,814) = 3.539$, $p< .001$) were significant. The effect of drug was not significant ($F(3,74) = 2.630$, $p = .056$) as would be expected since they had not received the drug at this point (Fig. 3.5B).

A four-way ANOVA with feeding, drug, and room as between-subjects factors, and time as a within-subjects factor on the test phase data revealed that food restricted rats now were more active overall ($F(1,74) = 4.036$, $p = .048$). However, food restricted rats were not more active in the habituation. Therefore this effect is unlikely to be a direct effect of food restriction on activity. Instead, this is rather a sign of an increased amphetamine-induced hyperactivity in food restricted rats. Furthermore, there was a main effect of drug ($F(3,74) = 34.588$, $p < .001$), and significant effect of room ($F(1,74) = 15.689$, $p < .001$) and time ($F(22,1628) = 15.578$, $p < .001$)(Fig. 3.5C). However, there was no drug by feeding interaction ($F(3,74) = 1.212$, $p = .311$), nor drug by feeding state by time interaction ($F(66,1628) = 0.611$, $p = .994$). Room interacted with multiple factors, a known problem of the two experimental rooms setup used, which was mitigated by the increased sample size in this experiment.

Post-hoc tests on the main effect of drug, with Sidak correction for multiple comparisons revealed that all doses of amphetamine increased activity levels

(veh-1mg/kg: $t(40) = 4.933$, $p < .001$; veh-2.5mg/kg: $t(40) = 10.029$, $p < .001$; veh-5mg/kg: $t(40) = 6.647$, $p < .001$) with the 2.5mg/kg group being more active than the 1 and 5mg/kg group (1mg/kg – 2.5mg/kg: $t(46) = 5.504$, $p < .001$; 2.5mg/kg – 5mg/kg: $t(46) = 3.652$, $p = .003$), which in turn did not differ from each other (1mg-5g/kg: $t(46) = 1.851$, $p = .258$).

Discussion

Amphetamine-induced hyperactivity at all doses independent of feeding state. 2.5mg/kg amphetamine led to the biggest increase in hyperactivity, indicating that this dose is closest to the peak of the inverted U-shaped function for locomotor activity. Food restricted animals were, overall, more active during the test phase while being less active during the habituation phase. Visual inspection of data suggests that this is likely driven by an enhanced response to AMPH. This suggests that changes in the feeding state increased the effect of amphetamine, thus reproducing Stuber et al. (2002).

Experiment 3f: Blood Amphetamine Levels were not Influenced by the Feeding State

Introduction

As described in the introduction to experiment 3e, one potential explanation for the effect of food restriction in experiment 3c is a changed absorption, distribution, metabolism, or excretion profile of d-amphetamine in food restricted rats compared to ad lib rats. It is important to note that the effect of food

restriction state on the amphetamine-induced activity levels does not necessarily have to be mediated through changes in striatal dopamine. For example, Stuber et al. (2002) showed that amphetamine-induced hyperactivity can be exacerbated by food restriction without changing striatal dopamine.

It is, however, possible that the absorption of the drug in the gut is changed and therefore, a different blood level of the drug is reached. This could lead to a subtle difference that cannot be detected in measurements of nucleus accumbens dopamine levels (Stuber et al., 2002). To test this hypothesis, blood levels of amphetamine were measured in food restricted and ad lib fed rats using dry blood spot sampling technique.

Methods

Blood levels of d-amphetamine were measured in a cohort of 24 male Sprague Dawley rats (Charles River, UK) injected with 1mg/kg, 2.5mg/kg or 5mg/kg d-amphetamine, half of which were food restricted. D-amphetamine sulphate was prepared at a concentration of 5 mg/ml (salt weight). From this stock solution, 2.5 and 1mg/ml solutions were prepared.

Blood samples were collected using the dried blood spot microsampling technique. Rats were restrained 15, 30, 60 and 120 minutes after dosing. The tail vein was pricked with a 23 gauge needle and ~20ul of blood collected with a capillary. This blood was transferred to a sample card, left to dry and then sent to Quintiles, USA for analysis using liquid chromatography–mass spectrometry (LC/MS/MS) techniques (Brewer and Henion, 1998).

In this technique, the physical separation abilities of liquid chromatography are used to separate molecules before channelling them into a two-stage mass spectrometer. In the first stage, molecules are selected based on their mass-to-charge ratio. In the second stage, the molecules of a determined mass-to-charge ratio are fragmented with high energy. The number and mass-to-charge ratio of these fragments are then recorded. From the identity of these fragments the identity and concentration of the whole molecule of interest can be determined. Thereby the concentration of d-amphetamine in a substance as complex as blood can be determined (Brewer and Henion, 1998).

Results

Blood amphetamine levels increased with greater doses of injected amphetamine, but were not influenced by feeding state.

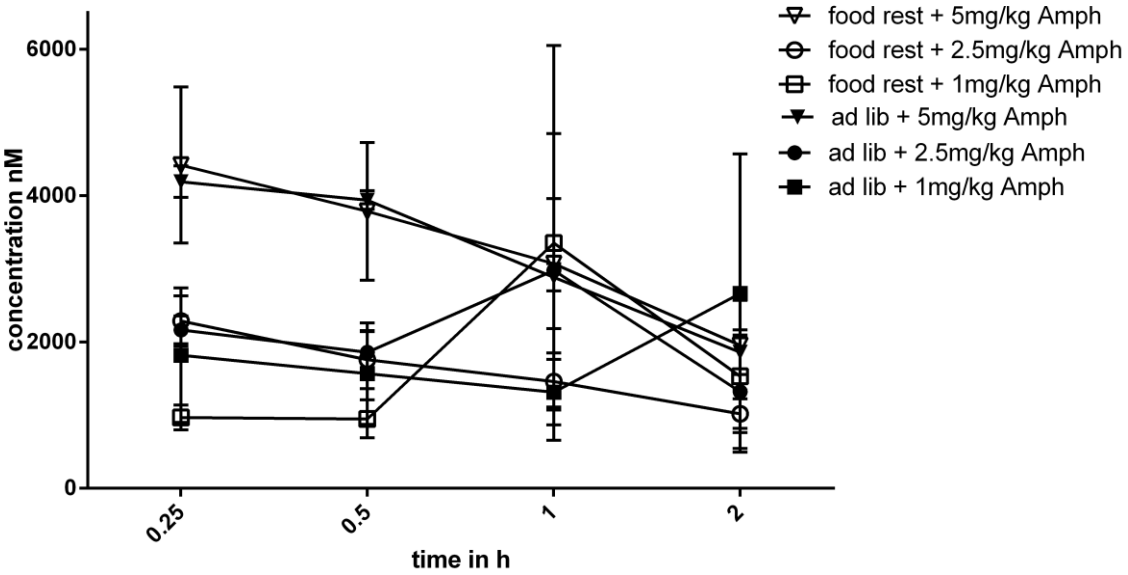


Figure 3.6: Unbounded blood concentration of d-amphetamine (1, 2.5, 5mg/kg; i.p.) at 4 time points during the 2 hours after injection (n=24). Data points represent mean ± SEM.

Analysis of blood amphetamine levels over 2 hours using a three-way ANOVA with feeding state and amphetamine dose as between and time as within-subjects factor revealed no significant effects (Feeding: $F(1,18) = 0.074$, $p = .789$; AMPH: $F(2,18) = 2.458$, $p = .114$; Feeding*AMPH: $F(2,18) = 0.065$, $p = .937$). However, the time by amphetamine interaction was close to significance ($F(6,54) = 1.964$, $p = .087$), and visual inspection of the data suggested that an analysis of the first half hour might show the expected amphetamine dose effect. As predicted, this analysis revealed a significant effect of amphetamine dose ($F(2,18) = 7.838$, $p = .004$) but again no effect of feeding ($F(1,18) = .152$, $p = .701$) and no interaction of feeding and amphetamine dose ($F(2,18) = .184$, $p = .834$) (Fig. 3.6).

Discussion

Feeding state had no influence on amphetamine blood levels which, as expected, rose with increased amphetamine dose, at least in the first half hour after injection. Therefore, it can be excluded that feeding state-induced changes in amphetamine absorption, metabolism, or excretion can account for the effect of feeding on the amphetamine by LY354740 interaction seen in the LMA tests (experiment 3e).

Experiment 3g: LY354740 Reduced Amphetamine-Induced Hyperactivity in Food Restricted but not ad lib fed Rats

Introduction

Experiment 3c and 3d revealed opposite effects of LY354740 on amphetamine-induced hyperactivity. LY3534740 had a tendency to increase amphetamine-induced hyperactivity in food restricted rats that had experienced various injections as part of previous experiments (experiment 3c), but reduced amphetamine-induced hyperactivity in naïve ad lib fed rats as reported previously in the literature (experiment 3d; Cartmell et al. (1999); Pehrson and Moghaddam (2010)).

This is a potentially interesting result, but as the comparison is made across two experiments it is therefore not as controlled as would be desirable. It is not clear exactly what experimental conditions are responsible for generating these different outcomes in experiment 3c and 3d. One possibility is that food restriction is an important factor. Thus, to test this food restriction was manipulated in this experiment to directly assess the effects of LY354740 on amphetamine-induced hyperlocomotion in a more controlled manner under different feeding states.

Methods

64 male Sprague Dawley rats (Charles River, UK), half of them food restricted, were injected with either veh/AMPH (2.5mg/kg) or LY354740 (10mg/kg)/AMPH

(2.5mg/kg) while being tested in the locomotor tracking setup used in experiment 3e. Drugs were prepared as described in experiment 3a and 3b.

Results

Surprisingly, LY354740 now reduced amphetamine-induced hyperactivity in food restricted but not ad lib fed rats (Fig. 3.7A).

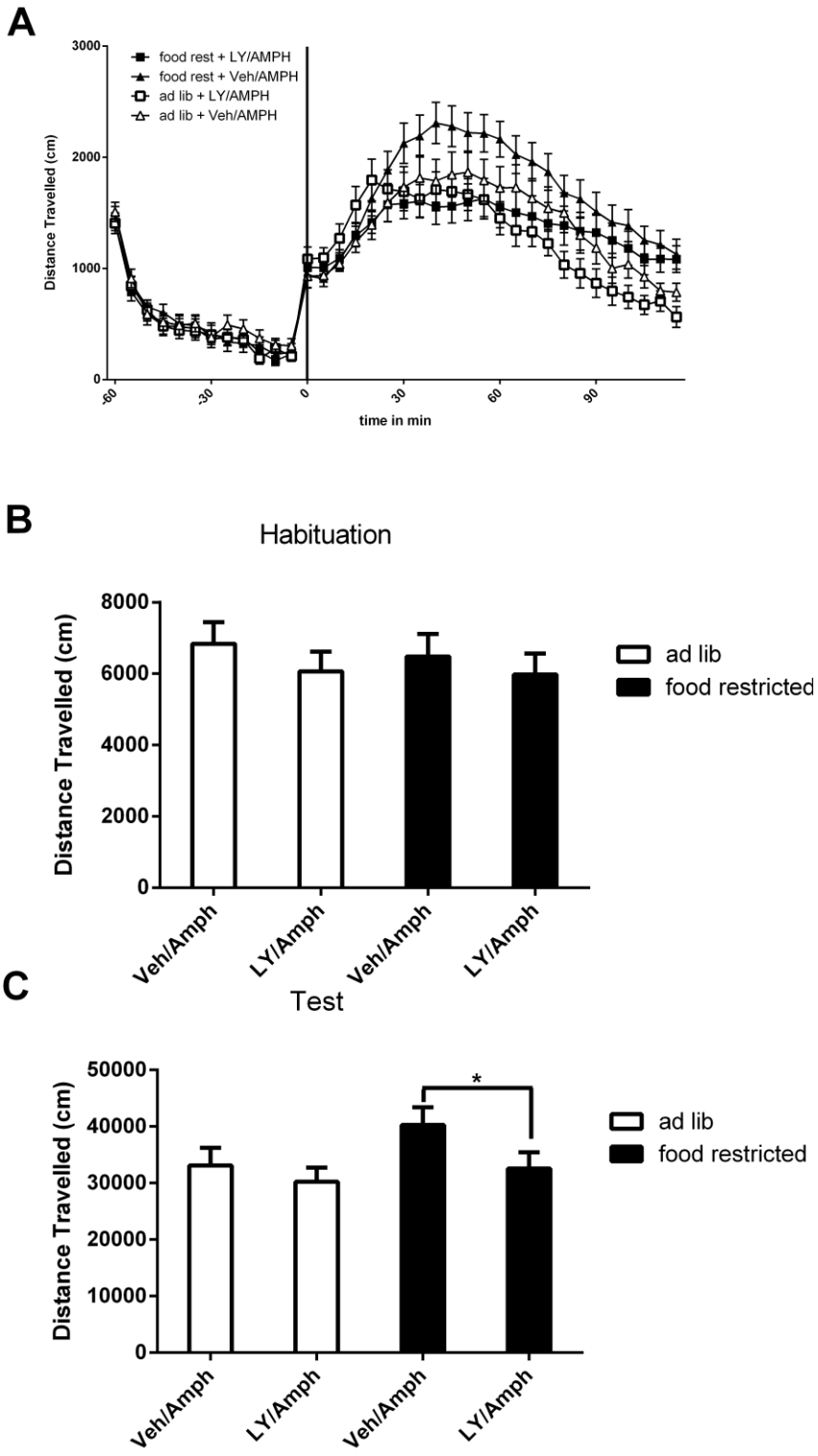


Figure 3.4: Locomotor activity test (LMA) demonstrating the effect of LY354740(LY) on d-amphetamine-induced hyperactivity in ad lib fed and food restricted rats. (A) Beam breaks during the LMA test in 5 min bins. The vertical line at t=0min denotes the time of dosing. (B) Total number of beam breaks during the pre-injection 60 min habituation period. (C) Total number of beam breaks during the 120 min post-injection test period (all n=64, n=16 per group). Data points represent mean ± SEM. * p < .05

A four-way ANOVA with feeding, drug, and room as between-subjects factors and time as a within-subjects factor on the habituation phase data revealed a significant effect of room ($F(1,56) = 85.886$, $p < .001$) and time ($F(11,616) = 133.085$, $p < .001$) but not of feeding ($F(1,56) = 0.328$, $p = .569$) and would-be drug condition ($F(1,56) = 2.711$, $p = .102$; Fig. 3.7B).

A four-way ANOVA with feeding, drug (LY/AMPH, veh/AMPH) and room as between-subjects factors and time as within-subjects factor on the test phase data revealed a significant effect of room ($F(1,56) = 10.079$, $p = .002$) and time ($F(23,1288) = 59.952$, $p < .001$) but not feeding ($F(1,56) = 3.058$, $p = .086$) or drug ($F(1,56) = 3.819$, $p = .056$). However, LY354740 showed a strong tendency to reduce amphetamine-induced hyperactivity overall. The interaction of feeding by drug ($F(1,56) = 0.789$, p .378) was not significant (Fig. 3.7C). This suggests that LY354740 had the same effect in both feeding conditions.

However, due to an a priori interest in the effects of time and drug in the two feeding states, further analyses in each of the levels of this variable were conducted. In ad lib rats there was no effect of LY354740 on amphetamine-induced hyperactivity ($F(1,28) = 0.521$, $p = .476$). However, in food restricted rats, LY54740 decreased amphetamine-induced hyperactivity ($F(1,28) = 4.441$, $p = .044$). Thus, in contrast to my previous experiment here LY354740 reduced amphetamine-induced hyperactivity in food-restricted rats but not in ad lib rats, although it is important to note that there was no drug by feeding interaction.

Discussion

LY354740 reduced amphetamine-induced hyperactivity in food restricted but not ad lib fed rats. This pattern opposes that seen in experiment 3c and 3d. Furthermore, food restriction did not decrease baseline activity during the habituation period, in contrast to the results of Experiment 3e. A further difference in the food restricted group is that rats in experiment 3c previously took part in an appetitively motivated task. Therefore, these rats may have begun to expect food rewards when being handled by the experimenter and taken to the suite of testing rooms, thereby potentially increasing their dopamine levels (Schultz et al., 1997).

Surprisingly, no effect of LY354740 was found in the ad lib group in experiment 3g. This lack of effect is potentially due to an increase of variance due to the experiment being conducted in two different rooms. This potentially reduced the power to a point that the numerical reduction of activity in ad lib fed rats due to LY354740 was not significant.

Experiment 3h: LY354740 Reduced Amphetamine-Induced Hyperactivity in ad lib fed Rats but not Food Restricted Rats that Expected Food

Introduction

Experiment 3c and 3d revealed opposite effects of LY354740 on amphetamine-induced hyperactivity. LY354740 had a tendency to increase amphetamine-

induced hyperactivity in food restricted rats that had experienced various injections as part of previous experiments (experiment 3c) but reduced amphetamine-induced hyperactivity in naïve ad lib fed rats (experiment 3d). Experiment 3g showed no amelioration of amphetamine-induced hyperactivity in ad lib fed rats but a reduction of amphetamine-induced hyperactivity in food restricted rats.

Experiment 3g was conducted in a different research facility (Eli Lilly, Windlesham) in which activity levels were measured using video tracking rather than beam breaks to assess locomotor activity. The following experiment will again compare the interaction of LY354740 and d-amphetamine in ad lib fed and food restricted rats but using beam breaks, and conducted in the Biomedical Services Building in Oxford, in the same manner as experiments 3c and 3d. In addition to simply being food restricted, food restricted rats were fed in an experimental room in the suite of testing rooms, thereby potentially building an expectation of reward upon handling.

Methods

To investigate the effect of feeding state and the possible expectation of food more systematically, half the animals in a novel cohort consisting of 48 naïve male Sprague Dawley rats (Harlan Laboratories) were food restricted over 10 days to 85% of their free feeding weight. For feeding, all rats of this cohort were transported to an experimental room different from the locomotor activity test room and different from their colony holding room, where the food restricted

animals were fed. Ad lib fed rats were also transported to this “feeding room” to control for the effect of transportation to the laboratory. In the Biomedical Services Building holding and experimental rooms are located on different floors. Therefore, rats have to be transported in an elevator potentially changing their arousal levels. Since both groups were transported, both groups were habituated to the elevator.

To increase the power of the comparison of interest (an interaction of LY354740, amphetamine and food restriction), only two injection conditions were included. Half of the rats were injected with veh/AMPH (2.5mg/kg), the other half with LY354740 (10mg/kg)/AMPH (2.5mg/kg), counterbalancing drug and feeding state (food restricted vs ad lib). The locomotor test was conducted before the animals were fed on the respective day, thereby inducing the expectation of food in the rats at the time of locomotor testing. Drugs were prepared as described in experiment 3a and 3b. Again behaviour was scored manually by observation and no sign of stereotypy was found.

Results

Food restricted rats that expected food were more active during both the habituation and test phases regardless of drug condition. Under these conditions, LY354740 inhibited amphetamine-induced hyperactivity in ad lib but not food restricted rats (Fig. 3.8A).

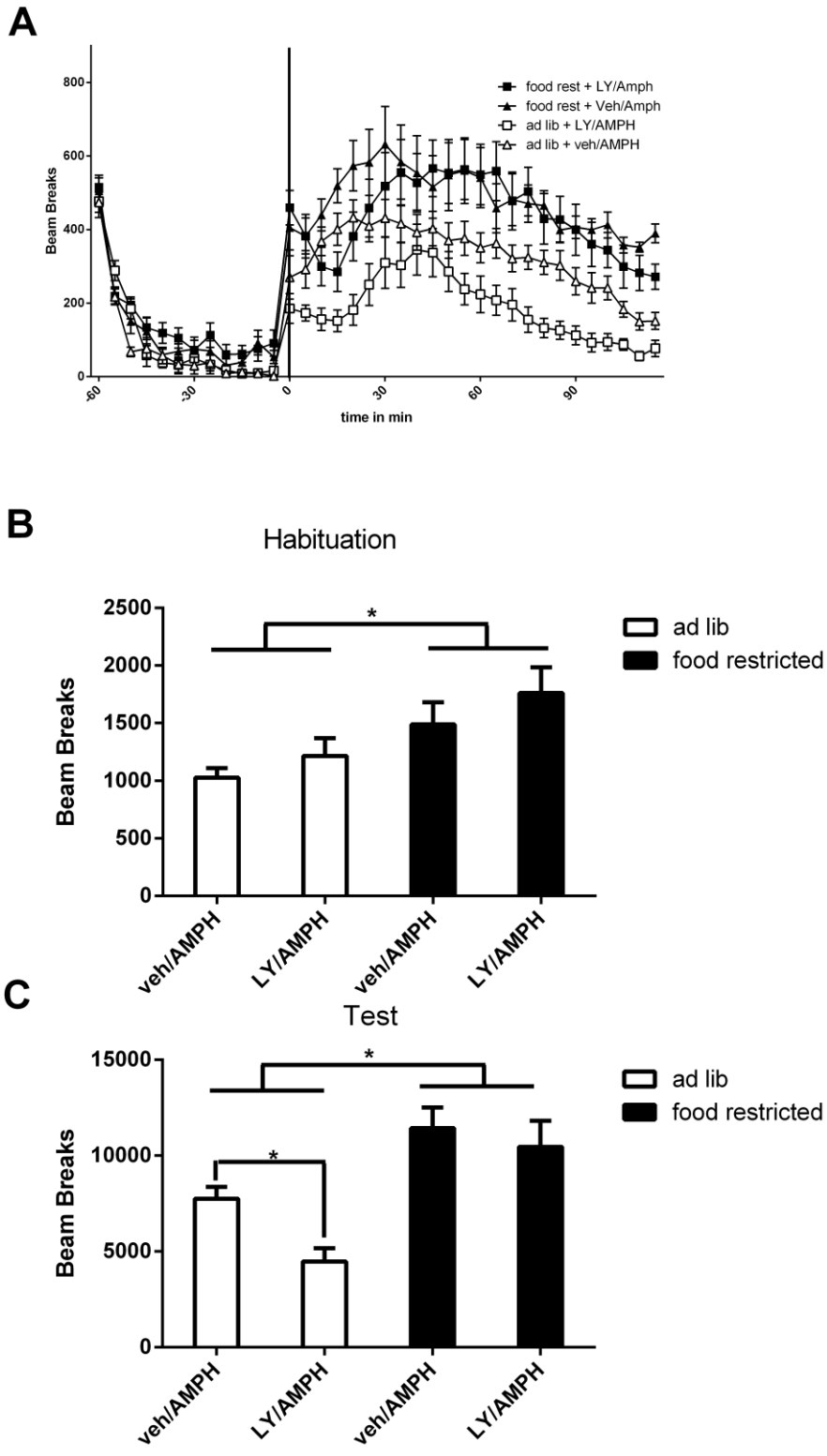


Figure 3.8: Locomotor activity test (LMA) demonstrating the effect of LY354740(LY) on d-amphetamine-induced hyperactivity in ad lib fed and food restricted rats. (A) Beam breaks during the LMA test in 5 min bins. The vertical line at t=0min denotes the time of dosing. (B) Total number of beam breaks during the pre-injection 60 min habituation period. (C) Total number of beam breaks during the 120 min post-injection test period (all n=48, n=12 per group). Data points represent mean ± SEM. * p < .05

During the habituation phase, before any injections took place, there were no differences in the would-be LY354740-injected groups compared to controls ($F(1,32) = 1.782$, $p = .191$). Food restricted rats were more active ($F(1,32) = 8.506$, $p = .006$). The interaction of feeding state and would be LY354740 condition was not significant ($F(1,32) = 0.061$, $p = .806$)(Fig. 3.8B).

A three-way ANOVA with time as within and, feeding state and drug as between-subjects factors over the 2 hour test period revealed that food restricted rats were significantly more active ($F(1,32) = 23.900$, $p < .001$), and that LY354740 reduced amphetamine-induced hyperactivity ($F(1,32) = 4.659$, $p = .038$). There was no feeding state by drug interaction ($F(1,32) = 1.368$, $p = .251$), suggesting that LY354740 reduced amphetamine-induced hyperactivity independently of feeding state (Fig. 3.8C).

A further two-way repeated-measures ANOVA of the effect of LY354740 on amphetamine-induced hyperactivity over 2h separately in ad lib fed rats was conducted based on an a priori interest in this relationship. This revealed a significant reduction of amphetamine-induced hyperactivity by LY354740 ($F(1,16) = 12.692$, $p = .003$) in agreement with Cartmell et al. (1999) and experiment 3d. This effect was clearly absent in food restricted animals ($F(1,16) = 0.313$, $p = .584$). So, LY354740 reduces amphetamine-induced hyperactivity in ad lib fed rats as reported previously, but not food restricted rats. Though, an absence of a feeding state by drug interaction cautions regarding the robustness of the conclusions that can be drawn.

Discussion

In ad lib fed rats LY354740 reduced amphetamine-induced hyperactivity reproducing Cartmell et al. (1999) and experiment 3d. LY354740 did not significantly reduce amphetamine-induced hyperactivity in food restricted rats, showing that the feeding state changed the amphetamine-LY354740 interaction potentially connecting the diverging results of experiment 3c and 3d. However, LY354740 did not exacerbate amphetamine-induced hyperactivity in food restricted rats that expected food, as might have been predicted from the results of experiment 3c. This experiment still points towards the possibility that expectation of food might play some role in the interaction of d-amphetamine with LY354740.

General Discussion

LY354740 impaired choice accuracy and exacerbated amphetamine-induced increases in latencies

LY354740 (10mg/kg) alone impaired T-maze rewarded alternation, replicating Aultman and Moghaddam (2001) and increasing running latencies. Amphetamine (2.5mg/kg) did not affect T-maze alternation choice accuracy on its own, but increased running latencies. This seems at first counterintuitive: Amphetamine induces hyperactivity, therefore rats might be expected to enter the goal arm more quickly. However, latencies do not necessarily reflect running speeds as amphetamine-injected rats, for example, might fail to start running

readily after being placed on the maze or explore the start arm rather than enter a goal arm. However, the current data set cannot elucidate the underlying factor responsible for changes in latencies.

Interestingly, LY354740 combined with amphetamine significantly impaired rewarded alternation choice accuracy compared to vehicle-injected controls whereas amphetamine by itself failed to have a significant effect. Actually, choice accuracy in the combined LY/AMPH condition in experiment 3b was comparable to choice accuracy in the 10mg/kg LY354740 condition in experiment 3a. Additionally, animals in the LY/AMPH condition displayed longer latencies than animals in the amphetamine and vehicle conditions.

This effect on latencies provided the first evidence for the hypothesis that under certain circumstances LY3534740 can exacerbate the amphetamine effect. This is surprising, since LY354740 reduces amphetamine-induced increases in dopamine as measured by microdialysis (Pehrson and Moghaddam, 2010), and should therefore attenuate the effects of AMPH.

However, since amphetamine alone did not affect rewarded alternation, and the psychological construct measured by latencies is unclear, the nature of the amphetamine-LY354740 interaction could not further be elucidated based on the T-maze data. Therefore, in subsequent studies locomotor activity was used to assess the amphetamine-LY354740 interaction.

LY354740 affected amphetamine-induced locomotor hyperactivity in a state-dependent manner

In all tests of locomotor activity, amphetamine reliably induced hyperactivity. Interestingly, the results showed that the effect of amphetamine was influenced by the internal state of the animal (ad lib vs food restricted), as was the interaction of amphetamine and LY354740 (Table 3.2).

Experiment	Rats used/state of rats	Facility	Measurement System	Light	Design	Results
3c	Food restricted Handled Expected food Received AMPH and LY354740 previously SD rats(Harlan)	BSB, Oxford	26cm x 16cm Beam Break System, bedding on floor	on	veh/veh veh/AMPH LY/veh LY/AMPH	LY had a tendency to exacerbate AMPH-induced hyperactivity
3d	Naive, ad lib fed SD rats(Harlan)	BSB, Oxford	26cm x 16cm Beam Break System, bedding on floor	on	veh/veh veh/AMPH LY/veh LY/AMPH	LY ameliorated AMPH-induced hyperactivity
3e	SD rats (Charles Rivers), half ad lib fed, half food restricted	Eli Lilly Research Centre	Video Tracking (Ethovision, Noldus) in 40cm x 40cm box, no bedding	off	Ad lib: 0, 1, 2.5, 5mg/kg AMPH Food restricted: 0, 1, 2.5, 5mg/kg AMPH	Food restriction increased AMPH-induced hyperactivity
3g	SD rats (Charles Rivers), half ad lib fed, half food restricted	Eli Lilly Research Centre	Video Tracking (Ethovision, Noldus) in 40cm x 40cm box, no bedding	off	Ad lib: Veh/AMPH LY/AMPH Food restricted: Veh/AMPH LY/AMPH	LY reduced AMPH-induced hyperactivity in food restricted but not ad lib fed rats
3h	SD rats (Harlan), half ad lib fed, half food restricted and expecting food	BSB, Oxford	26cm x 16cm Beam Break System, bedding on floor	on	Ad lib: Veh/AMPH LY/AMPH Food restricted: Veh/AMPH LY/AMPH	LY reduced AMPH-induced hyperactivity in ad lib fed rats but not food restricted rats that expected food

Table 3.2: Summary of the locomotor activity tests conducted in chapter 3.

Published literature suggested that LY354740 has a tendency to suppress amphetamine-induced hyperactivity (Cartmell et al., 1999). However, when assessed in the handled and food restricted rats used in the previous T-maze experiments which expected food when they were handled, LY354740, if anything, exacerbated amphetamine-induced hyperactivity (experiment 3c).

Since this was surprising, an attempt was made to reproduce the published literature in a cohort of naïve rats. This revealed the expected amelioration of amphetamine-induced hyperactivity by LY354740. It is of interest to note that Cartmell et al. (1999) do not specify whether or not rats used in their studies were experimentally naïve. Therefore, experiments previously conducted in the cohort used by Cartmell et al. could have changed the internal state of the rats. This could explain their failure to see a significant effect.

One possible explanation for the inconsistent effects of LY354740 is that, as (Zinberg, 1984) suggested, the internal state of an organism (set) influences the effects of the psychoactive substance amphetamine. This has previously been shown to be the case in humans. Depressed patients, for example, show an increased amphetamine response (Tremblay et al., 2002, Tremblay et al., 2005). Such findings also extend beyond clinical populations. Anxious distress in healthy volunteers, for example, predicts greater amphetamine-induced positive mood (Kirkpatrick et al., 2016). This suggests that individual differences can change the internal state (set) in such a way that the set influences the amphetamine response.

This, however, does not explain how the effect of LY354740 on amphetamine-induced hyperactivity can change its direction from inhibiting to exacerbating based on the set. One explanation is that due to a non-linear relationship between dopamine and locomotor activity levels, a reduction of dopamine by LY354740 levels leads to different effects depending on the state of the animal. As described in the introduction, the effect of dopamine on locomotion follows an inverted U-shape function. Increases in dopamine increase locomotion up to a peak, following which further increases in dopamine reduce rats' locomotor activity as a result of the emergence of competing stereotypical behaviours (McNamara et al., 2006).

In rats with low baseline dopamine (e.g. naïve rats on ad lib food like in experiment 3d) a 2.5mg/kg injection of AMPH would let the rat remain on the rising part of the inverted U-shape function in which higher dopamine levels lead to more locomotion. If LY354740 reduces dopamine levels (Pehrson and Moghaddam, 2010), this would lead to less activity in response to the same dose of amphetamine.

Food restriction and expectation of food are known to increase dopamine cell firing (Schultz et al., 1997, Branch et al., 2013). For rats with dopamine levels presumably increased as a result of food restriction and expectation of food (e.g. in experiment 3c), the same dose of amphetamine could move them towards an area of the curve in which more dopamine leads to less locomotion. If LY354740 were to reduce dopamine in this range, this would lead to more activity. I, however, did no observe stereotypy in the rats. They could, still, have displayed more subtle stereotypy not observable by visual monitoring.

In terms of the inverted U-shaped function this can be expressed as the free feeding group being on the rising arc of the function, where more dopamine produces more exploratory locomotor activity. The food restricted rats, due to their higher baseline dopamine levels are pushed towards a part of the inverted U-shaped function where lower dopamine levels lead to more exploratory activity. Therefore LY354740 potentially has the ability to increase activity levels in the food restricted rats.

I therefore conducted experiments to test the effects of food restriction on amphetamine-induced hyperactivity. Experiment 3e showed that food restriction increased amphetamine-induced hyperactivity, reproducing Stuber et al. (2002). Experiment 3f showed that an increase of amphetamine-induced hyperactivity by food restriction as seen in experiment 3e was not due to peripheral factors such as drug absorption. By showing that blood amphetamine levels were not affected by food restriction it was shown that the increased effect of amphetamine is likely due to changes in the central nervous system. However, Stuber et al. (2002) already showed that amphetamine-induced dopamine release in the nucleus accumbens is not changed by food restriction. Other potential explanations would be:

1) Food restriction can modulate amphetamine-induced dopamine release, but not in the nucleus accumbens.
2) Food restriction's effect on locomotor activity is mediated via other neurotransmitter systems or downstream of dopamine.

This discussion has covered the apparently inconsistent effect of LY354740 on amphetamine-induced hyperactivity in experiment 3c and 3d which potentially was due to an increased amphetamine effect in food restricted rats. However, so far this comparison has to be made between experiments. In experiment 3g and 3h I manipulated the feeding state within one experiment. In experiment 3g LY354740 reduced amphetamine-induced locomotor hyperactivity in food restricted rats but not in ad lib fed rats. Experiment 3h gave the opposite results. Here food restricted rats in addition expected food. In these rats, LY354740 did not reduce amphetamine-induced locomotor hyperactivity (similar to the effect seen in experiment 3c). LY35740, however, ameliorated the hyperactivity in ad lib fed rats in experiment 3h.

Combined, these experiments suggest an influence of the internal state on the interaction of amphetamine and LY354740. However, the differences in the results of the different locomotor tests in this chapter are also potentially affected by a whole host of other factors. They were run in different facilities, the Eli Lilly Research Centre and the Biomedical Science Building of the University of Oxford. Therefore, the differences between experiment 3g and 3h don't necessarily have to be due to the effect of an expectation of food. They could be due to a plethora of other differences in the conditions between the Eli Lilly Research facility and the University of Oxford.

1) Experimental Environment: Experiments in Oxford were run with the room lights on. Experiments in the Lilly facility were run in the dark, albeit still during the light phase (i.e. lights in the holding room were on). The area in which rats

could move was smaller in Oxford and had bedding on the ground, whereas rats were moving on Perspex in the Lilly facility.

2) Rat Supplier: In Oxford SD rats supplied by Harlan Inc. were used. In the Lilly facility SD rats supplied from Charles River were used.

3) Measuring system: In Oxford a beam break system was used. In the Lilly facility video tracking was used.

However, visual inspection of the data does not suggest a completely different pattern of results. LY354740 numerically reduced amphetamine-induced hyperactivity in both groups in both experiments. This is reflected in a lack of an interaction of drug and feeding state in experiment 3g and 3h. Still, there is potentially a hidden variable moving the rats along some non-linear function modulating the effect of LY354740. Group II metabotropic glutamate receptors are described to play an important role in arousal related processes (Lyon et al., 2011). Therefore, food restriction, reward expectation, amphetamine sensitization, injection stress, and previous handling might all modulate an arousal process (or processes) that influence the effect of LY354740.

However, continuing to investigate all the potential higher-order interactions using locomotor activity tests would be highly impractical. The sample size used in experiments 3 e, g, and h is already impractically high. Systematic testing of the interaction would mean using a naïve cohort in every experiment due to a potential role of amphetamine sensitization, which by itself interacts with factors

such a novelty (Badiani et al., 1998). This is impossible due to ethical and monetary considerations.

So far, LY354740 is hypothesized to have reduced dopamine independently of the state of the animal in this chapter. However, this reduction in dopamine had a different effect on locomotor activity levels as predicted by the inverted U-shaped function dependent on the state of the animal. In animals with low dopamine levels (e.g. experimentally naïve, ad lib fed animals), the reduction in dopamine reduced locomotor activity levels. In animals with high dopamine levels such a reduction of dopamine, could have reduced subtle stereotypies that were difficult to assess thereby increasing overall locomotor activity. So far locomotor activity levels were used as proxy for dopamine activity. In the next chapter a more direct measure of dopamine levels will be used to assess this hypothesis.

Chapter 4: The Effect of LY354740 on Phasic Dopamine and Tissue Oxygen

Introduction

The previous chapter (chapter 3) investigated the effect of LY354740 on the dopamine system using d-amphetamine to evoke changes in dopamine that could be assessed using behavioural measures, primarily locomotor activity. A state-dependent effect of LY354740 was found. LY354740 reduced amphetamine-induced locomotor hyperactivity in experimentally naïve animals, reproducing Cartmell et al. (1999). In food restricted animals that expected food a surprising tendency of LY354740 to increase amphetamine-induced hyperactivity was found. I suggested that LY354740 reduced dopamine levels independent of the state of the animal. The unexpected exacerbation of the amphetamine-response was hypothesized to occur due to a decrease in subtle stereotypies rather than an increase in dopamine levels.

To evaluate this hypothesis, a different method to assess the effect of LY354740 on the dopamine system more directly was needed. Previously it has been shown that mGluR2/3 double knockout mice have reduced dopamine levels in the striatum (Lane et al., 2013). However, systemically administered LY354740 did not affect baseline dopamine levels in the NAc (Moghaddam and Adams, 1998) or if anything reduced baseline dopamine levels (Karasawa et al., 2006). As discussed in the previous chapter, Pehrson and Moghaddam (2010) found that LY354740 inhibits amphetamine-induced dopamine release in the nucleus

accumbens as measured by microdialysis. However, when dopamine levels were increased by systemic injection of L-DOPA, a dopamine precursor, or electrical stimulation of dopamine neurons in the VTA, LY354740 had no effect. This suggests that the group II mGluR agonist's effect is not due to an effect on the dopaminergic neurons directly. That is to say the effect of LY354740 is not dependent on changes to dopamine release, reuptake or synthesis (Pehrson & Moghaddam, 2010). LY354740 instead would have to exert its effect upstream of dopaminergic synapses.

These studies used microdialysis which is able to measure absolute dopamine levels, but has a low temporal resolution in the range of seconds even in the best case (Wang et al., 2008). That makes it difficult to capture certain behaviourally-evoked dopamine changes like the phasic dopamine response to unexpected reward delivery or to punctate cues (Schultz et al., 1997). Fast-scan cyclic voltammetry as described in chapter 2, however, enables me to measure transient dopamine changes with a sub-second temporal resolution. Therefore, reward delivery can be used to induce activity in the dopamine system, to assess the effect of LY354740 on phasic dopamine.

Ultimately, the aim of the rodent studies in this thesis is to gain insight into the human brain. To do so, a set of measures and methods that can be compared between rodents and humans is needed. This thesis will use another electrochemical method in rodents to investigate a physiological variable usually measured in humans. This variable is the blood-oxygen level dependent (BOLD) response (Ogawa et al., 1990). When neurons in a brain area are active they require more oxygen than when they are inactive. Therefore, oxygen levels in

this brain area decline temporarily. However, as early as 40ms after the neural activation started, a haemodynamic response sets in that supplies more oxygenated blood to the active area, overcompensating for the oxygen demand of neural activity (Li et al., 2011). Therefore oxygen levels increase when a brain area is active. In humans this BOLD response is usually assessed using functional magnetic resonance imaging (fMRI). fMRI has also been applied in rodents (Brydges et al., 2013). However, it is very costly and rodents have to be head-fixed to measure brain activity. Instead 0_2 levels can also be measured via an electrode that can detect oxygen electrochemically, a method called oxygen amperometry (Lowry et al., 1997). This enables researchers to detect changes in 0_2 levels more economically and in freely moving, behaving rodents.

In this chapter the effect of LY354740 on the dopamine system is investigated. Rats performed a task in which unsignalled rewards of different magnitudes had to be collected from a food magazine while the phasic dopamine response in the nucleus accumbens (NAc) core was recorded. Based on the results of chapter 3 and Pehrson and Moghaddam (2010) a reduction of reward-evoked phasic dopamine was predicted. To investigate the effect of LY354740 on a wider network, tissue amperometry levels were recorded in a separate cohort in a similar unsignalled reward task. This cohort was implanted with amperometry electrodes into in the NAc, the medial prefrontal cortex (mPFC), the dorsal hippocampus (dHPC), and the retrosplenial cortex (RSC). This allowed comparing the phasic dopamine signal in the NAc to O_2 signals in the same task. mPFC, RSC and dHPC are known to be involved in short-term memory and regulating locomotor activity levels (Bannerman et al., 2004, Castner et al., 2004,

Vann et al., 2009). Therefore, the effect of LY354740 on these regions might suggest an underlying network explaining the effect of LY354740 seen in chapter 3.

Methods

Behavioural Tasks

Rats were placed in Med Associates Boxes (30.5 × 24.1 × 29.2 cm) and pellets were delivered to them at random intervals independent of head entry into the food magazine. The behavioural test consisted of a 120s [90-150s] variable interval reward schedule with 12 trials for the FCV cohort (experiment 4a) and 60 trials for the oxygen amperometry cohort (experiment 4b). Trials consisted of the unsignalled delivery of either one 45mg sucrose pellet (low), two pellets (medium) or 4 pellets (high). Due to limitations of the reward dispenser, it was only possible to deliver pellets every 0.5s. Hence the delivery of the high reward condition lasted approximately 2 seconds. During the behavioural task the total number of head entries was recorded in experiment 4a and 4b. Latencies were only recorded in experiment 4a.

Experiment 4a LY354750 in FCV

12 male SD rats were bilaterally implanted with carbon fibre electrodes at the University of Oxford from which phasic dopamine could be recorded using FCV (see Chapter 2). Rats were food restricted to no less than 85% of their free feeding weight for more than 3 months as part of a previous instrumental

conditioning experiment in which levers had to be pressed to gain food rewards (45mg sucrose pellets).

The dopamine signal of 16 electrodes passed the criterion for inclusion as defined in Chapter 2. For the analysis of behaviour in this task, one subject had to be removed due to a missing behavioural data set.

Experiment 4b LY354750 in Oxygen Amperometry

41 male Wistar rats (Charles River UK) aged approximately 12 months at the start of the experiment were housed in standard housing conditions (24 hour light/dark cycle, 0700-1900 light phase, with ad libitum access to water). Rats were food restricted to no less than 85% of their free feeding weight. Prior to this experiment rats were injected with various substances to assess changes in baseline oxygen levels. In the weeks prior to this experiment rats received three sessions with a 60s [30s-90s] variable interval (VI) schedule and one session of 120s [90s-150s] VI random reward schedule.

Oxygen Amperometry

O_2 levels were measured by constant potential amperometry (CPA) as described in (McHugh et al., 2011, Francois et al., 2015). Neural activity leads to a neurovascular response increasing blood flow to, and thereby oxygenation of, the active area. An increase in O_2 is therefore an indicator of neural activity. O_2 plays an important role in the metabolic pathway by which ATP is formed due to its ability to accept electrons. This electroactivity can be used to measure the O_2

concentration in tissue. To measure O_2 concentration a carbon paste electrode (CPE) (working electrode), an auxiliary electrode and a silver reference electrode are implanted into the brain. If a potential of -650mV is applied between the working and the reference electrodes O_2 will electrochemically reduce via a two-step function on the working electrode shown below:

$$O_2 + 2H^+ + 2e^- \leftrightarrow H_2O_2$$

$$H_2O_2 + 2H^+ + 2e^- \leftrightarrow 2H_2O$$

This leads to a negative current in the circuit linearly related to the change in oxygen concentration.

Electrodes

CPEs were built as described by Francois et al. (2012). The Teflon insulation of a 8T (200μm bare diameter, 270μm coated diameter) Teflon®-coated silver wire (Advent Research Materials, Suffolk, UK) was slid along the wire to create an approximately 2 mm deep cavity, which was then packed with carbon paste. The carbon paste was a mixture of 7.1g of carbon graphite powder and 2.5ml of silicone oil (both from Sigma-Aldrich, UK). Reference and auxiliary electrodes were silver wires prepared by removing the Teflon insulation from the same 8T wire.

Electrode Calibration

Before implantation, the sensitivity of the electrodes to changes in O_2 was tested. To that end, electrodes were placed in 15ml phosphate-buffered saline solution which was saturated with nitrogen (N_2) gas, atmospheric air, or pure O_2, varying the concentration from 0µM (N_2-saturated), over 240µM (air-saturated) to 1260µM (O_2-saturated) oxygen. Only electrodes that produced currents which were linearly related to the O_2 concentration (least square linear regression, $R^2 \geq 0.98$) were implanted.

Surgery

Implantation of electrodes was conducted by Charles River UK. Animals received Rimadyl (Carprofen 5mg/kg s.c.) pre-operatively and post-operatively. Induction and maintenance of anaesthesia was achieved by isoflurane. CPE electrodes were implanted into the infralimbic region of the prefrontal cortex (from bregma: AP = +3.2, ML = 0.7, DV (from dura) = -4.2) , the dorsal hippocampus (from bregma: AP = +3.0, ML = 2.0, DV (from dura) = -2.8), the nucleus accumbens (from bregma: AP = +1.9, ML = 0.8, DV (from dura) = -6.9) and the retrosplenial cortex (from bregma: AP = -3.2, ML = 0.5, DV (from dura) = -1.5) of the right hemisphere. The reference electrode was implanted into the posterior cortex at a depth of 2mm. The auxiliary electrode was connected to a skull screw placed over the posterior cortex. These electrode locations were used as part of a previous experiment in this cohort concerned with changes in the resting state of the rats.

All electrodes were connected to gold connectors which were in turn cemented into six-pin plastic sockets (Plastic One, Roanoke, VA) during surgery. After completion of surgery rats were housed individually for 7 days to allow recovery and subsequently housed in groups of four.

In Vivo Calibration

To verify the sensitivity of the working electrodes in vivo, tissue oxygen concentration was varied by changing the O_2 content of the air inhaled by the rat, thereby inducing mild hypoxia and hyperoxia. Rats were exposed to gaseous O_2 (BOC medical, Manchester, UK) and N_2 (BOC gases, Guildford, UK) by polyurethane tubing, connected to the appropriate gas cylinder in front of the snout for 30s at a flow rate of 1l/min (Fig. 4.1).

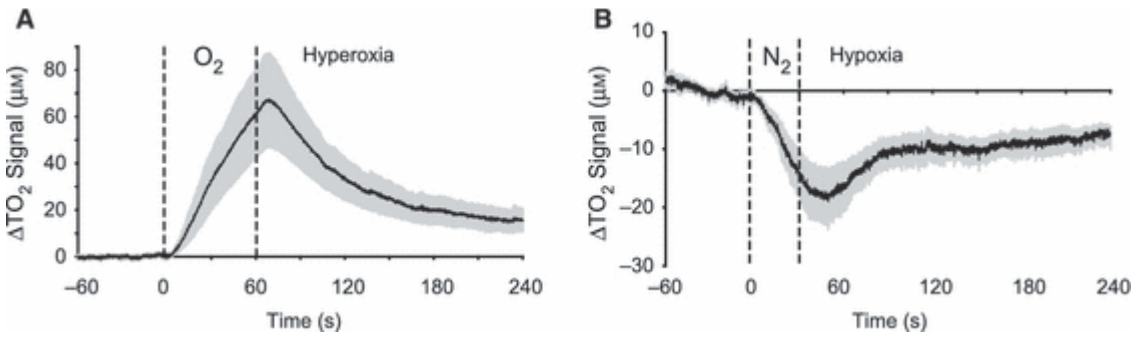


Figure 4.1: In vivo calibration of the working electrode. (A) Exposure to gaseous oxygen from t=0s to t=60s increases tissue oxygenation (hyperoxia) (B) Exposure to nitrogen from t=0s to t=30 decreases tissue oxygenation (hypoxia) from Li et al. (2011)

Data Recording

The 6-pin socket (Plastics One, Roanoke, VA, USA) mentioned above was connected to a four channel potentiostat (Biostat, ACM Instruments, Cumbria,

UK) via a flexible screened 6-core cable (Plastics One). A PowerLab 8/30 was used for analogue/digital conversion and data were collected on a PC running Chart_v5 software (AD Instruments, Oxford, UK).

Data Pre-Processing and Analysis

Data for each of the 4 working electrodes were analysed separately. Visual inspection of the raw data, while blind to the drug condition, suggested that a number of electrodes were broken and had to be excluded (number of broken electrodes: NAc=2, mPFC=3) based on the instability of the signal (Fig 4.2).

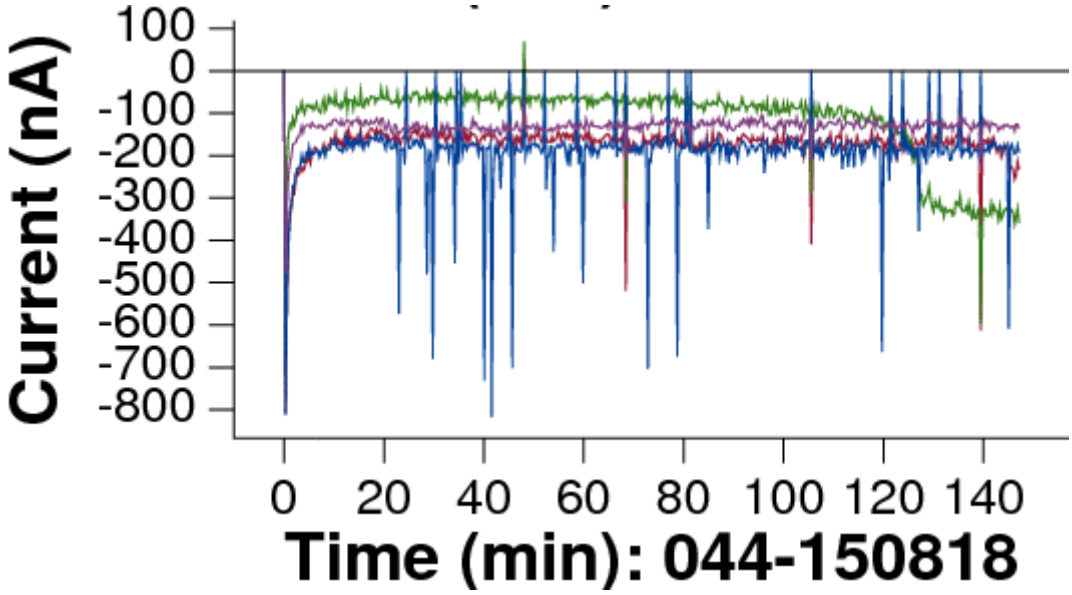


Figure 4.2: Example of the current output at the 4 implanted working electrodes of one animal over the session lasting approximately 140 minutes. Around t=0 all channels displayed a strong upward drift which reduced over the first 15 minutes as the electrodes settle. Two of the electrodes (blue and red) showed rapid transient discontinuities in the current throughout the session and were therefore excluded. The two remaining channels were included into the analysis even though the green channel displayed a slow drift starting around t=120min.

Missing data points were estimated using linear interpolation. Data were pre-processed with custom written Matlab and Bash scripts. Fast noise artefacts

were removed using a standard Butterworth filter (low-pass 0.1 Hz). Subsequently, the data were downsampled into 0.5s bins. Trials were extracted, centred on reward delivery, and baselined. Baselining was conducted by subtracting the last time bin before reward delivery from all other time bins. Hence, relative changes in the O_2 concentration in response to reward delivery were measured. After averaging over all traces of one animal, the area under the curve (AUC) was computed by summing up the data points from the point of reward delivery until 90s after reward delivery. Peak amplitude was calculated by taking the maximal current during the 90s after reward delivery. Latency to reward was defined as the time difference between reward delivery and location of the peak amplitude of the oxygen response.

Drug

LY354740 (provided by Eli Lilly U.K.) was made up in water at a concentration of 10mg/ml. The pH was adjusted to approximately pH 7 by spiking with small aliquots of 2.5M NaOH. All injections were given intraperitoneally 30-45min before the start of the experiment (i.e. before the first reward delivery) at a volume of 1ml/kg, in order to achieve final doses of 10mg/kg. Experiment 4a followed a within-subjects design and experiment 4b was a between-subjects design (vehicle: n = 21, LY354740: n=20).

Results

Experiment 4a: LY354740 Increased Phasic Dopamine in the Nucleus Accumbens Core

LY354740 increased phasic dopamine release in the nucleus accumbens core (n=12) in response to unsignalled rewards (Fig 4.3). A two-way ANOVA with reward magnitude (low, middle, high) and drug (vehicle, LY354740) as within-subjects factors for the area under the curve (AUC) for the 5 seconds after reward delivery revealed that LY354740 increased the dopamine response ($F(1,15) = 6.883$, $p = .019$), and bigger reward magnitude led to a larger dopamine response ($F(2,30) = 21.718$, $p < .001$). A bigger effect of LY354740 in the high reward condition led to a drug by reward magnitude interaction ($F(2,30) = 7.226$, $p = .003$).

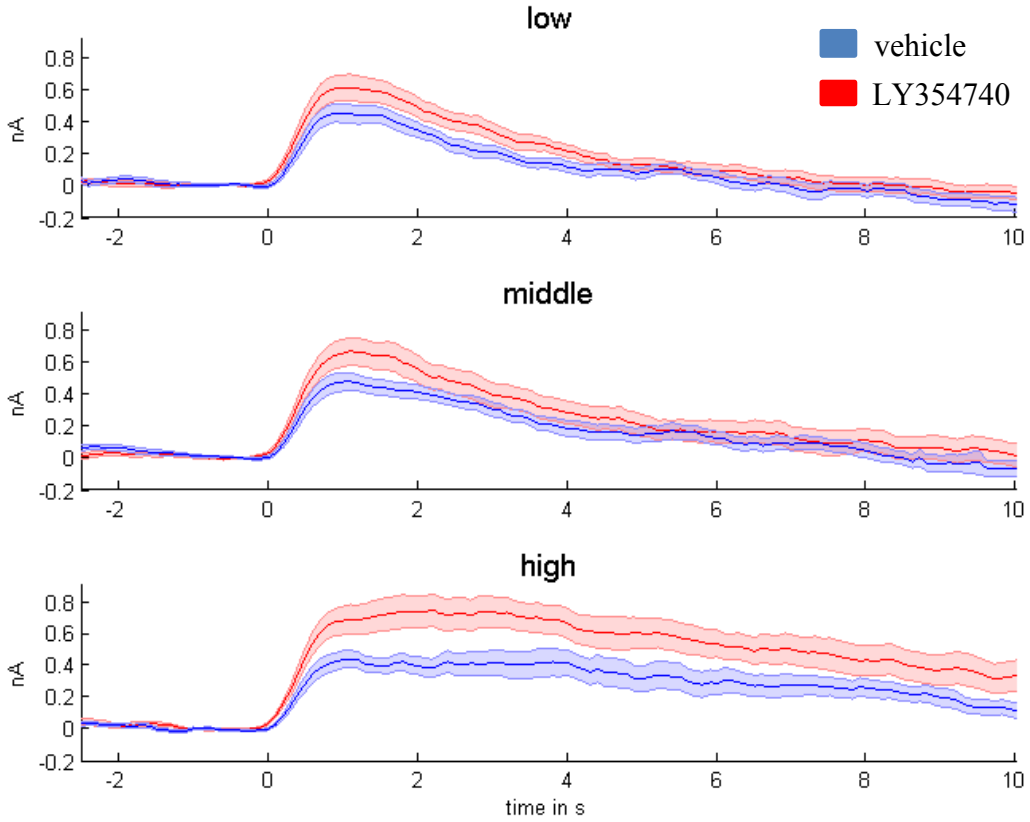


Figure 4.3: Effect of 10mg/kg LY354740 on phasic dopamine responses to different unsignalled reward magnitudes in the nucleus accumbens core. Upper panel: low reward magnitude, middle panel: middle reward magnitude, lower panel: high reward magnitude. Reward delivery began at t=0 (n=12). Data points represent mean ± SEM.

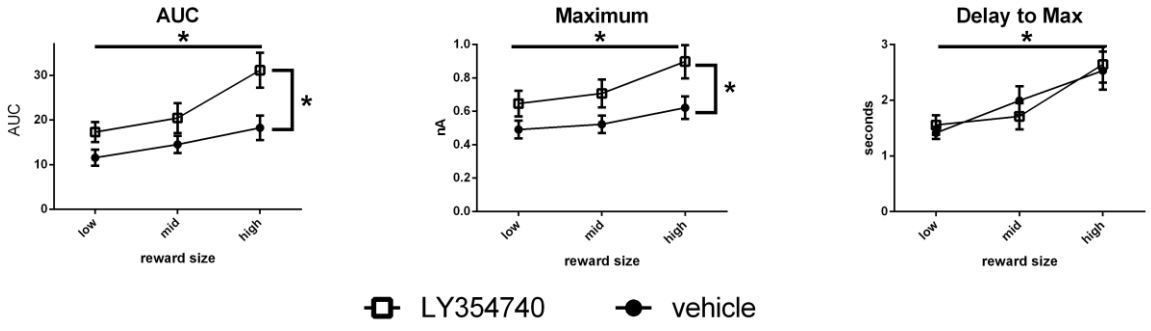


Figure 4.4: Effect of 10mg/kg LY354740 on phasic dopamine responses to unsignalled reward of varying magnitudes: Area under the curve (AUC), maximum, and delay to maximum of the FCSV response to three different reward magnitudes in the NAc core (n=12). Data points represent mean ± SEM. * p < .05

Next, the peak dopamine current in the 5 seconds after reward delivery was analysed. The analysis of the maxima broadly matched the AUC analysis, with an increased peak height in the LY354740 condition ($F(1,15) = 6.385$, $p = .023$), and an increase of the maxima with increasing reward magnitude ($F(2,30) = 15.940$, $p < .001$), but no interaction of drug and reward ($F(2,30) = 2.774$, $p = .078$). LY354740 did not influence the latency to peak ($F(1,15) = 0.002$, $p = .965$) which increased with reward magnitude ($F(2,30) = 9.477$, $p = .001$) (Fig 4.4). Potentially this increase with reward magnitude was due to the slow delivery of reward pellets. Due to limitations of the reward dispenser, it was only possible to deliver pellets every 0.5s. Hence the delivery of the high reward condition lasted approximately 2 seconds.

LY354740 did not change the total number of head entries ($t(10) = 0.008$, $p = .994$) during the session or latencies to collect rewards ($t(10) = -0.168$, $p = .870$) (Fig. 4.5).

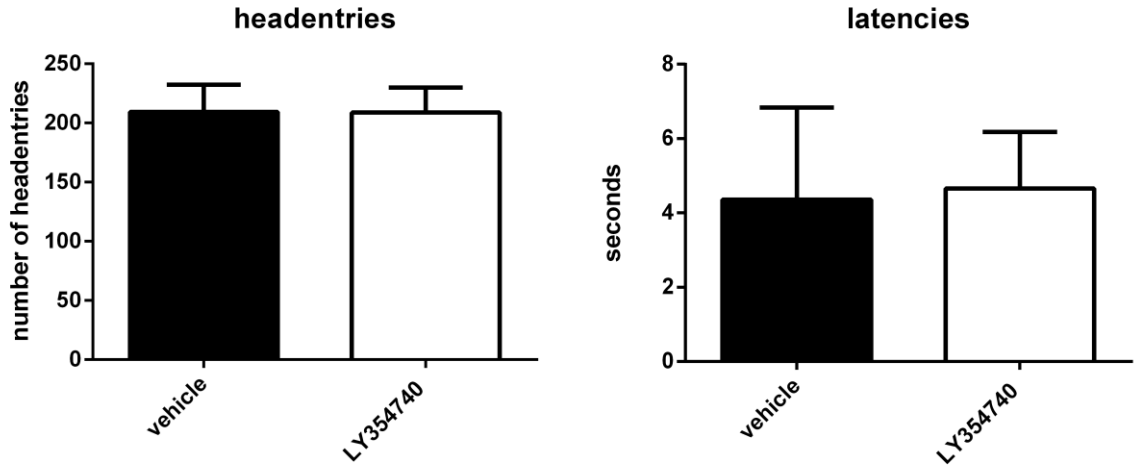


Figure 4.5: Effect of 10mg/kg LY354740 on behaviour during the unsignalled reward task. Total number of head entries during the session (left). Latency to collect reward (right) (n=12). Data points represent mean ± SEM.

Experiment 4b: LY354740 Decreased the O_2 Response in the Retrosplenial Cortex and Dorsal Hippocampus

LY354740 decreased the O_2 response to unsignalled reward in the retrosplenial cortex (RSC) and dorsal hippocampus (dHPC), but not in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). In NAc, mPFC, dHPC, and RSC bigger reward magnitudes were associated with larger O_2 responses.

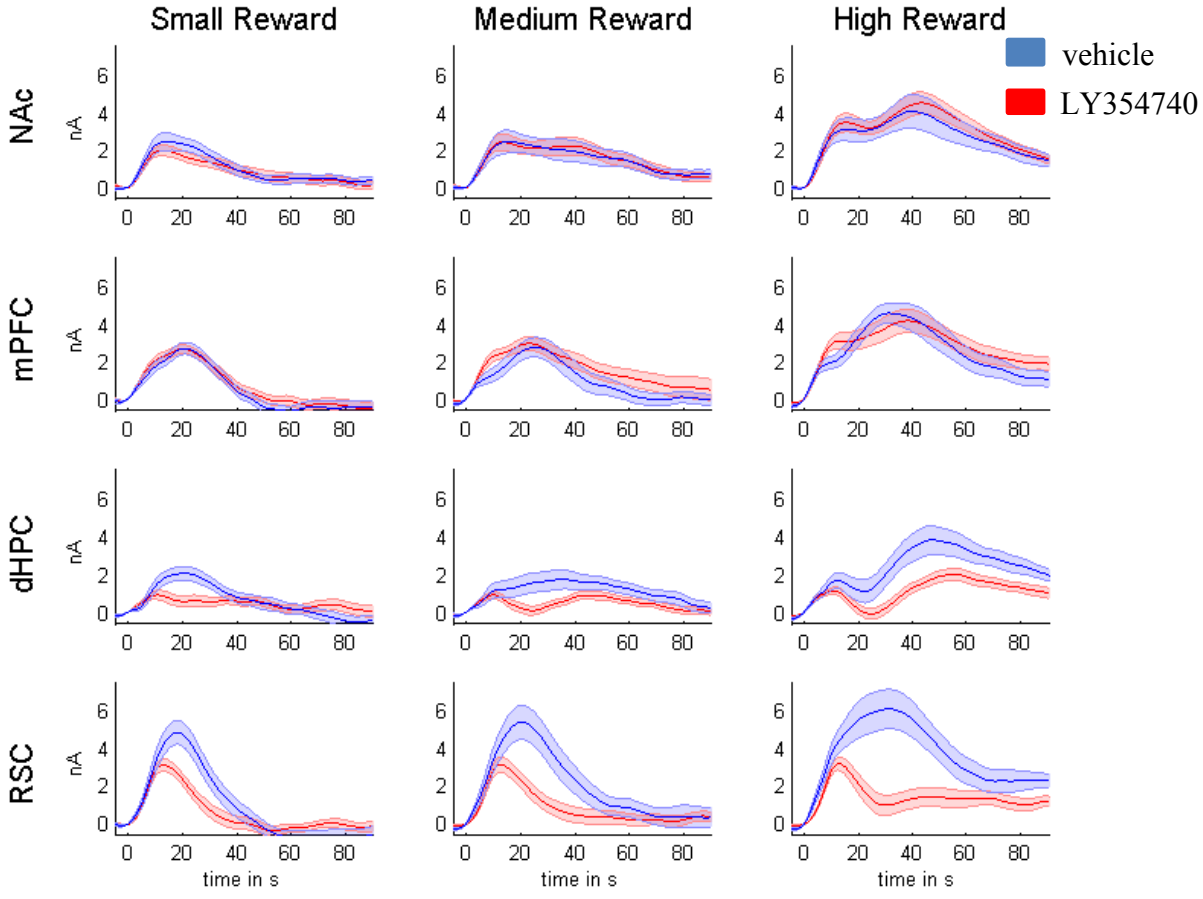


Figure 4.6: The effect of 10mg/kg LY354740 on the O_2 response to varying reward magnitudes in NAc (1st row (veh: n = 19, LY: n=20)), mPFC (2nd row (veh: n = 19, LY: n=19)), dHPC (3rd row (veh: n = 21, LY: n=20)), and RSC (4th row (veh: n = 21, LY: n=20)). 1st column: reward responses to low reward magnitude, 2nd column: reward responses to medium reward magnitude, 3rd column: reward responses to high reward magnitude (n=12). Reward delivery started at t = 0. Data points represent mean ± SEM.

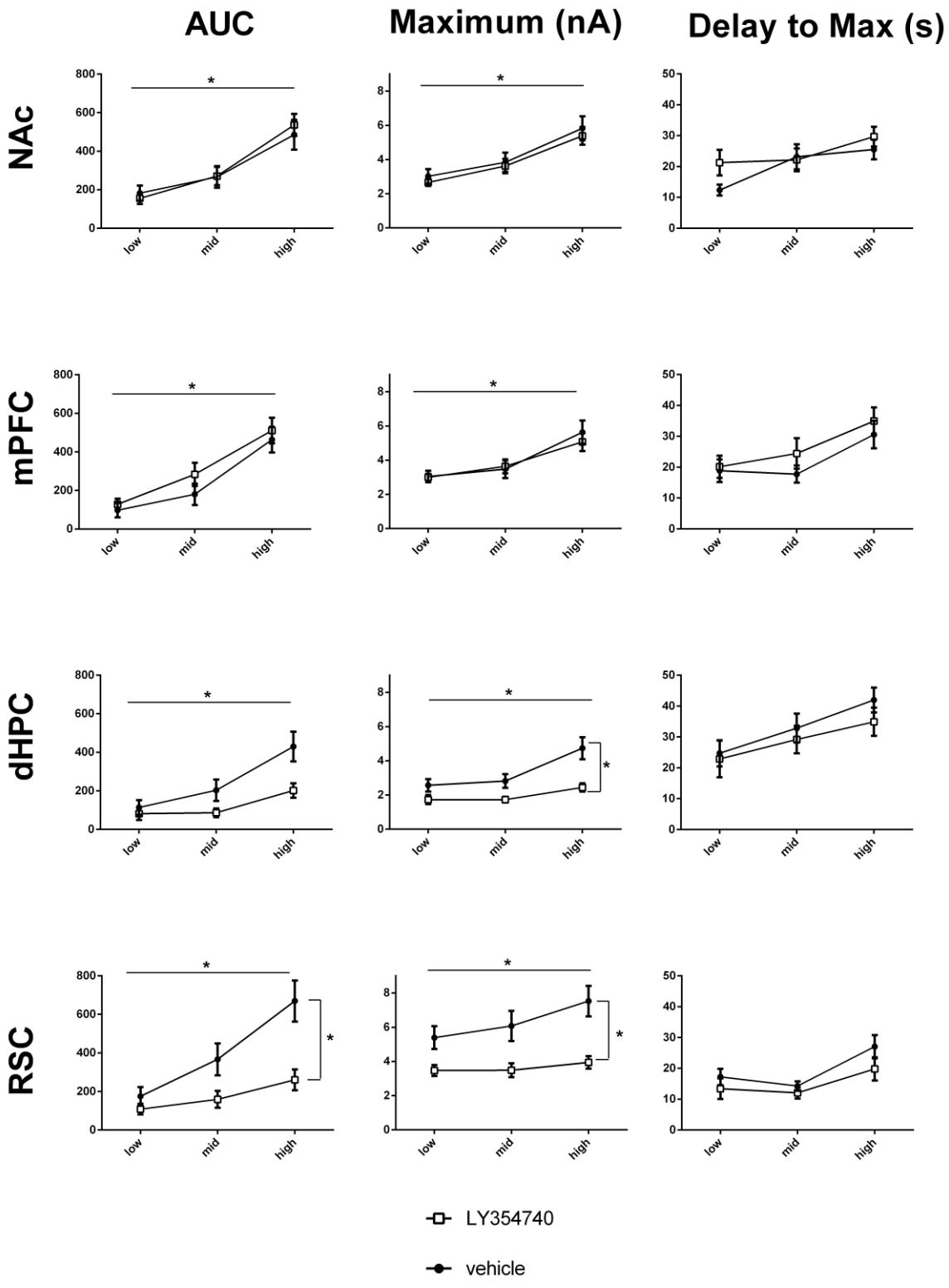


Figure 4.7: The effect of 10mg/kg LY354740 on area under the curve (AUC, 1st column), maximum (2nd column), and delay to maximum (3rd column) of the O_2 response to three different reward magnitudes (low, middle, high) in NAc (1st row (veh: n = 19, LY: n=20)), mPFC (2nd row (veh: n = 19, LY: n=19)), dHPC (3rd row (veh: n = 21, LY: n=20)), and RSC (4th row (veh: n = 21, LY: n=20)). Data points represent mean ± SEM. * p < .05

A three-way ANOVA was conducted with reward magnitude (low, middle, high) and brain region (NAc, mPFC, dHPC, RSC) as within-subjects factors and drug (vehicle, LY354740) as a between-subjects factor on the area under the curve (AUC) after reward delivery (veh: n=17, LY: n=19). This revealed a significant main effect of brain region ($F(3,102) = 3.645$, $p = .015$), primarily driven by a big response in the RSC (Fig. 4.6). Furthermore, the oxygen response increased with reward magnitude ($F(2,68) = 42.953$, $p < .001$) but the main effect of drug just failed to reach significance ($F(1,34) = 3.122$, $p = .086$). However, brain regions differed in their responses to the drug as illustrated by a highly significant drug by brain region interaction ($F(3,102) = 5.012$, $p = .003$).

Analysis of the maxima revealed a significant main effect of drug ($F(1,34) = 7.751$, $p = .009$) (Tab. 4.1, Fig 4.7), brain region ($F(3,102) = 12.179$, $p < .001$), and reward ($F(2,68) = 43.129$ $p < .001$). As in the AUC analysis, brain region interacted with drug ($F(3,102) = 5.178$, $p = .002$).

Analysis of the latency to maxima data revealed no effect of drug ($F(1,34) = 0.032$, $p = .860$) but an effect of brain region ($F(3,102) = 10.042$, $p < .001$) and reward magnitude ($F(2,68) = 24.887$, $p < .001$) (all interactions $p > 0.1$). The effect of brain region is not surprising since neurons in different brain regions show different latencies to respond to stimuli (Boynton et al., 1996, Talavage et al., 2004).

	AUC		Maximum		Latency to Max	
	F	P	F	p	F	p
Brain Region	F(3,102) = 3.645	**.015**	F(3,102) = 12.179	**<.001**	F(3,102) = 10.042	**<.001**
Brain Region *Drug	F(3,102) = 5.012	**.003**	F(3,102) = 5.178	**.002**	F(3,102) = 1.952	.126
Reward	F(2,68) = 42.953	**<.001**	F(2,68) = 43.129	**<.001**	F(2,68) = 24.887	**<.001**
Reward *Drug	F(2,68) = 1.418	.249	F(2,68) = 2.741	.072	F(2,68) = .363	.697
Brain Region * Reward	F(6,204) = 1.806	.099	F(6,204) = 4.882	**<.001**	F(6,204) = 1.030	.407
Brain Region *Reward *Drug	F(6,204) = 4.166	**.001**	F(6,204) = 2.025	.064	F(6,204) = .684	.663
Drug	F(1,34) = 3.122	.086	F(1,34) = 7.751	**.009**	F(1,34) = .032	.860

Table 4.1: Results of a three way ANOVA with reward magnitude (low, middle, high) and brain region (NAc, mPFC, dHPC, RSC) as within-subjects factors and drug (vehicle, LY354740) as a between-subjects factor on the area under the curve (AUC), the maximum, and the latency to maximum of the reward-evoked tissue oxygen response in experiment 4b.

Single Brain Regions

To investigate the interactions seen in the AUC and maxima data, each brain region was analysed separately with a two-way ANOVA with both drug and reward magnitude as factors.

NAc

The haemodynamic response in the NAc (veh: n = 19, LY: n=20) was stronger to higher reward magnitudes ($F(2,74) = 46.407$, $p < .001$) but showed absolutely no effect of drug ($F(1,37) = 0.587$, $p = .559$) nor drug by reward magnitude interaction ($F(2,74) = 0.023$ $p = .879$) in the AUC. This result was corroborated by the analysis of the maxima (Tab. 4.2).

	AUC		Maximum	
	F	p	F	P
Reward	F(2,74)=46.407	<.001	F(2,74)=45.148	<.001
Drug	F(1,37) = .587	.559	F(1,37) = .072	.931
Drug * Reward	F(2,74) = .023	.879	F(2,74) = .316	.577

Table 4.2: Results of a two-way ANOVA with both drug and reward magnitude as within-subjects factors on the area under the curve and maximum of the reward-evoked tissue oxygen response in the NAc.

mPFC

The O_2 response in mPFC (veh: n = 19, LY: n=19) displayed the same pattern as the NAc. The mPFC responded stronger to higher reward magnitude ($F(2,72) = 32.697$, $p < .001$) and showed no effect of drug ($F(1,36) = 0.324$ $p = .724$), or drug by reward magnitude interaction ($F(2,72) = 1.269$, $p = .267$) in the AUC. This result was again corroborated by the analysis of the maxima (Tab. 4.3).

	AUC		Maximum	
	F	P	F	P
Reward	F(2,72)=32.697	<.001	F(2,72)=1.658	<.001
Drug	F(1,36) = .324	.724	F(1,36) = .744	.479
Drug * Reward	F(2,72) = 1.269	.267	F(2,72) = .067	.798

Table 4.3: Results of a two-way ANOVA with both drug and reward magnitude as within-subjects factors on the area under the curve and maximum of the reward-evoked tissue oxygen response in the mPFC.

dHPC

LY354740 decreased the haemodynamic response in the dHPC(veh: n = 21, LY: n=20). Investigation of the AUC revealed an effect of reward magnitude ($F(2,78) = 15.615$, $p < .001$) and a reward magnitude by drug interaction ($F(2,78) = 6.690$, $p = .014$). Visual inspection of the data (Fig 4.2) suggests that LY354740 had a big effect on higher rewards and a small to no effect on the small reward which explains the failure of the overall main effect of drug to reach significance ($F(1,39) = 2.868$ $p = .063$). Analysis of the maxima did reveal a similar pattern with the difference that the maxima displayed a main effect of drug ($F(1,39) = 4.910$ $p = .010$) (Tab. 4.4).

	AUC		Maximum	
	F	p	F	P
Reward	F(2,78)=15.615	<.001	F(2,78)=20.720	<.001
Drug	F(1,39) = 2.868	.063	F(1,39) = 4.910	.010
Drug * Reward	F(2,78) = 6.690	.014	F(2,78) = 9.142	.004

Table 4.4: Results of a two-way ANOVA with both drug and reward magnitude as within-subjects factors on the area under the curve and maximum of the reward-evoked tissue oxygen response in the dHPC.

RSC

The O_2 response in the RSC(veh: n = 21, LY: n=20) was decreased by LY354740 injection ($F(1,39) = 6.352$, $p = .003$), and increased with rising reward magnitude ($F(2,78) = 22.878$, $p < .001$). The interaction of LY354740 with reward magnitude was significant ($F(2,78) = 8.730$, $p = .005$). Visual inspection of this

interaction revealed that LY354740 abolished the effect of reward magnitude seen in the vehicle group. The results of the maxima agreed with the analysis of the AUC (Tab. 4.5).

	AUC		Maximum	
	F	P	F	P
Reward	F(2,78)=22.878	<.001	F(2,78)=13.838	<.001
Drug	F(1,39) = 6.352	.003	F(1,39) = 5.303	.007
Drug * Reward	F(2,78) = 8.730	.005	F(2,78) = 9.851	.003

Table 4.5: Results of a two-way ANOVA with both drug and reward magnitude as within-subjects factors on the area under the curve and maximum of the reward-evoked tissue oxygen response in the RSC.

Behaviour

LY354740 injected rats made more head entries into the food magazine across the whole session ($t(40) = 2.324$, $p = .025$) than vehicle treated rats (Fig. 4.8) (veh: n=21, LY: n=20). At face value, this was surprising considering the apparent sedative properties reported in earlier chapters. However, observations of the rats during the task using a camera, suggested that LY354740-injected rats mainly remained in front of the food magazine whereas control animals continued to explore the box.

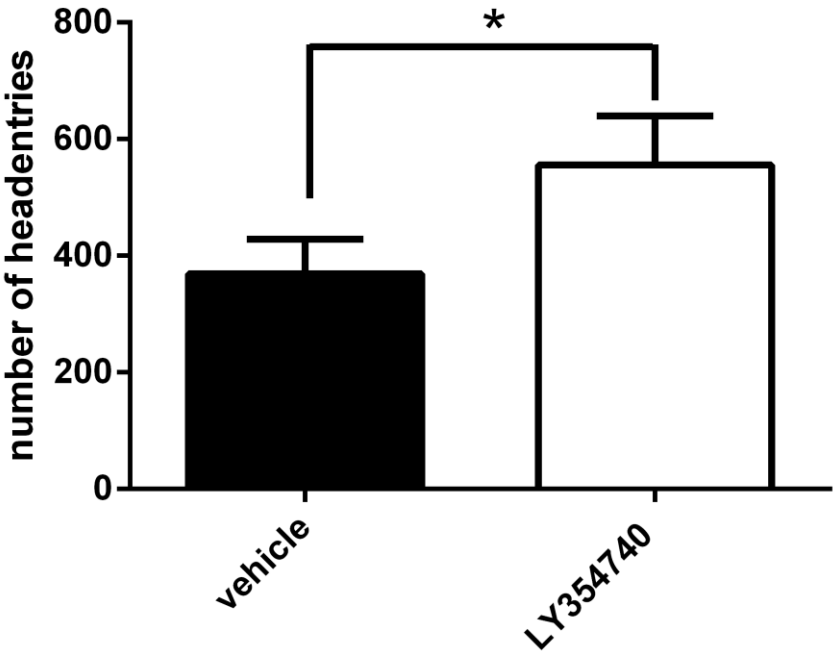


Figure 4.8: The effect of 10mg/kg LY354740 on the total number of head entries during the unsignalled reward test session (vehicle: n = 21, LY354740: n=20). Data represent mean ± SEM. * p < .05

Discussion

LY354740 increased the phasic dopamine response in the nucleus accumbens core to unsignalled rewards

In chapter 3 locomotor activity was used as readout of dopamine levels. LY354740 showed a state-dependent effect on amphetamine-induced locomotor activity. LY354740 decreased hyperactivity in naïve rats and exacerbated hyperactivity in food restricted animals that expected food. I hypothesized that LY354740 reduced dopamine levels independent of the state of the animal. I argued that the exacerbation of amphetamine-induced hyperactivity could be explained by the reduction of subtle stereotypies rather than an increase in

dopamine levels. However this hypothesis rested on the presence or absence of stereotypies, which are difficult to monitor. Therefore, phasic dopamine levels were directly measured in this chapter. Surprisingly, this experiment showed that LY354740 increased the phasic dopamine response in the nucleus accumbens core to unsignalled rewards. In light of this new evidence, it seems more likely that LY354740 has different effects on amphetamine-evoked dopamine level increases based on the state of the animal. In ad lib fed animals LY354740 would reduce amphetamine-induced increases of the dopamine levels as shown by Pehrson and Moghaddam (2010) using microdialysis. In food restricted rats that expect reward, however, LY354740 increases dopamine levels as shown in experiment 4a.

One limitation of this study however is the missing control condition of naïve ad-lib fed rats. Therefore, it cannot be concluded what led LY354740 to increase the dopamine response. It is possible that LY354740 increases phasic dopamine even in naive ad lib fed rats. However, several factors make it unpractical to run such a control condition with FCV in ad lib fed rats:

- Rats have to be extensively handled to habituate them to being plugged into the recording equipment so they will never be comparable to experimentally naïve animals in this regard.
- It is questionable whether ad lib fed rats would be motivated to readily collect food rewards or would show a phasic dopamine response to the rewards at all.

Another limitation is that FCV can only measure phasic changes of the dopamine levels. Currently absolute dopamine concentrations around the FCV electrode cannot be assessed. However, LY354740 does not affect absolute baseline dopamine levels as measured by microdialysis in ad lib fed rats (Moghaddam and Adams, 1998). It would therefore be surprising if LY354740 were to affect baseline levels in food restricted rats. However, the possibility cannot be excluded.

LY354740 did not affect reward-evoked tissue oxygen responses in the NAc or mPFC, but did affect responses in the dHPC and RSC

The oxygen response to unsignalled reward in the NAc, however, was not affected by LY354740. This seems surprising given the strong binding of LY354740 in the striatum (Higgins et al., 2004) and the above reported increase in dopamine release. On the other hand, LY354740, did not affect local glucose use in the NAc of conscious rats in an open field (Lam et al., 1999), suggesting activity levels in this region remained unchanged by LY354740. This raises the question how big an effect dopamine has on the neural activity in the NAc given the clear effect of LY354740 on phasic dopamine in the FCV study. It has previously been shown that dopamine release drives neurons in the NAc (Cheer et al., 2007). However, dopamine is a modulatory neurotransmitter. Therefore it cannot be taken for granted that relatively short transient increases in dopamine (ca 5s) can drive the neuronal activity to such a degree as to affect tissue oxygenation on the time scale measured this experiment (ca 90s).

The duration of the oxygen signals in experiment 4b are by themselves interesting. Francois et al. (2012) report reward-evoked tissue oxygen level responses in the NAc lasting approximately 30s. This is roughly similar to NAc responses in the small reward condition (Fig. 4.6, uppermost left panel). However, the duration of these responses increased massively as reward magnitude increased. The increased duration of reward delivery cannot explain this, as delivery of the high reward magnitude only lasted 2s and the tissue oxygen response in the NAc in this condition lasted approximately 90s. No video recordings of the animals were made during experiment 4b. Therefore their behaviour could not be investigated further to find potential behaviours correlated with these longer lasting tissue oxygen responses.

The comparison of phasic dopamine (experiment 4a) and tissue oxygen levels (experiment 4b) between experiments rests on the assumption that underlying physiology of the rats in both experiments was the same. We just observed different readouts in the two experiments. That is to say, if we were to measure tissue oxygen levels and phasic dopamine responses in the same animal we would make the same finding of increased dopamine and unchanged oxygen levels. However, experiment 4a (FCV) and experiment 4b (oxygen amperometry) differed in multiple ways apart from being run in different research establishments:

- Experiment 4a was conducted in SD rats whereas experiment 4b was conducted in Wistar rats.

- Because the O_2 response was so long lasting, experiment 4b was conducted as a VI 120 rather than a VI 60. This, however, should not have fundamentally changed the psychological or physiological process underlying the activity changes.
- The O_2 cohort (experiment 4b) displayed higher head entry levels in the LY354740 condition. No such difference was found in the FCV cohort (experiment 4a). This suggests that LY354740 potentially had a different effect in the animals in the two experiments.
- The FCV cohort was highly trained over more than 3 months in a previous experiment whereas the oxygen amperometry cohort was only trained for 3 weeks prior to the experiment.
- Experiment 4a consisted of 12 trials lasting 12 minutes. Experiment 4b consisted of 60 trials over two hours. This ought not to be problematic since LY354740 has a long half-life (personal communication with Gary Gilmour) and the analysis of the first 30 minutes of the amperometry experiment gave similar results to the analysis of all 60 trials across two hours (data not shown).
- Due to the rats used in experiment 4b being still in use as part of a different study, electrode placement could not be verified histologically as of yet.

Still, the tasks used in experiment 4a and 4b were broadly the same. Hence, the effect of LY354740 on the animals should be the same. However, the lack of electrode placement data should be considered when interpreting the O_2 data from experiment 4b. O_2 responses in the dHPC and RSC were reduced by

LY354740 which had no effect in the mPFC and NAc. Given the literature, the reduction in activity in the dorsal hippocampus by LY354740 is not surprising. There is strong expression of group II mGluRs in the hippocampus (Shigemoto et al., 1997) which act as spill over detectors in this region (Scanziani et al., 1997), reducing glutamate release when activated. And indeed in the hippocampus LY354740 decreases glutamate release (Kilbride et al., 1998, Higgins et al., 2004, Price et al., 2005), local glucose use in conscious rats (Lam et al., 1999) and anxiety-induced neural activity (Linden et al., 2004). Therefore, given these various measures of reduced neural activity in the hippocampus following LY354740, it is not surprising that tissue oxygen responses were also reduced in this region. LY354740 also decreased activity in the retrosplenial cortex, an area which has been related to memory function (Vann et al., 2009) and which shows reward responses (Tabuchi et al., 2005).

In this chapter I showed that LY354740 can under some conditions increase phasic dopamine release in the nucleus accumbens core. So far all licensed antipsychotics act as antagonists on the dopaminergic D2 receptor (Seeman and Lee, 1975, Kapur and Mamo, 2003) thereby inhibiting the dopaminergic system. Even though group II mGluR agonists would be the first antipsychotics without activity on the D2 receptor they would still be expected to reduce dopaminergic activity in the striatum. Therefore, the evidence presented in this and the previous chapter are problematic for the role of LY354740 as a putative antipsychotic.

Chapter 5: The Effect of LY354740 on the GluA1 Knockout Mouse

Introduction

In chapter 3 and 4 of this thesis I described the impact of LY354740 on the dopamine system. Somewhat surprisingly, it appeared that under some circumstances LY354740 exacerbated the effect of amphetamine and could result in enhanced phasic dopamine responses. This starts to question the utility of LY354740 as an antipsychotic.

However, widespread interest into group II mGluR agonists derived originally from their ability to ameliorate the effect of acute pharmacological glutamatergic manipulations relevant to schizophrenia. Moghaddam and Adams (1998) showed that LY354740 partially rescued PCP-induced working memory deficits in rewarded alternation. Moghaddam and Adams (1998) used PCP because the compound was known to induce psychosis in humans and target the NMDA receptors which are involved in the pathology of schizophrenia (Javitt and Zukin, 1991, Moghaddam and Javitt, 2012). However, schizophrenia is a neurodevelopmental disorder in which genetics play an important role. Humans that are born with a certain genetic make-up are more likely to develop schizophrenia. Twin studies suggest a heritability of schizophrenia of around 80% (Cardno et al., 1999, Mueser and McGurk, 2004). Acute pharmacological manipulation cannot catch this aspect of the disorder. Therefore, genetic manipulations in mice were used in this chapter to investigate the effect of

LY354740 on behavioural changes caused by glutamatergic dysfunction relevant to schizophrenia.

As described in chapter 1 the GluA1 knockout mouse shows some behavioural changes relevant to schizophrenia (Barkus et al., 2014). GluA1 KO mice have a deficit in short-term memory and so do not show short-term habituation (Sanderson et al., 2007, Sanderson et al., 2010, Sanderson et al., 2011a, Sanderson et al., 2011b).They cannot habituate their responses to recently presented stimuli. Thus, they pay inappropriately high and prolonged levels of attention to them. Such changes may also contribute to the aberrant salience seen in schizophrenia (Kapur, 2003).

This chapter will test whether LY354740 can rescue this deficit in short-term memory in GluA1 KO mice as assessed by rewarded alternation on a T-maze (the task used by Moghaddam and Adams (1998)). LY354740 has already been shown to be able to ameliorate the working memory deficits evoked by another glutamatergic manipulation, systemic PCP injection. Furthermore, LY354740 reduces hippocampal glutamate levels (Kilbride et al., 1998, Higgins et al., 2004, Price et al., 2005) which are known to be increased in hippocampal tissue samples from GluA1 KO mice (Chourbaji et al., 2008). This suggests that it may be possible for LY354740 to ameliorate the deficits in short-term memory seen in GluA1 KO mice.

In addition, similarly to chapter 3, locomotor activity tests were conducted to assess the effect of LY354740 on the hyperactivity in the GluA1 KO mouse. Notably, this effect has already been investigated. Procaccini et al. (2013) have

shown that LY354740 reduces hyperactivity in GluA1 KO mice at doses of 15m/kg and 30mg/kg. They found that this suppression of hyperactivity was stronger in the male KO mice than in female KO mice. This effect was driven by higher baseline activity in the male KO mice compared to female KO mice thereby giving male KO mice a greater window for the drug to produce a reduction in locomotor activity. Additionally, Procaccini et al. (2013) showed a reduction in c-Fos, a marker of neural activity, in the CA1 subunit of the dorsal hippocampus by LY354740. This effect was again driven by male GluA1 KO mice.

So, LY354740 has been shown to ameliorate hyperactivity in GluA1 KO mice (Procaccini et al., 2013) and to reverse the short-term memory impairment induced by a glutamatergic manipulation (Moghaddam and Adams, 1998). Therefore, I investigated if a group II mGluR agonist (LY354740) would rescue the spatial working memory deficit in GluA1 KO mice measured in a rewarded T-maze alternation task at a dose where it has been shown to reduce the locomotor hyperactivity seen in GluA1 KO mice.

Methods

Experiment 5A-C: The Effect of the group II mGluR Agonist LY354740 on Spatial Short-Term Memory during Rewarded Alternation Testing in GluA1 KO mice.

In Experiment 5A rewarded alternation was tested using a within-subjects procedure as described in Chapter 2 with an inter-trial interval of 6 to 10 minutes. Wildtype (WT) (female: N = 6; male: N = 5) and GluA1 KO mice (female: N = 7; male: N = 8) first received 30 trials of drug-free testing (five trials per day for six days). Each animal was then tested in the T-maze following an injection of either LY354740 (15mg/kg) or vehicle. After injection animals were returned to their home cages for 30 minutes before behavioural testing commenced. Each animal was then given 10 trials of rewarded alternation in the T-maze. Starting twenty-four hours after the first round of drug testing the animals were retrained in the absence of any drug treatment (with 3 sessions of 5 trials per session in Experiment 5A, and one session of 10 trials in Experiments 5B and 5C) to test whether LY354740 had any long lasting effects on rewarded alternation. On the day following retraining, mice that had previously received LY354740 now received vehicle injections, and vice versa for the remaining mice. The order of drug exposure was counterbalanced within genotype and sex as far as possible given the numbers of mice. The experimenter was blind to the genotype and drug allocations of the animals throughout testing. One male GluA1 KO mouse had to be excluded during testing due to a lack of locomotion in the drug condition.

In Experiment 5B, male wildtype (N = 7) and GluA1 KO mice (N = 7) underwent the same procedure as for Experiment 5A but now they received either vehicle or 30mg/kg LY354740. The rewarded alternation experiments conducted so far in this chapter have a longer inter-trial interval than the ones conducted in chapter 3 or by Moghaddam and Adams (1998). This changes the degree to which memory traces from previous trials interfere with performance on the current trials, the so called proactive interference effect. Therefore subsequently, in Experiment 5C, to investigate the potential effects of proactive interference the procedure used in Experiment 5B was repeated in the same mice using the same drug dose (30mg/kg), but now using a modified testing protocol in which the interval between trials was reduced to 20s. This procedure more closely matches that used by Moghaddam and Adams (1998) in which LY354740 reversed the effects of the non-competitive NMDAR antagonist PCP.

Experiment 5D-F: The Effect of the group II mGluR agonist LY354740 on Spontaneous Locomotor Activity in GluA1 KO mice.

In Experiment 5D, mice that had previously been tested in Experiment 5A were allowed ad libitum access to food and were then tested for spontaneous locomotor activity in clear plastic cages (26cm x 16cm x 17cm), containing clean sawdust. Activity levels were assessed by an array of infrared photobeam sensors as described in chapter 2. Immediately after an injection of either 15mg/kg LY354740 or vehicle mice were placed individually into test cages. The number of beam breaks was recorded in 24 bins of 5 minutes over a 2-hour

session. After the first round of testing (and following a washout period of 7 days), mice were tested in the locomotor cages with the vehicle/drug allocations reversed. In the second test mice were allocated a cage that was different from the first test session. The order of drug/vehicle injections was counterbalanced for sex and genotype as far as possible.

In Experiment 5E experimentally naïve wildtype (female: N = 6; male: N = 6) and GluA1 KO mice (female: N = 6; male: N = 5) that had been maintained with ad libitum access to food were also tested for locomotor activity using an identical protocol as in Experiment 5D but now with vehicle and 30mg/kg LY354740. Finally, in Experiment 5F the mice used in Experiments 5B and 5C were maintained on food restriction (to resemble the conditions used for T-maze testing) and locomotor activity assessed with either the 30mg/kg dose of the drug or vehicle as described above.

Drugs

LY354740 was made up in sterile water (Norbrook) to a final concentration of 1.5 or 3 mg/ml with the pH being adjusted with 1M NaOH to pH 7. Injections were given intraperitoneally at 10ml/kg resulting in a dose of either 15mg/kg or 30mg/kg. These doses were chosen following the observation by Procaccini et al. (2013) that 15 and 30mg/kg reduced the hyperactivity of male GluA1 KO mice with minimal effect on WT controls.

Results

Experiment 5A: LY354740 (15mg/kg) had no Effect on Rewarded Alternation Testing in GluA1 KO and WT mice.

GluA1 KO mice were impaired during the pre-training stage on the rewarded alternation T-maze task (mean percent alternation: WT = 70.91 ±3.24 SEM; GluA1 KO mice = 53.11% ±1.80 SEM; $F(1,22) = 24.017$ $p < .001$, WT: n=11, KO: n=15, Fig. 5.1A) as has been shown previously (Reisel et al., 2002, Taylor et al., 2011). There was no main effect of sex ($F(1,22) = 0.572$, $p = .458$) or genotype by sex interaction ($F(1,22) = 0.153$, $p = .700$). During the drug testing phase (Fig. 5.1B) GluA1 KO mice were still impaired compared to WT mice ($F(1,21) = 4.743$, $p = .041$), but there was no significant effect of drug and it did not significantly interact with genotype (both F values < 1, $p > .9$). There was also no significant main effect of sex ($F(1,21) = 0.154$, $p = .698$) and sex did not interact with other factors (F values < 1, p values > 0.30). The 15mg/kg dose of the drug had no effect on the running latencies of the mice on either the sample ($F(1,21) = 1.121$, $p = .302$, Fig. 5.1C) or choice runs ($F(1,21) = 0.767$, $p = .391$, Fig. 5.1D), and there was no significant interaction between drug and genotype (F values < 1, p values > 0.50). However, GluA1 KO mice took longer to complete both sample and choice runs (F values > 10.00, p values < 0.005). There were no other significant main effects or interactions (p values > 0.06).

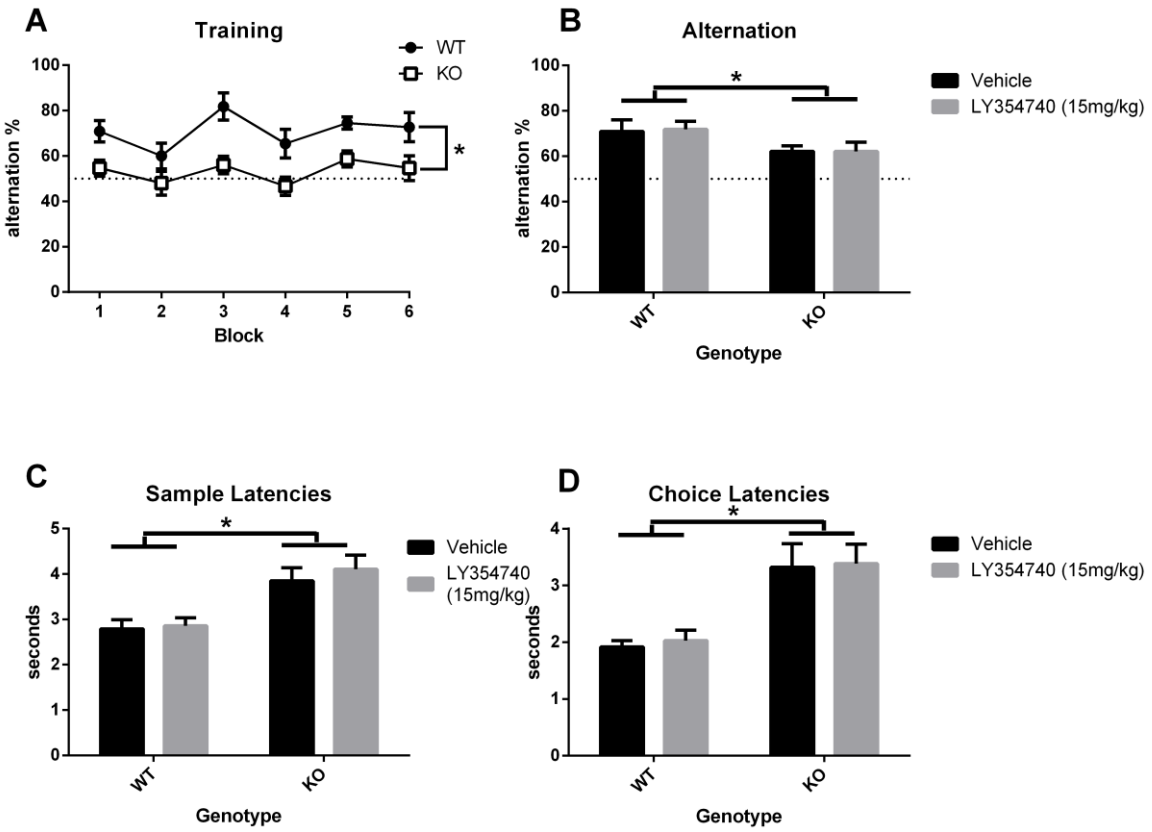


Figure 5.1: Rewarded alternation in GluA1 KO and WT mice in 15mg/kg LY354740 and a vehicle condition: (A) alternation during the training phase. The dotted line represents chance performance at 50% (B) alternation during the drug phase. (C) Latency in seconds to consume reward during the sample run. (D) Latency in seconds to enter the goal arm during the choice run (WT: n=11, KO: n=15). Error bars indicate ± SEM. * p < .05

In the off-drug retraining sessions (conducted between drug/vehicle sessions) mice performed at levels comparable to pre-drug training (mean percent alternation: WT = 74.58 ±5.45 SEM; GluA1 KO mice = 63.11% ± 2.75 SEM; $F(1,22) = 5.141$, $p = .034$). There was once again no effect of sex ($F(1,22) = 2.722$, $p = .113$), nor genotype by sex interaction ($F(1,22) = 2.438$, $p = .133$).

Experiment 5B: LY354740 (30mg/kg) had no Effect on Rewarded Alternation Testing in GluA1 KO and WT mice.

Since LY354740 showed no effect on alternation performance or latencies at a dose of 15mg/kg (WT: n=7, KO: n=7), the same T-maze task was conducted with an LY354740 dose of 30mg/kg in a separate naïve cohort. As found previously (experiment 5A), the GluA1 KO mice failed to alternate during the pre-training phase (mean percent alternation: WT = 80.95% ±2.15 SEM; GluA1 KO mice = 46.67% ±2.30 SEM; $F(1,12) = 118.718$, $p < .001$, Fig. 5.2A). The 30mg/kg dose of LY354740 also failed to affect alternation performance ($F(1,12) = 2.043$, $p = .178$) and drug treatment did not interact with genotype ($F(1,12) = 1.149$, $p = .305$, Fig. 5.2B). The pronounced deficit in the GluA1 KO mice, however, remained throughout testing with drug and vehicle ($F(1,12) = 24.608$, $p < .001$). A 30mg/kg dose prolonged running latencies in both the sample ($F(1,12) = 9.805$, $p = .009$ Fig. 5.2C) and choice runs ($F(1,12) = 12.539$, $p = 0.004$, Fig. 5.2D). GluA1 KO mice now completed trials quicker than WT mice, but the effect of genotype failed to reach significance ($F(1,12) < 4.20$, p values > .055). The interaction between drug and genotype also failed to reach significance ($F(1,12) < 2.40$, p values > 0.14). In the off-drug retraining session mice performed at levels comparable to pre-drug training (mean percent alternation; WT = 84.29 ±2.98 SEM, GluA1 KO mice = 51.43% ±2.61 SEM, $t(12) = 8.307$, $p < .001$).

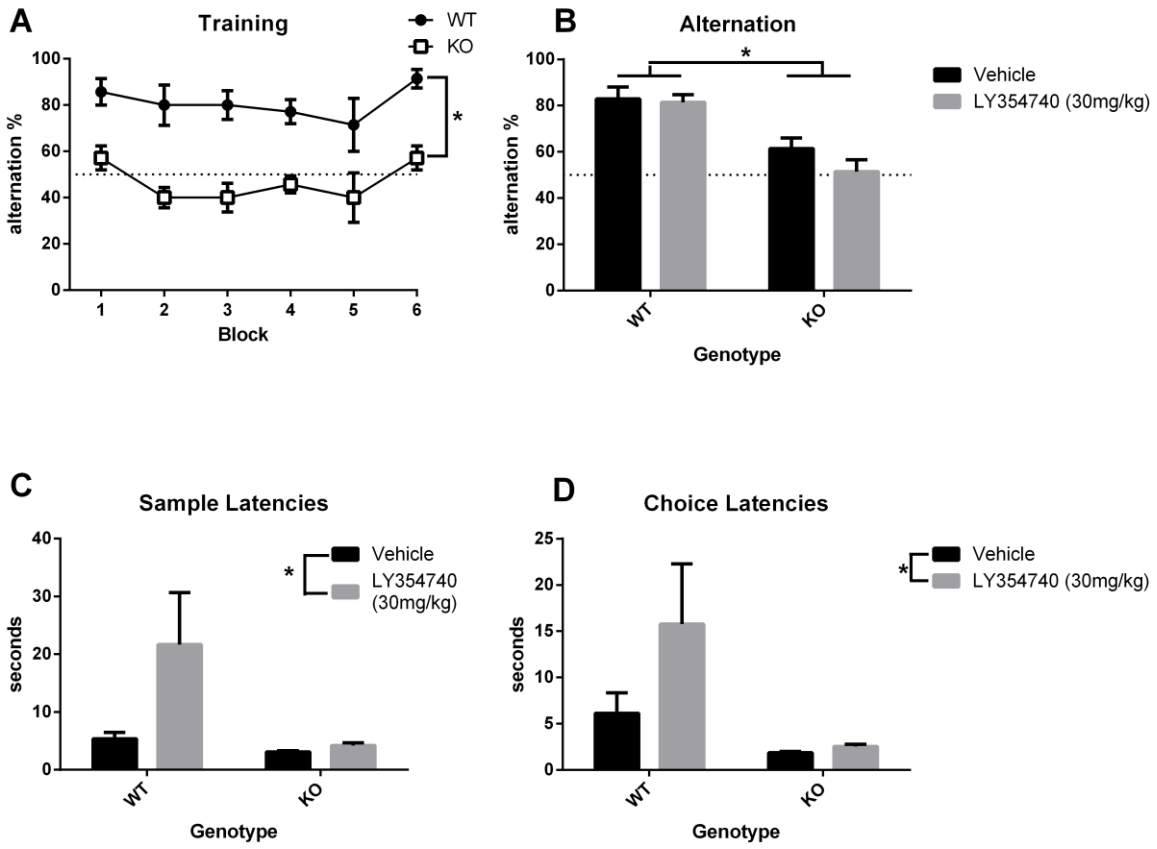


Figure 5.2: Rewarded alternation in GluA1 KO and control mice in the 30mg/kg LY354740 and vehicle condition: (A) Alternation during the training phase. The dotted line represents chance performance at 50% (B) alternation during the drug phase. (C) Latency in seconds to consume reward during the sample run. (D) Latency in seconds to enter the goal arm during the choice run (WT: n=7, KO: n=7). Error bars indicate ± SEM. * p < .05

Experiment 5C: LY354740 (30 mg/kg) had no Effect on Rewarded Alternation Testing with a Short Inter-Trial Interval in GluA1 KO and WT mice.

Given the lack of any effect of the group II mGluR agonist on spatial working memory performance, the rewarded alternation testing paradigm was changed to more closely resemble that used by Moghaddam and Adams (1998), in which LY354740 ameliorated the effect of the non-competitive NMDAR antagonist,

PCP, on rewarded alternation. This was achieved by reducing the inter-trial interval from 6-10 min to 20 s. The pronounced deficit in the GluA1 KO mice was still evident with the short inter-trial interval during the drug-free training stage (WT = 73.33% ±4.24SEM; GluA1 KO mice = 49.52% ±3.89 SEM; $F(1,12) = 17.16$, $p = .001$; WT: n=7, KO: n=7). During the drug test sessions, there was still no significant main effect of drug ($F(1,12) = 1.278$, $p = .280$), and no interaction with genotype ($F(1,12) = 0.888$, $p = .365$). However, there was still an overall substantial main effect of genotype ($F(1,12) = 15.517$, $p = .002$, Fig. 5.3A), reflecting the continued and persistent short-term memory deficit in the knockout mice. Given the results of Aultman and Moghaddam (2001), and the results of experiment 3a, there was an a priori prediction that LY354740 would impair performance in wildtypes. A paired sample T-test, however, found no reduction of alternation in the wildtypes in the drug condition compared to vehicle ($t(6) = 1.506$, $p = .183$). This may reflect the relatively small number used in this study and hence the lack of power.

The 30mg/kg dose of LY354740 did, nevertheless, result in a significant increase in running latencies on the sample run ($F(1,12) = 10.400$, $p = .007$) and the choice run ($F(1,12) = 20.895$, $p = .001$). The effect of drug did not significantly interact with genotype ($F(1,12)$ values < 1.50, p values > 0.25). GluA1 KO mice had significantly lower latencies in the choice run ($F(1,12) = 5.821$, $p = .033$) but not the sample run ($F(1,12) = 3.710$, $p = .078$). In the off-drug retraining sessions mice performed at levels comparable to pre-drug training (mean percent alternation; WT = 75.71 ± 6.49 SEM, GluA1 KO mice = 55.71% ±6.85 SEM, $t(12) = 2.119$, $p = .056$).

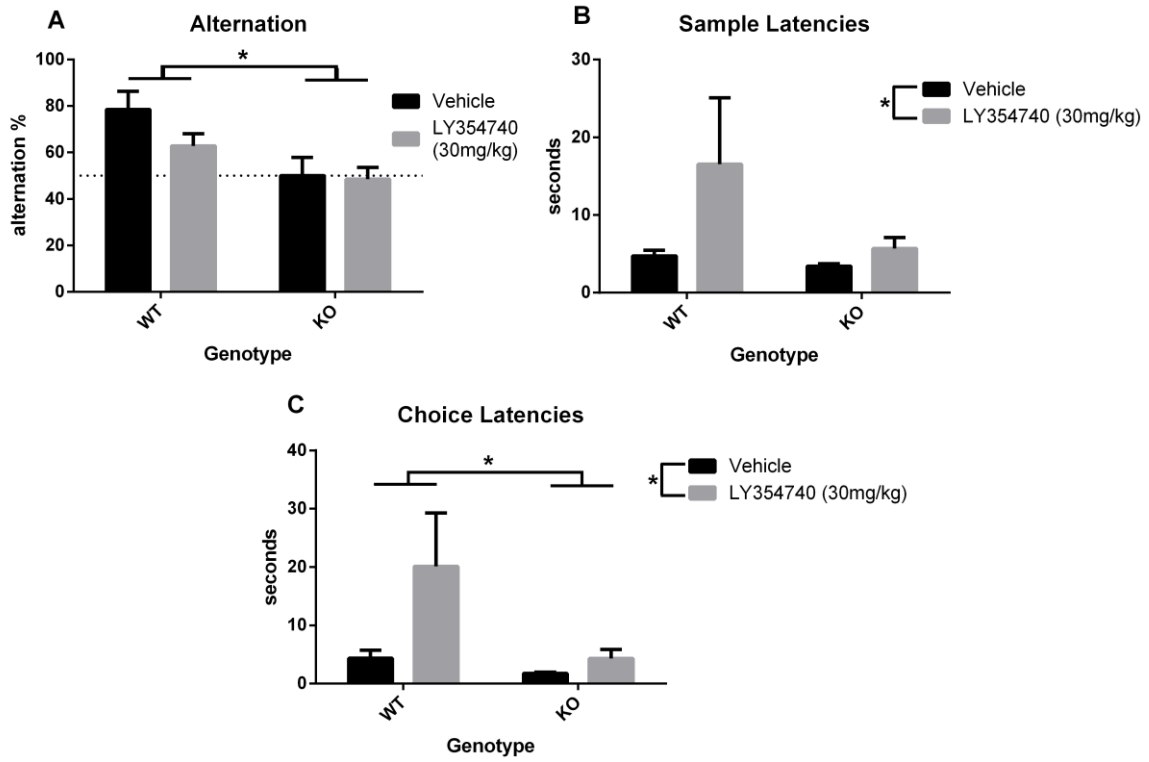


Figure 5.3: Rewarded alternation in GluA1 KO and control mice in the 30mg/kg LY354740 and a vehicle condition with a reduced inter-trial interval. (A) Alternation performance during the drug phase. (B) Latency in seconds to consume reward during the sample run. (C) Latency in seconds to enter the goal arm during the choice run (WT: n=7, KO: n=7). Error bars indicate ± SEM. * p < .05

Experiment 5D: 15mg/kg LY354740 Reduced Spontaneous Locomotor Activity in WT and GluA1 KO mice.

To test whether 15mg/kg can ameliorate the hyperactivity seen in GluA1 KO mice a locomotor activity test was conducted (WT: n=11, KO: n=15). 15mg/kg LY354740 reduced spontaneous locomotor activity in both wildtype and GluA1 KO mice. GluA1 KO mice were hyperactive compared to WT mice ($F(1,22) = 6.156$, $p = .021$, Fig. 5A). The 15mg/kg dose of LY354740 reduced locomotor activity ($F(1,22) = 5.421$, $p = .029$), but the effect of drug did not interact with genotype ($F(1,22) = 0.302$, $p = .588$). Activity declined over time bins ($F(23,506)$

= 9.416, p < .001) and the effect of genotype significantly interacted with time bin (F(23,506) = 1.773, p = .015). Time also interacted with drug (F(23,506) = 2.475, p < .001) and sex (F(23,506) = 1.653, p = .029), with the decline in activity over time being greater for male than female mice. There were no other significant main effects or interactions (p values > 0.1).

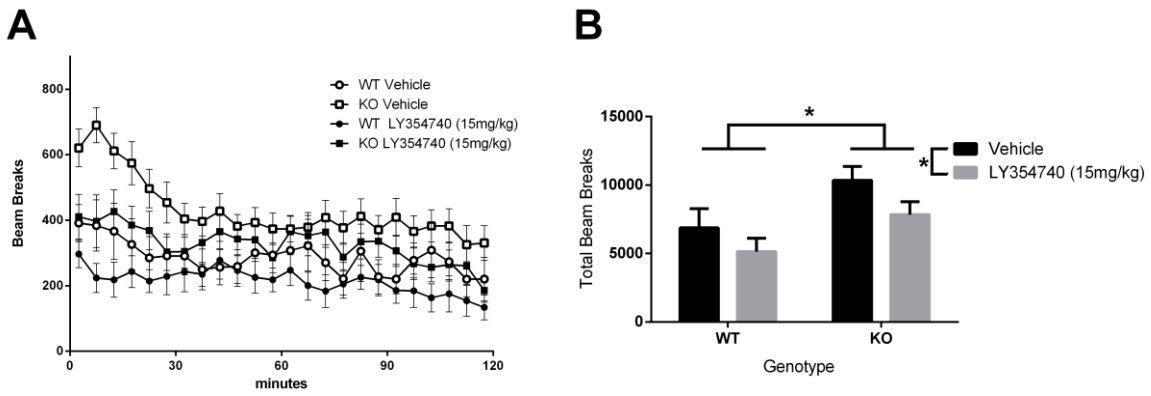


Figure 5.4: Locomotor activity test in GluA1 KO and WT mice with 15mg/kg LY354740 or vehicle injection. (A) Locomotor activity as assessed by beam breaks in 5min time bins. (B) Total beam breaks over 2 hours (WT: n=11, KO: n=15). Error bars indicate ± SEM. * p < .05

Experiment 5E: 30mg/kg LY354740 Reduced Spontaneous Locomotor Activity in ad lib fed WT and GluA1 KO mice.

Next, the effect of 30mg/kg LY354740 on locomotor activity was tested in ad lib fed mice (WT: n=12, KO: n=11). LY354740 reduced spontaneous locomotor activity in wildtype and GluA1 KO mice. GluA1 KO mice were again hyperactive (F(1,19) = 9.126, p = .007) and the 30mg/kg dose of LY354740 substantially reduced activity (F(1,19) = 50.535, p < .001), but, as before, the effect of drug did not significantly interact with genotype (F(1,19) = 2.699, p = .177, Fig. 5.5). There was a two-way interaction of genotype and sex (F(1,19) = 6.166, p =.023) and a

three-way genotype, sex and drug interaction (F(1,19) = 5.447, p = .031). This interaction, however, reflected a failure to find the usual significant hyperactivity effect in male GluA1 KO mice in the vehicle condition. Once again the effects of genotype, sex and drug each interacted with time bin (F values > 2.80, p values < 0.05).

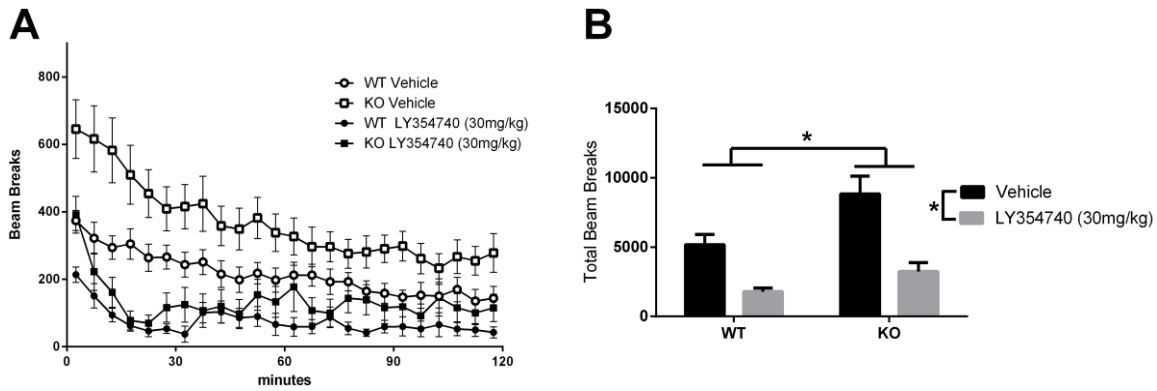


Figure 5.5: Locomotor activity test in ad lib fed GluA1 KO and WT mice with 30mg/kg LY354740 or vehicle injection. (A) Locomotor activity as assessed by beam breaks in 5min time bins. (B) Total beam breaks over 2 hours (WT: n=12, KO: n=11). Error bars indicate ± SEM. * p < .05

Experiment 5F: 30mg/kg LY354740 Reduced Spontaneous Locomotor Activity in Food Restricted WT and GluA1 KO mice.

Chapter 3 showed the influence of feeding state on the effect of LY354740. Therefore the effect of 30mg/kg LY354740 on locomotor activity was assessed in food restricted mice to resemble more closely condition used in the T-maze task (WT: n=7, KO: n=7). This revealed that 30mg/kg LY354740 also reduced spontaneous locomotor activity in food restricted wildtype and GluA1 KO mice. 30mg/kg of LY354740 significantly reduced locomotor activity (F(1,12) = 51.260, p < .001), GluA1 deletion resulted in pronounced hyperactivity (F(1,12) = 38.795, p < .001), and on this occasion the effect of drug significantly interacted with

genotype (F(1,12) = 11.303, p = .006, see Fig. 5.6). The interaction reflected the fact that the effect of drug was larger for GluA1 KO mice (F(1,12) = 55.35, p < .001) than for WT mice (F(1,12) = 7.21, p = .02). This presumably at least in part reflects a greater window for the drug to produce a reduction in locomotor activity. In these animals there was also a significant effect of time bin (F(23,276) = 9.190, p < .001) and significant three-way interaction of factors (F(23,276) = 2.758, p < .001).

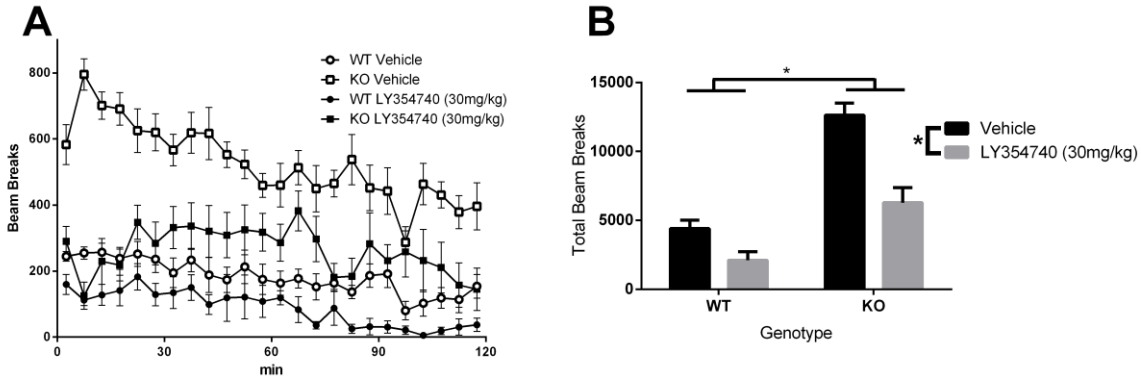


Figure 5.6: Locomotor activity test in food restricted GluA1 KO and WT mice with 30mg/kg LY354740 or vehicle injection. (A) Locomotor activity as assessed by beam breaks in 5min time bins (B) Total beam breaks over 2 hours (WT: n=7, KO: n=7). Error bars indicate ± SEM. * p < .05

Discussion

A group II mGluR agonist failed to rescue short-term memory performance in a transgenic mouse model of glutamatergic hypofunction

The group II mGluR agonist LY354740 was without effect on spatial working/short-term memory performance during T-maze rewarded alternation testing in both wildtype and GluA1 KO mice. Neither 15 nor 30mg/kg doses of the drug had any effect on choice accuracy, despite both doses resulting in clear reductions in locomotor activity levels in photocell activity cages and increases in running latencies in both genotypes. Therefore, these drug doses were not without a behavioural effect. Thus, there were clear reductions in locomotor activity that were not accompanied by any rescue of spatial short-term memory processes. The lack of any rescue effect in the GluA1 KO mice was also highly unlikely to be due to any negative performance effects of LY354740, masking any positive effects of the drug on memory performance, as the drug was also without any significant effect in the wildtype controls.

The failure of a group II mGluR agonist to rescue the spatial working memory deficit in a genetic model of glutamatergic hypofunction (GluA1 KO mice), stands in contrast to the successful rescue of the deficit with pharmacological models of glutamatergic hypofunction, for example with non-competitive NMDAR antagonists such as phencyclidine or MK801 (Moghaddam and Adams, 1998, Blot et al., 2015). There are several overt differences between these pharmacological interventions and the GluA1 KO mouse that could explain these different outcomes.

For one, these manipulations target different ionotropic glutamate receptors. In addition, the pharmacological interventions used were acute manipulations. Only on the day of testing a single dose of the antagonist was injected systemically. The GluA1 knockout, however, was present from the embryonic stage onwards. Potentially, this led to a developmentally driven dysfunction that cannot be corrected by LY354740.

It could be suggested that rodents have to learn to alternate (i.e. acquire a win-shift rule) in order to perform well in the rewarded alternation T-maze task. GluA1 KO mice, being a constitutive receptor knockout mouse, were consistently at chance levels of performance throughout the experiment, including the pre-training phase. This could be taken to suggest that these animals have never learned to alternate (i.e. acquired the win-shift rule). It may, therefore, be unrealistic to expect LY354740 to then rescue alternation performance in these animals if they haven't acquired the rules of the task.

Again it is important to point out that wildtype mice usually exhibit high levels of choice accuracy from the very beginning of training (e.g. Fig. 5.2 >80% choice accuracy in block 1). Furthermore, it is well known that animals alternate spontaneously (e.g. they display a novelty preference). Therefore, it has been suggested that wildtype rodents solve the T-maze rewarded alternation task using an innate preference for the relatively more novel over the relatively more familiar arm based on short-term memory/habituation (Sanderson et al., 2010). It seems therefore unlikely that wildtype mice learn a win-shift rule and so the lack of rescue in GluA1 KO mice is unlikely to be due to a failure of them to acquire

the win-shift rule. Still, a one-trial Y-maze task (Sanderson et al., 2007) could be used to assess short-term memory in the absence of any rule learning.

Another possible explanation for the difference in the effect of LY354740 on PCP and GluA1 knockout-induced working memory deficits might be the mode of action of PCP. One possible account of why LY354740 ameliorates the behavioural effects of PCP relates to the use dependent nature of the PCP-induced blockade of NMDA receptor function. PCP blocks the NMDA receptor by binding within the channel (Lodge and Johnson, 1990). For this binding site to be accessible to channel blockers, the receptor has to be activated. Therefore only active receptors can be blocked (MacDonald et al., 1987). LY354740 activates group II mGluR autoreceptors in pre-synaptic terminals. This reduces glutamate release which in turn could lead to less NMDA receptor channels being active. This would give PCP less opportunity to get into the NMDA receptor channels and block them. This would obviously not apply to GluA1 subunit knockouts.

A prediction based on this hypothesis is that if LY354740 was tested with a competitive NMDA receptor antagonist like APV it would not rescue performance. It may even exacerbate the effect of the competitive antagonist by reducing competing glutamate levels.

No sex specific effect of LY354740 on locomotor activity

Procaccini et al. (2013) saw an interaction of sex with the effect of LY354740 in measures of locomotor activity when investigating GluA1 KO mice. LY534740 reduced hyperactivity in GluA1 KO mice, but the effect was stronger in males

than in females. This was replicated in experiment 5d at a dose of 15mg/kg, but not at a dose of 30g/kg (experiment 5e). However, to formally investigate a potential three-way interaction of sex, genotype and drug, much bigger sample sizes would be necessary.

Conclusion

LY354740 could not reverse the cognitive deficits of a genetic glutamatergic manipulation with relevance to schizophrenia. Notably, this genetic manipulation was present throughout the lifetime of the animal as is the case in the genetic risk factors of schizophrenia. However, genetic changes in schizophrenia are unlikely to be equivalent to a complete knockout of a protein. Still, the results of this chapter suggest a limitation of LY354740 as an antipsychotic. This adds to the findings of chapter 3 and 4 in which LY354740 was found to increase dopamine levels under certain conditions. The opposite of what would be expected from an antipsychotic.

Chapter 6: The Phasic Dopamine Response to Neutral Stimuli and Rewards in GluA1 KO mice

Introduction

This chapter will further investigate the putative aberrant salience phenotype observed in GluA1 mice. To appreciate how salience can be understood in different ways it is instructive to see how Schultz (2016) divided salience into multiple components:

- *physical salience* which is driven by the physical properties of the stimulus in question such as size, form, colour and position
- *novelty and surprise salience* which derives from comparison of the presented stimulus in question with previously presented stimuli
- *motivational salience* which, according to Berridge (2007), is the ability of a stimulus to draw attention due to its rewarding or aversive properties.

The Habituation of the Orienting Response to Neutral Stimuli (HORNS) paradigm (Sanderson et al., 2011b) is a task (see fig. 1.3 in the 1st chapter) that allows me to investigate physical and novelty/surprise salience. In this task, pairs of lights were presented. In these pairs, the second stimulus is either the same or different from the first stimulus. Wildtype mice oriented less towards the second stimulus if it is same as the first presentation in the pairing than if the second

stimulus is different (i.e. they habituate to the light in a stimulus-specific manner). By contrast, GluA1 knockout mice respond more to the second stimulus if it is the same as the first one, compared both to the presentation of a different stimulus or to the first stimulus presentation (i.e. they sensitize) consistent with the deficit in short-term habituation in these animals.

The HORNS task would provide a good behavioural assay for investigating stimulus-evoked dopamine responses using FCV. However, the effect seen in wildtype mice has so far not been replicated. Furthermore, the aberrant salience seen in the GluA1 KO mice has not been reproduced with another glutamatergic modulation. Therefore, the HORNS task was validated in two aspects. Wildtype mice were run to test whether the stimulus-specific habituation seen by Sanderson et al. (2011b) could be replicated. In addition, a group of MK-801 injected mice was run on the HORNS task. MK-801 is an NMDA receptor antagonist (Wong et al., 1986) with a profound influence on perceptual and cognitive processes reminiscent of schizophrenia (Javitt and Zukin, 1991, Moghaddam and Javitt, 2012) and it produces deficits in short-term memory (Blot et al., 2015). Thereby, the habituation of the orienting response was replicated in wildtype mice and the effect of an established glutamatergic manipulation with relevance to schizophrenia on the HORNS task was investigated.

Kapur (2003) stated that aberrant salience seen in schizophrenia is due to aberrant dopamine signalling. Considering that GluA1 is implicated in schizophrenia (Barkus et al., 2014), it is possible that GluA1 mice also have aberrant dopamine signalling when displaying aberrant salience even though previous studies have found no differences in baseline dopamine levels using

tissue level in the home cage between GluA1 KO and wildtype mice (Wiedholz et al., 2008). Testing this hypothesis, Wiedholz et al. (2008) injected dopamine into the nucleus accumbens of anesthetized GluA1 KO mice and littermate controls and measured the extracellular dopamine levels with a voltammetric technique. These measurements showed that extracellular dopamine levels decreased slower in GluA1 KO mice compared to littermate controls. This suggested a decrease of dopamine clearance in GluA1 KO mice. While being of value for showing how the kinetics of dopamine uptake might be altered after deletion of GluA1, such an experimental setup in anesthetized animals cannot assess how the dopamine system reacts in a behavioural task. FCV, on the other hand provides the ability to measure behaviourally-evoked dopamine responses. Therefore, GluA1 mice and littermate controls were implanted with carbon fibre electrodes to examine their phasic dopamine response while performing behavioural tasks.

After the HORNS task was validated at a behavioural level, it was run in GluA1 KO mice and littermate controls while the phasic dopamine response was recorded in the nucleus accumbens core using FCV. Finally, a variable reward procedure, comparable to that described in Chapter 4, was used to investigate the effect of GluA1 knockout on reward-elicited phasic dopamine release.

Methods

Animals

For experiments 6a and 6b (MK801 experiments) a cohort of 24 male C57/Bl6 mice, ~12 weeks old, were obtained from Harlan Olac Ltd (Oxon, United Kingdom). All mice were housed in groups of 6, in a vivarium, where temperature and humidity were controlled. The animals were exposed to a 12 hr light-dark cycle (0700 to 1900) and behavioural testing was carried out during the light phase. Mice were maintained at above 85% of their free-feeding weight throughout testing by receiving a restricted diet.

For experiment 6c and 6d (FCV experiments) 51 mice (26 WT, 25 KO) aged 8 to 20 weeks were implanted with FCV electrodes aimed at the nucleus accumbens core as described in Chapter 2. 26 (13 WT, 13 KO) of these were unilateral implantations into the left hemisphere. As the surgery was refined, 25 (13 WT, 12 KO) bilateral implantations were conducted. Of these 51 mice, 35 mice (16 KO, 19 WT) completed at least one testing session in which the dopamine signal was recorded and their data were included in this chapter.

Analysis of behaviour during experiment 6c (the HORNS task) was based on 35 mice (19 WT, 16 KO) of which 12 wildtypes and 14 knockouts completed both sessions of the HORNS task resulting in 31 sessions in wildtypes and 30 sessions in knockouts. The remaining mice were not run after the first session due to lack of a dopamine signal. Due to this lack of a second session in a subset of the mice, a full factorial model with session as a within-subjects factor

is not possible. Therefore each session will be analysed as its own data point. Stimulus order was counterbalanced as far as possible considering the low sample size.

In these 61 behavioural sessions, 21 sessions (13 WT, 8 KO) were found to have a dopamine signal. Since 3 WT sessions and 1 KO session were in mice that displayed a dopamine signal on both electrodes the analysis presented here was based on the electrodes rather than mice. This resulted in a sample size of 25 recording sessions (16 WT, 9 KO) which were recorded in 14 different mice (9 WT, 5 KO).

For experiment 6d after validating that their FCV signal contained dopamine, 6 WT and 5 KO mice were included into the analysis. Since in one animal per group both working electrodes displayed a dopamine signal, the analysis of the FCV data was based on a sample size of 13 electrodes (7 WT, 6 KO).

Equipment

During experiment 6a mice were tested in operant chambers (15.9 x 14.0 x 12.7 cm; ENV-307A, Med Associates), enclosed in sound attenuating cubicles (ENV-022MD, Med Associates), using a task programmed with Med-PC IV software (Med Associates). 20% sucrose solution could be dispensed into a magazine (2.9 x 2.5 x 1.9 cm; ENV-303HD, Med Associates) via a single speed syringe pump (PHM-100A-UK). Breaks in an infrared beam (ENV-303HDM, Med Associates) across the bottom of the magazine were used to measure magazine activity. Two LED (ENV-321M, Med Associates) lights were positioned on the

same wall as the food magazine to the left and to the right of the magazine. A house light (ENV-315M, Med Associates) was placed on top of the conditioning box facing away from the chamber floor. Each chamber was equipped with a fan (ENV-025AC, Med Associates) that was turned on for the duration of the session.

For experiment 6c and 6d, chambers were similar except that a custom-made gravity-based reward-delivery system was used to deliver sucrose solution into a custom-built food magazine via a spout. The food magazine in experiments 6c and 6d extended into the box as opposed to the standard food magazine in the above setup which was embedded into a wall of the operant box. This modification was made to give head-capped mice clear access to the reward in the food magazine.

HORNS Task

The Habituation of the Orienting Response to Neutral Stimuli (HORNS) task is based on the paradigm developed by Sanderson et al. (2011b) for mice which, in turn, was based on the work of Marshall et al. (2004) in rats.

In order to obtain a quantitative measure of the strength of orienting, animals were first trained to nose poke in a food magazine. Before the first day of training, mice had access to sucrose solution (20% to volume) in their home cage overnight to habituate them to the reward. During the first training session, mice were habituated to the conditioning boxes with a single 0.5ml sucrose solution aliquot placed in the food magazine for 30min. Mice were then trained to

nose-poke in a food magazine to gain sucrose rewards. During the second training day, 30 sucrose rewards (22µl) were delivered on a random interval (RI) schedule. This meant that reward was delivered as soon as a head entry was made to the food magazine after a programmed time period had elapsed. The schedule was based on a 60s Fleshler schedule (Fleshler and Hoffman, 1962), a schedule in which the probability of a reward is independent of the period of time since the last reward. For training sessions 3-6, 24 sucrose solution rewards were presented according to a 120s Fleshler schedule.

During the HORNS task each mouse was again presented with 24 rewards according to a 120s RI Fleshler schedule with reward delivery being dependent on head entry into the magazine. However, in addition, during each session of this task, two different visual stimuli were presented for 10s each independently of the reward delivery schedule. One consisted of two flashing (0.909Hz) LED lights positioned to the left and to the right of the food magazine and the other was a continuously illuminated house light placed on top of the conditioning box facing away from the mouse. To assess stimulus-specific short-term habituation, each session consisted of 8 pairs of stimulus presentations (delay between 1st and 2nd stimulus presentations of 30sec) with an inter-trial interval (ITI) of 310s. The first 4 trials consisted of a pseudorandom permutation of the 4 possible trial types: house→house, LED→LED, house→LED or LED→house, which was then repeated for the second half of the trials. If the mice orient to a stimulus rather than continuing to nose-poke, there will be a reduction in the duration of “head entries” to the food magazine. Hence, suppression of head entry is a measure of orienting. If a mouse habituated to the first stimulus it would show weaker head

entry suppression to the familiar stimulus (SAME) than to the alternative stimulus (DIFFERENT).

Experiment 6a: HORNS Task with 0.1mg/kg MK-801

Each mouse received four sessions of the HORNS task. Across the 4 sessions of the task each mouse completed two sessions after receiving 0.1mg/kg MK-801 and two after receiving the vehicle (fully counterbalanced within-subjects design), with each animal receiving the same treatment before session 1 and 4, and session 2 and 3 respectively.

Experiment 6b: Locomotor Activity Test with 0.1mg/kg MK-801

Spontaneous locomotor activity was measured as described in Chapter 2. Locomotor testing commenced in the same cohort two days after completion of the HORNS task (see above) to test whether MK-801 had an overall effect on locomotor activity. Mice remained on food restriction. After 30min of habituation to the locomotor apparatus mice were injected with MK-801 or saline in a between-subjects design and immediately placed back into the activity cages for 2 hours.

Drug

MK-801 maleate (Tocris) for experiment 6a and 6b was made up in 0.9% saline solution to a stock concentration of 0.025mg/ml and injected at a volume of 4ml/kg to reach a final dose of 0.1mg/kg. Injections (i.p.) were performed 25min before mice were tested in the operant box (i.e. 30 minutes before the first light

was presented) in experiment 6a. In experiment 6b mice were immediately placed in activity cages after injections took place.

Fast-Scan Cyclic Voltammetry in GluA1 Knockout Mice

Overview of Experimental Procedure

Mice were implanted with carbon fibre electrodes into the nucleus accumbens core. After surgery mice were given at least 2 weeks to recover and were then set on a food restricted diet limiting them to 85% of their free feeding weight. Subsequently, they were habituated to Med Associates boxes in which all of the following experiments took place. They were magazine trained with sucrose solution similar to the training in experiment 6a during which no FCV signal was recorded. During this period cycling took place. As described in Chapter 2 this is a necessary session in which the cyclic potential is applied over a prolonged period of time to settle the working electrode. After the quality of the electrode was established, two sessions of the HORNS task were conducted as described above.

This was followed by a single session of unsignalled, variable magnitude reward delivery during which the FCV signal was recorded. In the unsignalled variable reward task, mice were placed in Med Associates Boxes and 20% sucrose solution was delivered to them at random time points but also dependent on head entry into the food magazine. The behavioural test consisted of a 120s [90-150s] variable interval reward schedule with 24 trials. Trials consisted of the unsignalled delivery of 10µl (low), 22µl (medium) or 48µl (high) of sucrose

solution. During this behavioural task the duration and total number of head entries was recorded.

The dopamine response to unrewarded head entries was also extracted by selecting head entries that were at least 30s before and at least 10 after a reward delivery. To reduce the influence of bouts of strong magazine activity a dead time of 10s after a detected unrewarded head entry was imposed during which head entries were ignored. Despite these seemingly restrictive criteria at least 51 unrewarded head entries were included into the analysis per electrode. At the end of testing, mice were transcardially perfused and their brains were extracted as described in chapter 2.

Validation of the Dopamine content in the FCV signal

Electrodes were validated as outlined in chapter 2. Whereas dopamine responses to unsignalled reward delivery have been shown to be useful for validation in rats, they have proven less predictive of dopamine responses in the HORNS task in mice. For example, some mice in the HORNS task displayed strong dopamine responses to neutral stimuli but only barely detectable responses to rewards. If the validation were to be purely based on reward responses, these mice could not be included. Therefore, the validation criteria (see chapter 2) were also applied to the first occurrence of neutral stimulus presentations during the experiment.

Results

Experiment 6a: 0.1mg/kg MK-801 Abolished Stimulus-Specific short-term habituation

The HORNS task was conducted in wildtype mice (n=24) after injections of either saline or the NMDA receptor antagonist MK-801. Orienting was measured as a change in magazine responding during the stimulus compared to the 10s before stimulus presentation. In the 10s pre-stimulus baseline period, all mice were spending a proportion of their time in the food magazine, which reduced when the stimuli were first presented, indicative of orienting. A three-way within-subjects ANOVA on the head entry duration for the first stimulus presentation of each pair (i.e. each trial) with drug (vehicle vs MK-801), stimulus type (LED vs house light) and stimulus period (pre-stimulus baseline vs stimulus on) revealed a main effect of stimulus period ($F(1,23) = 89.125$, $p < .001$) which reflected less time in the magazine during presentation of the stimuli. On average, this effect was bigger in response to the house light than to the LED (stimulus type*stimulus period: $F(1,23) = 12.541$, $p = .002$) (Fig. 6.1). Importantly, there was also an interaction between drug and stimulus period, caused by greater head entry suppression (orienting) in the MK-801 condition (drug*stimulus period: $F(1,23) = 8.093$, $p = .009$). However, post-hoc tests showed that this may have been partly driven by higher baseline responding levels in the MK-801 condition ($t(23) = 35.379$, $p < .001$).

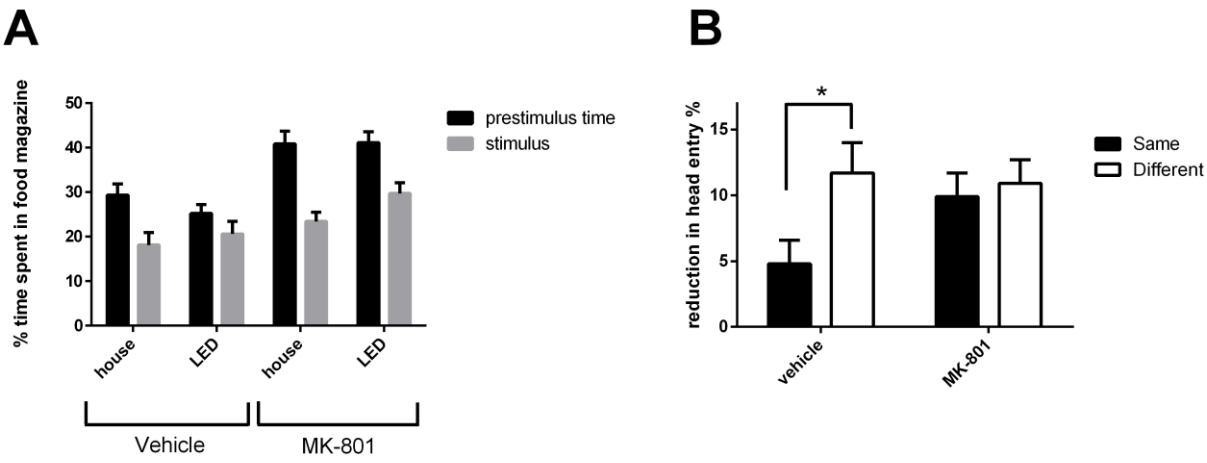


Figure 6.1: The effect of 0.1mg/kg MK-801 on orienting to a neutral stimulus. (A) Percentage time spent in the food magazine during the first stimulus and its pre-stimulus baseline period. (B) Suppression of head entry (pre-stimulus head entry – stimulus head entry) in response to the 2nd stimulus grouped by trial type (SAME vs DIFFERENT from the 1st stimulus) (n=24). Data points represent mean ± SEM. * p < .05

To account for the effect of the baseline differences in the drug conditions, for all subsequent analyses magazine activity was converted to a difference score by subtracting the percentage of magazine activity during the stimulus from the percentage of magazine activity during the pre-stimulus baseline period. Thus, scores greater than zero indicate that the stimulus presentation resulted in suppression of head entry duration (i.e. orienting), whereas scores equal to zero indicate no change in responding.

After establishing that the task was able to pick up orienting, I then analysed the change in orienting upon second presentation of either the same or the different stimulus. Analysis of head entry suppression revealed stimulus-specific habituation (i.e. reduced orienting in the SAME compared to the DIFFERENT condition) after vehicle but not after MK-801 injection (see Fig. 6.1b). The suppression of head entry data for the 2nd stimulus was analysed with a three-way within-subjects ANOVA with drug (MK-801, vehicle), trial type (SAME vs

DIFFERENT) and stimulus type (house vs LED) as factors. Stimulus type was based on the physical identity of the 2nd stimulus in the pair. The ANOVA revealed overall stronger suppression of head entry during the DIFFERENT stimulus than the SAME stimulus ($F(1,23) = 5.055$, $p = .034$) i.e. there was evidence of stimulus-specific short-term habituation, but no effect of drug ($F(1,23) = 1.142$, $p = .296$) and stimulus type ($F(1,23) = 0.804$, $p = .379$). The interaction of drug and stimulus type was significant ($F(1,23) = 4.674$, $p = .041$), showing the importance of including the stimulus type of the 2nd stimulus into the analysis. However, none of the other interactions reached significance, including the trial type x drug interaction ($F(1,23) = 1.952$, $p = .176$).

Despite the lack of drug by trial type interaction, given the *a priori* interest as to whether there was evidence of stimulus-specific habituation in the controls, I ran a further simple main effects analysis comparing trial type for each drug condition. This showed that, after vehicle injections, there was a significant effect of trial type ($F(1,23) = 5.357$, $p = .030$), thus validating the task as a test of short-term habituation. However, this effect was absent in the MK-801 condition ($F(1,23) = 0.153$, $p = .699$).

Together these analyses show that stimulus-specific short-term habituation to neutral stimuli can be observed in control mice. Moreover, this habituation is no longer present after injection of 0.1mg/kg MK-801. However, it is also important to note that there was no significant drug * trial type interaction in the main ANOVA.

Experiment 6b: 0.1mg/kg MK-801 did not Induce Hyperactivity

Injection of 0.1mg/kg MK-801 did not affect locomotor activity (veh: n=12, MK-801: n=12; Fig. 6.2). An ANOVA with drug as between-subjects factor and time as within-subjects factor during the post-injection period showed no main effect of drug ($F(1,22) = 0.032$, $p = .860$) or drug x time interaction ($F(23,506) = 1.150$, $p = .287$). The overall main effect of time was significant ($F(23,506) = 1.575$, $p = .044$), reflecting habituation to the testing environment across the session.

However, while during the pre-injection habituation period there was no effect of group (i.e. subsequent drug condition; $F(1,22) = 1.352$, $p = .257$), there was a near significant interaction of drug by time ($F(5,110) = 2.296$, $p = .050$) caused by the would-be vehicle group displaying higher baseline activity just prior to the injections, thereby possibly masking a potential MK-801 induced hyperactivity. This was despite the pseudorandom allocation of mice to the two drug condition. To test this possibility the post-injection data were expressed as percentage of baseline activity (i.e. percentage change in locomotor activity). This ANOVA confirmed the lack of MK-801 induced hyperactivity as there was no main effect of drug ($F(1,22) = 1.069$, $p = .313$) or drug by time interaction ($F(23,506) = 1.218$, $p = .222$).

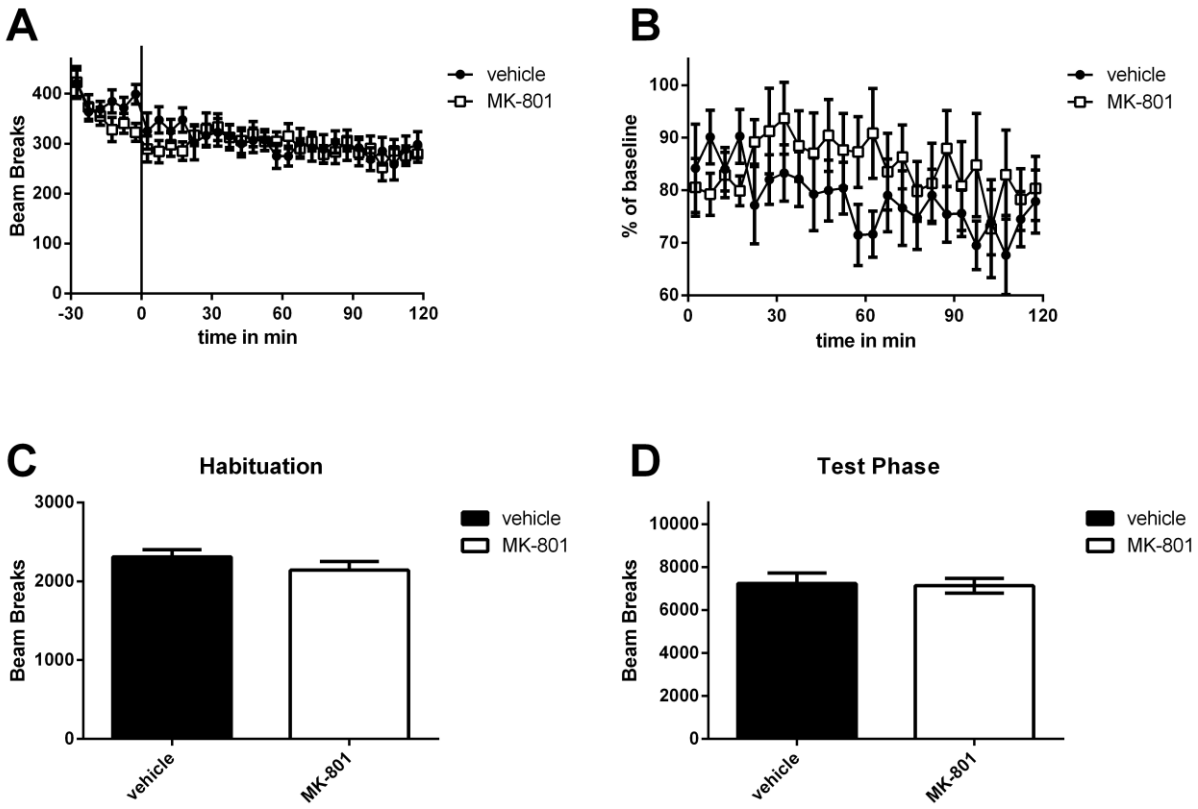


Figure 6.2: The effect of 0.1mg/kg MK-801 on locomotor activity. (A) Locomotor activity in 5 minute time bins. The vertical line at t=0 denotes the time of dosing. (B) Locomotor activity normalised to the pre-injection baseline during the testing phase. t=0 denotes dosing. (C) Total locomotor activity during the habituation phase. (D) Total locomotor activity during the testing phase (veh: n=12, MK-801: n=12). Error bars represent ± SEM.

Experiment 6c: GluA1 KO Abolished Habituation of the Dopamine Response to Neutral Stimuli.

Behaviour

In the next experiment, GluA1 KO mice and WT controls (WT: n=31, KO: n=30), implanted with FCV electrodes into the nucleus accumbens, were run on the HORNS task while dopamine levels were monitored.

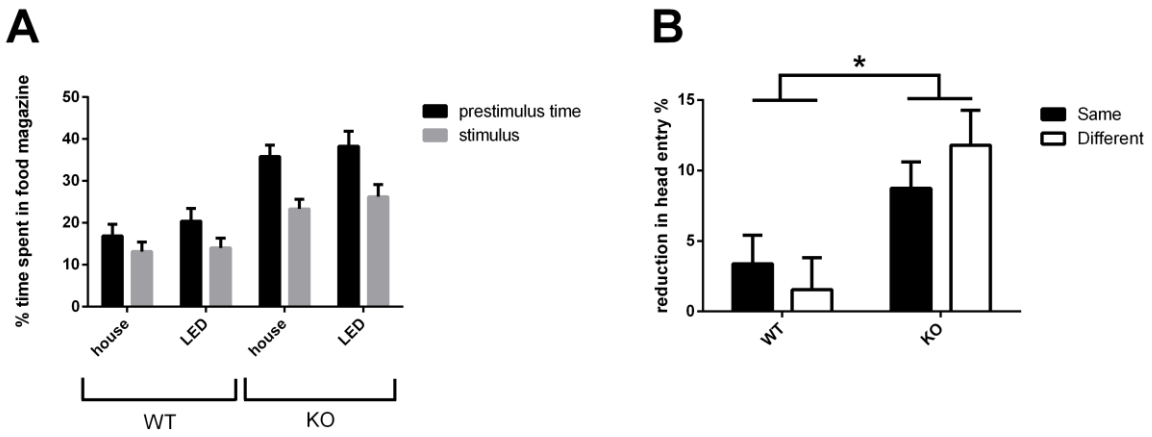


Figure 6.3: The behavioural effect of GluA1 knockout on orienting towards neutral stimuli. (A) Percentage time spent in the food magazine during the first stimulus and its pre-stimulus baseline period. (B) Suppression of head entry in response to the 2nd stimulus grouped by trial type (SAME vs DIFFERENT) (WT: n=31, KO: n=30). Error bars represent ± SEM. * p < .05

As in experiment 6a, both groups of mice oriented to the lights as shown by a reduction of head entries when the first light in each trial was presented compared to a 10s pre-stimulus baseline period. This was confirmed by a repeated-measures ANOVA for the first stimulus presentation with genotype (WT vs KO) as between-subjects factor and stimulus type (LED vs house light) and stimulus period (pre-stimulus baseline vs stimulus on) as within-subjects factors which showed a main effect of stimulus period ($F(1,59) = 50.765$, $p < .001$). Importantly, this effect was bigger in knockout mice than in the wildtypes (Fig. 6.3A), resulting in an interaction between genotype and stimulus period ($F(1,59) = 8.893$, $p = .004$). Post-hoc tests showed that wildtypes and knockouts showed head entry suppression when the lights were presented (WT: $F(1,30) = 8.448$, $p = .007$, KO: $F(1,29) = 46.276$ $p < .001$) and there was increased baseline head entry duration in the knockouts during the pre-stimulus period ($F(1,59) = 31.931$, $p < .001$). There was also a significant overall increase in head entry duration in the knockouts (pre-stimulus and stimulus combined; $F(1,59) = 29.576$, $p < .001$).

However, unlike in experiment 6a, there was no effect of stimulus type ($F(1,59) = 1.272$, $p = .264$).

Interpretation of the analysis of the suppression of head entry by the 2nd Stimulus as conducted in this experiment was difficult. The suppression of head entry data for the 2nd stimulus was analysed with a three-way ANOVA with trial type (SAME vs DIFFERENT), and stimulus type (house light vs LED) as within-subjects factors and genotype as between-subjects factor. Analysis of head entry suppression in response to the 2nd stimulus revealed that knockouts show stronger head entry suppression. There was a significant main effect of genotype ($F(1,59) = 10.653$, $p = .002$), but no effect of trial type ($F(1,59) = 0.172$, $p = .690$) or stimulus type ($F(1,59) = 0.841$, $p = .363$). In fact, wildtypes did not show any discernible suppression of head entry (Fig. 6.3b, mean %± SEM: 2.47%± 1.54; t-test against zero: $t(30) = 2.417$, $p = .131$) whereas knockouts displayed robust head entry suppression (mean% ± SEM: 10.03% ± 1.714; $t(29) = 5.763$, $p < .001$). The three-way interaction of genotype * trial type * stimulus type had a trend towards significance ($F(9,531) = 2.093$, $p = .080$), again suggesting a role of the stimulus type in the response to the 2nd stimulus. However, the absence of significant head entry suppression in wildtypes made an interpretation of this result difficult. No other interaction displayed any trends or significance ($F < 1.8$, $p > .190$).

Next, general habituation of the orienting response to LEDs and house lights over the session (as opposed to short-term stimulus-specific habituation: see below) was analysed (Fig. 6.4). To that end, the session was divided into a 1st and 2nd half. All stimuli, independent of whether they were the 1st or 2nd stimulus

of a pairing, were included in this analysis. A three-way ANOVA with stimulus type (LED vs house light) and halves (1st and 2nd half of session) as within-subjects factors and genotype (WT vs KO) as a between-subjects factor was conducted on this dataset. There was no effect of stimulus type consistent with what had been observed for the first stimulus of each pair ($F(1,59) = 0.044$, $p = .834$). Furthermore, there was still a tendency towards stronger orienting in the knockouts ($F(1,59) = 3.585$, $p = .063$). As seen above, this might be reflecting higher baseline levels. However, there was no main effect of halves ($F(1,59) = 1.271$, $p = .264$) or interaction between genotype and halves ($F(1,59) = 0.521$, $p = .473$), showing that the overall levels of orienting in both groups on average stayed constant over the session. Nonetheless, visual inspection of the data in Fig. 6.3 suggested a different pattern of habituation between LED and house lights in the knockouts. This was reflected in a trend towards significance in the three-way stimulus type by genotype by halves interaction ($F(1,59) = 3.219$, $p = .078$). (Note also that this response pattern in this experiment is reminiscent of the pattern of phasic dopamine that will be found Fig. 6.11 and 6.12). Therefore, orienting responses were compared between wildtypes and knockouts on each level of the factors halves and stimulus type. This exploratory analysis revealed that knockouts oriented more strongly to house lights in the 2nd part of the session than the wildtypes ($t(29) = 2.133$, $p = .037$). None of the other pairwise comparisons were significant ($t < 1.4$, $p > 0.19$).

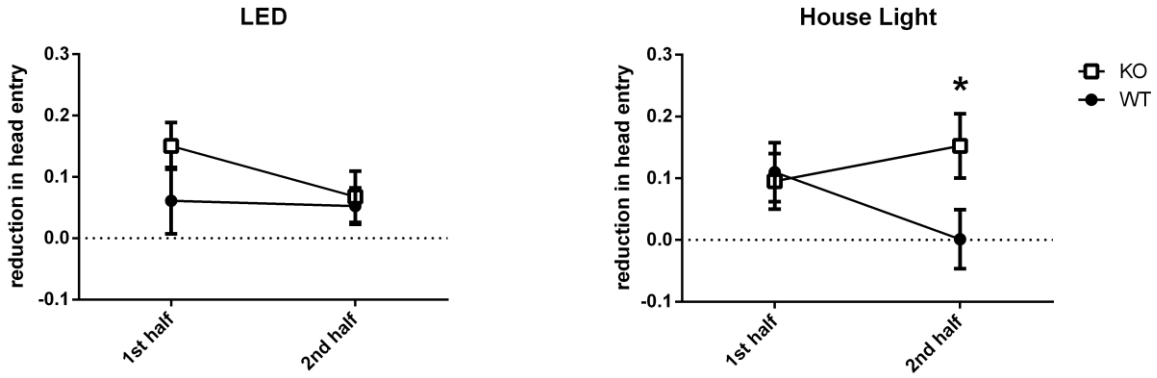


Figure 6.4: Average orienting to neutral stimuli in the first and second half of a session in the HORNS task. Data are divided by stimulus type into LED responses (left) and house light responses (right) (WT: n=31, KO: n=30). Error bars indicate ± SEM. * p < .05

GluA1 KO mice showed increased dopamine responses to the LED and slower decay of the responses to the house light

To assess the phasic dopamine response during the HORNS task, GluA1 KO and wildtype mice (WT: n=16, KO: n=9) were implanted with carbon fibre electrodes into the nucleus accumbens core (Fig. 6.5).

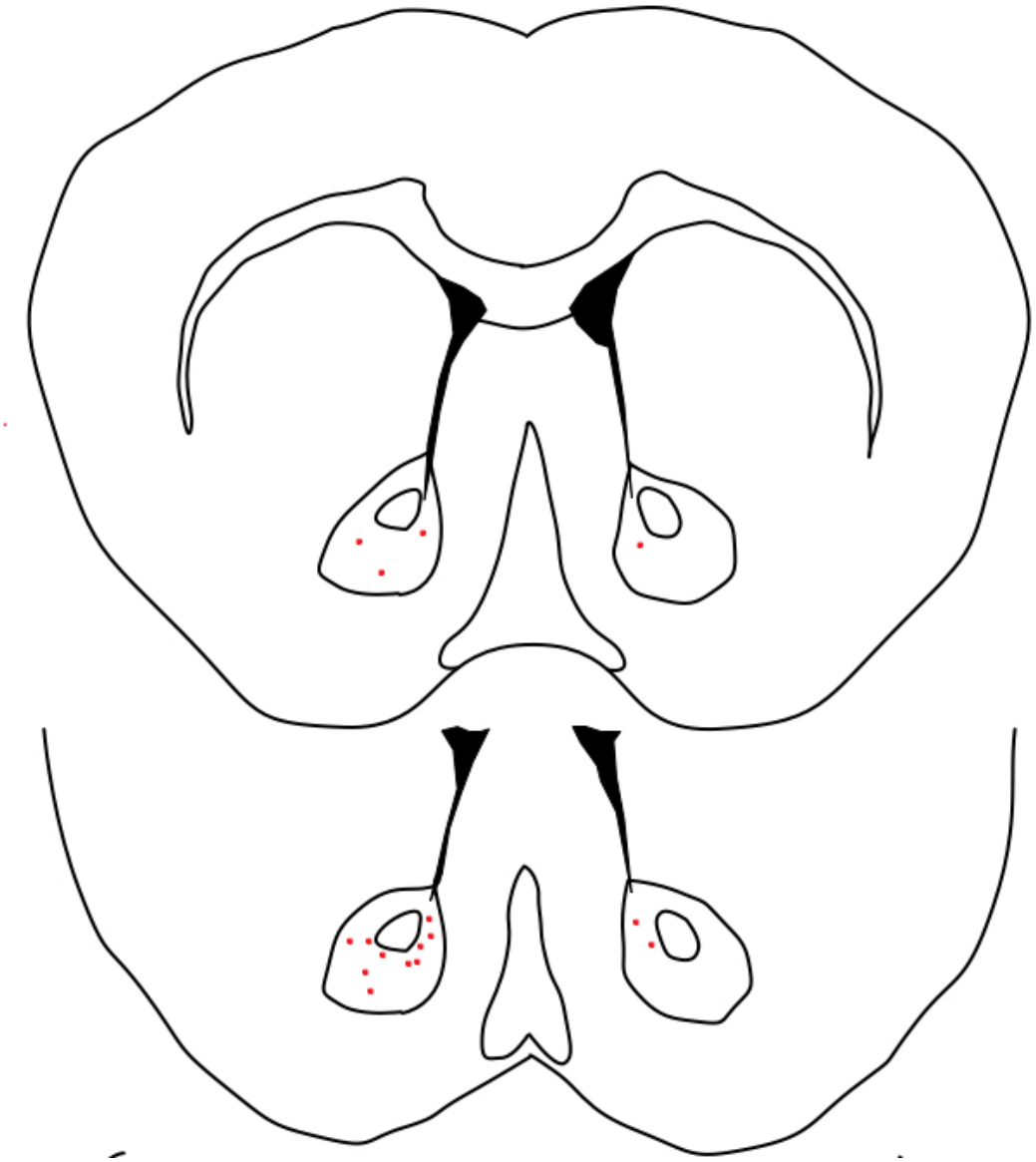


Figure 6.5: Locations of each carbon fibre electrode in the nucleus accumbens core depicted on coronal sections at +0.98mm (top) and +1.18mm (bottom) from bregma (Paxinos and Franklin, 2001).

Before analysing the dopamine response to SAME and DIFFERENT trial types for the 2nd stimuli, it was important firstly to establish whether there were differential responses based on the identity of the stimulus. Therefore, responses to the 1st stimuli were initially analysed. Overall, knockouts and wildtypes had a

similar dopamine response to the first house light presentation of each trial. However, knockouts had a stronger response to the flashing LED than to the house light (Fig. 6.6).

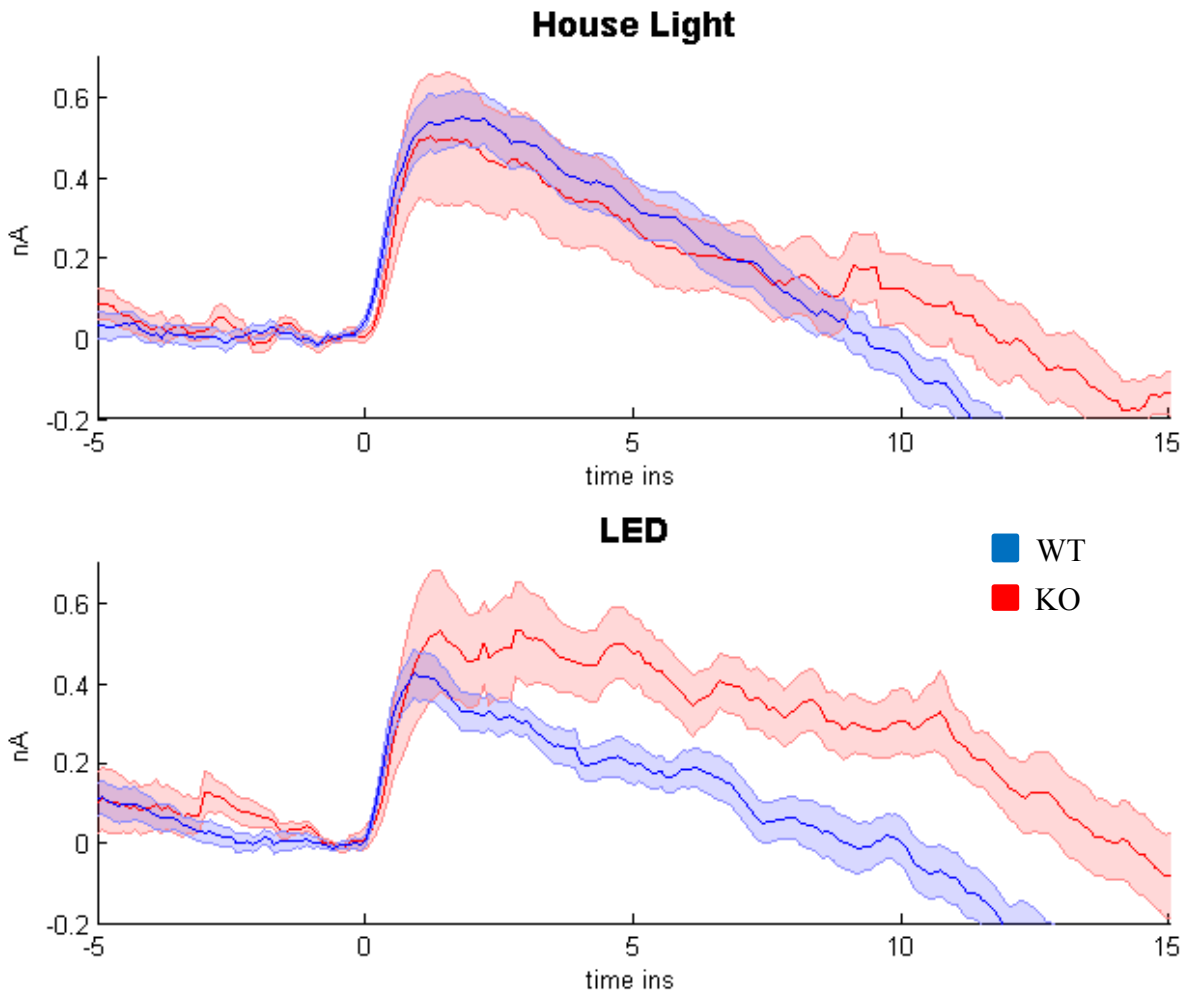


Figure 6.6: Average transient phasic dopamine response to the first neutral light (house or LED) in each pairing. The upper panel shows the average response to a continuously presented house light. The lower panel shows the response to a flashing LED. Both panels compare wildtypes (blue) to knockouts (red). Both lights were presented from t = 0s to t = 10s (WT: n=16, KO: n=9). Error bars indicate ± SEM.

A two-way ANOVA of the peak dopamine current following presentation of the light stimuli in each group was performed, with genotype (WT vs KO) as the

between-subjects factor and stimulus type (house light vs LED) as a within-subjects factor. This revealed an interaction of stimulus type by genotype ($F(1,23) = 11.209$, $p = .003$) without a main effect of stimulus type ($F(1,23) = 0.334$, $p = .569$) or genotype ($F(1,23) = 0.288$, $p = .597$) (Fig. 6.7). To investigate the interaction of stimulus type and genotype further a simple main effects analysis of the responses of wildtypes and knockouts to house and LED respectively were conducted. This showed that there was no genotype effect in the peak dopamine responses to the house light ($t(23) = 1.305$, $p = .265$) or the LED ($t(23) = 1.642$, $p = .114$). However, wildtypes showed stronger house light-evoked dopamine responses than LED-evoked dopamine responses ($t(15) = 3.142$, $p = .007$). Knockouts, on the other hand, showed a tendency for stronger dopamine responses to the LED than the house light ($t(8) = 1.886$, $p = .096$).

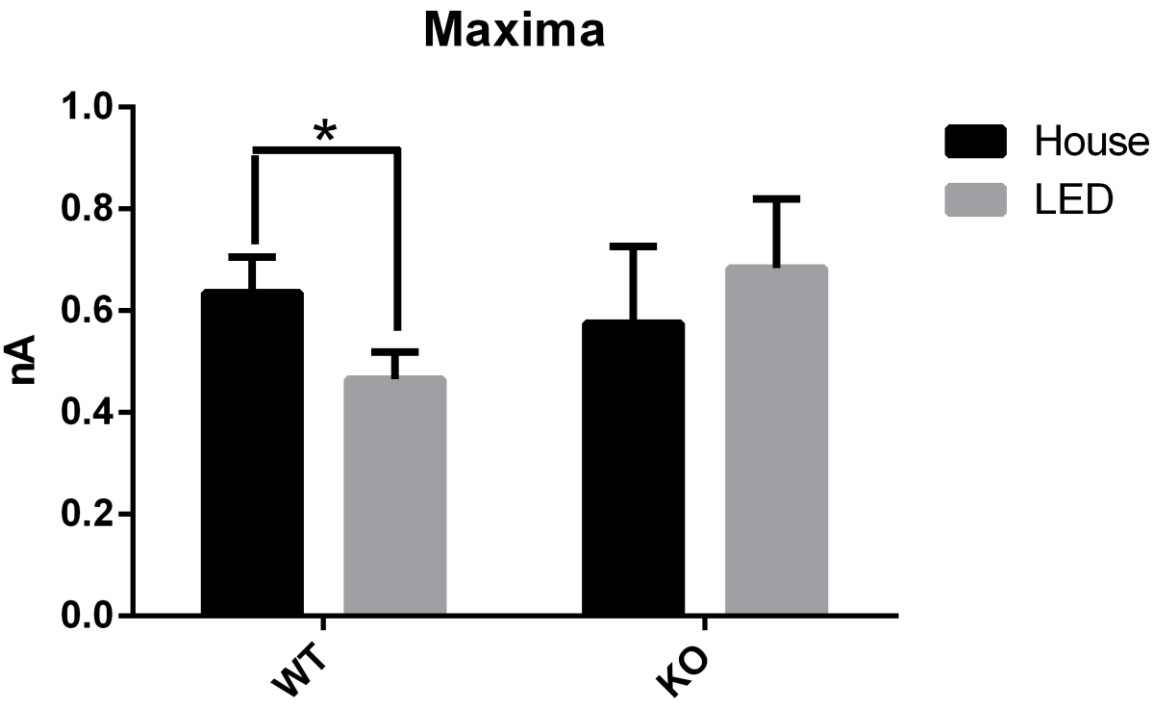


Figure 6.7: Maximal (peak) dopamine response evoked during the first stimulus in each stimulus pair (house light vs LED) (WT: n=16, KO: n=9). Error bars indicate ± SEM. * p < .05

To capture the change in dopamine levels over the stimulus period, the area-under-the-curve was calculated for the first and second halves of the stimulus presentation. A three-way ANOVA was conducted with stimulus type (house vs LED) and time bin (1^{st} vs 2^{nd} half of stimulus) as within-subjects factors and genotype (WT vs KO) as a between-subjects factor. This again revealed an interaction of stimulus type by genotype ($F(1,23) = 6.625$, $p = .017$) without a main effect of stimulus type ($F(1,23) = 0.028$, $p = .868$) and genotype ($F(1,23) = 1.342$, $p = .259$) (Fig. 6.8). To investigate this interaction, a simple main effects analysis of the responses of wildtypes and knockouts to house and LED respectively were conducted. This revealed that knockouts had a stronger dopamine response to the LED ($F(1,23) = 2.661$, $p = .014$) but that there was no difference in the response to the house light ($F(1,23) = 0.155$, $p = .879$). The dopamine response was overall less pronounced in the 2^{nd} part of the stimulus (main effect of time bin: $F(1,23) = 27.375$, $p < .001$) and this decay of the signal was stronger in the house light than in the LED (interaction of stim type * time bin: $F(1,23) = 8.037$, $p = .009$). However, there was no interaction of genotype by time bin ($F(1,23) = 0.325$, $p = .574$).

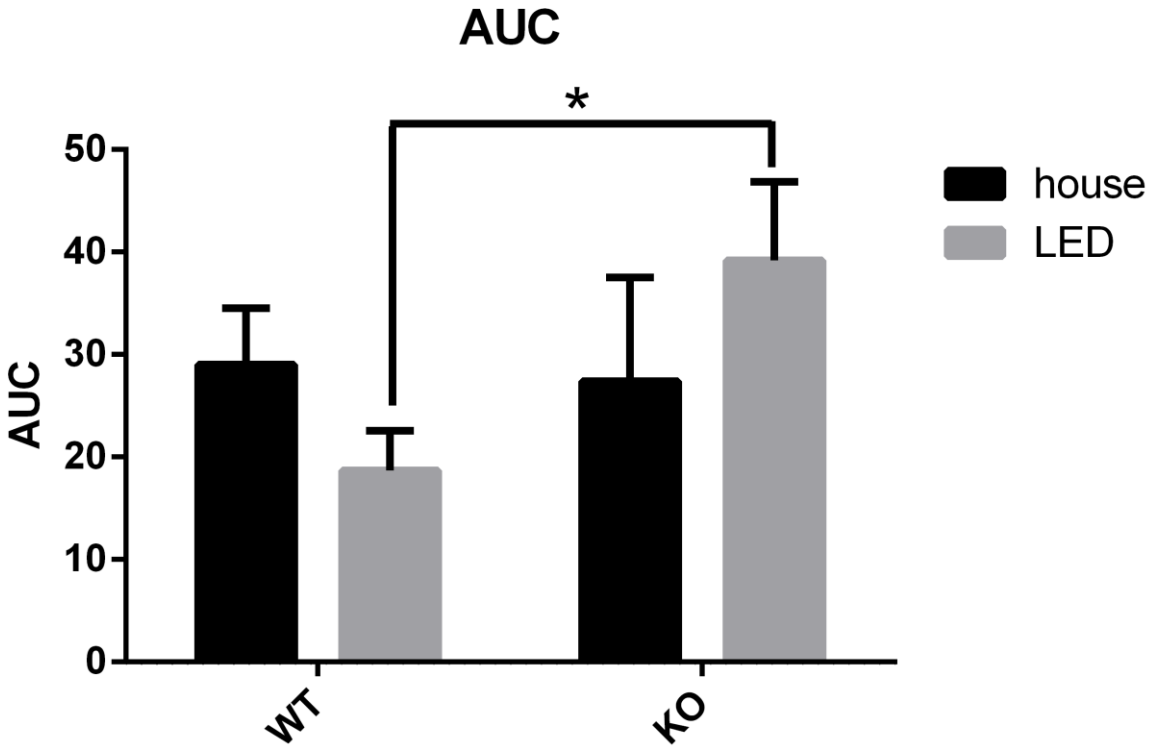


Figure 6.8: Area under the curve from t=0s to t=10s of the dopamine response evoked during the first stimulus in each stimulus pair (house light vs LED) (WT: n=16, KO: n=9). Error bars indicate ± SEM. * p < .05

Slope of Decay

To analyse the decay of the dopamine signal after its peak, the local maximum of the signal within the first two seconds was determined. The trace from this time point until the end of the stimulus at t=10s was extracted and a linear function was fitted to the trace. The slope of this function is reported in the following analysis.

Analysis of the house light showed that the decay of the dopamine signal was slower in knockouts compared to wildtypes ($t(23) = 2.481$, $p = .021$). Visual examination of the signal showed that the dopamine response to the LED was

non-linear, and therefore cannot be captured by a linear function. This is potentially due to the flashing nature of the stimulus which potentially leads to a dopamine release more distributed over time (Fig. 6.9).

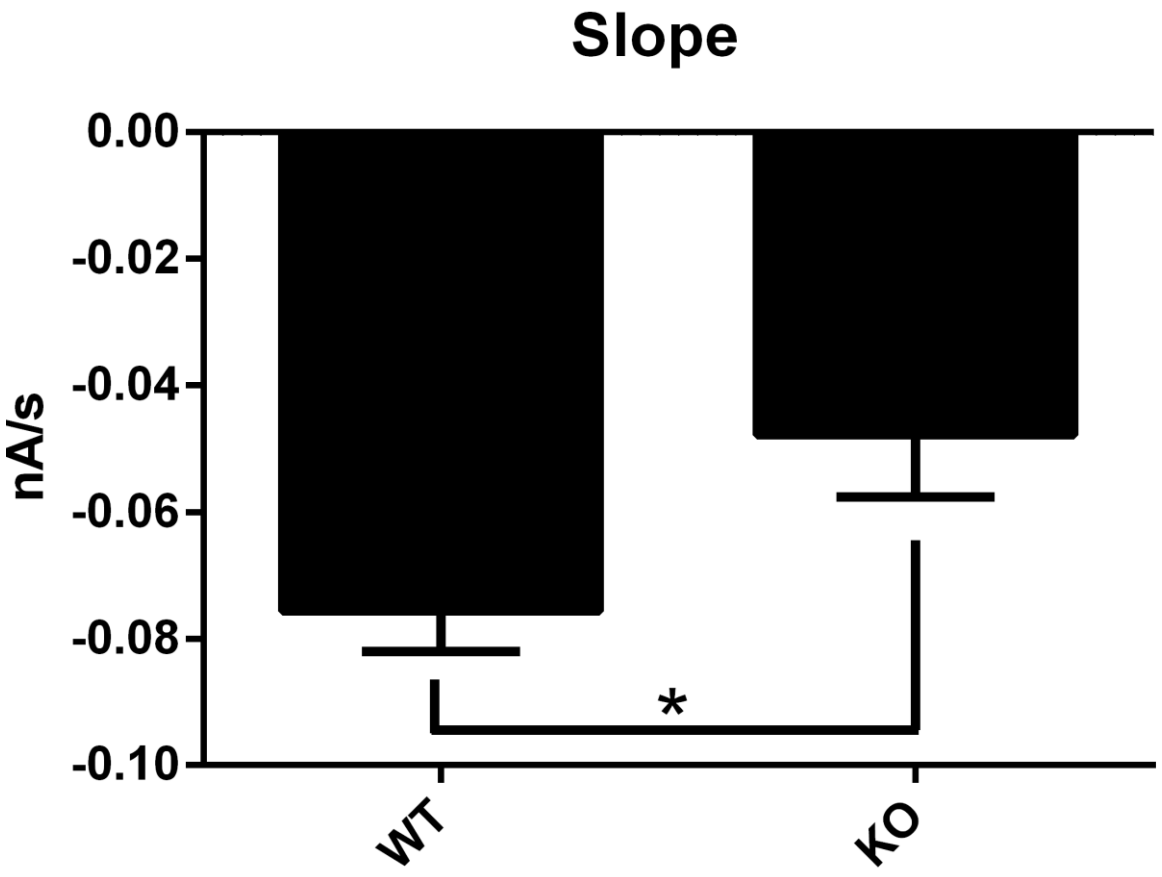


Figure 6.9: Slope of the dopamine response from the local maximum of the response during the first 2s to the end of the stimulus (at t=10) in the house light condition (when the house light was the first stimulus in the stimulus pair) (WT: n=16, KO: n=9). Error bars indicate ± SEM. * p < .05

The Dopamine Response of wildtype mice to neutral stimuli habituated over the session, but failed to habituate in GluA1 KO mice

The habituation of the dopamine signal to light stimuli over the session was analysed next (WT: n=16, KO: n=9). This analysis was conducted across both

stimuli in each light pairing, comparing all light stimuli in the first half with all light stimuli in the second half of the session. This analysis revealed a habituation of the dopamine response to neutral stimuli in wildtypes but not GluA1 knockout mice over the session (Fig. 6.10).

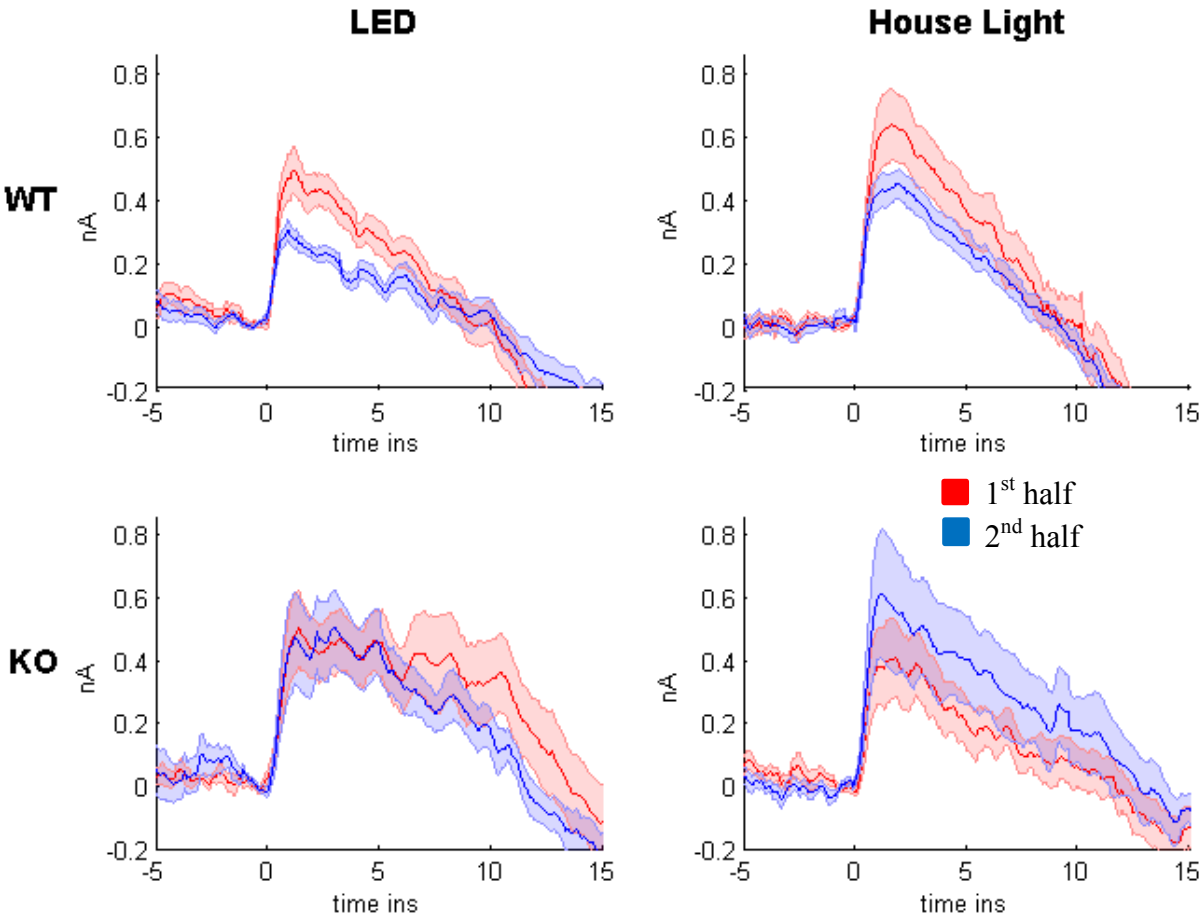


Figure 6.10: Average transient dopamine responses to the neutral lights (house and LED) in the first and second half of the experiment. The upper row shows the average response in wildtypes. The lower panel shows the average response in knockouts. The left column shows responses to the LED. The right column shows responses to the house light. Both panels compare responses in the 1st half of the session (red) with the 2nd half of the session (blue). Both lights were presented from t = 0s to t = 10s (WT: n=16, KO: n=9). Error bars indicate ± SEM.

Peak (maxima) dopamine responses to neutral stimuli were analysed with a three-way ANOVA with genotype as a between-subjects factor and halves (1st half vs 2nd half of session) and stimulus type (LED vs house light) as within-subjects factors (Fig. 6.11). This analysis revealed a significant genotype by halves interaction ($F(1,23) = 6.256$, $p = .020$). Subsequently, analysis of the simple main effects was conducted. This revealed that wildtypes had a reduced dopamine response in the second half compared to the first half ($F(1,15) = 9.853$, $p = .007$). Knockout mice showed no such reduction ($F(1,8) = 1.924$, $p = .203$). Furthermore, visual inspection of the data suggested a sensitization of knockouts to presentation of the house light. However, a separate ANOVA in the knockouts showed no effect of halves ($F(1,8) = 1.355$, $p = .278$). Still, the pattern of responding with a putative rise of the response of knockouts to presentation of the house light over the session shows remarkable similarity with the behavioural response (see Fig. 6.4). Furthermore, there were no overall main effects of genotype ($F(1,23) = 0.458$, $p = .505$), stimulus ($F(1,23) = 0.630$, $p = .436$), or halves ($F(1,23) = 0.960$, $p = .337$), although there was a significant interaction between genotype and stimulus type ($F(1,23) = 8.895$ $p = .007$), reproducing the analysis of the first stimulus of each pairing. Again, this interaction was driven by a tendency towards stronger dopamine responses in the knockouts to the LED (simple main effect: $F(1,23) = 3.253$, $p = .084$). The interactions of halves by stimulus ($F(1,23) = 2.083$, $p = .162$) and halves by stimulus by genotype ($F(1,23) = 1.844$, $p = .188$) were not significant. This showed that the habituation process over the session were not different in both stimulus types as measured by maxima.

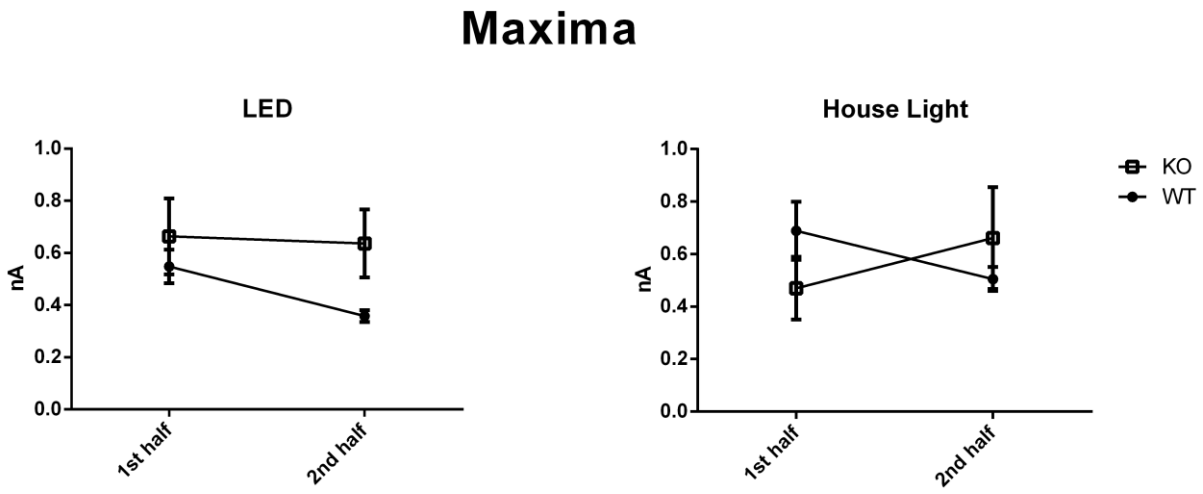


Figure 6.11: Average peak (maxima) of the dopamine responses to neutral stimuli in the first and second half of a session of the HORNS task. Data are divided by stimulus type into LED responses (left) and house light responses (right) (WT: n=16, KO: n=9). Error bars indicate ± SEM.

Area under the curve of the dopamine signal displayed a similar pattern as the maxima data. However, due to a higher variance in this measure, none of the effects or interactions were significant. The area under the curve (AUC) of the dopamine responses to neutral stimuli was analysed with a three-way ANOVA with genotype as a between-subjects factor and halves (1^{st} half vs 2^{nd} half of session) and stimulus type (LED vs house light) as a within-subjects factor (Fig. 6.12). This revealed no main effect of genotype, stimulus type, or halves ($F(1,23) < 1.5$, $p > 2.3$). In this analysis the genotype by halves and genotype by stimulus type interaction narrowly failed to reach significance (genotype by halves: $F(1,23) = 3.991$, $p = .058$; genotype by stimulus type: $F(1,23) = 3.827$, $p = .063$). However, since the analysis of the maxima showed a significant interaction of genotype by halves, a simple main effects analysis was still conducted. This revealed the same pattern. Wildtypes showed a significantly reduced area under

the curve in the second half (F(1,15) = 5.768, p = .030) whereas knockouts did not (F(1,8) = .539, p = .484).

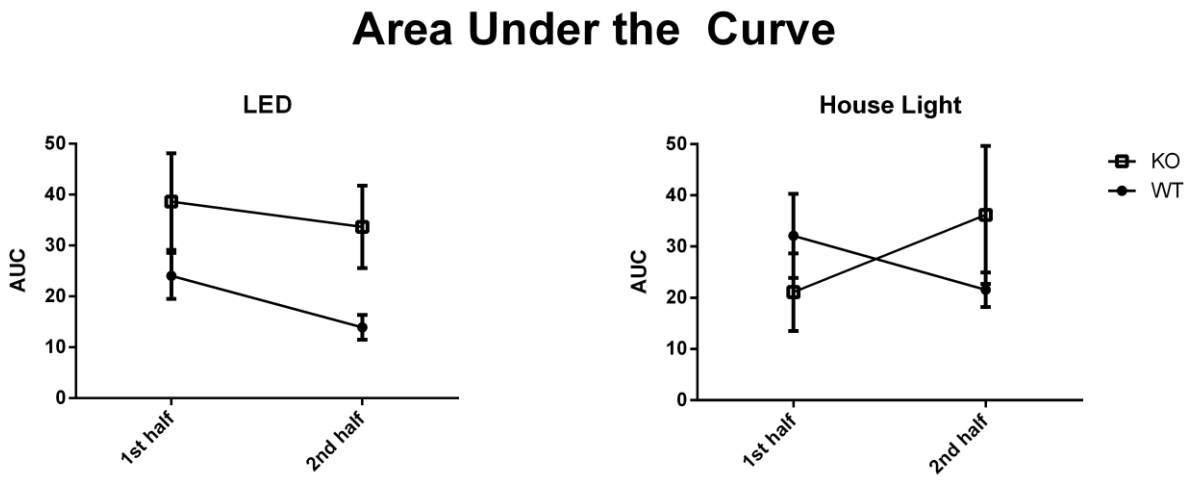


Figure 6.12: Average area under the curve of the dopamine responses to neutral stimuli in the first and second half of the HORNS task. Data are divided by stimulus type into LED responses (left) and house light responses (right) (WT: n=16, KO: n=9). Error bars indicate ± SEM.

Wildtypes showed stimulus-specific habituation of the dopamine response if the 2nd stimulus is an LED

Wildtypes showed stimulus-specific habituation when the 2nd stimulus in a pairing was an LED but not if it was a house light. Knockouts did not show stimulus-specific habituation to either stimulus (Fig. 6.13).

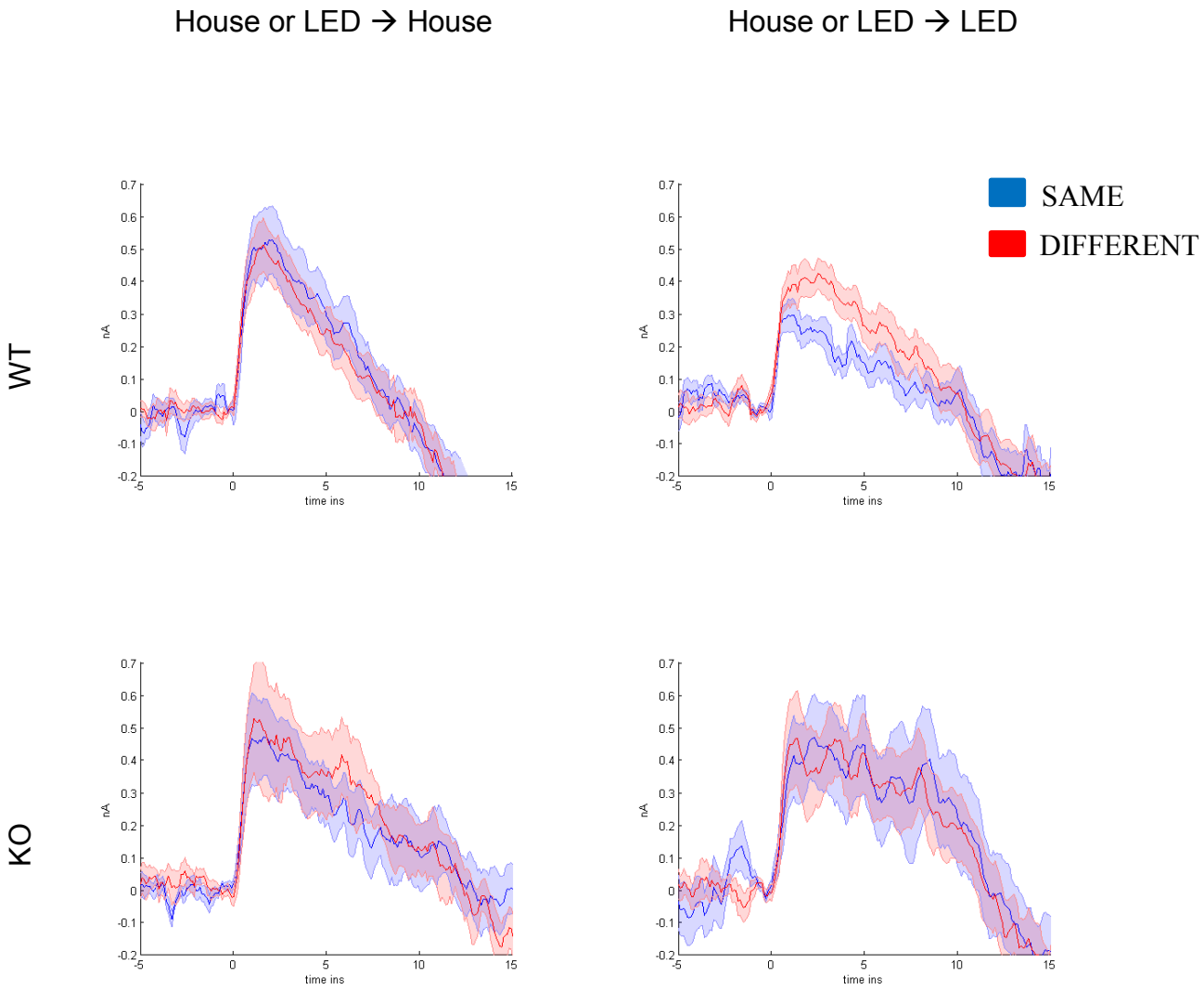


Figure 6.13: Average transient dopamine responses to the 2nd neutral light stimuli in each pairing. Dopamine traces are grouped by genotype (1st row: WT, 2nd row: KO) and stimulus type of the 2nd stimulus (1st column: house light, 2nd column: LED). This allows comparison of the effect of trial type (SAME (blue) vs DIFFERENT (red)) on each level of genotype and stimulus type (WT: n=16, KO: n=9).

A three way ANOVA with stimulus type (house vs LED) and trial type (SAME vs DIFFERENT) as within-subjects factors and genotype (WT vs KO) as the between-subjects factor was conducted (Fig. 6.14). This did not show a main effect of genotype ($F(1,23) = 0.459$, $p = .505$), stimulus type ($F(1,23) = 0.385$, $p =$

.541) or trial type (F(1,23) = 0.200, p = .659). The absence of an effect of trial type suggests that there was no overall stimulus-specific habituation to the first stimulus (i.e. mice oriented similarly in the SAME and DIFFERENT conditions). There was also no trial type by genotype interaction (F(1,23) = 0.011, p = .919). This suggested that knockouts and wildtypes displayed the same response pattern in the SAME and DIFFERENT conditions.

However, the analysis also revealed a significant three-way trial type by genotype by stimulus type interaction (F(1,23) = 4.334, p = .049). Visual inspection of the data showed that this interaction was driven by a higher dopamine response in the DIFFERENT trial than in the SAME trial in wildtypes when the 2nd stimulus was a LED, which was not present in the knockouts (Fig. 6.13 right panel). Therefore, an ANOVA on wildtype mice was performed with stimulus type (house vs LED) and trial type (SAME vs DIFFERENT) as within-subjects factors. This revealed a stimulus type by trial type interaction (F(1,15) = 7.767, p = .014). Due to this interaction a paired t-test on wildtypes when the 2nd stimulus was a LED was performed. This t-test revealed a strong tendency (t(15) = 2.097, p = .053) for the DIFFERENT trial type to evoke a stronger dopamine response than the SAME trial type.

A similar ANOVA on knockout mice with stimulus type (house vs LED) and trial type (SAME vs DIFFERENT) as within-subjects factors revealed no effect of stimulus type, trial type, and no stimulus by trial type interaction (F < 0.5, p > .5). This suggested that wildtypes displayed stimulus-specific habituation if the first stimulus was an LED, but not if the first stimulus was a house light. Knockout mice never showed stimulus-specific habituation.

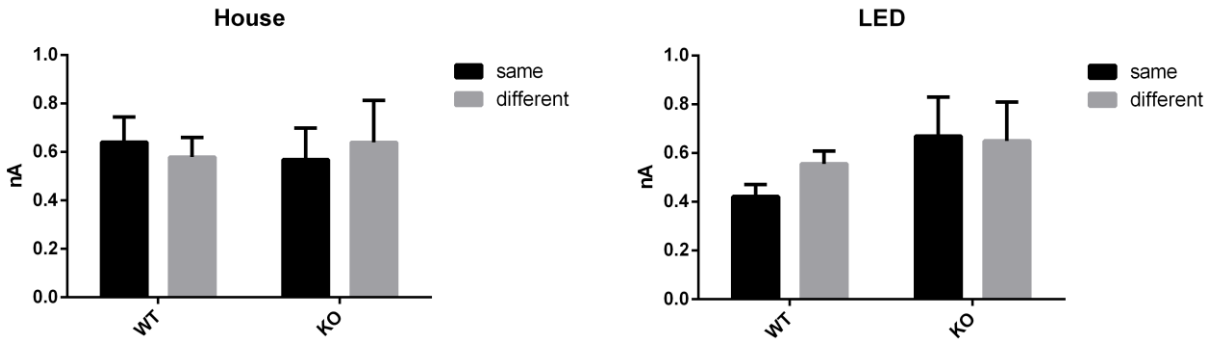


Figure 6.14: Peak dopamine response to the 2nd neutral stimulus in each trial based on stimulus type (house: left graph, LED: right graph), genotype (WT vs KO), and trial type (SAME vs DIFFERENT) (WT: n=16, KO: n=9). Error bars indicate ± SEM.

A similar analysis was conducted for the area under the curve. This analysis suggested that wildtype mice show stimulus-specific habituation when the first stimulus was a LED, but not when the first stimulus was a house light. GluA1 KO mice never showed stimulus-specific habituation. A four-way ANOVA with stimulus type (house vs LED), trial type (SAME vs DIFFERENT) and time bin (1st half of stimulus (0s-5s) vs 2nd half of stimulus (5s-10s)) as within-subjects factors and genotype (WT vs KO) as the between-subjects factor was conducted on the area under the curve of the dopamine signal for the duration of the stimulus (Fig. 6.15). This revealed an overall reduction of the signal in the 2nd time bin ($F(1,23) = 28.853$, $p < .001$) and no main effect of genotype $F(1,23) = 1.318$, $p = .263$), trial type ($F(1,23) = 0.281$, p .601), or stimulus type ($F(1,23) < 0.001$, $p = .996$). Knockouts had a tendency to show longer lasting responses (genotype*time bin: $F(1,23) = 4.190$, $p = .052$) and the LED induced a longer lasting response than the house light (stimulus type*time bin: $F(1,23) = 12.835$ $p = .002$). This response pattern is strikingly similar to the responses to the first stimulus reported above (Fig. 6.6).

Importantly, as in the maxima data, this analysis of the AUC revealed a three-way trial type by genotype by stimulus type interaction ($F(1,23) = 5.675$, $p = .026$), which inspection of Fig. 6.13 suggests might be driven by a greater response in the DIFFERENT than in the SAME condition in wildtypes when the 2nd stimulus is a LED (Fig. 6.13 upper right panel). To formally investigate this interaction a three-way within-subjects ANOVA was conducted on wildtypes with stimulus type (house vs LED), trial type (SAME vs DIFFERENT) and time bin (1st half of stimulus vs 2nd half of stimulus) as factors. This revealed a stimulus type by time bin interaction ($F(1,15) = 13.416$, $p = .002$) as was present in the main ANOVA and the analysis of the 1st stimulus. The 1st half of the dopamine response in the wildtypes was investigated. A further two- way within-subjects ANOVA on the wildtypes during the first 5 seconds of the stimulus, with stimulus type (house vs LED) and trial type (SAME vs DIFFERENT) as factors was conducted. This analysis showed a significant interaction of stimulus type and trial type ($F(1,15) = 10.581$, $p = .005$). This gave evidence to investigate the LED condition separately in wildtypes. Comparing the first five seconds of the WT response if the second stimulus was an LED, showed that wildtypes' dopamine system responded stronger in the DIFFERENT condition than in the SAME condition ($t(15) = 2.649$, $p = .018$). This effect was not present ($t(15) = 1.729$, $p = .104$) when the whole 10s were inspected due to the reduction of the signal in the 2nd half of the DIFFERENT trial.

To investigate if this effect was present in knockouts, a three-way within-subjects ANOVA was conducted on knockouts with stimulus type (house vs LED), trial type (SAME vs DIFFERENT) and time bin (1st half of stimulus vs 2nd half of

stimulus) as factors. There was no effect of trial type ($F(1,8) = 0.046$, $p = .835$) and trial type did not interact with another factor ($F(1,8) < 0.6$, $p > 0.4$). None of the other effects or interactions was significant ($F < 3.2$, $p > .11$). Notable, there was also no effect of trial type during the first 5s of the stimulus when the first stimulus was an LED ($t(8) = 0.048$, $p = .963$). In summary, this analysis suggested that wildtype mice show stimulus-specific habituation when the first stimulus was a house light. GluA1 KO mice never showed stimulus-specific habituation.

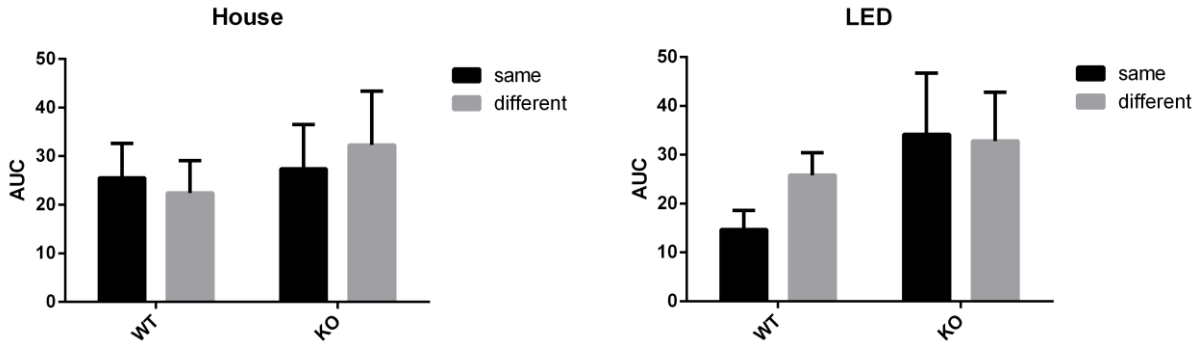


Figure 6.15: Area under the curve over of the dopamine response over the full 10s stimulus duration to the 2nd neutral stimulus in each trial based on stimulus type (house vs LED), genotype (WT vs KO), and trial type (SAME vs DIFFERENT) (WT: n=16, KO: n=9). Error bars indicate ± SEM

Unsignalled Reward during HORNS

Although the HORNS task was designed to examine stimulus-based habituation separate to motivational salience, rewards were nonetheless periodically delivered on a RI schedule throughout the session in order to maintain food magazine responses. Such unsignalled reward presentation reliably causes increases in activity of midbrain dopamine neurons (Mirenowicz and Schultz,

1994, Cohen et al., 2012) and also increases dopamine release in the NAc core (Gan et al., 2010). Therefore, we could in addition examine dopamine signals in response to these unsignalled rewards (WT: n=16, KO: n=9) to determine whether these were also modulated in the KO mice.

As can be observed in Fig. 6.16, in fact peak dopamine responses to these instrumentally-derived, unpredictable reward deliveries were on average more than twice as large in KO mice compared to controls (WT peak: 0.27nA ± 0.051 SEM, KO peak mean: 0.62nA ± 0.084). Both the peak dopamine and average change in dopamine levels following reward (in an 8 second window after reward delivery) were significantly increased in the knockouts (peak: $t(23) = 3.785$, $p = .001$, average: $t(23) = 3.239$, $p = .004$). Therefore, not only were there stimulus-specific changes in dopamine responses to neutral stimuli, but there were also striking increases in dopamine release following action-contingent reward deliveries in the GluA1 KO mice.

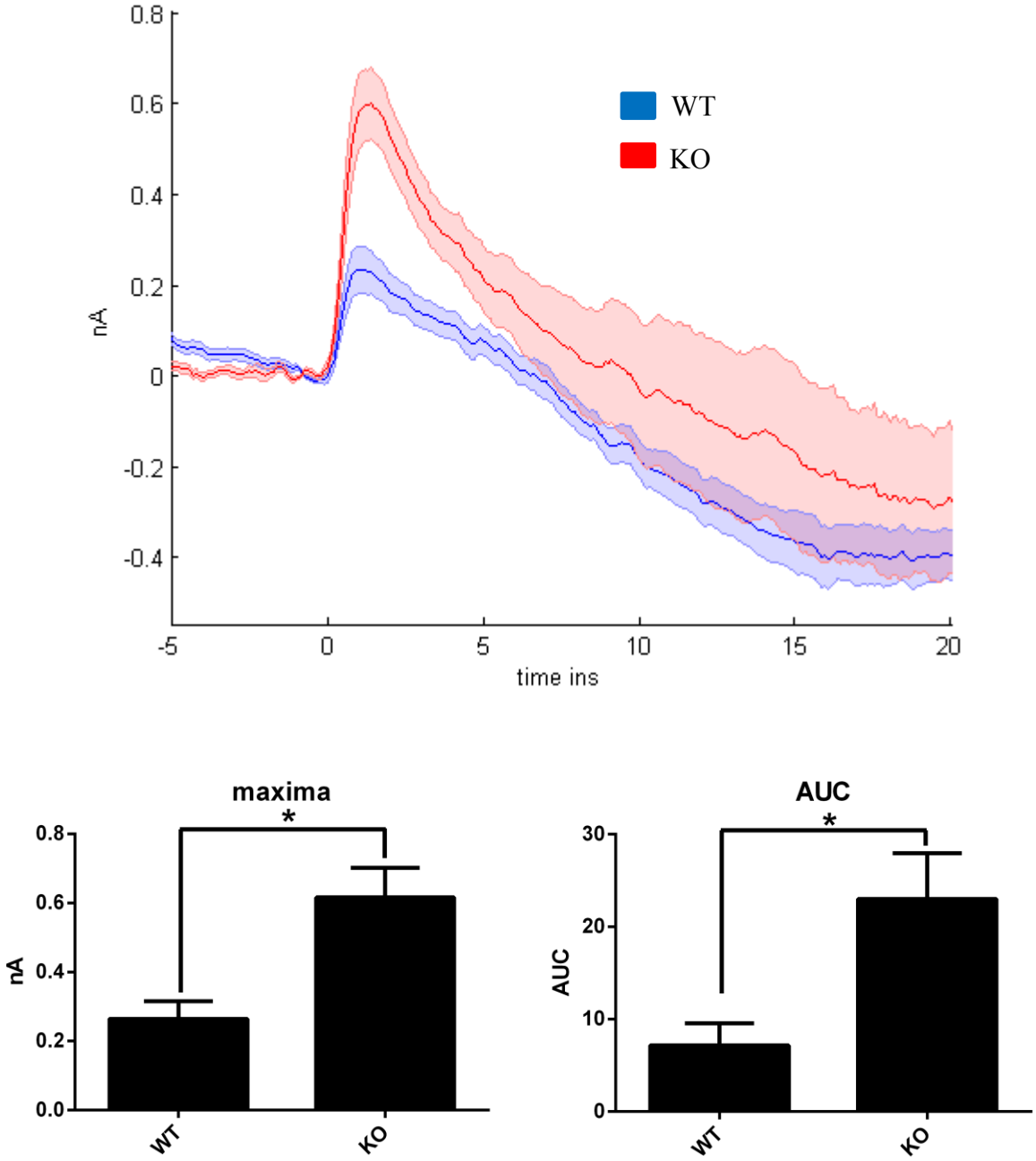


Figure 6.16: Dopamine responses to rewarded head entries during the HORNS task. The upper panel displays the average transient dopamine response to reward delivery (t = 0) in WT (blue) and KO (red) mice. The lower panels show peak (left) and area under the curve (right) calculated over 8s after reward delivery (WT: n=16, KO: n=9). Error bars indicate ± SEM. * p < .05

Experiment 6d: GluA1 Knockout mice had an Increased Dopamine Response to Head Entry-dependent Reward Delivery which did not Decrease across Session

To further investigate this striking increase of the dopamine response in GluA1 knockout mice to unsignalled reward delivery, 11 mice (6 WT, 5 KO) went on in a separate session to perform a task where three different magnitudes of unsignalled reward were delivered (10µl (low), 22µl (medium) or 48µl (high), again on a 120s variable interval schedule. The medium reward magnitude was equivalent to the reward given during the HORNS task. Again, reward delivery was contingent on head entry into the food magazine following the allotted time interval.

Behaviour

As in the HORNS experiment knockouts showed higher head entry duration levels. Interestingly, whereas the head entry duration reduces over the session in wildtypes it stayed constant in the knockouts (Fig. 6.17). A two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and time block (1st half vs 2^{nd} half of session) as the within-subjects backed up these observations, with knockouts spending considerably more time in the food magazine overall ($F(1,9) = 19.795$, $p = .002$). While there was an overall effect for mice to spend less time in the food magazine during the 2^{nd} part of the session ($F(1,9) = 32.179$, $p < .001$), there was also a time block by genotype interaction ($F(1,9) = 12.054$, $p = .006$). Post-hoc analysis of the reduction of head entry duration during the

session in each of the genotypes showed that it is present in the wildtypes (t(5) = 6.219, p = .002) and absent in knockouts (t(4) = 1.678, p = .169).

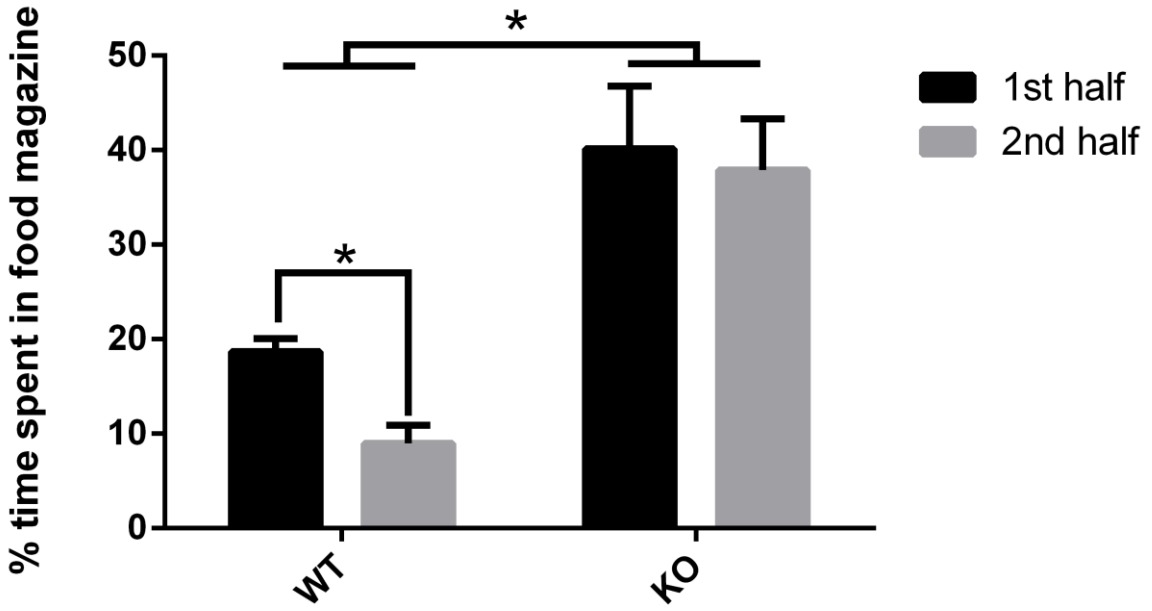


Figure 6.17: Percentage time spent in the food magazine during the unsignalled variable reward experiment (experiment 6d) (WT: n=7, KO: n=6). Error bars indicate ± SEM. * p < .05

Dopamine Responses

As can be observed in Fig. 6.18, there were rapid increases in dopamine following reward delivery in both groups WT: n=7, KO: n=6) and the average dopamine levels post-reward positively scaled with increasing reward magnitude. Importantly, as observed in the previous experiment (Exp. 6c), these increases were substantially greater in the knockouts. A three-way ANOVA with time block (1st half vs 2nd half of session) and reward magnitude (low, mid, high) as within-subjects factors and genotype (WT vs KO) as the between-subjects factor was conducted on the peak dopamine response (Fig. 6.19). This revealed an overall increase in dopamine responses in the knockouts ($F(1,11) = 7.491$, $p = .019$), though not an interaction between genotype and magnitude ($F(2,22) = 0.362$, $p =$

.700) suggesting that in both genotypes dopamine coded the reward magnitude. Interestingly, there was also a main effect of time block ($F(1,11) = 17.538$, $p = .002$) and an interaction between time-block and genotype ($F(1,11) = 12.876$, $p = .004$). Post-hoc tests showed that this was caused by a significant decrease in dopamine release across the session in the wildtypes but not the knockouts (simple main effect of time block: WTs: $F(1,6) = 48.931$, $p < .001$; KOs: $F(1,5) = 0.119$, $p = .744$).

A similar pattern of results was found when analysing the area under the curve of the dopamine response in a 8s window post-reward (Fig. 6.20). However, here there was now an overall significant increase in the dopamine response with increasing reward magnitude ($F(2,22) = 5.886$, $p = .009$). By contrast, the interaction of genotype by time block failed to reach significance ($F(1,11) = 2.293$, $p = .158$) and the interaction of genotype and reward magnitude was also not significant ($F(2,22) = 0.650$, $p = .532$). However, given the findings from peak data, the simple main effect of time block was nonetheless investigated separately in the two genotypes. As found for the peak dopamine response, wildtypes displayed a strong reduction between the 1st and the 2nd half (simple main effect of block: $F(1,6) = 22.637$, $p = .003$) whereas knockouts did not (simple main effect of block: $F(1,5) = 0.919$, $p = .382$).

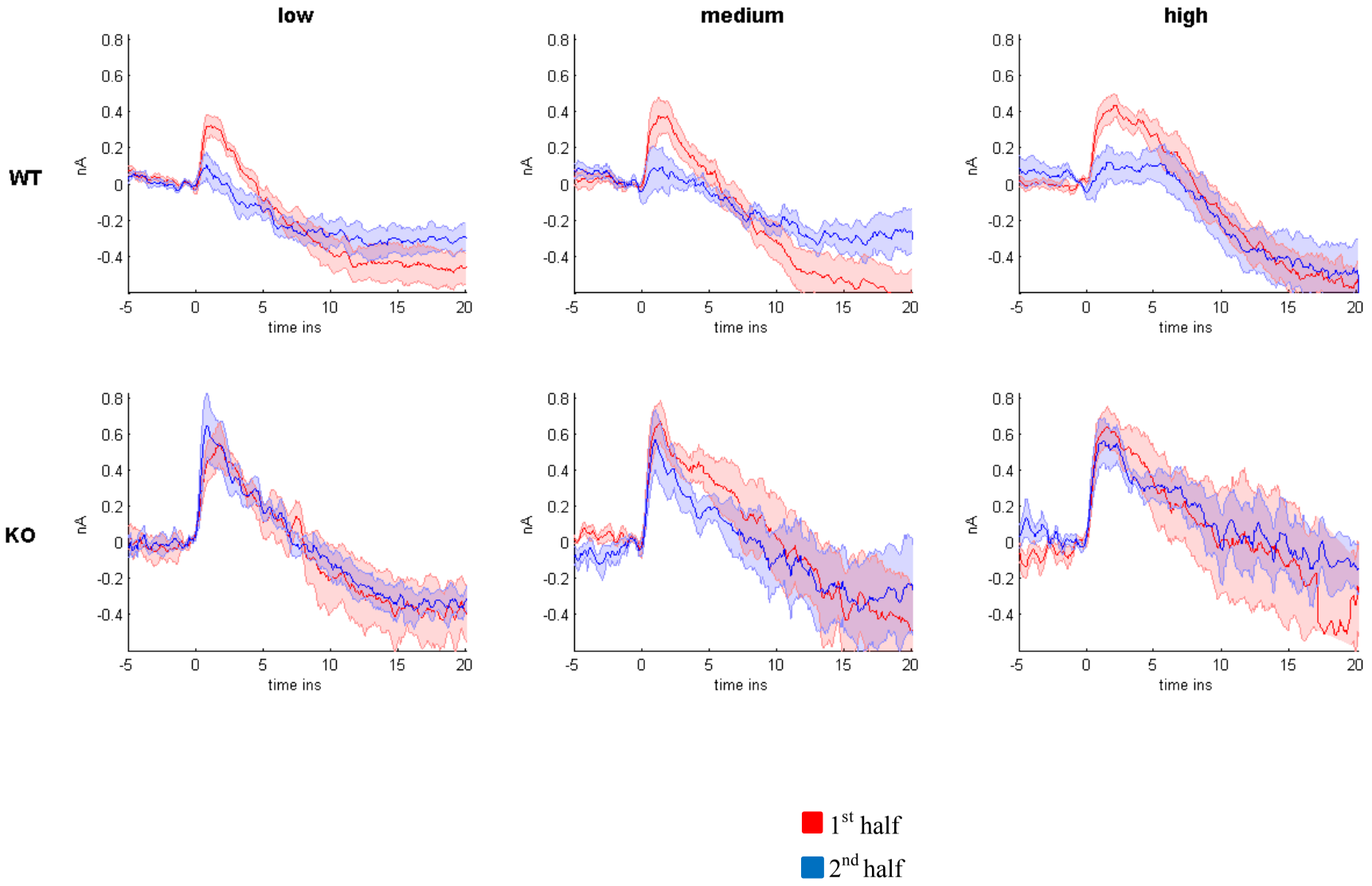

low
medium
high
WT
KO
nA
time ins
1st half
2nd half

Figure 6.18: Average transient dopamine response to unsignalled reward delivery upon head entry. Dopamine traces are grouped by genotype (1st row wildtypes, 2nd row: knockout) and reward magnitude (1st column: low, 2nd column: medium, 3rd column: high). This allows comparison of the effect of halves of the session (1st half (red) vs 2nd half (blue)) at each level of genotype and reward magnitude(WT: n=7, KO: n=6). Error bars indicate ± SEM.

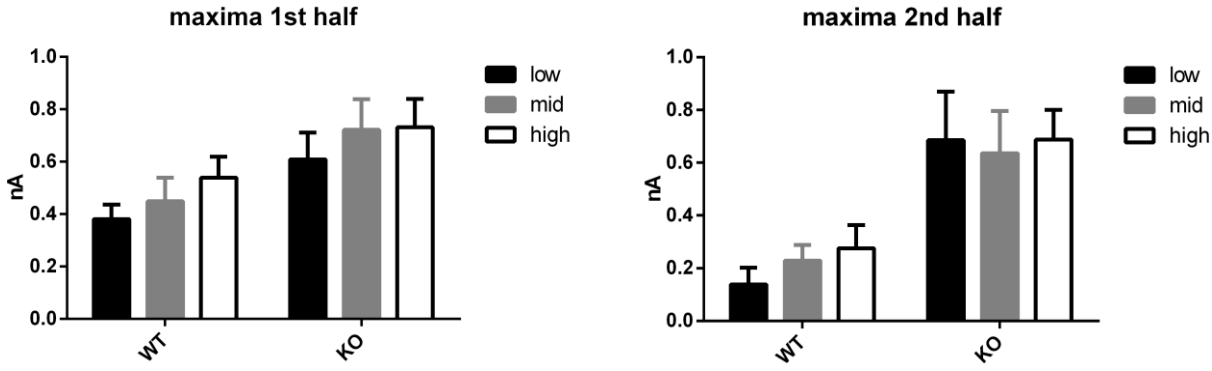


Figure 6.19: Average peak (maximum) of the transient dopamine response during 8s after unsignalled reward delivery. Grouped by time bin (left: 1st half of session, right: 2nd half of session) and reward magnitude (1st column (black): low, 2nd column (grey): medium, 3rd column (white): high in each triad of bars) (WT: n=7, KO: n=6). Error bars indicate ± SEM.

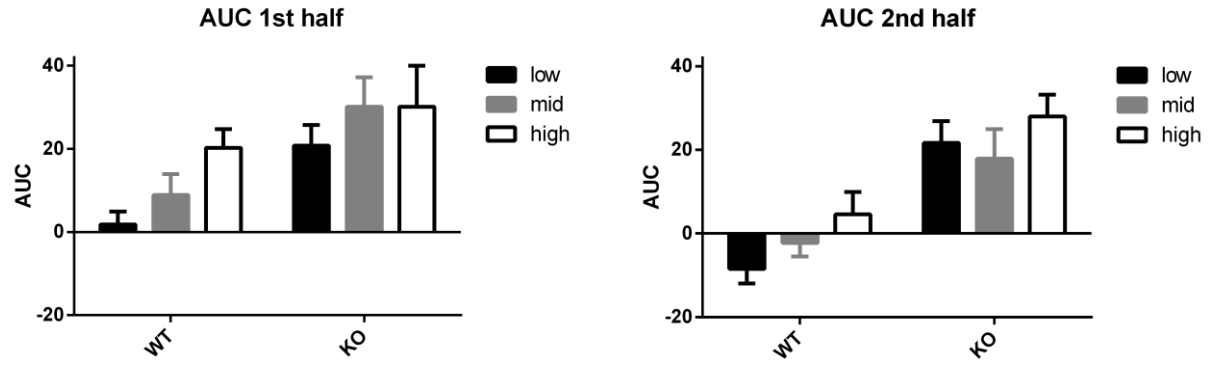


Figure 6.20: Average area under the curve of the transient dopamine response during 8s after unsignalled reward delivery. Grouped by time bin (left: 1st half of session, right: 2nd half of session) and reward magnitude (1st column (black): low, 2nd column (grey): medium, 3rd column (white): high in each triad of bars) (WT: n=7, KO: n=6). Error bars indicate ± SEM

Unrewarded Head Entries

GluA1 KO mice showed stronger instrumental responding (i.e. higher head entry levels). A potential underlying reason for this increase could be the stronger dopamine response to reward seen in GluA1 KO mice, which could act as a reinforcer of the instrumental response (Niv and Montague, 2008). However, instrumental responding is not only controlled by positive feedback for responses that yield more reward than expected. If a response yields less reward than expected, a negative reward prediction error is generated which makes the behaviour less likely to be repeated.

To investigate the occurrence of such negative feedback in the two genotypes, I examined changes in dopamine levels time-locked to magazine head entries that did not yield reward. Unrewarded head entries were associated with pronounced dips in dopamine, consistent with negative prediction error responses in the dopamine signal in WT. By contrast, these dips were strongly attenuated and arguably completely absent in the knockouts. This was supported by a two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and time block (1st half vs 2^{nd} half of session) as a within-subjects factor on the area under the curve over the 8s after an unrewarded head entry (Fig. 6.21). This showed that the dips were bigger in the wildtypes than in the knockouts ($F(1,11) = 11.115$, $p = .017$). There was no effect of time block ($F(1,11) = 0.054$, $p = .770$) and no time block*genotype interaction ($F(1,11) = 2.304$, $p = .157$).

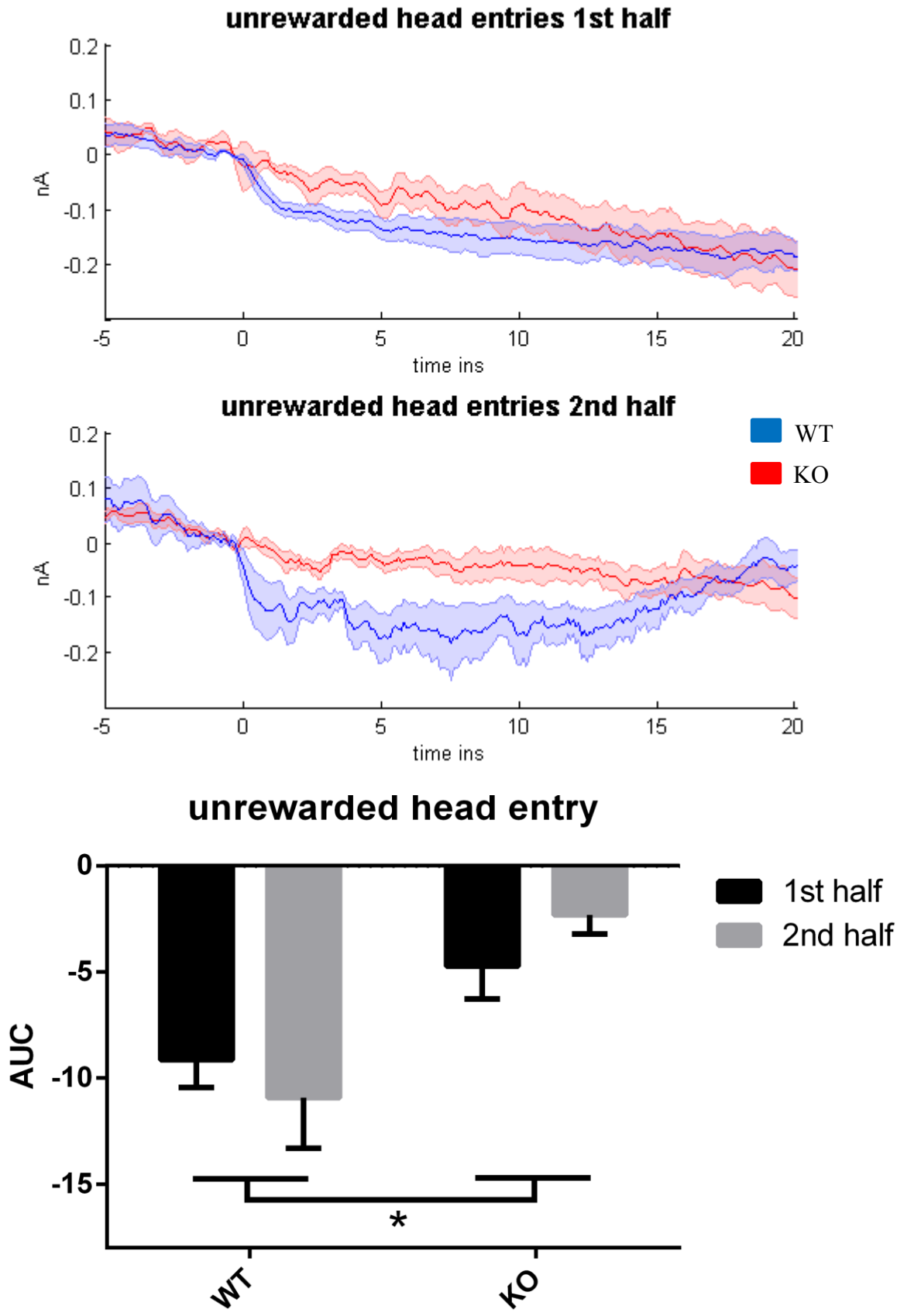


Figure 6.21: Dopamine response to unrewarded head entries during the variable unsignalled reward experiment. The upper panels shows the average transient dopamine responses to unrewarded head entry in the first and second half of the session (top and middle) respectively. The lower panel shows the AUC calculated for the 8s after head entry (WT: n=7, KO: n=6). Error bars indicate ± SEM. * p < .05

Discussion

GluA1 KO mice showed a lack of habituation of the dopamine response to neutral stimuli. Additionally, they showed increased reward-induced dopamine responses that did not decrease across the session as wildtypes' dopamine responses did. Thus, there was evidence for a hyperdopaminergic phenotype in these mice with a primary glutamatergic dysfunction.

Behavioural Response to Neutral Stimuli

First, the HORNS task, a behavioural assessment of habituation to neutral stimuli, was validated in c57/Bl6 mice (experiment 6a). This showed that wildtype mice display stimulus-specific habituation of the orienting response. Additionally, this experiment was conducted after systemic injections of the NMDA receptor blockers MK-801. MK-801 abolished stimulus-specific habituation at a dose that did not induce hyperactivity in a locomotor activity test (experiment 6b). The thusly-validated HORNS task was then conducted in GluA1 KO mice and littermate controls while the dopamine response was measured using FCV (experiment 6c).

Sanderson et al. (2011b) showed that in the HORNS task wildtype mice orient less towards the second stimulus if it is the same as the first presentation in the pairing than if the second stimulus is different (i.e. they habituate to the light in a stimulus-specific manner). By contrast, GluA1 knockout mice respond more to the second stimulus if it is the same as the first one, compared both to the

presentation of a different stimulus or to the first stimulus presentation (i.e. they sensitize) consistent with the deficit in short-term habituation in these animals.

In experiment 6c KO mice displayed stronger suppression of the head entry duration by neutral stimuli in the HORNS task. This increase in head entry suppression was potentially due to increased baseline head entry duration. This gave KO animals more room to display a suppression of head entry duration during the light stimuli. Further analysis of the behavioural responses in this task was difficult due to low orienting in wildtypes.

One reason for these low levels of orienting in the wildtypes, compared to Sanderson et al. (2011b) and experiment 6a, might be that mice had headcaps which were connected to a commutator. However, visual inspection suggested that headcapped mice moved indistinguishably from non-implanted mice. While the possibility of an influence of headcap and tether on behaviour cannot be completely discounted, another factor appears to be more likely to influence orienting. The food magazine in both Sanderson et al. (2011b) and experiment 6a was the standard MED Associates magazine, which is embedded into the wall of the operant chamber. Therefore, when mice placed their snout in this version of the food magazine the wall partially hid the house light and LED from view. Hence, even considering the increased visual field in mice compared to humans, mice would have to exit the magazine to see the lights. Experiment 6c, however, used a custom-made food magazine that extended into the operant box. In this food magazine, the lights were still in full view of the mice while their snout was in the magazine. Therefore, animals did not have to remove their head from the magazine to see the stimuli. This presumably decreased the magnitude of any

orienting response and hence suppression of head entry into the food magazine by light stimuli.

GluA1 KO mice still displayed higher baseline head entry levels than wildtypes. This differed from Sanderson et al. (2011) where reward delivery was not dependent on head entry and KO and WT showed the same head entry duration. In experiment 6c reward delivery was dependent on head entry, making collection of reward into an operant task. Notably, Johnson (2005) already found that GluA1 KO mice nose poke more to receive food reward in an instrumental task.

GluA1 KO mice displayed increased dopamine responses evoked by neutral stimuli that do not display short-term habituation

GluA1 KO mice displayed stronger phasic dopamine release in response to neutral stimuli than wildtypes. This increase expressed itself differently dependent on stimulus identity. To flashing LED stimuli, GluA1 KO mice exhibited an outright increase in dopamine release as reflected in higher peak dopamine responses and an increased area under the curve. In response to a continuous house light KO mice displayed peak dopamine responses similar to wildtypes but a slower decay of the signal. This could potentially be explained by the different nature of the two stimuli. The continuous house light led to dopamine release at stimulus onset, which then decreased linearly in both genotypes but at different rates. The flashing LED, however, was changing across the 10s stimulus duration. In effect, these flashes could be seen as eleven individual presentation of a short LED. GluA1 KO mice are known to have

deficits in short-term habituation (Sanderson et al., 2007, Sanderson et al., 2010, Sanderson et al., 2011b). So, whereas wildtypes habituate to the flashing, GluA1 KO mice continued to pay attention to the stimulus, leading to a phasic dopamine response that stayed constant for the first half (0-5s) of the stimulus presentation (Fig. 6.6).

More direct evidence for a deficit in habituation of the dopamine responses comes from the analysis of the phasic dopamine response across the session. Wildtypes showed an overall decreased dopamine response to neutral stimuli in the 2nd half of the session compared to the 1st half. Knockouts, however, showed no such decrease. In the case of the house light there is even preliminary evidence for an increase of the dopamine response. This would fit with the behavioural analysis, which showed weak evidence for an increase of the orienting response to the house light in the 2nd half compared to the 1st half of the session in KO mice. However, the associated statistical comparison was not significant.

Finally, wildtype mice showed stimulus-specific habituation of the dopamine response when the 2nd stimulus was an LED but not if it was a house light. GluA1 KO mice did not show stimulus-specific habituation to either stimulus. This, in part, follows the behavioural habituation seen by Sanderson et al. (2011b). However, due to low baseline head entry in wildtypes, interpretation of the orienting responses in this experiment was difficult. Nevertheless, wildtype mice showed habituation to neutral stimuli at the level of the dopamine response, albeit only if the first stimulus was an LED. This habituation is lost in GluA1 KO mice. However, no sensitization of the response was found in GluA1 KO mice at

either the behavioural or the physiological level. This suggests the necessity to replicate the behavioural effect seen by Sanderson et al. (2011b).

GluA1 KO mice show increased reward-evoked, phasic dopamine signals and decreased dips in the dopamine signals during non-rewarded head entries into the food magazine.

GluA1 KO mice had an enormously increased dopamine response to unsignalled reward delivery during the HORNS task compared to WT mice. This interesting finding was investigated further using a dedicated unsignalled reward paradigm in which reward delivery was again dependent on head entry (experiment 6d). Once more, knockout mice displayed increased head entry levels and an increased reward-induced dopamine response. Intriguingly, wildtype mice displayed phasic dips in the dopamine levels upon non-rewarded head entries, which were absent in GluA1 KO mice. Potentially, these two findings combined might explain the increased head entry levels of GluA1 KO mice during instrumental tasks observed in both experiment 6c and 6d, as well as by Johnson et al. (2005).

However, so far the role of dopamine as reward prediction error signal has only been presented in Pavlovian conditioning tasks in which a cue had to be associated with a reward delivery and no actions are necessary. In these Pavlovian tasks dopamine neurons in the VTA initially fire at reward delivery when the cue is not yet associated with the reward. As the cue gets associated with the reward, dopamine neurons start to be active when the cue is presented leading to a phasic dopamine signal at the cue presentation. If, however, the cue

is presented and the predicted reward is not then given, dopamine cell activity will dip below baseline and we would see a dip in the phasic dopamine response in the nucleus accumbens (Schultz et al., 1997).

In instrumental conditioning, animals have to learn about actions and their consequences (e.g. did this head entry lead to reward?) rather than about the associations between stimuli. So called actor/critic models can explain the role a dopaminergic reward prediction error signal could have in instrumental conditioning (Niv and Montague, 2008). In this model the actor initiates actions (e.g. a head entry) on the outside world based on a policy (e.g. spend 10% of the session in the food magazine). However, the actor does not have access to the "real world" to evaluate the outcome of its actions. This is the role of the critic in this model. The critic evaluates the state the animal is in. If an action leads to a better state than expected (e.g. a head entry leads to an unsignalled reward), a positive reward prediction error (a phasic dopamine increase) is sent to the actor. Thereby, the action just performed (e.g. head entry) by the actor is reinforced and is therefore more likely to be repeated. If, however, an action is less rewarding than expected (e.g. a head entry that does not lead to reward delivery), a negative reward prediction error (a dip in dopamine) is sent to the actor. This would make the action (e.g. head entry) less likely to be repeated. Dopaminergic activity is believed to act as the reward prediction signal in this model (Joel et al., 2002). Therefore, increased reward-evoked dopamine responses could give GluA1 KO mice stronger reinforcement signals, increasing their instrumental behaviour (i.e. head entries). And reduced dips in response to

unrewarded head entries could equally increase head entries by reducing negative feedback.

Experiment 6d also allowed investigating the change of the reward-induced dopamine response over a single session. Similar to the dopamine response to neutral stimuli, the reward-induced dopamine response in WT mice decreased over the session. This reduction, however, did not occur in knockouts. This is in accordance with Johnson et al. (2005). As discussed above Johnson et al. (2005) saw higher nose poking levels to gain reward in an instrumental task in GluA1 KO mice. This, however, was part of a more complex experiment. Knockout and wildtype mice were given the option to nose poke to receive one of two possible rewards (A or B) over multiple trials. Then reward A was devalued by giving mice unlimited access to this reward before the experimental session started. WT mice reduced their response to the devalued reward in this session. GluA1 KO mice, however, still consumed equal amounts of reward A and reward B. This suggests that KO mice were insensitive to the change in value of rewards as satiety set in in experiments 6c and 6c. This insensitivity to the devaluation of food reward by satiety was also reflected in the reward-evoked dopamine responses. Wildtype mice showed decreased reward-evoked dopamine responses in the second half compared to the first half, potentially reflecting satiety processes. This decrease was absent in knockout mice.

This chapter showed hyperdopaminergic responses in a mouse with a glutamatergic dysfunction relevant to schizophrenia. Wiedholz et al. (2008) has previously reported a hyperdopaminergic phenotype in GluA1 KO mice based on reduced dopamine clearance in the nucleus accumbens. However, in this study

anesthetized mice were locally infused with dopamine and the clearance of this extracellular dopamine was measured. Therefore, the observed change in clearance was not associated with any behaviour. This chapter, on the hand, showed increased behaviourally-evoked phasic dopamine responses in a task relevant to aberrant salience in schizophrenia. If one were to define an aberrant process as any process differing from wildtype mice, this chapter could be said to have shown aberrant dopaminergic activity in GluA1 KO mice in a task assessing salience attribution to neutral stimuli.

Chapter 7: The Phasic Dopamine Response during Pavlovian Conditioning in GluA1 Knockout Mice

Introduction

Chapter 6 investigated the phasic dopamine responses of wildtypes and GluA1 KO mice to neutral stimuli and instrumentally contingent, unsignalled rewards. This revealed striking changes in patterns of dopamine release in the GluA1 KO mice: a prolonged dopamine response elicited by neutral stimuli, a greatly increased dopaminergic response to reward delivery and an absence of dips in dopamine levels following an unrewarded action. The most widely held view on the role of dopamine is that it acts as a reward prediction error signal (Schultz et al., 1997, Schultz, 2016). Early evidence for this view came from the observation that dopaminergic cells in the VTA of macaques are responding to cues that predict reward rather than the reward itself, when an association between cue and reward has been acquired (Schultz et al., 1997).

These experiments were conducted as Pavlovian conditioning experiments (i.e. animals did not have to perform an action to get rewarded). Reward delivery in chapter 6, however, was contingent upon head entry. Therefore it is not clear how comparable the results of chapter 6 are to reward responses in Schultz et al. (1997). Reward prediction error signals in Pavlovian conditioning can be measured as changes in phasic dopamine in the nucleus accumbens core.

Parker et al. (2010) paired the presentation of a lever with reward delivery and recorded FCV signals from the nucleus accumbens core of mice. These mice displayed rising phasic dopamine responses as the CS (lever extension) was associated with rewards over multiple trials. This increase in the CS-evoked dopamine response over many CS-US pairings followed the predictions of Schultz et al. (1997). However, in contrast to Schultz et al. (1997) the US-evoked dopamine response in mice did not decrease as the CS-US association was acquired. Nevertheless, Parker et al. (2010) saw a phasic dopamine response to a cue that predicted reward suggesting dopamine still acts as reward prediction error signal in mice during Pavlovian conditioning. In the previous chapter, it was argued that the reward prediction error signal is increased in GluA1 KO mice as suggested by increased phasic dopamine responses to unsignalled reward. This suggested that GluA1 KO mice perceive the same reward as more rewarding (i.e. more reinforcing). The strength of association acquired in a Pavlovian task increases with the magnitude of the US (Bouton, 2007). Therefore, assuming dopamine in the nucleus accumbens core acts as a reward prediction error signal, the increased reward-induced dopamine response in the GluA1 KO mice should increase Pavlovian conditioning. However, Mead and Stephens (2003) showed that GluA1 KO mice learn the association between a tone (the conditioned stimulus, CS) and food delivery (the unconditioned stimulus, US) in a Pavlovian conditioning experiment at a rate equivalent to wildtypes.

This chapter will investigate how the phasic dopamine signal and learning in GluA1 KO mice is changed in an appetitive Pavlovian conditioning task. In this experiment this association of CS (8s of lever extension) and US (pellet delivery)

was evidenced by an approach of the mice to the food magazine while the CS was presented. Phasic dopamine was measured in the nucleus accumbens core throughout every session of conditioning using FCV.

Methods

Task

After completion of the variable reward experiment (experiment 6d), 19 mice (9 WT, 10 KO) went on to perform the appetitive Pavlovian conditioning task. Prior to starting this experiment, all animals were given a number of 20mg grain pellets in their home cage in order to habituate them to this new reward (as opposed sucrose solution). The mice were then given two sessions of 24 presentations of a single reward pellet on a 120s variable time schedule (range: 90-150s). Reward delivery was now not dependent on head entry into the food magazine. During these sessions no FCV signal was recorded.

This was followed by 8 sessions of Pavlovian conditioning. Each session consisted of 24 pairings of the conditioned stimulus (CS) with a one pellet reward as the unconditioned stimulus (US). The CS consisted of the extension of a lever positioned to the left of the food magazine. The lever acted purely as a CS; responses on this had no consequences for the task. The lever was extended for 8s and retracted coincided with reward delivery. Head entry duration during the CS and an equivalent 8s pre-CS baseline period were recorded.

FCV Data analysis

The analysis of the FCV data was conducted in two steps. First, responses to the CS and US on the first session only were investigated. Then the development of these responses across sessions was investigated to examine a potential reward prediction error with learning.

Analysis of the FCV data was conducted on slightly different sets of electrodes. Some electrodes showed a good dopamine signal in early sessions, but degraded before the experiment was completed. They therefore could not be included into an across-session analysis, but still gave valuable insight into the phasic dopamine response during the very first session. Analysis of the first session was therefore conducted on all 21 viable electrodes (13 WT, 8 KO).

For the analysis of the FCV signals over all 8 sessions, two consecutive sessions were combined into one block resulting in four blocks. In some animals, not all sessions passed our criteria for inclusion. Therefore, for our analyses of changes in patterns of dopamine across blocks, I averaged data from validated sessions for a given block. This meant that if one of the two sessions in a block was missing, only data from the single session within a block was included. However, if both sessions were present, the average dopamine signal across the two sessions was taken. This enabled the inclusion of 11 electrodes (6 WT, 5 KO) into the analysis of the dopamine signal across blocks. There was no difference in the number of excluded sessions between wildtypes and knockouts. Where appropriate, results of post-hoc tests were corrected for multiple comparisons using Sidak correction.

Results

Behaviour: Wildtypes and Knockouts Acquired the Association of CS and US

Wildtype and knockout mice (WT: n=9, KO: n=10) acquired a Pavlovian conditioning task at an equivalent rate over 8 training sessions. In order to track Pavlovian learning rates in the GluA1 KO and WT mice, the change in the amount of time spent in the food magazine during presentation of the CS was recorded. However, this measure can be influenced by overall response rates. Therefore, I initially examined time spent in the magazine during the pre-CS period. As observed in the previous chapter, overall magazine responding was generally higher in the knockouts than wildtypes. A two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and session (8 sessions) as the within-subjects factor was conducted on the 8s baseline period before presentation of the conditioned stimulus (Fig 7.1). This revealed a trend towards higher baseline head entry duration in knockouts ($F(1,14) = 4.136$, $p = .061$) as was seen in the previous experiments (experiment 6c and 6d). There was no effect of sessions ($F(7,98) = 0.528$, $p = .811$) or genotype by sessions interaction ($F(7,98) = 1.011$, $p = .429$).

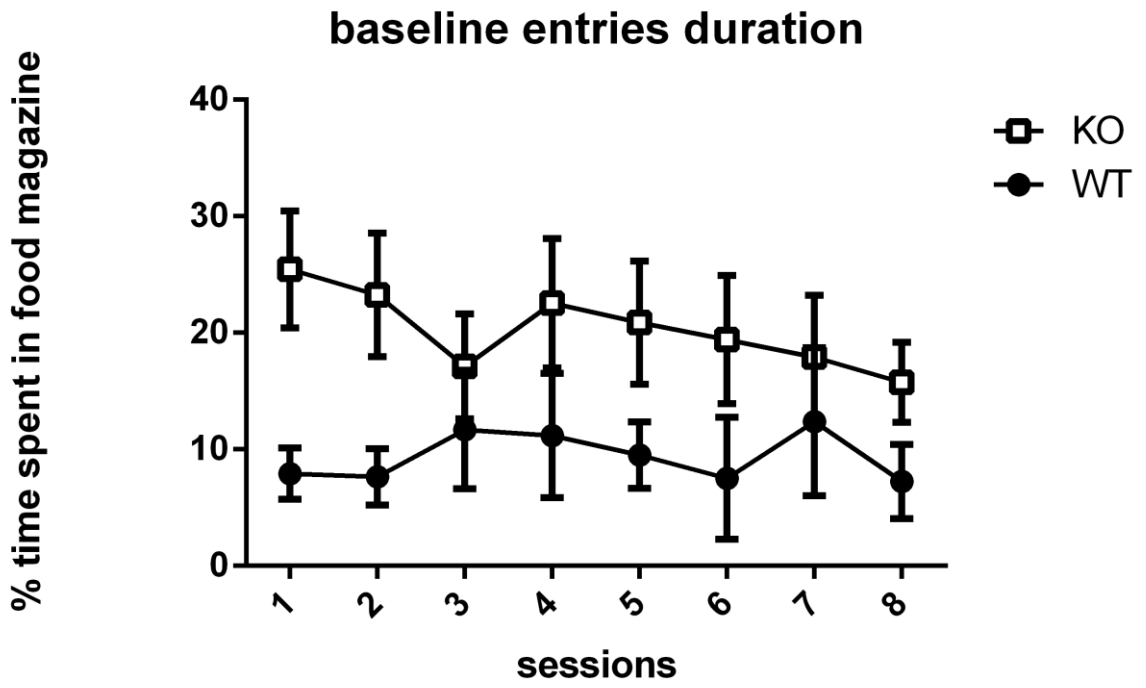


Figure 7.1: Percentage head entry duration during the 8s pre-CS baseline period over 8 sessions of Pavlovian conditioning (WT: n=9, KO: n=10). Error bars indicate ± SEM.

In order to account for this difference in baseline head entry duration, head entry duration during the baseline was subtracted from head entry duration during the CS (CS head entry – baseline head entry). A two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and sessions (8 sessions) as a within-subjects factor was conducted on the difference score (CS head entry – baseline head entry; Fig 7.2). This revealed learning occurring, as quantified by an increase of head entry during the CS relative to baseline over the 8 sessions (main effect of session: $F(7,98) = 3.232$ $p = .004$) . This learning was not different between genotypes (main effect of genotype: $F(1,14) = 0.083$, $p = .778$, genotype by sessions: $F(7,98) = 0.579$, $p = .771$). Separate analyses for each genotype showed that both genotypes overall significantly increased their head entry duration in response to the CS compared to baseline (WT: $F(1,6) = 8.416$, $p = .027$; KO: $F(1,9) = 7.538$, $p = .025$).

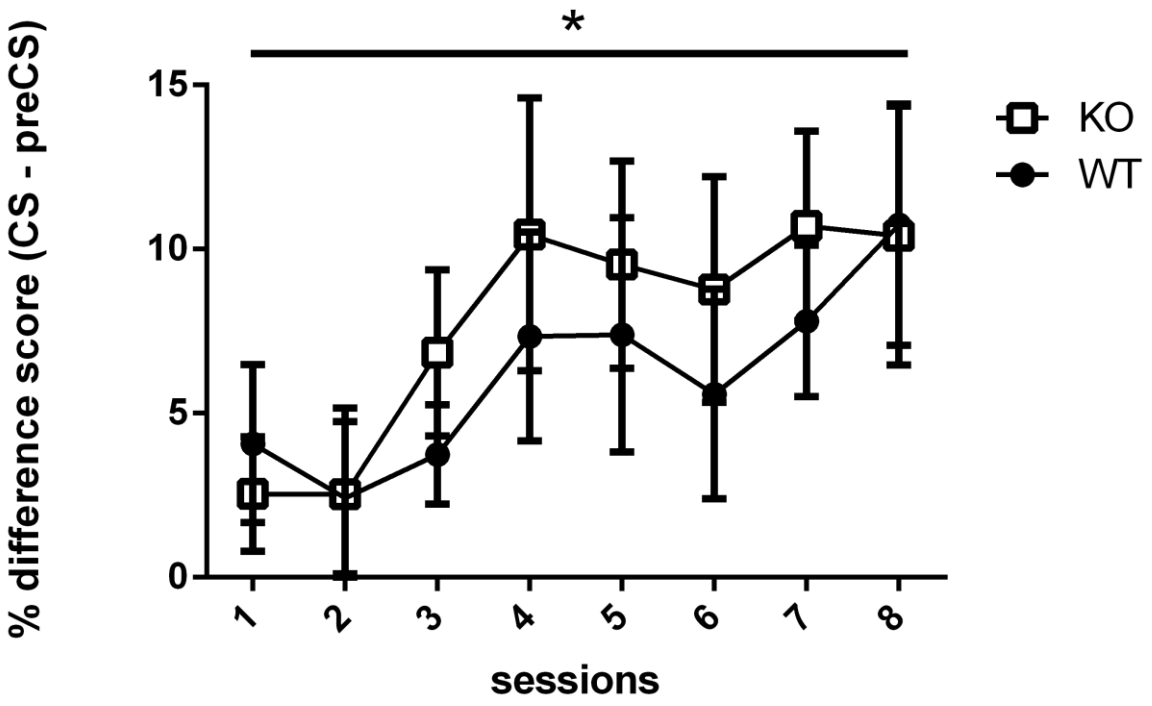


Figure 7.2: Difference score (CS - preCS) of head entry duration during the 8s CS over 8 sessions of Pavlovian conditioning. A change of head entry duration difference score of 0 would denote an absence of learning. Any increase in head entry duration shows learning (WT: n=9, KO: n=10). Error bars indicate ± SEM. * p < .05

FCV: no Genotype Difference in the Dopamine Responses during the first Session

Phasic dopamine responses to the CS (lever extension) and US (reward delivery) were recorded over 8 sessions (WT: n=13, KO: n=8. First, responses to the CS and US during the first session will be investigated. In the first session in the beginning the CS is, in effect, just another neutral stimulus, albeit a very different kind of stimulus from the lights used in chapter 6. Rewards in the first session can be expected to be not yet predicted by the CS at least in the beginning, therefore they are unsignalled rewards. However, in contrast to

chapter 6, reward delivery was not dependent on head entry into the food magazine in the current experiment.

Next, changes of the phasic dopamine response across sessions were investigated to show how the reward prediction error signal moves to the CS. However, since recording sessions were missing in some animals due to the instability of the FCV signal, two consecutive sessions were combined into one block resulting in four blocks for analysis.

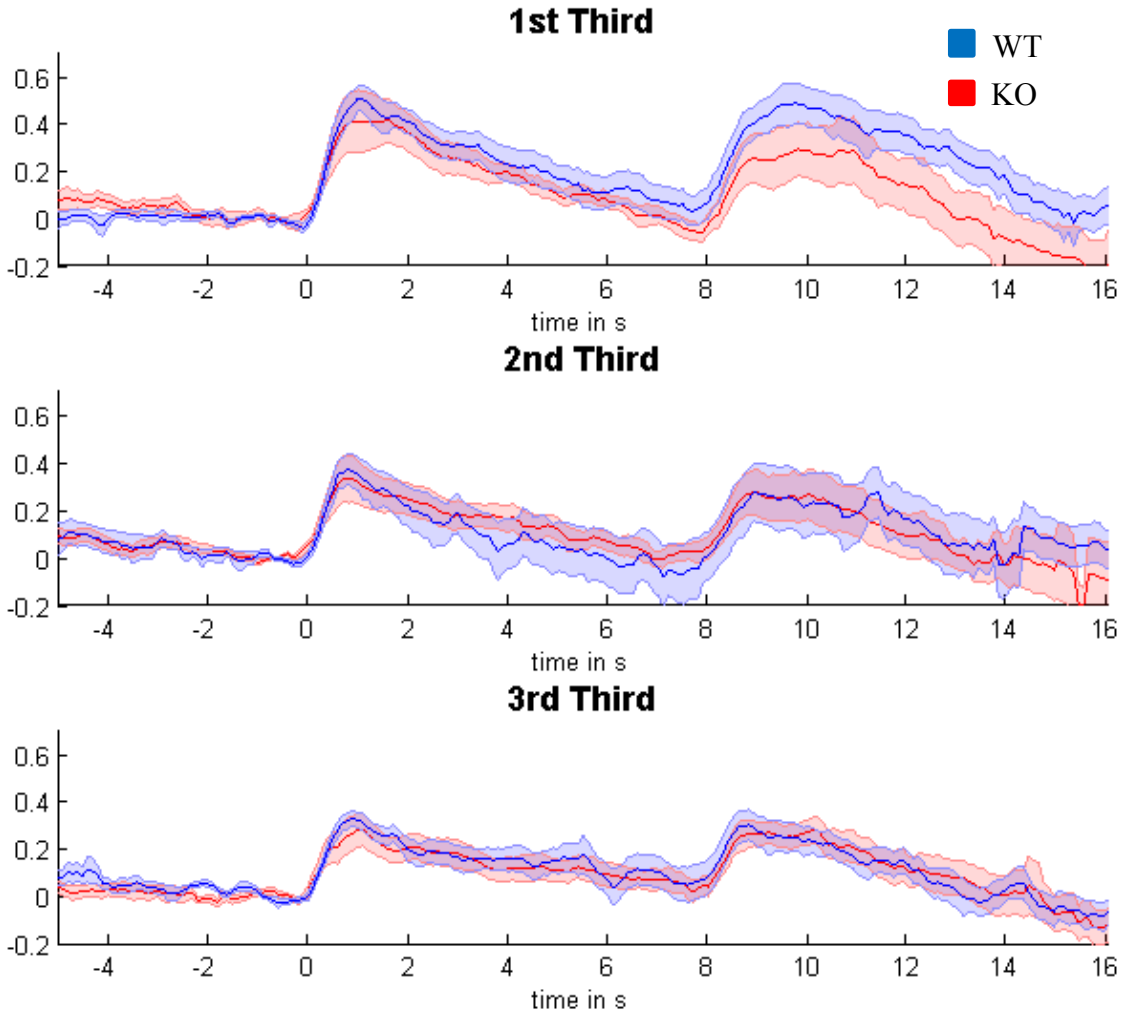


Figure 7.35: Average transient dopamine response during the 1st session (24 trials) of Pavlovian conditioning. The session is divided into three thirds of 8 trials each (Upper panel: 1st third, middle panel: 2nd third, lower panel: 3rd third). The CS (extension of lever) is presented at t=0. The US (grain pellet) is presented at t=8 (WT: n=13, KO: n=8). Error bars indicate ± SEM.

First, a two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and time bin ($1^{st}/2^{nd}/3^{rd}$ part of session) as the within-subjects factor on the maximum dopamine response to the lever in the first session was conducted (Fig 7.4) (Fig 7.3). This revealed a significant decrease of the maxima during the session (main effect of time bin; $F(2,38) = 11.509$, $p < .001$) and no effect of genotype ($F(1,19) = 0.535$, $p = .474$) or genotype by time bin interaction ($F(2,38) = 0.164$, $p = .849$). The same analysis was then conducted on the AUC of the dopamine responses during the lever presentation of the first conditioning session (Fig 7.5). Unlike the analysis of the maxima, reduction of the AUC over the session (effect of time bin; $F(2,38) = 2.555$, $p = .091$) just failed to reach significance. Again there was no effect of genotype ($F(1,19) = 0.015$, $p = .930$) or genotype by time bin interaction ($F(2,38) = 0.954$, $p = .394$).

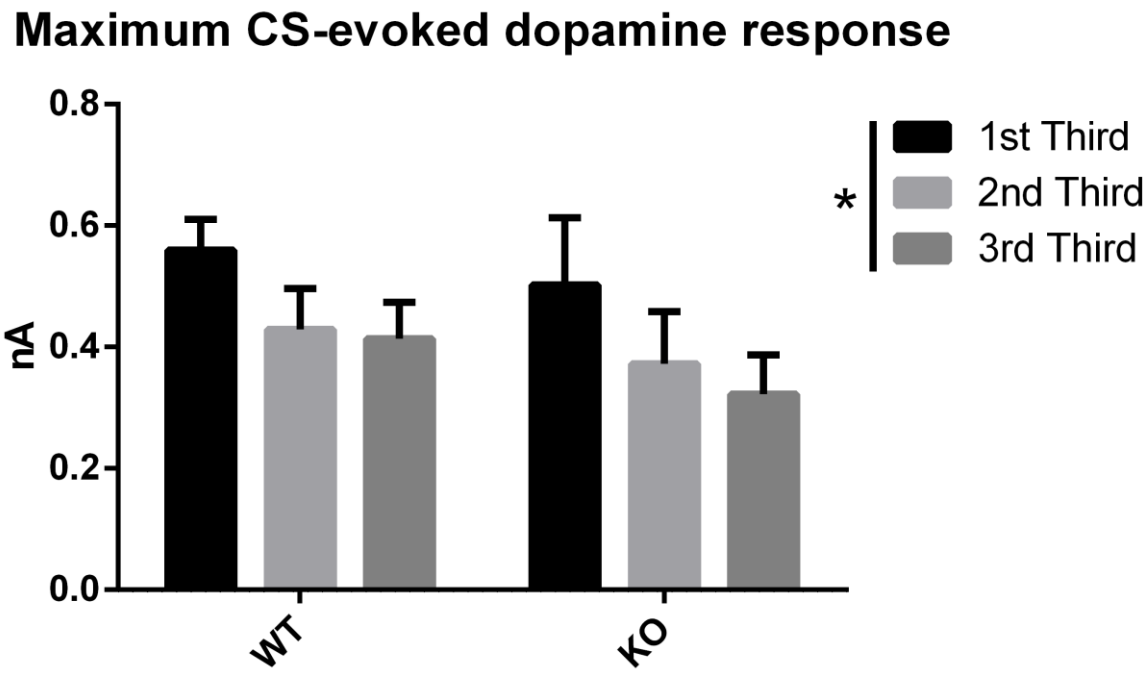


Figure 7.4: Average peak dopamine response (maximum peak height) to the lever during the first session of training. The session is divided into the first, middle and last third (8 trials each) (WT: n=13, KO: n=8). Error bars indicate ± SEM. * p < .05

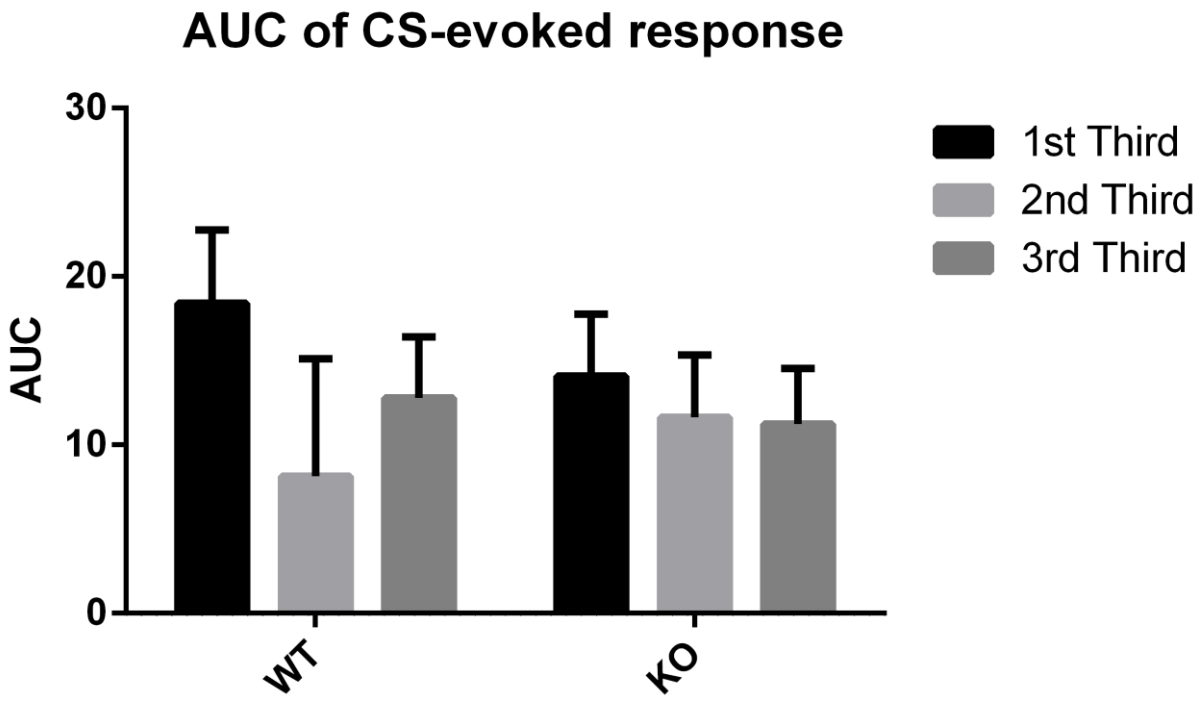


Figure 7.5: Average area under the curve of the dopamine response to the lever during the first session of training. The session is divided into the first, middle and last third (8 trials each) (WT: n=13, KO: n=8). Error bars indicate ± SEM.

Next, changes in the peak dopamine response following pellet delivery were examined using a two-way ANOVA with genotype (WT vs KO) as the between-subjects factor and time bin (3 levels) as the within-subjects factor response to the US (Fig 7.6). One WT and one KO mouse had to be removed from the analysis due to missing data points (the electrochemical model predicting the dopamine content of the model failed due to high noise levels). There was no effect of genotype ($F(1,19) = 0.376$, $p = .547$) and a tendency of the US response to diminish over time ($F(2,38) = 2.728$, $p = .078$). Interestingly, this effect of time had a tendency to interact with genotype. Visual inspection of the data seemed to suggest that the pattern of dopamine responses seen in experiment 6d was

partially replicated here, with a greater reduction in wildtypes than knockouts. However, the interaction between genotype and time bin just failed to reach significance ($F(2,38) = 2.458$, $p = .099$). The same analysis was then conducted on the AUC of the dopamine response after the reward delivery (Fig 7.7). As in the analysis of the maxima, there was no effect of genotype ($F(1,19) = 2.036$, $p = .172$) and a tendency for an interaction of genotype and time block ($F(2,34) = 1.801$, $p = 0.181$). In the AUC data, no effect of time block could be found ($F(2,34) = 0.637$, $p = .535$).

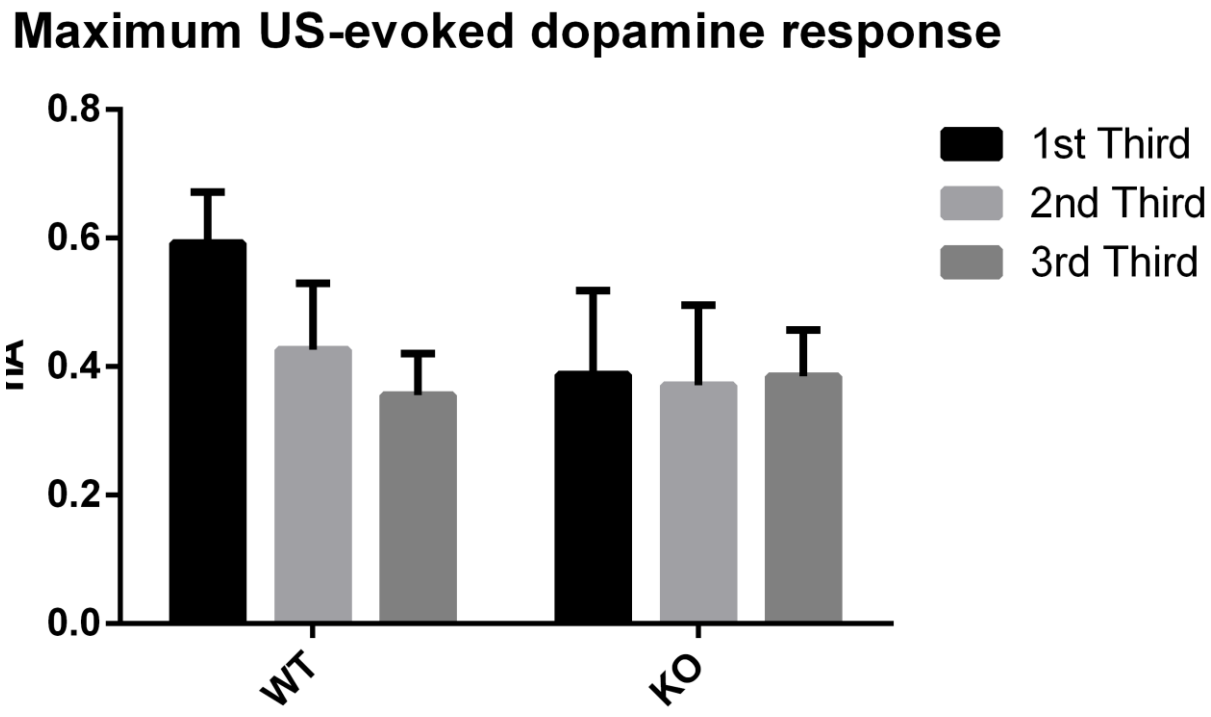


Figure 7.6: Average peak dopamine response to the US during the first session of training. The session is divided into the first, middle and last third (8 trials each) (WT: n=13, KO: n=8). Error bars indicate ± SEM.

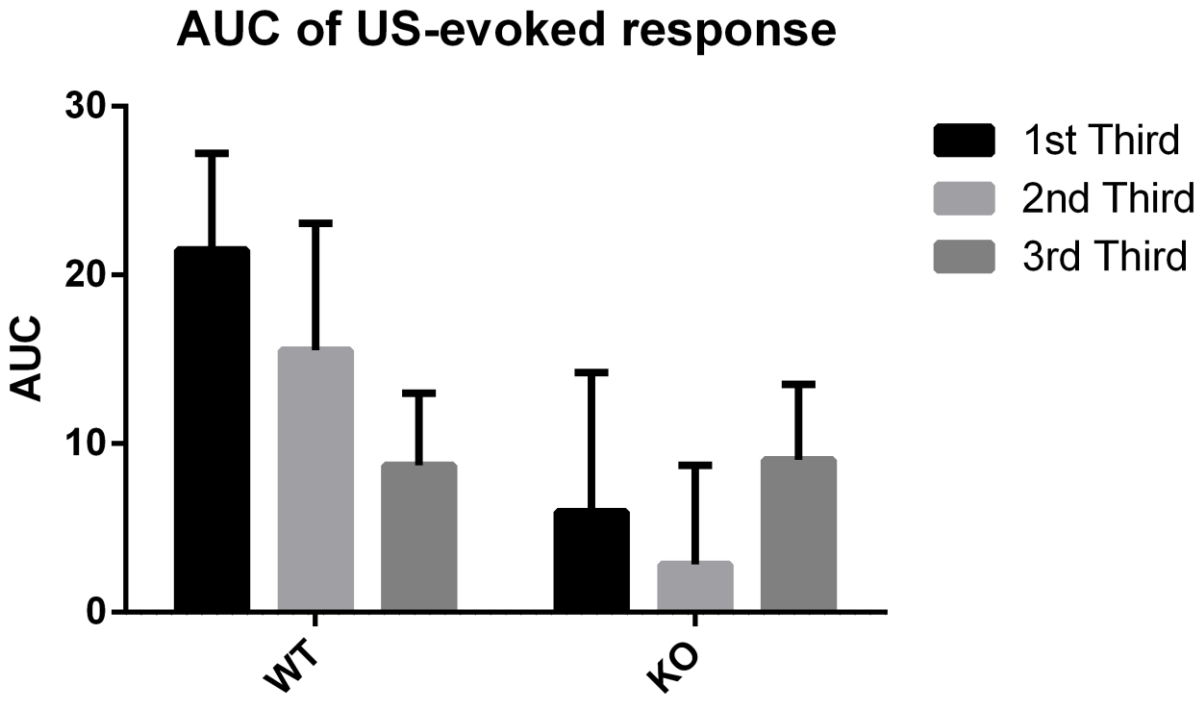


Figure 7.7: Average AUC of the dopamine response to the US during the first session of training. The session is divided into the first, middle and last third (8 trials each) (WT: n=13, KO: n=8). Error bars indicate ± SEM.

Dopamine responses to the CS increased over blocks in both genotypes

In order to determine how patterns of dopamine release to the CS and US adapted dynamically with learning, peak and average dopamine responses following these events were analysed using a three-way ANOVA including stimulus (CS vs US) and training blocks (sessions 1/2, 3/4, 5/6, and 7/8) as within-subjects factors, and genotype (WT vs KO) as the between-subjects factor (WT: n=6, KO: n=5).

The dopamine responses of both genotypes to the CS increased over block as measured by the AUC but not the maximal response. This increase in CS responding occurred due to a reduced decay of the dopamine response to the CS after the maximum was reached in later blocks (Fig 7.8). Overall, CS-evoked dopamine responses were higher than US-evoked responses in KO mice. Such a difference was not present in WT mice.

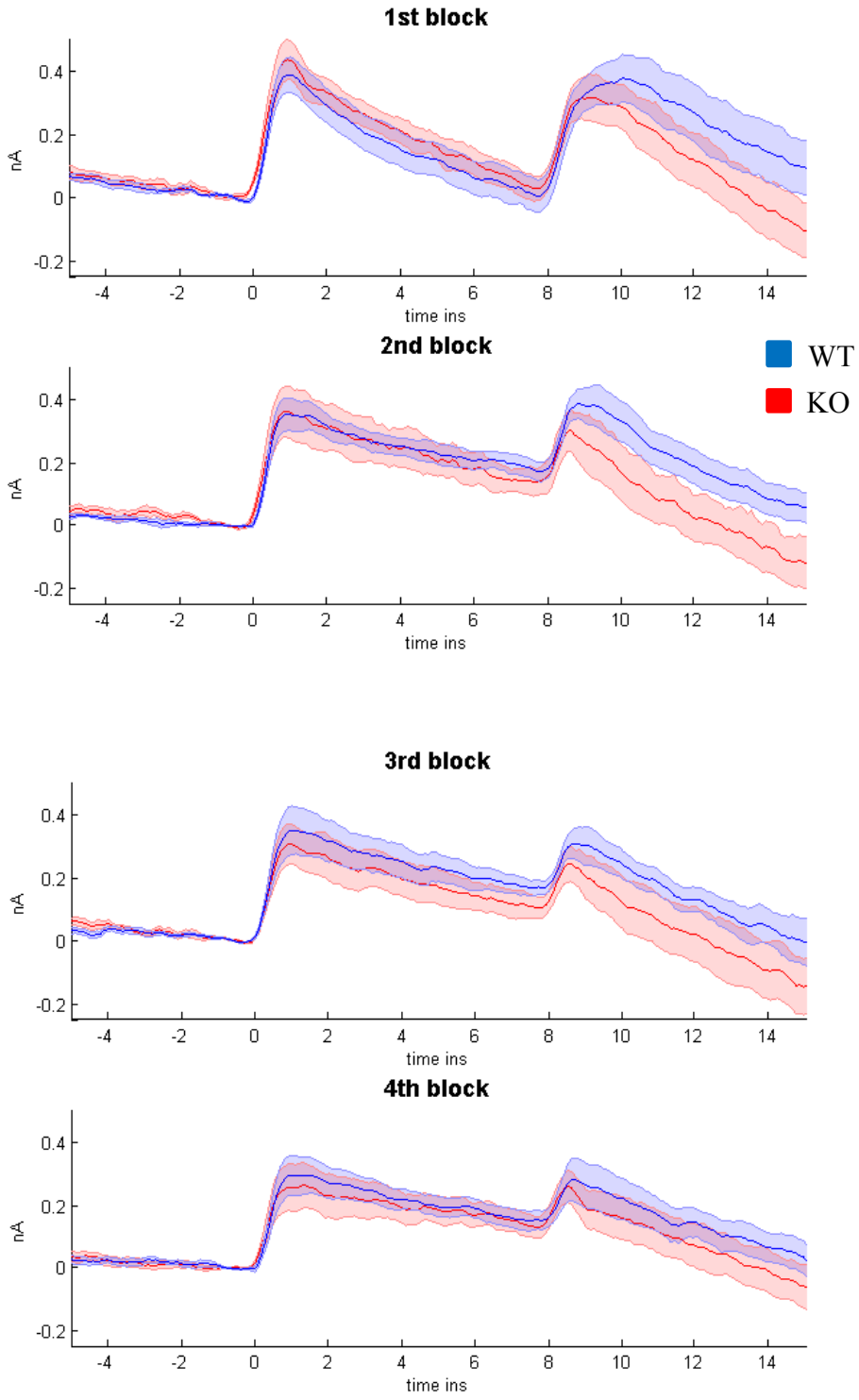


Figure 7.8: Average transient dopamine responses over 4 blocks (8 sessions) of Pavlovian conditioning. Each graph represents one block (two consecutive sessions). The CS (extension of lever) is presented at t=0. The US (pellet) is presented at t=8 (WT: n=6, KO: n=5). Error bars indicate ± SEM.

Analysis of the maxima revealed an overall reduction of the signal over blocks ($F(3,30) = 3.036$, $p = .044$) (Fig 7.9). Responses to the US were lower overall than to the CS ($F(1,10) = 8.519$, $p = .015$) with an interaction of genotype and stimulus ($F(1,10) = 11.254$, $p = .007$). Simple main effects analysis revealed that CS-evoked dopamine responses were stronger than US-evoked responses in KO mice ($F(1,5) = 22.340$, $p = .005$) , but not WT mice ($F(1,5) = 0.085$, $p = .782$). However, when the US-evoked responses were compared in WT and KO mice, the result was not significant ($F(1,10) = 0.872$, $p = .372$). Finally, the two-way interaction stimulus * block ($F(3,30) = 0.451$, $p = .719$) and the three-way interaction of stimulus * block * genotype ($F(3,30) = 0.780$, $p =.514$) were not significant, suggesting that the peak dopamine response does not consistently change during learning in either genotype.

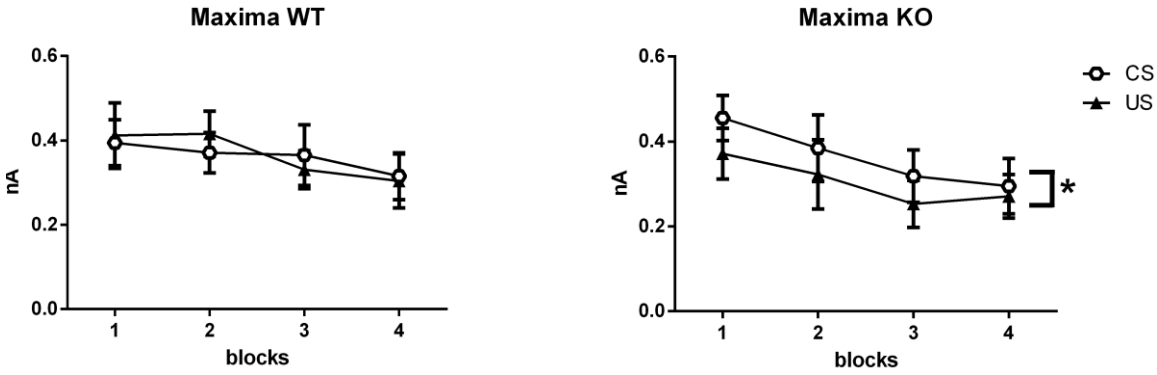


Figure 7.9: Average peak (maxima) of the dopamine response to the CS and US in wildtypes (left) and knockouts (right) over 4 blocks of Pavlovian conditioning (WT: n=6, KO: n=5). Error bars indicate ± SEM. * p < .05

Investigation of the AUC showed a significant effect of stimulus ($F(1,10) = 76.649$, $p < .001$). However, when analysing the AUC the dopamine levels did not reduce over blocks (main effect of block: $F(3,30) = 0.578$, $p = .634$) (Fig

7.10). Again the ANOVA revealed a significant stimulus by genotype interaction ($F(1,10) = 5.546$, $p = .040$). Simple main effects analysis again revealed a lower dopamine responses to the US compared to the CS in knockouts ($F(1,5) = 117.656$, $p < .001$), replicating the pattern of the peak dopamine response. However, now the wildtype mice also exhibited stronger dopamine responses to the CS compared to the US ($F(1,5) = 13.880$, $p = .014$). Simple main effects analysis comparing the dopamine responses of wildtype and knockout mice to the CS and US respectively, showed no significant differences between the genotypes (CS: $F(1,10) = 0.040$, $p = .846$; US: $F(1,10) = 1.753$, $p = .215$).

Additionally, this AUC analysis captured a potential learning correlate. Stimulus interacted with time block ($F(3,30) = 6.017$, $p = .002$). This suggested that the relative size of the dopamine signal to CS and US changed over the blocks. If the dopamine response to the CS increased relative to the US-induced dopamine response this would be consistent with a reward prediction error developing over blocks. To further investigate the interaction of stimulus by time block the effect of stimulus (CS vs US) was investigated in each block. This showed that the difference between CS and US in the first block is not significant ($t(11) = 1.414$, $p = .185$). However, in all following blocks the CS response was bigger than the US response ($t > 5.2$, $p < .001$). Finally, to investigate whether this change between the 1st to the 2nd block is due to an increase in CS responding or reductions in US responding, responses in the 1st and 2nd block were compared in isolation. This revealed a trend towards increased responding to the CS ($t(11) = 2.121$, $p = .057$) and no significant change to the US responses ($t(11) = 1.196$, $p = .257$). However, this learning correlate did not interact with genotype

(stimulus*block*genotype: F(3,30) = 1.644, p = .200) showing that genotype did not affect the overall pattern of CS- or US-evoked dopamine changes in the two groups.

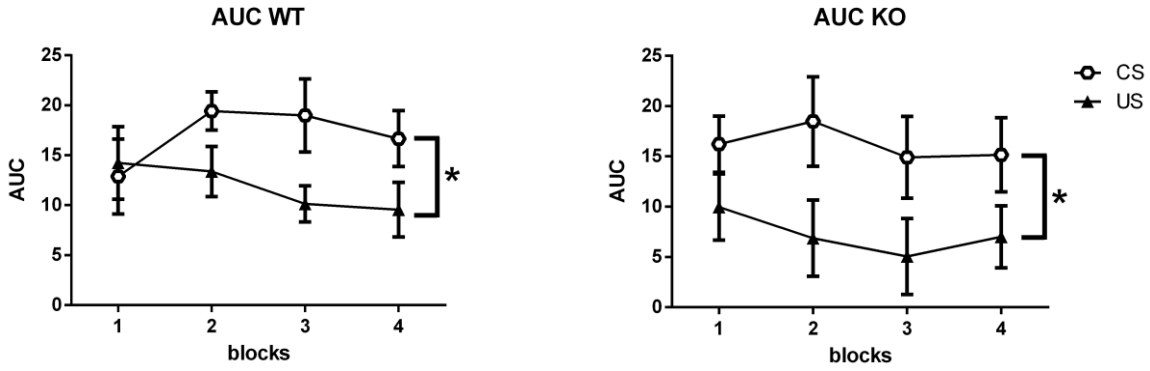


Figure 7.10: Average area under the curve for the dopamine response to CS and US in wildtypes (left) and knockouts (right) over 4 blocks of Pavlovian conditioning (WT: n=6, KO: n=5). Error bars indicate ± SEM. * p < .05

So far we have established from the data a general increase in dopamine levels during the CS period over blocks without an associated increase in the peak dopamine response. This suggests that the dopamine signal is more sustained as learning progresses. In order to capture this aspect of learning, the slope of a linear function fitted to the signal from its peak to the end of the stimulus was used.

This decay slope of the dopamine response to the CS was analysed with a two-way ANOVA with block as the within-subjects factor (4 levels) and genotype as the between-subjects factor (Fig 7.11). This revealed a significant decrease of the slope (i.e. slower decay of the dopamine signal) over time blocks (F(3,30) = 10.244, p < .001) and no effect of genotype (F(1,10) = 0.069, p = .799) and no genotype*time block interaction (F(1,10) = 0.524, p = .486).

To further investigate the main effect of block, post hoc tests between the four blocks were conducted. They revealed that the slopes in the first time block were significantly steeper than in all the other blocks ($p < .024$), which did not significantly differ between themselves ($p > .590$). This led to the conclusion that for both genotypes a significant change occurred between the first and the second time block, after which no further changes to the slope occurred. Notably, this correlates well with the learning curve (Fig 7.2). The biggest change in the difference score for head entries occurred between session 2 and 3, which corresponds to block 1 and block 2 in the FCV analysis.

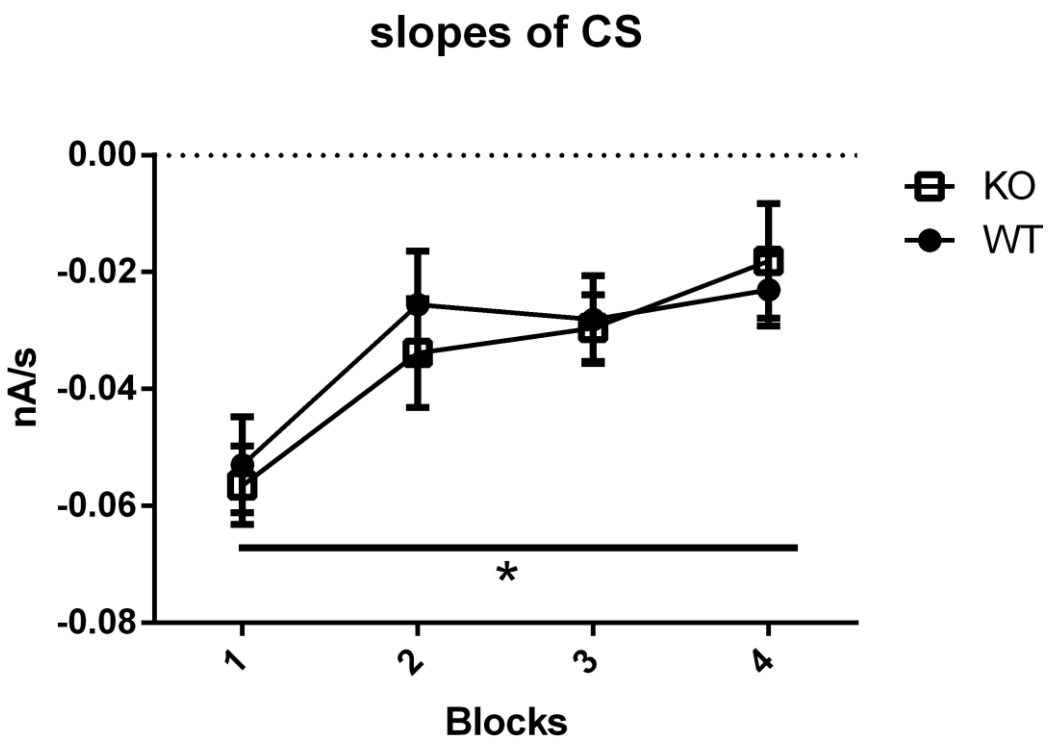


Figure 7.11: Average decay slope of the dopamine responses to the CS after the dopamine peak is reach over 4 blocks of Pavlovian conditioning (WT: n=6, KO: n=5). Error bars indicate ± SEM. * p < .05

Discussion

The rate of Pavlovian conditioning was not affected by GluA1 knockout, replicating the findings of Mead and Stephens (2003). However, GluA1 KO mice again displayed an, albeit non-significant, increase in head entry levels as seen previously in chapter 6. This was not fully expected as Sanderson et al. (2011b) did not find an increase in head entry levels in GluA1 KO mice during a HORNS task when reward delivery was independent of head entry. However, mice that were included in the current experiment had all previously completed the HORNS and Variable Reward tasks in which reward delivery was contingent upon head entry. Therefore, it cannot be excluded that previous instrumental conditioning in the same conditioning box (experiment 6c and d) influenced head entry duration here. On the other hand, Mead and Stephens (2003) reported increased baseline head entry levels in GluA1 KO mice during a Pavlovian task. This suggests that GluA1 mice show increased baseline head entry levels in appetitive Pavlovian conditioning paradigms and unsignalled reward delivery tasks when reward delivery was dependent on head entry. GluA1 KO mice, however, show no increased head entry levels if rewards are both unsignalled and are delivered independently of head entry into the food magazine (Sanderson et al., 2011b).

Dopamine responses to the CS and the US in wildtypes and knockout were largely indistinguishable during this experiment. Both genotypes displayed a longer lasting response to the CS as the association of the CS to the US was acquired over multiple sessions. Both genotypes displayed strong CS-induced

dopamine responses right from the start of training. This differs from the finding of Parker et al. (2010), where lever extension only elicited a dopamine response in wildtype mice as the stimulus became associated with the reward; only a negligible response was present during the first session. Similar studies of Pavlovian learning in rats have also shown that it can take several sessions for a CS to elicit dopamine release (Flagel et al., 2011).

Over sessions the dopamine response to the CS became longer lasting. This was reflected by a significant increase in CS-, but not US-induced dopamine levels in both genotypes without an increase in the peak amplitude of the response. Further analysis revealed that CS-evoked increases in dopamine levels were more sustained. This fits a recently expanded model of dopamine signalling by Schultz (2016). According to Schultz (2016) the dopamine response elicited by a stimulus sequentially codes multiple aspects of the stimulus. An early component codes the salience of the stimulus and is activated unselectively. A later component, however, codes for the precise value of the stimulus, for example whether it predicts reward. The more sustained CS-evoked dopamine response seen in this experiment might therefore be said to reflect the change in value as animals learn the association between the CS and subsequent US.

The classical reward prediction error studies in macaque monkeys observed a reduced US response as the CS-US association is acquired by the animal (Schultz et al. 1997). This reduction in US-evoked dopamine responding was not observed in the current study. However, similar response patterns in Pavlovian conditioning have already been observed in mice both with FCV recordings of

the phasic dopamine response in the nucleus accumbens core (Parker et al., 2010), and during single cell recordings of neural activity of dopaminergic neurons in the VTA (Cohen et al., 2012). One potential explanation for this might be that it takes much longer for mice to lose their dopamine reward prediction error response to the reward in Pavlovian conditioning. Potentially, if more conditioning sessions were run, dopamine responses to the US would eventually disappear.

Intriguingly, whereas GluA1 KO mice displayed longer lasting responses to neutral stimuli in chapter 6, this was not the case for CS responses in this chapter. One potential explanation is the different type of stimulus used in this chapter. The extension of the lever causes a noise and while the lever is extended the mouse can see and touch it. Therefore, the CS is a visual stimulus, an auditory stimulus and potentially a tactile stimulus if the mouse chooses to approach it. The neutral stimuli in chapter 6 on the other hand are lights and thereby only visual stimuli. However, previous experiments suggest that GluA1 KO mice show the same lack of habituation independent of the stimulus type being used. They, for example, fail to habituate to lights (Sanderson et al., 2011b) and objects (Sanderson et al., 2011a). This suggests GluA1 knockout disrupts habituation irrespective of modality. However, GluA1 KO mice were shown by Mead and Stephens (2003) to be able to acquire simple Pavlovian conditioning indistinguishable from wildtypes. Therefore, it is not surprising that the dopamine responses in knockouts were indistinguishable from wildtypes. This showed that the hyperdopaminergic response is not generalized in GluA1 KO mice, but that phasic dopamine is aberrant in a task in which GluA1 KO were

shown to be impaired (i.e. the HORNS task: Sanderson et al. (2011b), chapter 6) and indistinguishable in tasks in which GluA1 KO mice behave indistinguishable from wildtypes (i.e. Pavlovian conditioning).

Interestingly, GluA1 KO mice also did not show an increased dopamine response to the reward. This finding is in sharp contrast to the markedly increased reward-evoked dopamine response in chapter 6. There are at least two potential explanations for this difference. First, KO mice could perceive sucrose solution, used in the HORNS and variable reward task described in Chapter 6, but not grain pellets, used in the current experiment, as more rewarding than WT mice. However, this explanation can be discounted on the basis of Johnson et al. (2005). Johnson et al. (2005) presented GluA1 KO and WT mice with sucrose and grain pellets and found no difference in the preferences for the two rewards between genotypes. This suggests that GluA1 KO mice do not value sucrose solution or grain pellets differently from wildtype mice.

A second explanation for the difference could lie in the structure of the tasks used. In the HORNS and Variable Reward tasks, reward delivery was contingent on head entry. This instrumental component was not present in the current Pavlovian experiment. Here, rewards were delivered independently of head entry into the food magazine. The hypothesis that the contingency of reward delivery upon head entry (or the lack of such a contingency) influences the dopamine signals in wildtypes and GluA1 KO mice could be tested in the following experiment. GluA1 KO mice and littermate controls are implanted with FCV electrodes and receive unsignalled rewards. However, for one half of the cohort reward delivery is dependent on head entry into the food magazine. For the other

half it is not. The prediction based on the data in this and the preceding chapter would be the following: GluA1 KO mice should have an increased dopamine response to unsignalled reward delivery that is dependent on head entry. However, if the reward delivery is unsignalled there should be no increase in reward-evoked dopamine responses in the KO mice.

This chapter has investigated the effect of GluA1 knockout on appetitive Pavlovian conditioning. I have found that behaviour in this task was unchanged reproducing Mead and Stephens (2003). Additionally, I found that the phasic dopamine responses to the CS and the reward were unchanged in GluA1 KO mice. This was in contrast to the hyperdopaminergic response of GluA1 KO mice to neutral stimuli and to rewards in an instrumental task seen in chapter 6. This showed that the hyperdopaminergic phenotype seen in GluA1 KO is specific to certain tasks rather than a generalized increase in dopamine response to all stimuli. This again underlines the importance of investigating dopamine changes during behaviour rather than anesthetized preparations as was done by Wiedholz et al. (2008).

Chapter 8: General Discussion

This thesis investigated the interaction of the glutamate and the dopamine systems in tasks relevant to schizophrenia. This was achieved by manipulating the glutamate system mainly via two approaches while both the dopamine system and behaviour of the animal were monitored. One of these manipulations was a constitutive whole-brain GluA1 knockout. The other manipulation consisted of systemic injections of the group II metabotropic glutamate receptor agonist LY354740, a member of a class of drugs which has been suggested as potential antipsychotic.

LY354740 impaired spatial working memory as reflected in reduced rewarded alternation T-maze performance in rats, reproducing Aultman and Moghaddam (2001). Additionally, LY354740 reduced amphetamine-induced hyperactivity in naïve ad lib fed rats. Curiously, the agonist displayed a tendency to increase amphetamine-induced hyperactivity in food restricted, extensively-handled rats that expected to receive food and exacerbated the effect of amphetamine on T-maze running latencies. This gave preliminary evidence of a state-dependent propensity for LY354740 to increase dopamine activity (chapter 3).

To further investigate this ability of LY354740 to increase dopamine activity, phasic dopamine signals in the nucleus accumbens core were measured using FCV in response to unsignalled rewards in food restricted and extensively-handled rats that expected food rewards in the experimental context. This confirmed that LY354740 can increase phasic dopamine activity in the nucleus

accumbens core under certain conditions. Notably, LY354740 did not increase the reward-evoked tissue oxygen levels, a haemodynamic measure of neural activity, in the nucleus accumbens. This potentially suggests a disconnection of the dopamine and the overall neural activity as measured by the haemodynamic response although these signal changes were happening over very different timescales. However, LY354740 reduced reward-evoked tissue oxygen responses in the hippocampus albeit electrode locations still need to be confirmed in the tissue oxygen study (chapter 4).

LY354740 has been shown to ameliorate the effect of the NMDA-receptor antagonist PCP on spatial working memory on the T-maze (Moghaddam and Adams, 1998). The effect of PCP is thought to resemble the symptoms seen in schizophrenia. GluA1 KO mice also display a phenotype reminiscent of schizophrenia and display a spatial short-term memory deficit on the T-maze. However, this phenotype could only in parts be ameliorated by LY354740. The mGluR agonist reduced hyperactivity in GluA1 mice, but failed to correct their spatial working memory deficits on the T-maze (chapter 5).

In a final set of experiments GluA1 KO mice and littermate controls were implanted with FCV electrodes into the nucleus accumbens core and phasic dopamine responses were recorded in behaving mice. GluA1 KO mice showed a lack of habituation of the dopamine response to neutral stimuli. GluA1 knockouts showed overall increased phasic dopamine responses to neutral stimuli. This dopamine response habituated across the session in wildtypes, but not knockouts. Wildtype mice also showed stimulus-specific habituation of the dopamine signal if the first stimulus in a pair was a LED when comparing recently

and less recently presented stimuli. Knockouts did not display such stimulus-specific habituation. Additionally, knockouts showed massively increased reward-evoked dopamine responses when unsignalled food delivery was dependent on head entry into the food magazine. These responses did not decrease over the session in contrast to the reduction in dopamine responses to reward seen in WT mice (chapter 6). When wildtype mice made head entries that were not rewarded they exhibited a negative prediction error signal. This event was absent in GluA1 KO mice.

Pavlovian conditioning was not affected by GluA1 knockout on the level of behaviour or neurochemistry. Associated dopamine responses to the CS and US in wildtypes and knockouts were overall indistinguishable. Both genotypes displayed longer lasting dopamine responses to the CS as the association of the CS to the US was acquired over multiple sessions. Notably, GluA1 KO mice did not show increased reward-evoked dopamine responses when the reward delivery was not contingent on a head entry being made (chapter 7).

In the following chapter the general implications of these findings will be discussed. First potential mechanisms by which GluA1 KO and systemic LY354740 could increase the phasic dopamine response will be described. Then, the status of LY354750 as a putative antipsychotic will be discussed in the context of the heterogeneity seen in schizophrenia. Finally, different paradigms to assess attention and learning in rodents and humans will be discussed.

Two very different Glutamatergic Manipulations can induce Increased Phasic Dopamine Responses

The two main interventions used in this thesis to manipulate the glutamate system differ on multiple levels. GluA1 knockout is a genetic, whole-brain constitutive deletion of an AMPA receptor subunit. LY354740 on the other hand is a pharmacological manipulation which instead targets the group II metabotropic glutamate receptors. And whereas GluA1 knockout is present from the embryonic stage onwards, LY354740 was systemically injected acutely in adult rats on the day of testing. Despite these differences both of these interventions strongly increased phasic dopamine responses to unsignalled rewards in the nucleus accumbens core at least under some conditions.

GluA1-containing AMPA receptors are diffusely expressed throughout the brain (Martin et al., 1993) and LY354740 binds diffusely throughout the forebrain (Higgins et al., 2004). Yet, both GluA1 knockout and LY354740 appear to have a particularly profound impact on glutamatergic processes in the hippocampus. As discussed in the introduction, GluA1 knockout animals show reduced excitatory postsynaptic potentials (EPSP) (Jensen et al., 2003, Romberg et al., 2009) and lack the early component of LTP (Hoffman et al., 2002, Frey et al., 2009, Romberg et al., 2009, Erickson et al., 2010). LY354740 also has a profound influence on hippocampal activity. Chapter 4, showed that reward-evoked hippocampal tissue oxygen responses are decreased by LY354740, suggesting a reduction of responsiveness of the hippocampus. This adds to the finding of reduced glucose levels in the hippocampus (Lam et al., 1999). Both are evidence

of a reduction in overall baseline neural activity in the hippocampus. Notably, EPSPs were reduced by LY354740 via a process of presynaptic inhibition of glutamate release (Kilbride et al., 1998, Price et al., 2005) whereas the reduction of EPSPs in GluA1 KO mice presumably is due to a reduction of AMPA receptor density in the postsynapse (Romberg et al. 2009, but see Zamanillo et al. 1999). Furthermore, in vivo assessment of hippocampal activity of freely moving GluA1 KO mice and controls using c-Fos staining suggests an increase in activity levels in GluA1KO mice (Procaccini et al., 2011, Procaccini et al., 2013). Potentially, this increase could reflect changes to the wider neural network. For example, brain regions projecting to the hippocampus could be more active, thereby increasing hippocampal activity. Still, the reduction in glutamatergic EPSPs points towards a similar impairment in basal processing in the hippocampus due to either manipulation.

Lisman and Grace (2005) present a theory that potentially explains how changes in the hippocampus can influence firing of dopaminergic neurons in the VTA which, in turn, would increase dopamine release in the nucleus accumbens core. They propose a hippocampal-VTA loop which they argue regulates the entry of novel information into long-term memory. In this model, the hippocampus acts as a novelty detector comparing stored memory with the current sensory input (Vinogradova, 2001, Fyhn et al., 2002). This information is sent to the VTA via the subiculum, the nucleus accumbens, and the ventral pallidum or by an alternative pathway via the lateral septum (Luo et al., 2011). In the VTA, this novelty signal then increases the firing of dopaminergic neurons. Evidence for this hippocampus-controlled novelty-induced increase in dopamine release in the

NAc comes from multiple sources. One of them is the finding that a novel environment increases dopamine levels in the nucleus accumbens (Legault and Wise, 2001). This increase is blocked by temporally inactivating the subiculum, a major output structure of the hippocampus (Legault and Wise, 2001). The subiculum is not only necessary for novelty-evoked increases in dopaminergic activity, but electrically (Mitchell et al., 2000, Floresco et al., 2001) or chemically (Legault et al., 2000) increasing the activity of the subiculum is also sufficient to increase dopamine levels in the nucleus accumbens as measured by microdialysis. Therefore, the evidence shows that dopamine activity is increased by novelty and that neural activation of the subiculum is necessary and sufficient for increased dopamine release. The explanation so far covered only one part of the hippocampal-VTA loop that presumably allows subsequent memory processing. The other part of the loop consists of dopamine release into the hippocampus (Scatton et al., 1980) which facilitates the induction of LTP (Frey et al., 1990).

When analysing the VTA cell activity rather than dopamine levels in the nucleus accumbens, a more complex picture arises. Electrical stimulation of the subiculum increases the number of active neurons in the VTA, but does not affect phasic burst firing of these active neurons. This burst firing in active neurons is increased by stimulation of the pedunculopontine tegmental nucleus (PPTg). This stimulation of the PPTg, in turn, does not affect the number of active neurons (Lodge and Grace, 2006). In short, there is a double dissociation between the role of the PPTg and the subiculum. Subiculum controls the number of active neurons and the PPTg controls burst firing. Lodge and

Grace (2006) conclude from these data that hippocampal manipulations increase the general responsiveness of the VTA to stimuli rather than increasing stimulus-specific firing as stimulation of the PPTg does.

This would suggest that hippocampal manipulations affect responding independent of the stimulus (i.e. they produce a generalized increase in arousal). The response to every subsequent stimulus should be changed. However, this is not what was observed in GluA1 KO mice. GluA1 KO mice have a deficit in stimulus-specific habituation as observed in the HORNS task (Sanderson et al., 2011b) or the object-recognition task (Sanderson et al., 2011a). They demonstrate inappropriately high levels of attention/exploration specifically to a stimulus they have previously experienced. Additionally, Lisman and Grace (2005) would predict a reduced dopamine response, if the hippocampal neural processing is disrupted as is the case in GluA1 KO mice and systemic LY354740 injection. Normally, the hippocampus is detecting novelty. If novelty is detected the dopamine response is increased to facilitate processing of this potentially biologically relevant stimulus. If this detection is disrupted, no novelty signal should arrive in the VTA. Therefore, less dopamine should be released into the NAc. This thesis, however, found an increase in dopamine release in manipulations that disrupt hippocampal neural processing.

One potential explanation for this discrepancy is that the locus of the effect leading to an increase in dopamine is not actually in the hippocampus or at least not entirely. The effect of GluA1 knockout is unlikely to be completely mediated by the effects of the knockout on hippocampal function. Mice with a GluA1 knockout specific to the dorsal or ventral hippocampus still alternate significantly

above chance levels on a T-maze, albeit marginally impaired with respect to littermate controls (Freudenberg et al., 2013). This suggests that the deficit in short-term memory seen in GluA1 knockouts may not solely be due to the direct effect of the receptor knockout on hippocampal activity.

Another potential way how the glutamate and the dopamine system might interact is in the NAc itself. Sensitization to cocaine, a drug targeting the dopamine system among other targets, is associated with the insertion of GluA1 homomeric receptors in the NAc (Churchill et al., 1999, Boudreau and Wolf, 2005). This suggests that plasticity of the dopamine system is associated with changes in glutamate receptors. This plasticity was absent in GluA1 KO mice, showing the importance of GluA1 subunit in this process (Dong et al., 2004). Additionally, LY354740, infused locally into the NAc, decreases dopamine levels as measured by microdialysis whereas a group II antagonist increases dopamine (Karasawa et al., 2006). These two streams of evidence suggest that glutamate directly affects the dopamine system in the NAc. However, the glutamatergic manipulations used in this thesis were systemic. Drugs were given i.p. and the GluA1 knockout was a whole-brain knockout. Therefore, it is difficult to say in which brain regions and on which cell types these interventions had an effect.

While Lodge and Grace (2006) predict an effect of hippocampal activity on tonic baseline levels, this thesis found an effect of such manipulations on phasic stimulus-evoked dopamine signals. In fact, baseline dopamine levels in the nucleus accumbens are unaffected by systemic administration of LY354740 (Moghaddam and Adams, 1998) and GluA1 knockout (Wiedholz et al., 2008). This illustrates the importance of studying neural correlates of psychological

processes in behaving animals during the appropriate tasks, as opposed to studying unspecific home cage behaviours.

Heterogeneity in Schizophrenia

This thesis showed that two very different glutamatergic manipulations can have the same effect on phasic dopamine in the striatum at least under some conditions. This is of interest, because the aetiology of schizophrenia has a considerable genetic component, yet there is big variability in the potential risk genes, spanning the neurotransmitter systems dopamine and glutamate among others (Ripke et al., 2014). The clinical presentation equally displays high levels of heterogeneity (Moghaddam et al., 1997, Mueser and McGurk, 2004, van Os and Kapur, 2009). Furthermore, schizophrenia has a considerable overlap in genetic liability and symptomatology with other mental disorders such as bipolar disorder (Lichtenstein et al., 2009). This leads some researchers to go as far as to conclude that schizophrenia is not a useful label at all (Bentall, 2003). Still, undoubtedly there are patients with psychosis. But what causes delusions and hallucinations in these patients will most likely vary widely. The theory put forward by Howes and Kapur (2009) suggests that changes to the dopamine system are the final common pathway in how these various upstream alterations lead to psychosis. Therefore, it is highly interesting that two interventions with relevance to schizophrenia led to hyperdopaminergic responses in the striatum presumably through two very different mechanisms. Although it is less likely that a group II mGluR receptor agonistic function is responsible for schizophrenia.

Group II mGluR Agonists as Antipsychotics

Group II mGluR agonists have instead been suggested as a putative antipsychotic (Patil et al., 2007). The major pathobiological process associated with psychosis is an increase in dopaminergic activity in the striatum. Therefore an antipsychotic would be expected to reduce this dopaminergic activity. And so far every licensed antipsychotic is an antagonist of the dopaminergic D2 receptor. While Group II mGluR agonists don't show binding at the D2 receptor, they would still be expected to dampen dopamine activity in the striatum and previous evidence has been consistent with this possibility (Pehrson and Moghaddam, 2010). Therefore it is problematic for its role as an antipsychotic that LY354740 could actually increase phasic dopamine responses to reward (chapter 4).

However, results reported in chapter 3 suggest that this increase in dopamine activity is not always present. In naïve rats, LY354740 reduced amphetamine-induced hyperactivity, suggesting a reduction of dopamine activity by LY354740. However, in some states LY354740 failed to inhibit amphetamine-induced hyperactivity or even showed a tendency to increase hyperactivity and exacerbate the effect of amphetamine on running latencies on the T-maze. This shows a state-dependency of the effect of group II mGluR agonists. Notably, this state-dependency is somewhat reminiscent of the effect of a double knockout of the two group II mGluRs (mgluR2/3). mGluR2/3 double KO mice were impaired on a rewarded alternation T-maze task (Lyon et al., 2011). However, when arousal was increased by injection stress, alternation performance in mGluR2/3

double KO mice increased whereas alternation performance decreased in wildtype controls. Potentially, both the state dependent effect of LY354740 and the arousal effect seen in mGluR2/3 double KO mice, could point towards a role of group II mGluRs in mediating arousal's effect on other brain functions.

Additionally the state-dependent effect of LY354740 on amphetamine responses raises an important methodological question for locomotor activity tests as drug discovery assays. The inhibition of psychomimetic-induced hyperactivity is a standard screening test for putative antipsychotics. However, in chapter 3 a potential antipsychotic showed different effects on amphetamine-induced hyperactivity based on the history of the rat and the experimental setup used. Therefore, it is important to consider the reliability of suppression of drug-induced hyperactivity. For practical reasons, it is not uncommon to test multiple compounds in the same animals for drug discovery (personal communication with Gary Gilmour). The results obtained in this thesis suggest that this reuse of animals potentially changes the state of the animal and thereby the results of the locomotor test. At least, in the future, the history of the animals used should be documented in a more detailed manner than is currently the case. For example, Cartmell et al. (1999) did not report whether the rats used were completely naïve or took part in a previous experiment.

Modelling Schizophrenia in Rodents

On a broader level the validity of locomotor activity tests as a tool in antipsychotic drug-development is questionable. Its validity is based on the idea that the psychomimetics used to induce hyperactivity disrupt brain physiology in a way

reminiscent of schizophrenia. However, in schizophrenia speech and thought are disordered and patients subjectively perceive things that are not observable to others. This thesis used rodents to answer research questions relating to this disorder. But as the nature of its symptomatology makes clear, we cannot have a "schizophrenic mouse". Rodents cannot speak. We cannot know what they think or if they have a conscious perception.

Beyond these more conceptual difficulties, there are at the moment also no schizophrenia models in animals. A schizophrenia model would be a construct (i.e. an animal in which a certain set of intervention have been performed) which could predict the effectiveness of a potential antipsychotic in schizophrenia patients. Such a model, for example, would be injected with a putative antipsychotic and a set of test would be conducted on it. If the antipsychotic had a certain effect on these tests, this would then predict the efficacy of this compound as an antipsychotic in human. Again, considering the uniquely human aspect of schizophrenia with its effect on consciousness, it is questionable whether there will ever be a rodent model of schizophrenia. In the case of locomotor activity, not all drugs that ameliorate psychomimetic-induced hyperactivity, also act as antipsychotics. Central depressants such as barbiturates and opioids reduce amphetamine-induced hyperactivity without showing efficacy as antipsychotics. Therefore, reduction of drug-induced hyperactivity should not be seen as a valid or reliable model of schizophrenia.

GluA1 knockout mice are also not a schizophrenia model. A knockout of a single gene which displays a polymorphism upstream of its coding region in some schizophrenia patients does not capture a satisfactory proportion of the biology

of schizophrenia to predict drug effectiveness. For example, Boerner et al. (in preparation) showed that the working memory deficit in GluA1 KO mice cannot be ameliorated by the antipsychotic haloperidol. This test therefore does not predict antipsychotic action of this compound in humans. However, antipsychotics show limited efficacy in ameliorating cognitive symptoms in schizophrenia (Kapur and Mamo, 2003). Equally, the putative antipsychotic LY354740 could not rescue the working memory deficits of GluA1 KO mice. Still, LY354740 was able to reduce GluA1 knockout-induced hyperactivity. However, considering the lack of specificity of this effect discussed above, this is not conclusive evidence of an ameliorative effect on locomotor hyperactivity with relevance to schizophrenia.

Salience and PPI

GluA1 KO mice can still be a useful tool for schizophrenia research. As discussed in this thesis they show schizophrenia-related behaviours that suggest a state of aberrant salience or a potential precursor of aberrant salience. The nature of different types of salience will be discussed next. In the course of this discussion I will compare salience as measured in the HORNS task with other paradigms used to understand attentional abnormalities in schizophrenia.

From the earliest conceptualizations by Bleuler and Kraeplin, schizophrenia was thought to be associated with changes in the way the world is perceived (Angst, 2002). More recent theories like the aberrant salience account of schizophrenia (Kapur, 2003) frame this change in terms of attentional processes. Salience attribution can be driven by properties which can be ascribed to the stimuli in

question such as the physical properties of the perceptual input (e.g. its intensity) or the (relative) novelty of the stimulus. Salience attribution can however also be driven by the association of a stimulus to a biological meaningful outcome. In this case salience is a learned property given to a stimulus to motivate responses associated with the stimulus as described by Berridge (2007). Pavlov's dog, for example, might well hear the bell independent of any conditioning (innate or novelty salience). If the bell did not mean anything then the dog would habituate to it across multiple presentations and the bell would become less salient. However, the dog would pay special attention to the bell when it is associated with a food reward (motivational salience). This differentiation of innate or novelty salience and motivational salience is by no means a dichotomy. Both types of salience are most likely present in the response to most stimuli in our everyday environment. There are, however, tasks used in schizophrenia research that are potentially more selective for one rather than the other type of salience.

Additionally, an argument can be made that these different types of salience show aberrant behaviour at different times during the development of psychosis. According to Kapur (2003) aberrant dopamine firing induces aberrant salience. A so far healthy human (patient X) could develop a firing pattern of dopaminergic cells that is different from the typical pattern (for example due to upstream glutamatergic changes). This could make patient X attribute salience to stimuli that would not normally draw attention. This could in the beginning be a stimulus-driven process, because no motivation is induced by this stimulus. The world just looks brighter, sounds louder, feels more intense as has been described in case reports by patients in the prodromal phase of schizophrenia (Kapur, 2003).

However, as described by Berridge (2007), dopamine potentially turns stimuli from neutral pieces of information into desirable, attractive, and motivating stimuli. This could initiate aberrant learning processes. Through these processes, patient X would “learn” things about the world, healthy humans would describe as delusional. Both these processes that in reality will both make an important contribution have been addressed with experimental paradigms in animals and humans.

The most widely used of these paradigms is pre-pulse inhibition (PPI). In PPI a strong stimulus, for example a tone, is presented to evoke a defensive startle response (Swerdlow et al., 2008). This startle response is inhibited when a weaker stimulus, the pre-pulse, is presented between approximately 30-480ms before the startle-inducing stimulus (Geyer et al., 2002). Just like the orienting response in the HORNS task, the startle response and the inhibition of the startle by the pre-pulse are innate behaviours that do not have to be learned (Blumenthal, 1996, Braff et al., 2001). An organism is presented with a plethora of stimuli in the outside world and has to filter out the important ones. PPI tests this filtering or “gating” process called sensorimotor gating. This filtering can also be seen as a type of habituation. The pre-pulse habituates the subjects to the tone. Thereby the startle response is inhibited. Notably, PPI is happening over a very different time scale to other tests of habituation discussed in this thesis (object recognition, spatial novelty preference, HORNS task) and is sometimes described as pre-attentive (Levin and Buccafusco, 2006), although notably all of these assays are affected by GluA1 deletion (Barkus et al., 2014).

This PPI effect is lower in schizophrenia patients (i.e. the pre-pulse inhibits the startling response less than it does in healthy individuals; Braff et al. (1978)). However, the distributions of PPI in schizophrenia patients and healthy controls are overlapping. Therefore, this measure cannot be used as a diagnostic tool, but can still be utilized to assess sensorimotor gating effects of various manipulations in patients and healthy subjects (Braff et al., 2001). For example, there is evidence that atypical antipsychotics restore the PPI response in schizophrenia patients (Kumari and Sharma, 2002). This suggests that clinical efficacy of a drug might be correlated with an increase of PPI. PPI has also played an important role in preclinical studies because the startle response can be assessed in animals and humans by measuring the whole body flinch in rodents and the eye-blink response in humans. This made PPI one of the most widely used translational tools to assess the efficacy of novel putative antipsychotics (Swerdlow et al., 1994, Geyer et al., 2001, Geyer et al., 2002) .

Preclinical assays like PPI in rodents have the ultimate aim to help us understand the workings of human physiology and pathology. Therefore it is important that drugs used to model aspects of a disorder have similar effects in the model organism and humans. This thesis, for example, has used the NMDA receptor antagonist MK-801 to assess the workings of the HORNS task since this class of drugs is known to evoke behaviours reminiscent of schizophrenia in humans. The effect of another NMDA-antagonist ketamine on PPI levels has been investigated in humans and rats. In humans ketamine actually increases PPI (Duncan et al., 2001, Abel et al., 2003) but in rats it decreases PPI (Mansbach and Geyer, 1989). This suggests that extending conclusions from

rodent PPI studies to human behaviour is not straightforward. This, however, does not mean that PPI is not a useful tool. However, PPI has to be used, combined with other tests of attention and cognition, and wider considerations have to be taken into account. Swerdlow et al. (2008) warns of unrealistic expectations of PPI as an assay in schizophrenia research. A reduction or increase in PPI has so far not been shown to have an adaptive effect. Therefore, a reduction in PPI cannot be said to be a deficit or, by itself, without additional assessments, be evidence of schizophrenia. For example, men on average display higher levels of PPI than women at a given configuration of experimental parameters (Swerdlow et al., 1993, Aasen et al., 2005). It would be absurd to suggest that therefore, women show a more schizophrenia-like phenotype.

Just like PPI, the HORNS task tests habituation to a stimulus. However, PPI uses the reduction of the startle response to a tone, by a pre-pulse that is presented less than 500ms beforehand. This delay is too short for the pre-pulse and the pulse to be consciously perceived as too distinct stimuli. Therefore, PPI has been described as testing a pre-attentive process. The HORNS task, on the other hand, tests the habituation of the orienting response in the range of seconds. These delays are easily long enough to ascribe more formal attentional processes to the mice performing this task (as opposed to what could be considered pre-attentive), as was done in this thesis. In addition, this task allows the use of an internal control. As was done in chapter 6, a within-subjects comparison between the SAME and DIFFERENT condition can be made. This controls for two factors making the results more interpretable:

1) It makes the habituation effect observed in the SAME condition stimulus-specific. In PPI it is not certain whether the change induced by the pre-pulse is due to a stimulus-specific habituation or a general modulation of the arousal state. In the HORNS task, however, the habituation to the light stimulus observed in the SAME condition can be compared to the response in the DIFFERENT condition. This makes it possible to decide whether the first stimulus changed overall arousal (less orienting to the 2nd stimulus in both the SAME and DIFFERENT condition) or whether stimulus-specific habituation has occurred (less orienting in the SAME condition only).
2) The comparison of SAME vs DIFFERENT conditions allows controlling for changes in orienting and magazine entry. If, in contrast, there is a difference in baseline startle between groups, PPI differences between groups become difficult to interpret (Swerdlow et al., 2008). For example, if an intervention decreases baseline startle, a reduction in PPI could simply be due to a floor effect. Due to a lower baseline startle, it is harder to see a reduction in this startle. The HORNS task, in contrast, allows comparing suppression of head entry between conditions in the same animal. Therefore, changes in baseline responding are less problematic.

Motivational Salience in Schizophrenia

As described above, the aberrant salience observed in schizophrenia wholly goes beyond changes in stimulus-driven salience attribution. Motivational salience is also impaired. This impairment can be assessed in monetary

incentive delay tasks (Knutson et al., 2001, Deserno et al., 2013). These tasks measure how expectation of reward speeds up responding to a stimulus.

Roiser et al. (2009) investigated salience attribution to stimuli in schizophrenia patients and controls. If subjects did not respond quickly enough no reward was delivered. Before and during the presentation of the target stimulus T (a black square), stimuli were presented that predicted the probability of a reward being available in the particular trial. These stimuli varied along two dimensions, colour (blue or red) and shape (animal or household item), resulting in 4 stimulus types. One of these dimensions was informative for the reward probability whereas the other was not task-relevant. For example, when a blue object was presented 87.5% of trials were rewarded whereas only 12.5% of trials were rewarded if the object was red. In this example, it would not have mattered whether the object was an animal or a household object.

During this task the reaction time to the black square was measured to assess implicit salience by measuring how the prior presentation of different stimuli (e.g. a red animal) speeded up responding. In other words, it was tested how much the stimulus was able to motivate responding to the black square (i.e. speed it up or slow it down). In addition, participants were given a questionnaire at the end of the experiment in which they had to estimate the reinforcement probability associated with each stimulus type to assess explicit salience i.e. the subjects' conscious awareness of the probabilities.

This allowed Roiser et al. (2009) to measure four different types of salience:

- explicit adaptive salience: How accurately subjects judged the likelihood of reward along the informative dimension (red vs blue) in a questionnaire after the experiment
- implicit adaptive salience: How much the subject's response times adapted along the informative dimension (red vs blue)
- explicit aberrant salience: How accurately subjects judged the likelihood of reward along the non-informative dimension (animal vs household item) in a questionnaire after the experiment
- implicit aberrant salience: How much the subject's response times adapted along the non-informative dimension (animal vs household item)

Schizophrenia patients showed lower levels of explicit adaptive salience and implicit adaptive salience. This suggests, that patients could not use the information from the informative dimension to motivate their responding and could not estimate the reward probabilities predicted by the stimulus (e.g. red animal). A subgroup of schizophrenia patients with delusional symptomatology in addition showed higher levels of explicit aberrant salience, they thought that the non-informative dimension carried information about the reward property. In a later study Roiser et al. (2013) found aberrant salience in people with ultra-high risk of developing schizophrenia, suggesting that aberrant salience precedes the onset of psychosis.

This research gives preliminary evidence for aberrant salience in schizophrenia. However, as the authors acknowledge these studies were run in small samples on which many statistical tests were conducted, increasing the chance of false-

positives. This suggests that more studies in patients have to be conducted to allow a definitive conclusion.

On a broader level, it is important to consider that the definition of aberrant salience used by Roiser et al. (2009) was different from what has been used in this thesis. So far every deviation of attentional processes from the norm has been described as aberrant. Roiser et al. (2009), however, found what they described as reduced adaptive motivational salience in schizophrenia patients overall (including non-delusional patients). Patients did not show learning of a fact about the world which was completely unrelated to the task (i.e. they did not think that the uninformative dimension was predicting reward probabilities). Only delusional patients showed aberrant salience. Patients overall rather showed deficits in learning to motivate their actions in the appropriate way along the informative dimension.

Prediction Errors and Learning in Schizophrenia

This points towards a deficit in reinforcement learning which has been widely reported in schizophrenia (Deserno et al., 2013). Notably, aberrant salience in healthy subjects has been found to be correlated with increased striatal dopamine and reduced reward prediction error signals (Boehme et al., 2015). Subjects were tested in a salience attribution task similar to Roiser et al. (2009). The aberrant salience shown by a healthy subject in this task correlated negatively with reward prediction error signals in the ventral striatum during a separate operant conditioning task as measured by fMRI. Additionally subjects with higher aberrant salience had higher presynaptic dopamine levels in the

ventral striatum, suggesting a hyperdopaminergic state. Therefore, this study connected aberrant salience, with hyperdopaminergia and reduced reward prediction error signals albeit in healthy subjects.

In schizophrenia patients these striatal reward prediction errors, presumably based on a dopaminergic signal, are also reduced (Murray et al., 2008, Gradin et al., 2011, Deserno et al., 2013, Schlagenhauf et al., 2014), despite the striatal hyperdopaminergia widely reported in schizophrenia. One potential explanation for this apparent contradiction is that the hyperdopaminergia is reported to be present in the dorsal striatum (Kegeles et al., 2010) whereas the blunted dopaminergic reward prediction errors are observed in the ventral striatum. This thesis showed that LY354740 can increase dopaminergic reward prediction error in the ventral striatum in an unsignalled reward task under certain conditions. Potentially, this increase could correct the blunted reward prediction error signal seen in schizophrenia patients and thereby have a precognitive effect.

GluA1 KO mice provide an example of a mouse that displays aberrant salience and deficits in a form of learning, short-term habituation. This thesis also showed increased reward-evoked phasic dopamine responses in an instrumental task and a lack of negative feedback (dips in dopamine) if behaviour was not reinforced by reward (chapter 6). This neurochemical change could potentially underlie the increased response levels observed in GluA1 KO mice. This increased persistence of behaviour has already been seen in the lack of an effect of satiety-induced reinforcement deduction (Johnson et al., 2005) and slower extinction (Barkus et al., 2012) in GluA1 KO mice. This suggests that maybe as (potentially) in schizophrenia their aberrant salience keeps them from using the

dopamine signal as a reward prediction error to the same extend wildtype mice can.

Other Manipulations to be Assessed in the HORNS Task

This thesis has shown that GluA1 KO mice show a changed pattern of phasic dopamine responses related to their deficits in short-term habituation. Further FCV studies with this knockout mouse and other similar manipulations with relevance to schizophrenia would be of interest. Currently the habituation of the orienting response in a HORNS task has only been tested and shown to be changed by two manipulations: GluA1 knockout and systemic injection of MK-801. In addition Marshall et al. (2004) have shown that hippocampal lesions induce short-term sensitization in a related task. Further tests could be:

Since pharmacological manipulations of the NMDA receptors antagonist MK-801 abolished the stimulus-specific habituation (Chapter 6), it would be interesting to test a genetic approach. Whole brain constitutive GluN2A knockouts show a selective short-term habituation deficit remarkably similar to the GluA1 KO mice (Bannerman et al., 2008, Bannerman et al., 2012). Additionally, GluN2A has been widely associated with schizophrenia (Moghaddam and Javitt, 2012, Ripke et al., 2014). Therefore, a lack of stimulus-specific short-term habituation in the HORNS task would be expected with an associated change in phasic dopamine.

Finally, so far the effect of manipulations of the GluA1 AMPA receptor subunit has only been investigated in whole brain constitutive knockout mice in this thesis. Recently however, a novel approach based on antibodies has been developed. Lee (2014) developed an antibody that is specific to membrane bound GluA1 receptors. This antibody, when infused into a brain region, temporarily removes GluA1-containing AMPA receptors from cell membranes in this region. Thereby the role of GluA1 in a brain area can be investigated, without the confounding influence of developmental effects as are present in region-specific knockouts such as the hippocampal specific knockout used by Freudenberg et al. (2013).

Conclusions

This thesis investigated the effects of two manipulations of the glutamate system on dopamine in behaviours relevant to schizophrenia. These manipulations were a constitutive whole-brain knockout of the GluA1 AMPA receptor subunit and systemic injections of the group II mGluR agonist LY354740. This thesis made several advances to the state of knowledge concerning the interaction of glutamate and dopamine in tasks relevant to schizophrenia:

1) The effect of the group II metabotropic receptor agonist LY354740 is state-dependent. Dependent on the internal state of the animal LY354740 either had a tendency to increase amphetamine-induced hyperactivity or ameliorate amphetamine-induced hyperactivity (chapter 3).
2) LY354740 could, under certain circumstances, increase dopamine release in the nucleus accumbens core. Potentially related, LY354740 also

reduced hippocampal activity as measured using a haemodynamic tissue oxygen signal (chapter 4)

3) LY354740 failed to rescue cognitive deficits induced by GluA1 knockout, in marked contrast to previous reports of its effects in ameliorating deficits induced by non-competitive NMDAR antagonists. However, it could ameliorate hyperactivity in GluA1 knockout mice (chapter 5).
4) GluA1 KO mice had a hyperdopaminergic phenotype in tasks relevant to schizophrenia. GluA1 KO mice showed increased phasic dopamine responses to neutral stimuli and these dopamine responses were slower to habituate. Furthermore, they exhibited increased dopamine responses to unsignalled rewards in an instrumental task (chapter 6). Phasic dopamine responses in simple Pavlovian conditioning, however, were indistinguishable from controls (chapter 7).

Thus, both systemic LY354740 and GluA1 knockout could increase phasic dopamine release in the nucleus accumbens core, at least under some conditions. With respect to schizophrenia, this leads to different conclusions:

A group II mGluR agonist recently failed to reach its primary endpoint in a clinical trial for schizophrenia (Downing et al., 2014). Potentially, the state-dependent effect of LY354740 could explain the difficulties these agonists have in exerting an antipsychotic effect in clinical studies of schizophrenia. Based on my current data, it is even conceivable that under certain circumstances group II mGluR agonists could exacerbate the hyperdopaminergic state in some schizophrenia patients while ameliorating it in others.

GluA1 KO mice have been shown to display deficits in short-term habituation which could be a driver of aberrant salience. This thesis showed that this deficit in short-term memory is associated with a deficit in the habituation of phasic dopamine responses and thus with a hyperdopaminergic state under certain conditions. This adds further evidence that GluA1 KO mice have a phenotype relevant to schizophrenia. Therefore, additional studies in these knockout animals might elucidate the neurobiology of schizophrenia further.

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