Investigation of the behavioural and neurobiological effects of SSRI discontinuation in mice



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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Departments of Pharmacology and Experimental Psychology

Lincoln College

University of Oxford

Hilary Term 2023

Abstract

Helen M Collins, Lincoln College Oxford, DPhil, Hilary Term 2023

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Cessation of a selective serotonin reuptake inhibitor (SSRI) is associated with SSRI discontinuation syndrome, which includes symptoms such as anxiety and sleep disturbances. Symptoms typically appear within a week of stopping treatment, can be severe, and can last for several weeks or even months in some patients. The syndrome recently rose to prominence following a series of reviews finding that more than half of patients discontinuing SSRI treatment will experience symptoms. Yet, there have been few preclinical studies of the condition and its neurobiological basis is unknown. Thus, the aim of this thesis was to establish a model with which to investigate the behavioural and neurobiological effects of SSRI discontinuation in mice.

Behavioural studies found that two days after discontinuation from 12 days of treatment with the SSRI paroxetine, mice exhibited increased anxiety-like behaviour and behavioural inhibition on the elevated plus maze compared to saline-treated and continued paroxetine controls. These effects were short-lasting, having dissipated five days after discontinuation, and required at least 12 days of treatment before their emergence. They were evident in male but not female mice, and were also present, albeit to a lesser extent, following citalopram and venlafaxine discontinuation. Sleep and circadian rhythms were also assessed using continuous homecage monitoring, finding evidence of changes in sleep-wake activity for seven days after paroxetine cessation.

The 5-hydroxytryptamine (5-HT) system was then probed to investigate the neurobiological effects of SSRI discontinuation. Immunohistochemical experiments found evidence of a rebound increase in c-FOS immunoreactivity in 5-HT neurons in the dorsal raphe nucleus two and five days after paroxetine cessation, suggesting that discontinuation was associated with 5-HT neuron activation. *Ex vivo* analysis of regional brain tissue also found evidence of a rebound increase in 5-HT synthesis following paroxetine discontinuation. *In vivo* microdialysis experiments showed that while basal hippocampal extracellular 5-HT fell to saline control levels following discontinuation, depolarisation-evoked 5-HT release was increased. Gene expression analysis found evidence of a reduction in 5-HT_{1A} autoreceptor expression in the raphe nuclei following paroxetine discontinuation. It was therefore hypothesised that paroxetine discontinuation was associated with a fall in extracellular 5-HT, which led to the disinhibition of 5-HT neurons and thus a rebound increase in their excitability. Nonetheless, the behavioural effects of SSRI cessation could not be induced in mice receiving continued paroxetine by experimentally manipulating the 5-HT system with 5-HT_{1A} receptor ligands, suggesting that this approach could not be used to precipitate SSRI discontinuation.

In summary, these studies provide the first model of SSRI discontinuation syndrome in mice and offer novel insights into the effects of SSRI cessation on the 5-HT system. Further work is needed to determine if changes to 5-HT transmission are causal in the symptoms of SSRI discontinuation syndrome.

Acknowledgements

Firstly, I would like to thank my supervisors: Prof Trevor Sharp, Prof David Bannerman, and Dr Raquel Pinacho. I'd like to thank them all for their valuable insight into this project, as well as their ongoing encouragement and support. I am also very grateful to the Wellcome Trust for the financial input that enabled this work.

I'd like to thank Trevor for his day-to-day supervision and help with this thesis. I may not have found the process easy, but I've definitely become a better scientist and writer from my time in your lab. Thank you to David for encouraging me when I've found the work challenging. I've learnt so much from working with you, and I really appreciate your input into these experiments. A huge thank you to Raquel for her friendship and support. I've loved being your accomplice over the last few years, and you literally taught me everything I know about mice; I couldn't have done this without you.

I'd also like to thank the other members of the Sharp lab. I thank Judith Schweimer for teaching me microdialysis and for always being on hand to fix things in the lab. A big thank you goes to Cheryl So for her friendship throughout my DPhil, I'm so glad we could support each other through this process. Thank you to Sophie Gullino for being such a great friend and I wish you all the success with your DPhil. Thank you to Fuen Pilar Cuellar for all her work on the PCR experiments. I am also grateful to the students who have worked with me on this project: Lizzie Daly, Dersu Ozdemir, Yulia Sudarikova, Caroline Lazarenco and Cara Fuller. Special thanks go to Dersu for being such a good friend during her time in the lab, and to Caroline for stepping in and finishing one of the experiments when I had to isolate.

I am also grateful to everyone who has provided valuable insight into this project, and especially for Stuart Peirson's advice regarding the experiments in Chapter 7. I thank everyone who has helped or guided me during experiments, including Richard (OQF), Nina and Debbie (BMS) and Katie Hewitt (BNU). Thank you to Chris Barkus for all your 3Rs input and for listening to my (many) rants about 'experiments'. Thanks also go to the Anthony lab for their advice and the use of their equipment for the PCR experiments.

A special thank you goes to my friends, in particular the AHS gals and Lincoln pals. I'm sorry I've not been able to see you all nearly as much as I'd like, especially over the last year. Thank you for welcoming me back when I can actually come and see you, and for always asking me how the rats are doing. I'd particularly like to thank Maddy for being such a fun housemate for the first two years of my DPhil. An important thank you also goes to my GCSE Biology teacher Mrs Burt – thank you for inspiring me to study biology in the first place!

Thank you to my family, Mum, Dad, and James, for your ongoing love and support. I really appreciate the encouragement you've always given me. I'd also like to thank Granny and Nanna for always being there to call while I walk home from the lab.

And finally, my lovely Pol. Thank you for your endless patience, kindness, and support, and for being my biggest cheerleader. You've looked after me so much in the last few months, so now it's my time to look after you. We're the best team.

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Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine
5-HTT	5-HT transporter
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
ACSF	Artificial cerebrospinal fluid
ADS	Antidepressant Discontinuation Syndrome
ANOVA	Analysis of variance
AOF	Aversive open field
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycle
DA	Dopamine
DESS	Discontinuation Emergent Signs and Symptoms (scale)
DG	Dentate gyrus
DH	Dorsal hippocampus
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
DRN	Dorsal raphe nucleus
EPM	Elevated plus maze
GAD	Generalised anxiety disorder
GI	Gastrointestinal
GOI	Gene of interest
HKG	Housekeeping gene
HPLC-ED	High performance liquid chromatography with electrochemical detection
I.p.	Intraperitoneal
IQR	Interquartile range
IS	Interday stability
IV	Intraday variability

IVC	Individually ventilated cages
LDB	Light/dark box
LDT/PPT	Laterodorsal and pedunculopontine tegmental nuclei
LMA	Locomotor activity
LSD	Least Significant Difference
LTC	Light tight chamber
MAO	Monoamine oxidase
MAOI	MAO inhibitor
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
MRN	Median raphe nucleus
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NICE	National Institute for Health and Care Excellence
NIH	Novelty-induced hyponeophagia
NREM	Non-rapid eye movement (sleep)
NSF	Novelty-suppressed feeding
OF	Open field
PAG	Periaqueductal grey
PBS	Phosphate buffered saline
PFC	Prefrontal cortex
PIR	Passive infrared
PLC	Prelimbic cortex
qPCR	Quantitative real-time polymerase chain reaction
RCT	Randomised control trial
REM	Rapid eye movement (sleep)
RN	Raphe nuclei
RNA	Ribonucleic acid
S.c.	Subcutaneous
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNRI	Serotonin noradrenaline reuptake inhibitor

SPSS	Statistical Product and Service Solutions (IBM)
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TPH2	Tryptophan hydroxylase 2
VH	Ventral hippocampus
ZT	Zeitgeist time

Publications

Parts of the work included in this thesis have been presented in the following publications and conference abstracts:

Helen M Collins, Raquel Pinacho, Dersu Ozdemir, David M Bannerman, Trevor Sharp. Effect of SSRI discontinuation on anxiety-like behaviours in mice. *J Psychopharmacol.*, 2022; 36(7):794-805.

Helen M Collins, Raquel Pinacho, David M Bannerman, Trevor Sharp. Immunohistochemical evidence that paroxetine discontinuation in mice is associated with increased activation of 5-HT neurons. *Neuroscience Applied*, 2022; 1, Suppl. 1: 100652.

- Early Career Excellence Award, *35th ECNP Congress – Vienna 2022*

Helen M Collins, Raquel Pinacho, David M Bannerman, Trevor Sharp. Immunohistochemical evidence that paroxetine discontinuation in mice is associated with increased activation of 5-HT neurons. Selected Abstracts from Pharmacology 2022. *Br J Pharmacol*, 2022. doi: 10.1111/bph.15944.

- Dom Spina Prize for Experimental Design, *Pharmacology 2022*

Helen M Collins, Dersu Ozdemir, Yulia Sudarikova, Elizabeth Daly, Raquel Pinacho, David M Bannerman, Trevor Sharp. Effect of paroxetine discontinuation on 5-HT neurochemistry in mice ex vivo and in vivo. Special Issue: 34th ECNP Congress – Lisbon 2021 Hybrid. *Eur Neuropsychopharmacol*, 2021; 53, Suppl. 1: S407-S408.

- Best Poster Prize, *34th ECNP Congress – Lisbon 2021*

Helen M Collins, Raquel Pinacho, David M Bannerman, Trevor Sharp. The effect of discontinuation from selective serotonin reuptake inhibitors on anxiety in mice. Selected Abstracts From Pharmacology 2020. *Br J Pharmacol*, 2021;178(2):381-486.

- Best Oral Communication, *Pharmacology 2020*

Helen M Collins, Raquel Pinacho, Dersu Ozdemir, David M Bannerman, Trevor Sharp. Investigation of the behavioural effects of paroxetine discontinuation in mice. Special Issue: 33rd ECNP Congress – Vienna 2020. *Eur Neuropsychopharmacol*, 2020; 40, Suppl. 1: S136-S137.

Contributions

In Chapter 3, Raquel Pinacho conducted the initial citalopram discontinuation experiment.

In Chapter 5, Yulia Sudarikova ran the regional brain tissue samples from the two day discontinuation (12 days once-daily paroxetine) experiment. Lizzie Daily ran the samples from the five day discontinuation (12 days twice-daily paroxetine) experiment. Fuen Pilar Cuellar ran the qPCR experiments for the two and five day paroxetine discontinuation experiments and helped with tissue dissection. Sophie Gullino dosed the mice for the two day paroxetine discontinuation repeat qPCR experiment and helped with tissue dissection and qPCR experiments.

In the first precipitation experiment in Chapter 6, Caroline Lazarenco helped with the repeated injections and tested all mice on the EPM.

Chapter 1
Introduction

1 Scope of this thesis

Selective serotonin reuptake inhibitors (SSRIs) are currently the first-line pharmacological treatments for major depressive disorder (MDD) and several anxiety disorders. Abruptly stopping treatment with an SSRI can produce a debilitating discontinuation syndrome, typified by symptoms such as anxiety and sleep disturbances (Haddad, 1997; Fava et al., 2015). Although clinicians have been aware that SSRIs can produce discontinuation reactions since the 1990s, SSRI discontinuation syndrome rose to prominence in 2018 following a series of reports that suggested that up to half of patients will experience discontinuation symptoms (Davies & Read, 2018; Davies et al., 2018; Davies & Read 2019). These reports also claimed that symptoms can be more severe and persistent than clinical guidelines currently suggest. Since then, there has been renewed interest in understanding the syndrome, for example who is likely to experience the symptoms and how best to avoid them.

There is also a need to better understand the neurobiological basis of the discontinuation symptoms. One preclinical study has shown that SSRI discontinuation increased anxiety-like behaviour in rats (Bosker et al., 2010), mirroring one of the core symptoms in patients. Changes in the 5-hydroxytryptamine (5-HT) system have also been reported in the days following SSRI cessation (Caccia et al., 1993; Trouvin et al., 1993; Neumaier et al., 1996; Anthony et al., 2000). Nonetheless, there has yet to be a systematic investigation of how SSRI discontinuation affects rodent anxiety-like behaviour, or an attempt to understand how these effects may be mediated by altered 5-HT transmission.

This thesis therefore aimed to establish a mouse model of SSRI discontinuation with which to investigate its behavioural and neurobiological effects. The following text reviews our current understanding of the clinical discontinuation syndrome and includes an overview of the previous preclinical studies. It also discusses the central 5-HT system, its modulation of anxiety-like behaviours and the mechanism of action of SSRIs, as well as the current theories for the neurobiological basis of discontinuation symptoms.

2 SSRI discontinuation syndrome

2.1 Clinical definition

The abrupt cessation of SSRIs can produce a debilitating discontinuation syndrome. Typical symptoms include dizziness, anxiety, sleep disturbances, and nausea (Gabriel & Sharma, 2017; Davies & Read, 2018; Horowitz & Taylor, 2019). Symptoms typically appear within a few days of treatment cessation, or even a reduction in dose, and generally last for a couple of weeks (Haddad, 1997; NICE, 2009; Iacobucci, 2019). Clinicians have historically thought of the syndrome as comprising of "mild, transient symptoms that can lead to impairments in functioning or productivity" (Haddad, 1997; Haddad, 2001), but not as a condition that will cause serious harm to the patient. Nonetheless, recent evidence suggests that the symptoms can be severe and persist for several months in some patients (Hengartner et al., 2020). To acknowledge the large variation in experiences, the National Institute for Health and Care Excellence (NICE) recently updated its guidelines to say that the syndrome can be "more difficult, with symptoms lasting longer, in some cases several weeks, and occasionally several months" (NICE, 2022).

2.2 Symptoms

The symptoms of SSRI discontinuation syndrome fall into six categories, as described by the FINISH pneumonic: Flu-like symptoms, Insomnia, Nausea, Imbalance, Sensory symptoms, and Hyperarousal (Berber, 1998; Gabriel & Sharma, 2017). Dizziness is the most common symptom of SSRI cessation (Black et al., 1993; Leiter et al., 1995; Oehrberg et al., 1995; Haddad, 1997; Lejoyeux & Adès, 1997; Haddad et al., 1998). It forms part of a class of symptoms known as 'disequilibrium', which also includes light-headedness, vertigo, and discoordination (Warner et al., 2006; Horowitz & Taylor, 2019).

Affective symptoms, including anxiety and agitation, are also common following SSRI cessation, even in patients who did not experience them during their previous illness. Anxiety was first reported in an open-label study of 14 patients in the two weeks following fluvoxamine discontinuation (Black et al., 1993), and has since been observed as early as two days after SSRI cessation (Debattista & Schatzberg, 1995; Fava & Grandi, 1995; Benazzi, 1998; Michelson et al., 2000; Baldwin et al., 2006a; Bhanji et al., 2006; Shelton, 2006; Belaise et al., 2014). The Royal College of Psychiatrists estimated that anxiety affects 25% of patients for at least three months following SSRI discontinuation (RCP, 2012), including 42% of people after paroxetine discontinuation and 16% after escitalopram discontinuation (Montgomery et al. 2004a). Other mood symptoms common after SSRI cessation include crying (Shelton, 2006), increased depression scores (Benazzi, 1998; Milliken & Cooper, 1998; Delgado, 2006; Tint et al., 2008), suicidal thoughts (Tint et al., 2008; Lerner & Klein, 2019) and panic attacks (Benazzi, 1998).

Sleep disorders, including insomnia and sleep disturbances, are also common following SSRI cessation (Haddad, 1997). Rapid eye movement (REM) sleep rebound has also been demonstrated following SSRI discontinuation and has been linked to the

vivid and abnormal dreaming often reported by patients (van Bemmel et al., 1993) (discussed in Chapter 7 section 1.1). There are also a host of somatic symptoms, including gastrointestinal (GI) disturbances, such as nausea and vomiting, as well as flu-like symptoms such as headaches, lethargy and fatigue, cramps, and muscle aches (Barr et al., 1994; Koopowitz & Berk, 1995; Haddad, 1997; Yasui-Furukori & Kaneko, 2011). Unusual sensory symptoms can also occur, including paraesthesia (abnormal feelings such as skin tingling and prickling) and 'electric shock-like' sensations (Mallya et al., 1993; Frost & Lal, 1995; Coupland et al., 1996; Haddad, 1997). Cognitive symptoms such as confusion, amnesia and 'brain zaps', sudden feelings of extreme tiredness and the inability to concentrate, are also common (Haddad, 1997; Shoenberger, 2002).

Neither SSRI discontinuation nor the broader Antidepressant Discontinuation Syndrome (ADS) are included in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Consequently, these syndromes lack formal diagnostic criteria. To study the effects of SSRI discontinuation in clinical settings, several scales have been developed to identify patients experiencing discontinuation effects. The Discontinuation Emergent Signs and Symptoms (DESS) scale is a self-rated or clinician-led questionnaire based on the common symptoms, which has been validated as a useful experimental tool. For example, patients undergoing paroxetine discontinuation scored higher on the DESS scale (indicating more symptoms) than patients maintained on paroxetine treatment (Montgomery et al., 2004b).

2.3 Incidence

Although it was previously thought to only affect a small proportion of patients, SSRI discontinuation syndrome is now recognised as a common occurrence after

stopping SSRI treatment. There have been several reviews attempting to quantify the proportion of patients that experience adverse effects when discontinuing their medication. Coupland et al. (1996) was the first retrospective assessment of medical notes of patients discontinuing the SSRIs fluoxetine, fluvoxamine and paroxetine, which estimated that 12% of patients experienced symptoms. The first randomised, placebo-controlled trial of SSRI discontinuation found that 34.5% of patients experienced symptoms in the two weeks following cessation compared to only 14% of patients that were maintained on treatment (Oehrberg et al., 1995). Other studies have suggested that discontinuation rates may, in fact, be much higher, for example between 46-59% of patients in studies of fluvoxamine, sertraline and paroxetine (Rosenbaum et al., 1998; van Geffen et al., 2005; Fava et al., 2007; Tint et al., 2008; Read et al., 2014). A large patient study from the Royal College of Psychiatrists supported these conclusions, finding that 63% of patients experienced SSRI discontinuation symptoms (RCP, 2012).

The question of how many people experience SSRI discontinuation syndrome recently rose to prominence. In 2018, a series of reviews (published in conjunction with an All Party Parliamentary Group report on Prescribed Drug Dependence) reiterated that SSRI discontinuation symptoms are more prevalent than had previously been recognised. Based on data from clinical trials, case studies, clinician surveys and even analysis of online forums, these reports estimated that 56% of patients may experience discontinuation symptoms (Davies & Read, 2018; Davies et al., 2018; Davies & Read, 2019). Nevertheless, the latter studies have been criticised for relying too heavily on biased patient samples and for failing to include several studies reporting low rates of discontinuation symptoms, leading to inflated estimates of their incidence (Jauhar et al., 2019). Despite the methodological flaws, however, these recent data highlight that a much

greater proportion of patients experience discontinuation symptoms than had previously been realised.

The incidence of the discontinuation syndrome varies between SSRIs, with paroxetine being the most likely to cause symptoms (Fava et al., 2015; Gastaldon et al., 2022). Randomised control trials (RCT) estimate that between 16% and 100% of patients discontinuing paroxetine will experience at least one discontinuation symptom (Keuthen et al., 1994; Oehrberg et al., 1995; Coupland et al., 1996; Hindmarch et al., 2000; Bogetto et al., 2002; Judge et al., 2002; Lader et al., 2004; Tint et al., 2008; Read et al., 2014). The risk of discontinuation symptoms after paroxetine treatment was estimated to be ten times greater than following citalopram or fluvoxamine, and one hundred times more likely than fluoxetine (Price et al., 1996). Studies directly comparing paroxetine to other SSRIs also found it was more likely to cause, and produce more severe, symptoms (Benazzi, 1998; Hindmarch et al., 2000; Markowitz et al., 2000; Sir et al., 2005; Baldwin et al. 2006a; Baldwin et al. 2006b). In contrast, less than 5% of patients experienced symptoms after fluoxetine discontinuation (Coupland et al., 1996; Lejoyeux & Adès, 1997; Bogetto et al., 2002; Judge et al., 2002). Notably, the variation in the risk of different SSRIs producing symptoms likely contributes to the large variation in the estimates of the overall incidence of the syndrome.

Paroxetine is thought to be particularly problematic due to its short half-life. Paroxetine has a half-life of 21 h in humans (Bourin et al., 2001) compared to four to six days for fluoxetine and its metabolite norfluoxetine (Sanchez et al., 2014). This produces a more rapid reduction in plasma paroxetine concentrations following treatment cessation (Henry et al., 2000; Michelson et al., 2000). Moreover, paroxetine has non-linear metabolism kinetics because it inhibits its own metabolising enzyme, cytochrome P450

2D6 (CYP2D6) (Jessurun et al., 2017). Thus, when plasma paroxetine levels fall, its metabolism increases and its washout is accelerated (Fava & Grandi, 1995).

2.4 Severity

SSRI discontinuation symptoms are typically considered to be mild (Oehrberg et al., 1995; Price et al., 1996); yet, between one fifth and one third of patients described their symptoms as severe (Sir et al., 2005; Cartwright et al., 2016). The recent reports on the incidence of SSRI discontinuation also found that symptoms were severe for 42% of patients (Read & Williams, 2018; Davies & Read, 2019).

Qualitative reports of patients' experiences of SSRI discontinuation further demonstrate the hitherto unappreciated severity of the symptoms. One patient described their experience as: "it seems like I'm about five times as anxious/depressed as I was pre-paroxetine" (Pestello & Davis-Berman, 2008). Another described their symptoms as "so severe I'm lucky I did not end my life" (Davies et al., 2018). Case studies also provide examples of how symptoms have been so severe that patients have needed to restart their treatment (Price et al., 1996), been unable to work (Koopowitz & Berk, 1995), and even been hospitalised (Bhanji et al., 2006).

Nonetheless, it is hard to determine the objective severity of symptoms, as reports are usually based on subjective and self-reported symptoms. Biased patient samples may also have overestimated the severity of the syndrome. For example, Davies and Read (2019) included studies of comments left on online forums about SSRI discontinuation (Read et al., 2014). Nonetheless, while the current studies may be of limited quality, there is growing evidence that SSRI discontinuation symptoms can be more severe than previously recognised.

2.5 Duration

Discontinuation symptoms can appear as early as 24 h after the final dose of an SSRI, and they commonly emerge within two to three days of treatment cessation (Black et al., 1993; Bloch et al., 1995; Debattista & Schatzberg, 1995; Fava & Grandi, 1995; Frost & Lal, 1995; Kasantikul, 1995; Milliken & Cooper, 1998; Black et al., 2000). Symptoms typically peak after five days (Black et al., 1993; Bogetto et al., 2002) and usually resolve within two to three weeks (Coupland et al., 1996; Haddad, 1997; Milliken & Cooper, 1998; Fava et al., 2015).

Yet, there is also evidence that symptoms can last for much longer. For instance, a third of patients discontinuing paroxetine and escitalopram still had symptoms one month later (Fava et al., 2007; Prakash & Dhar, 2008). A placebo-substitution trial also found that 40% of patients had symptoms six weeks after fluoxetine discontinuation (Zajecka et al., 1998). In another study, symptoms lasted for 90 weeks on average in patients discontinuing an SSRI after at least five years of treatment (Stockmann et al., 2018), and one study even reported a mean symptom duration of 2.5 years (Belaise et al., 2012).

Notably, these reports face similar patient biases as studies of the incidence and severity of symptoms. It is also difficult to assess the average length of the syndrome as many patients restart their medications due to the persistence of their symptoms (Price et al., 1996). Nevertheless, the existence of reports of prolonged discontinuation symptoms suggests that the syndrome is not always as short-lived and self-limiting as clinical guidelines currently suggest.

One explanation as to why the duration of symptoms can vary so greatly between patients is that different symptoms emerge at different times following treatment cessation. Firstly, there are new, somatic symptoms that appear immediately following

discontinuation. There are also rebound symptoms including anxiety and depression that may have been present in the underlying disorder that worsen 36 to 96 h after cessation and can last for up to six weeks. Finally, post-withdrawal syndrome, also called protracted withdrawal, describes any symptoms that persist for longer than six weeks (Belaise et al., 2014; Chouinard & Chouinard, 2015; Henssler et al., 2019; Cosci et al., 2021). In patients with post-withdrawal syndrome, symptoms lasted for 37 months on average, with one patient reporting symptoms for 79 months (Hengartner et al., 2020). Thus, some patients may only experience early somatic or rebound symptoms, which dissipate within a couple of weeks. Conversely, post-withdrawal syndromes may persist for much longer.

2.6 Risk factors

Aside from identifying paroxetine as an SSRI that is likely to cause symptoms, the greatest risk factor for experiencing SSRI discontinuation syndrome is thought to be abrupt treatment cessation (Lejoyeux & Adès, 1997; Himei & Okamura, 2006). In a study of patients discontinuing from different SSRIs, abrupt cessation was more likely to cause discontinuation symptoms, and produce more symptoms, than gradual tapering (van Geffen et al., 2005). Moreover, while patients experienced severe symptoms within two to three days of abrupt paroxetine discontinuation, gradual tapering allowed the same patients to later stop paroxetine without any symptoms (Dominguez & Goodnick, 1995). Tapering SSRIs at the end of treatment has therefore been suggested to reduce the risk of the discontinuation syndrome and has recently been recognised in clinical guidelines (NICE, 2023). Several clinical trials are currently underway to identify the best tapering strategy to minimise discontinuation symptoms (Khan et al., 2014; Berwian et al., 2017; Horowitz & Taylor, 2019; Ruhe et al., 2019).

Prolonged SSRI treatment also appears to be a risk factor (Jha et al., 2018; Read & Williams, 2018; Horowitz et al., 2022). For example, one week of SSRI treatment was not sufficient to produce symptoms in patients taking SSRIs for premenstrual dysphoric disorder (Yonkers et al., 2015). On the other hand, clinical trials regularly report symptoms after just six to eight weeks of treatment (Robert et al., 1995; Markowitz et al., 2000; Montgomery et al., 2004b; Sir et al., 2005; Baldwin et al., 2006b). There is also evidence that younger patients are more likely to experience discontinuation symptoms than older patients (Bogetto et al., 2002; Himei & Okamura, 2006; Fava et al., 2015; Read & Williams, 2018; Gastaldon et al., 2022), although this may reflect the tendency for young patients to discontinue treatment more quickly (Jha et al., 2018). Having previously experienced discontinuation symptoms may also predispose an individual to experiencing them again (Jha et al., 2018; Horowitz et al., 2022).

There is also evidence that sex and gender may be factors in determining an individual's risk of SSRI discontinuation syndrome. On one hand, women are more than twice as likely to take an SSRI than men (HSCIC, 2014), and most case studies of SSRI discontinuation have been in women. A trial of paroxetine and fluoxetine cessation also found that women experienced more discontinuation symptoms than men (Bogetto et al., 2002). Conversely, a recent pharmacovigilance study found that men were more likely to experience antidepressant discontinuation symptoms than women (Gastaldon et al., 2022). A retrospective assessment of medical notes also found that 21.2% of men but only 9.9% of women experienced SSRI discontinuation symptoms (Coupland et al., 1996). These data suggest that men are more likely to experience discontinuation symptoms than women. Nonetheless, very few RCTs have stratified results by sex; thus, the influence of sex and gender on discontinuation symptoms is still poorly understood.

There may also be genetic risk factors that have not been systematically investigated. For instance, a small study of patients with MDD and generalised anxiety disorder (GAD) found a weak association between the C(-1019)G polymorphism in the 5-HT_{1A} receptor gene and the risk of experiencing SSRI discontinuation symptoms (Murata et al., 2010). Nonetheless, there has yet to be a large scale study implicating variation in 5-HT_{1A} receptor gene expression in the incidence or severity of discontinuation symptoms. It is also not known if other polymorphisms, such as in the enzymes that metabolise SSRIs (Sindrup et al., 1992; Özdemir et al., 1999), alter the risk of discontinuation symptoms.

There are currently no socioeconomic or demographic risk factors linked to SSRI discontinuation syndrome (Gabriel & Sharma, 2017). Nonetheless, the existing studies overwhelmingly include white, European patients (e.g. Baldwin et al. 2006a). The apparent absence of demographic or socioeconomic risk factors may therefore reflect the poor diversity of study populations rather than a genuine lack of effect.

Overall, there is a poor understanding of the risk factors for SSRI discontinuation syndrome. The ongoing Optimal Personalised Antidepressant Use (OPERA) trial aims to understand the factors that determine a patient's risk of developing discontinuation symptoms to better identify potential sufferers before they discontinue their SSRI treatment (Vinkers et al., 2021).

2.7 The difference between discontinuation syndrome and relapse

It is important to note that SSRI discontinuation syndrome is distinct from relapse (Lejoyeux & Adès, 1997; Horowitz & Taylor, 2022). Relapse is defined as the "insidious return of depressive symptoms" (Jha et al., 2018), whereas discontinuation reactions are

of “predictable onset” and include “psychological and bodily symptoms not previously complained of by patients” (Lader, 1983). For instance, somatic symptoms such as dizziness and nausea are not characteristic of relapse but are some of the most common discontinuation symptoms.

Another difference between relapse and discontinuation is the time course of symptoms. As noted above, discontinuation symptoms typically emerge within days of treatment cessation, and data suggest that most patients have recovered a year later (Koopowitz & Berk, 1995; Black et al., 2000; Fernando & Schwader, 2000). In contrast, relapse of depression is not common in the first week after discontinuation and the risk of relapse gradually increases over time (Haddad et al., 1998; Vinkers et al., 2021). Additionally, discontinuation symptoms tend to resolve immediately if treatment is reinstated (Dominguez & Goodnick, 1995; Coupland et al., 1996), whereas it can take several weeks for SSRI treatment to improve symptoms of relapse (see section 4.3).

Nonetheless, the similarity between the symptoms of SSRI discontinuation syndrome and relapse can make it difficult to differentiate between these conditions (Rosenbaum et al., 1998). It is also challenging to distinguish between the long-lasting post-withdrawal syndrome and relapse, as both can be present several months after treatment cessation (Chouinard & Chouinard, 2015; Vinkers et al., 2021). The misdiagnosis of discontinuation symptoms as relapse of depression has likely led clinicians to overestimate the risk of relapse (Fava, 2020), potentially resulting in patients taking SSRIs for longer than is necessary. Differentiating between SSRI discontinuation syndrome and relapse is therefore important for studying the condition, as well as to avoid the sub-optimal treatment of patients (Fornaro et al., 2023).

2.8 SSRI ‘discontinuation’ versus ‘withdrawal’

There has also been controversy regarding the name of the syndrome, and specifically over the use the term ‘withdrawal’. Withdrawal is a highly stigmatised term due to its association with psychotropic drugs. This term also implies that a drug is addictive, defined as compulsive drug taking, and may produce dependence, tolerance, and cravings (Haddad et al., 1998). SSRIs are not considered addictive and they do not cause cravings (Davies & Read, 2018; Jauhar et al., 2019; Lerner & Klein, 2019); hence, the term discontinuation syndrome is more commonly used (Nielsen et al., 2012).

Nonetheless, there is evidence that patients taking SSRIs do develop tolerance to the drugs, since treatment becomes less effective over time (Haddad, 2005; Nielsen et al., 2012). Patients also describe themselves as addicted. For instance, 33-55% of antidepressant users in patient surveys believed they were addicted to their SSRI (Hoencamp et al., 2002; Read et al., 2014). It has also been argued that the term ‘discontinuation syndrome’ minimises the severity of the symptoms some patients experience, which are more akin to withdrawal reactions from other drugs (Guy et al., 2020). Moreover, since patients can experience symptoms following single missed doses and reductions in dose, ‘discontinuation’ is not necessary for symptoms to emerge (Pies, 2019). Thus, while the syndrome is currently referred to as SSRI or antidepressant discontinuation syndrome, the debate over its name, and what it implies, continues.

2.9 Discontinuation from other antidepressants

Tricyclic antidepressants (TCA) and monoamine oxidase (MAO) inhibitors (MAOI) also produce discontinuation syndromes (Andersen & Kristiansen, 1959; Kramer et al., 1961), including symptoms such as GI disturbances, headaches and sleep disruption

(Nielsen et al., 2012). Unlike SSRIs, however, TCA and MAOI cessation can also produce movement disorders including akathisia and parkinsonism, as well as behavioural symptoms such as mania, panic attacks, and delirium (Dilsaver et al., 1987). On the other hand, affective symptoms such as anxiety are rarely reported (Haddad, 1997; Milliken & Cooper, 1998), suggesting that the mechanisms underlying SSRI discontinuation symptoms may differ to those of TCA or MAOI withdrawal.

Serotonin-noradrenaline reuptake inhibitors (SNRIs) such as venlafaxine and duloxetine also cause discontinuation symptoms. Venlafaxine is particularly problematic, producing similar rates of discontinuation symptoms to paroxetine (Tint et al., 2008; Fava et al., 2015; Davies & Read, 2019). The propensity of venlafaxine to cause symptoms also likely arises from its short half-life (only 3-4 h in humans; Schweizer et al., 1997). Like SSRIs, SNRI discontinuation produces symptoms such as anxiety and sleep disturbances (Montgomery et al., 2004a). Reports also suggest that the symptoms can be severe (Sir et al., 2005; Read et al., 2014) and can last for several weeks (RCP, 2012).

3 The 5-HT system

3.1 Discovery of 5-HT

The molecule now known as 5-HT was first extracted from enterochromaffin cells of the GI tract (Vialli & Erspamer, 1933, 1937; Erspamer, 1940), where it was associated with smooth muscle contraction (Kosterlitz & Robinson, 1957; Lembeck, 1958). It was soon detected in blood plasma and linked to vasoconstriction (Rapport et al., 1948). Yet, it was not until the 1950s that these compounds were identified as one and the same (Rand & Reid, 1951; Reid & Rand, 1952). The subsequent discovery of 5-HT in the mammalian

brain suggested it may be involved in neurotransmission (Erspamer & Asero, 1952; Twarog & Page, 1953).

3.2 Anatomy of the 5-HT system

The first evidence of 5-HT neurons in the rat midbrain came from studies by Dahlström & Fuxe (1964), which were later refined by immunohistochemical studies (Steinbusch et al., 1981; Köhler & Steinbusch, 1982). The soma of 5-HT neurons are located in discrete nuclei called the raphe nuclei (Baker et al., 1991), of which the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) are the best characterised. In rodents, the DRN is located ventral to the cerebral aqueduct and periaqueductal grey (PAG), and the MRN is ventral to the DRN (Steinbusch, 1981; Jacobs & Azmitia, 1992; Paxinos & Franklin, 2007) (*Fig. 1.1*).

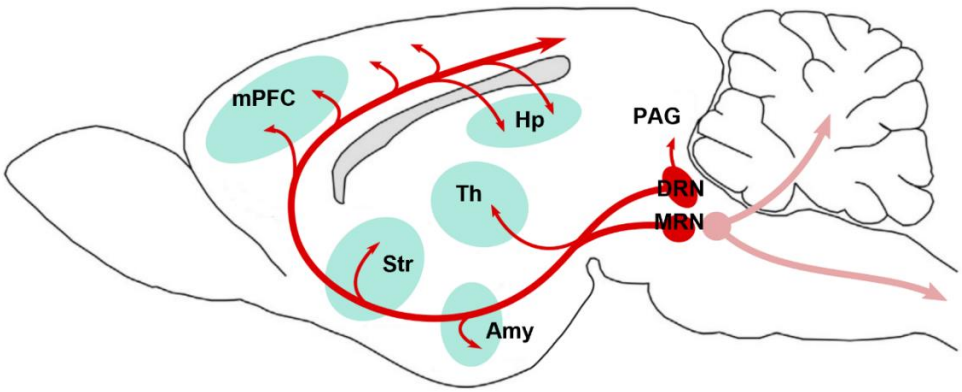


Figure 1.1 Mouse 5-HT system. Rostral group, dark red. Caudal group, pale red. Amy, amygdala; DRN, dorsal raphe nucleus; Hp, hippocampus; mPFC, median prefrontal cortex; MRN, median raphe nucleus; PAG, periaqueductal grey; Str, striatum; Th, thalamus. Adapted from Lesch et al., (2012).

5-HT neurons send diffuse projections throughout the brain (Muzerelle et al., 2016). The DRN and MRN form part of the rostral group of 5-HT neurons that innervate forebrain structures such as the frontal cortex, thalamus, hippocampus, and amygdala (Hornung, 2003). The DRN preferentially innervates the cortex, striatum, and dorsal

hippocampus (DH), whereas the MRN innervates the ventral hippocampus (VH) and septum (Azmitia & Segal, 1978; Lowry et al., 2005) (*Fig. 1.1*). Nonetheless, the DRN and MRN have overlapping terminal fields – for instance, the VH receives 5-HT projections from the MRN and the DRN (Köhler & Steinbusch, 1982; Jacobs & Azmitia, 1992; Muzerelle et al., 2016). In contrast, 5-HT neurons in the caudal group of raphe nuclei innervate the cerebellum, brain stem and spinal cord (Steinbusch et al., 1981; Jacobs & Azmitia, 1992; Nestler et al., 2015) (*Fig. 1.1*). In association with its far-reaching projections, the 5-HT system has been implicated in the modulation of many behaviours. These include emotion and mood, sleep, arousal, attention, cognition, appetite, thermoregulation and sensory processing (Jacobs & Fornal, 1991).

3.3 5-HT synthesis and metabolism

5-HT is synthesised from the essential amino acid tryptophan, which crosses the blood-brain barrier via the large neutral amino acid transporter. Tryptophan is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). L-amino acid decarboxylase then converts 5-HTP into 5-HT.

TPH is the rate-limiting enzyme in 5-HT synthesis (Fitzpatrick, 1999). There are two gene isoforms of TPH, *TPH1* and *TPH2*. TPH2 is predominately expressed in the CNS in humans and rodents, whereas TPH1 is expressed in peripheral tissues (Walther et al., 2003). TPH2 is synthesised in the soma of 5-HT neurons but is also transported to axonal terminals for local 5-HT synthesis (Deguchi & Barchas, 1972; Koubi et al., 2001). Its activity is regulated by neuronal activation, via phosphorylation by calcium calmodulin-dependent kinases, forming a feedback loop between 5-HT neuronal activity and synthesis (Yamauchi & Fujisawa, 1983; Hrdina, 1987; Ehret et al., 1989).

5-HT is metabolised by monoamine oxidase (MAO) on mitochondrial membranes and in the extracellular space. There are two isoforms of the gene encoding MAO, *MAO-A* and *MAO-B* (Grimsby et al., 1991). MAO-B is predominantly expressed in adult 5-HT neurons (Levitt et al., 1982; Westlund et al., 1988). After being oxidised by MAO, aldehyde dehydrogenase forms the main metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) (Bortolato et al., 2010; Nestler et al., 2015).

3.4 5-HT transmission

5-HT is loaded into vesicles in neuronal terminals by the vesicular monoamine transporter (VMAT2) (Nickell et al., 2014) (*Fig. 1.2*). 5-HT is released by depolarisation of the presynaptic terminal, opening voltage gated calcium channels, and triggering exocytosis. Putative 5-HT neurons were initially shown to have slow, regular firing patterns (Aghajanian & Haigler, 1974; Aghajanian et al., 1978), fire at frequencies between 1-5 Hz, have broad action potentials and large after-hyperpolarisations (Vandermaelen & Aghajanian, 1983). These electrophysiological properties were later confirmed in *in vivo* juxtacellular-labelling studies in the rat DRN (Allers & Sharp, 2003). Nonetheless, there is also evidence that 5-HT can be released by burst firing (Hajós & Sharp, 1996) and is regulated by cortical activity (Schweimer et al., 2011). 5-HT is also involved in volume transmission, as it is released from en passant synapses and thus acts on extra-synaptic sites (Descarries et al., 2006; Descarries & Riad, 2012). This reflects the role of 5-HT as a neuromodulator, as opposed to the fast transmission typical of neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA).[1]

[1] Notably, subpopulations of 5-HT neurons also co-release glutamate and GABA, which act as classical fast neurotransmitters (Luo et al., 2015; Sengupta et al., 2017). The role of this co-transmission, however, is poorly understood and is the beyond the scope of this thesis.

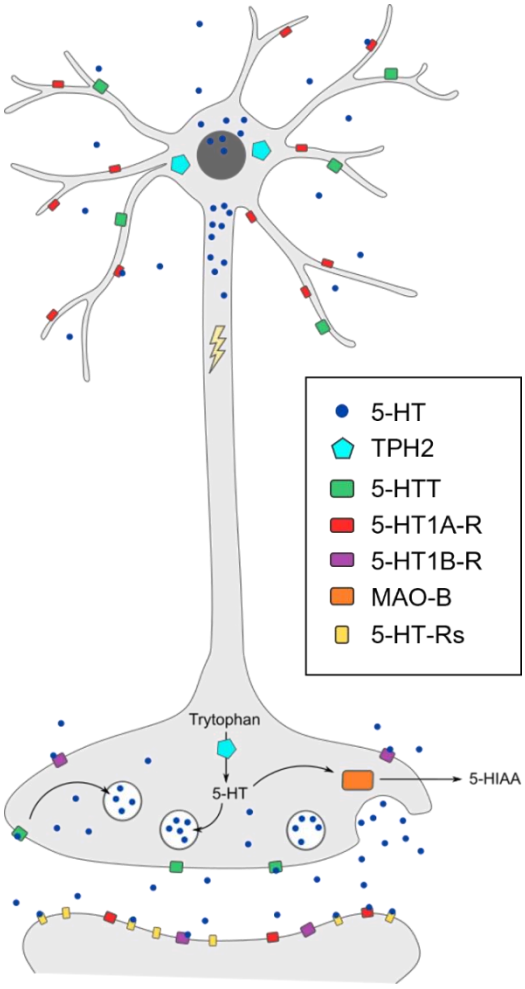


Figure 1.2 Anatomy of a 5-HT neuron. TPH2, tryptophan hydroxylase 2; 5-HTT, 5-HT transporter; 5-HT1A-R, 5-HT_{1A} receptor; 5-HT1B-R, 5-HT_{1B} receptor; MAO-B, monoamine oxidase B; 5-HT-Rs, postsynaptic 5-HT receptors. Based on diagram in Harvey & Slabbert (2014).

5-HT transmission is terminated by reuptake into the presynaptic neuron by the 5-HT transporter (5-HTT, also known as the serotonin reuptake transporter, SERT). 5-HTT is a sodium-dependent transporter encoded by the *SLC6A4* gene (Kanner & Schuldiner, 1987; Lesch et al., 1994; Bradley & Blakely, 1997). 5-HTT is expressed in the axons and terminals of 5-HT neurons (Mecca, 2019) but also on the soma and dendrites in the raphe nuclei (Fujita et al., 1993; Malagié et al., 1995; Bengel et al., 1997) (*Fig 1.2*). After reuptake by the 5-HTT, 5-HT is reloaded into vesicles or degraded by MAO (Bortolato et al., 2010).

3.5 5-HT receptors

There are 14 molecularly identified 5-HT receptors, comprising of seven receptor families (Hoyer et al., 2002) (*Table 1.1*). Thirteen of the 14 receptors are metabotropic and signal via G-protein coupled receptors (GPCRs). The 5-HT_1 and 5-HT_5 families are $G_{i/o}$-coupled inhibitory receptors. On the other hand, 5-HT_2 receptors are $G_{q/11}$-coupled, meaning that they activate phospholipase C to produce inositol triphosphate, and close potassium channels. The 5-HT_4, 5-HT_6 and 5-HT_7 families are also excitatory but are G_s-coupled. The 5-HT_3 receptor is the only ionotropic 5-HT receptor (for reviews see Barnes & Sharp, 1999, and Sharp & Barnes, 2020). There are several subtypes of 5-HT receptor within each family, each with distinct affinities for 5-HT, distributions, and functions (summarised in *Table 1.1*). The various signalling cascades regulated by receptor activation add to the complexity of 5-HT transmission. Furthermore, there is growing evidence that 5-HT receptors are coupled to non-canonical signalling pathways, which can produce structural changes and alter neural plasticity (Sharp & Barnes, 2020).

Table 1.1 5-HT receptors. Adenylyl cyclase, AC; Cyclic adenosine monophosphate, cAMP; extracellular signal-regulated kinase, ERK; phospholipase A2, PLA_2; protein kinase A, PKA; voltage-gated calcium channels, VGCCs**.** Non-canonical signalling pathways are detailed in brackets. Details from de Almeida and Mengod (2008); Millan et al. (2008); O'Leary and Cryan (2010); López-Muñoz and Alamo (2011); Rang et al. (2011); Sharp and Barnes (2020).

Receptor	Distribution	Mechanism	Effect of receptor activation
5-HT_1			
5-HT_{1A}	Presynaptic in 5-HT neurons in the raphe nuclei (autoreceptor) Postsynaptic in the hippocampus, cortex, limbic areas (heteroreceptor)	$G_{i/o}$-coupled	AC inhibition, reduced cAMP PKA activation Indirect hyperpolarisation by opening potassium channels Inhibition of VGCCs (ERK activation)
5-HT_{1B} (high expression in rodents)	Presynaptic 5-HT terminals Post-synaptic in the basal ganglia, substantia nigra	$G_{i/o}$-coupled	AC inhibition, reduced cAMP PKA activation Indirect hyperpolarisation by opening potassium channels Inhibition of VGCCs (ERK activation) (β-arrestin-dependent signalling)
5-HT_{1D} (high expression in other mammals)	Raphe nuclei 5-HT neurons Trigeminal nucleus, substantia nigra, nucleus accumbens, hippocampus	$G_{i/o}$-coupled	AC inhibition, reduced cAMP PKA activation Indirect hyperpolarisation by opening potassium channels Inhibition of VGCCs
5-HT_{1E}	Trigeminal nucleus	$G_{i/o}$-coupled	AC inhibition, reduced cAMP PKA activation
5-HT_{1F}	Raphe nuclei 5-HT neurons Trigeminal nucleus, hippocampus, cortex	$G_{i/o}$-coupled	AC inhibition, reduced cAMP PKA activation
5-HT_2			
5-HT_{2A}	Cortex, limbic areas, basal ganglia, claustrum	$G_{q/11}$-coupled	Phospholipase C activation, increased inositol triphosphate (PLA_2 activation) (ERK activation) (β-arrestin-dependent signalling)
5-HT_{2B}	Hypothalamus, cortex, amygdala	$G_{q/11}$-coupled	Phospholipase C activation (PLA_2 activation) (ERK activation) (β-arrestin-dependent signalling)
5-HT_{2C}	Raphe nuclei GABA interneurons Cortex, hippocampus, limbic areas, basal ganglia, hypothalamus, substantia nigra	$G_{q/11}$-coupled	Phospholipase C activation (PLA_2 activation) (ERK activation) (β-arrestin-dependent signalling)

5-HT_3	Cortex, limbic areas, vagus nerve, area postrema, hippocampus, brainstem nuclei	Ligand-gated ion channel	Sodium, potassium, and calcium influx Fast neuronal excitation
5-HT_4	Cortex, limbic areas, basal ganglia, hippocampus, striatum, substantia nigra, nucleus accumbens	G_s-coupled	AC activation, increased cAMP Slow membrane depolarisation L-type calcium channel facilitation PKA activation (ERK activation)
5-HT_5			
5-HT_{5A}	Frontal cortex, raphe nuclei 5-HT neurons, hippocampus, cerebellum	$G_{i/o}$-coupled	AC inhibition, reduced cAMP Hyperpolarisation (ERK activation) (mTOR and Cdk5-dependent signalling)
5-HT_{5B} (rodents only)	Raphe nuclei, hippocampus, habenula	$G_{i/o}$-coupled	AC inhibition, reduced cAMP
5-HT_6	Cortex, limbic areas, basal ganglia, striatum	G_s-coupled	AC activation, increased cAMP
5-HT_7	Suprachiasmatic nucleus, hypothalamus, cortex	G_s-coupled	AC activation, increased cAMP Slow membrane depolarisation L-type calcium channel facilitation (ERK activation)

The 5-HT_{1A} receptor was first identified by Pedigo et al. (1981) and is expressed throughout the rodent brain (Lein et al., 2007). Receptor autoradiography and immunohistochemical studies first showed that 5-HT_{1A} autoreceptors are expressed in the dendrites and soma of 5-HT neurons in the DRN and MRN (Vergé et al., 1986; Kia et al., 1996; Descarries & Riad, 2012). Moreover, 5-HT neuron ablation with 5,7-dihydroxytryptamine halved 5-HT_{1A} receptor expression in the DRN (Vergé et al., 1986), confirming its presynaptic expression. The action of the 5-HT_{1A} autoreceptors can be demonstrated by applying 5-HT to rodent midbrain slices, which hyperpolarises 5-HT neurons and reduces their firing rate (Aghajanian & Lakoski, 1984; Sprouse & Aghajanian, 1987).

There are several highly selective 5-HT_{1A} receptor agonists that have proved crucial in elucidating 5-HT_{1A} receptor function, such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Middlemiss & Fozard, 1983; Kalipatnapu et al., 2004). On the other hand, 5-HT_{1A} receptor antagonists include WAY-100135, WAY-100635 and NAD-299 (Fletcher et al., 1996) (discussed further in Chapter 6 section 1).

By comparison, the 5-HT_{1B} receptor is expressed in 5-HT terminals, where its activation reduces 5-HT release (Sharp et al., 1989b; Fink & Göthert, 2007). There are also several potent 5-HT_{1B} receptor agonists and antagonists (Hoyer et al., 1994; Skingle et al., 1995; Barnes & Sharp, 1999).

Both 5-HT_{1A} and 5-HT_{1B} receptors are also expressed on postsynaptic neurons, where they function as heteroreceptors. 5-HT_{1A} heteroreceptors are extensively expressed in the rodent forebrain, including the medial prefrontal cortex (mPFC), amygdala, and hippocampus (Glaser et al., 1985; Riad et al., 2000). In comparison, 5-HT_{1B} heteroreceptors are predominantly found in the hippocampus and basal ganglia (O'Leary & Cryan, 2010). It is important to note, however, that the drugs described above activate both the autoreceptors and the heteroreceptors (Yocca, 1990).

4 SSRI antidepressants

4.1 SSRIs

SSRIs are currently the first-line pharmacological treatments for depression and several anxiety disorders (NICE, 2009). Fluvoxamine was the first SSRI to be approved for the treatment of MDD in 1986 and was advantageous over older antidepressants due to its improved adverse effects profile (Anderson, 1998; Feighner, 1999; Ferguson, 2001).

Five other SSRIs have since been approved: paroxetine, citalopram, escitalopram, sertraline, and fluvoxamine. Paroxetine was the first SSRI approved for the treatment of GAD, although other SSRIs are also now used as anxiolytics (Allgulander, 2009).

SSRIs selectively inhibit the 5-HTT thereby blocking 5-HT reuptake into the presynaptic neuron and increasing extracellular 5-HT (Aghajanian & Lakoski, 1984; Chaput et al., 1986; Hajós et al., 1995; Malagié et al., 1995; Czachura & Rasmussen, 2000). These effects persist with chronic SSRI treatment (Hajós-Korcsok et al., 2000; Malagié et al., 2000; Popa et al., 2010; Guiard et al., 2012).

Nonetheless, the pharmacological profiles of the SSRIs vary. Escitalopram and citalopram, for example, are more selective for the 5-HTT than paroxetine, whereas paroxetine has a higher affinity for the 5-HTT. Notably, paroxetine also has higher affinities for the noradrenaline (NA) transporter (NET) and M1 muscarinic receptors than other SSRIs (Sanchez et al., 2014). SSRIs are metabolised by cytochrome P450 enzymes (Sanchez et al., 2014), such as CYP2D6 for paroxetine and CYP2D19 for citalopram (von Moltke et al., 1999; Jornil et al., 2010).

4.2 Monoamine theory of depression

For the last 60 years, the monoamine depletion theory has been the predominant theory of the neurobiological basis for depression. The first evidence that a monoamine deficiency may cause depression came from the serendipitous discovery that isoniazid produced rapid improvements in mood in patients with tuberculosis (Salzer & Lurie, 1953; Kuhn, 1958). It was later deduced that these drugs increased synaptic concentrations of NA, dopamine (DA) and 5-HT by blocking their reuptake, or by suppressing their metabolism, respectively (Glowinski & Axelrod, 1964; Ross & Renyi,

1969). From this, it was implied that a deficit in monoaminergic neurotransmission caused depression (Schildkraut, 1965; Coppen, 1968).

Subsequent studies have specifically linked low levels of 5-HT to depressed mood. For instance, lower concentrations of 5-HIAA and tryptophan were reported in the blood of depressed patients compared to healthy controls (Coppen et al., 1978; Cowen et al., 1989). Accordingly, depleting plasma tryptophan levels (and hence 5-HT synthesis) in patients taking SSRIs, or those with a history of depression, produced a rapid reduction in mood compared to vehicle-treated controls (Delgado et al., 1990; Smith et al., 1997; Delgado et al., 1999). Moreover, positron emission topography (PET) studies have reported lower 5-HT_{1A} receptor and 5-HTT binding in the brains of depressed patients than in healthy controls (for example, Lesch et al., 1996, Reimold et al., 2008, and Selvaraj et al., 2011), further implicating low 5-HT transmission in depression.

Nonetheless, the 5-HT theory of depression is increasingly being called into question. Exemplifying this growing scepticism, a recent (albeit greatly criticised) umbrella review found no significant association between low 5-HT and depression (Moncrieff et al., 2022). Consequently, while there is much evidence to suggest that 5-HT is depleted in depressed brains, it is not clear whether this is the main factor underlying the pathophysiology of the disorder.

4.3 Mechanism of SSRI action

Despite near 100% occupancy of the 5-HTT with acute SSRI treatment (Meyer et al., 2004; Parsey et al., 2006), it can take several weeks of treatment before patients report an improvement in mood (Posternak & Zimmerman, 2005). This suggests that SSRIs may produce neuroadaptive changes that are ultimately responsible for their therapeutic

effects. In the 1980s, it was hypothesised that a reduction in 5-HT_{2C} receptor and β-adrenoreceptor sensitivity was responsible for this delay (Blier & Bouchard, 1994). Nonetheless, blockade of these receptors was not sufficient to produce antidepressant effects in humans (Artigas et al., 1996), suggesting that these changes may simply be homeostatic responses to elevated extracellular monoamine levels.

In the 1990s, it was posited that 5-HT_{1A} autoreceptor desensitisation may be the neuroadaptation necessary to produce antidepressant effects. Repeated SSRI treatment results in the persistent elevation of extracellular 5-HT and a loss of sensitivity of the 5-HT_{1A} autoreceptors over time (reviewed in Raymond et al., 1991, and Descarries & Riad, 2012). Exemplifying this effect, citalopram, paroxetine and fluoxetine acutely reduced 5-HT neuronal firing, but firing rates recovered to vehicle-control levels after two weeks of repeated treatment (Chaput et al., 1986; el Mansari et al., 1995; Le Poul et al., 1995; Czachura & Rasmussen, 2000).[2]

There is also evidence that 5-HT_{1A} receptors are necessary for the antidepressant effects of SSRI administration in rodents. For instance, knocking out 5-HT_{1A} receptor expression in mice prevented the behavioural effects of repeated fluoxetine treatment, i.e., reducing the latency to start eating on the novelty-suppressed feeding (NSF) test (Santarelli et al., 2003). Moreover, the co-administration of pindolol, a non-selective 5-HT_{1A} receptor antagonist, was shown to accelerate the onset of the therapeutic effects of SSRIs in clinical trials (Portella et al., 2010). In contrast, other studies have demonstrated antidepressant effects of SSRI treatment in 5-HT_{1A} receptor knockout (KO) mice

[2] While it is beyond the scope of this chapter to review the mechanisms of 5-HT_{1A} receptor desensitisation, it is thought to occur through several mechanisms, including phosphorylation of the receptor (Raymond et al., 1991; Ramamoorthy et al., 1998), internalisation (Riad et al., 2000) and uncoupling from G proteins (Li et al., 1996), but not changes in its expression (Hervás et al., 2001; Riad et al., 2008).

(Richardson-Jones et al., 2010), suggesting the receptors are not necessary for the behavioural effects to emerge. Together, these findings suggest that 5-HT_{1A} autoreceptors may be involved in the mechanism of action of SSRIs but that they are not the sole mediators of their effects.

Changes in neuroplasticity have also been linked to antidepressants (Castrén, 2005; Castrén & Hen, 2013; Kraus et al., 2017). Preclinical studies have found that stress reduces markers of plasticity, and there is evidence of similar changes in the post-mortem brains of depressed patients (reviewed in Pittenger & Duman 2008, and Duman & Aghajanian, 2012). On the other hand, SSRIs regulate a host of plasticity genes and transcription factors to enhance neuroplasticity (Conti et al., 2002; Sillaber et al., 2008). Increased neurogenesis was also evident in the hippocampi of mice treated repeatedly with fluoxetine (Santarelli et al., 2003; Hodes et al., 2010). Preclinical evidence further suggests that changes in neural plasticity are necessary and sufficient to evoke antidepressant effects (for example, Conti et al., 2002, Lindholm et al., 2014, and Santarelli et al., 2003). Thus, SSRIs may gradually reverse deficits in neuroplasticity over time, explaining the delay in onset of their therapeutic effects.

The neuropsychological view of SSRI action is that these drugs improve an individual's affective cognitive bias (Harmer et al., 2017). Affective biases can be thought of as the effect of emotion on cognitive processes or decision making (Hales et al., 2014). In humans, this can be probed with the facial emotional recognition task, in which depressed patients interpret neutral expressions as more negative than healthy controls (Dalili et al., 2015). Interestingly, a single dose of citalopram ameliorated this negative bias, and produced a positive bias in healthy controls (Harmer et al., 2003a; Bhagwagar et al., 2004; Harmer et al., 2009). Functional magnetic resonance imaging (fMRI) studies

have also shown that acute SSRI administration reduced the reactivity of components of the limbic system, such as the amygdala and PFC, in depressed patients and produce similar effects in healthy controls (Del-Ben et al., 2005; Murphy et al., 2009). It has therefore been hypothesised that a more positive emotional bias interacts with increased neuroplasticity during SSRI treatment to help patients learn more positive emotional associations with their environments. Thus, SSRIs are thought to provide the neural substrate for enhanced positive emotional processing, which improves mood over time (Harmer et al., 2017).

4.4 Issues with SSRI treatment

As well as a delay in the onset of therapeutic effects, SSRIs lack efficacy for many patients. Although antidepressants are generally thought to be more efficacious for treating depression in adults than placebo treatment (Cipriani et al., 2018), the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial found that 50-66% of patients do not fully recover with antidepressant treatment (Warden et al., 2007; Al-Harbi, 2012). Depression is also a highly heterogeneous disorder, with its physiological and psychological symptoms and causes varying considerably between individuals. Consequently, pharmacological treatment for depression may work better for some patients than others (Lynch et al., 2020). SSRI efficacy is further complicated by environmental factors. For example, evidence suggests that living in a low-income area is predictive of lower treatment response rates (Cohen et al., 2006). Even in mice, the behavioural effects of SSRIs were dependent on the quality of the living environment (Branchi et al., 2013). These findings suggest that environmental influences may be critical in determining the efficacy of SSRIs, not just their pharmacological properties.

Despite their improved tolerability and side-effect profile compared to older tricyclic antidepressants (Cipriani et al., 2018), SSRIs still produce side effects for many patients. These include anxiety, nausea, sexual dysfunction, and increased risk of suicide (Ferguson, 2001; Ramic et al., 2020) (although the incidence of suicidality is contested; for example, Fergusson et al., 2005, and Gunnell et al., 2005). Side effects are particularly common in the early stages of SSRI therapy (NICE, 2009), but can persist for the duration of treatment in some patients (Cartwright et al., 2016).

Emotional blunting is a particularly prevalent side effect. Between 40 and 60% of patients report having their emotions dulled during SSRI therapy (Langley et al., 2023). Although this is considered useful in suppressing negative emotions, it can also constrain positive feelings, with patients describing how they no longer find pleasure in previously enjoyable activities (Ma et al., 2021). Although the neuropsychological mechanism of emotional blunting is poorly understood, increased extracellular 5-HT in the frontal cortex and modulation of midbrain dopaminergic transmission have been postulated (Barnhart et al., 2004; Sansome et al., 2010; George et al., 1992). Interestingly, the rapid reversal of this emotional suppression could contribute to mood symptoms associated with SSRI discontinuation syndrome, in that after prolonged emotional blunting, the sudden ability to feel stronger emotions may be perceived as withdrawal symptoms.

Thus, while SSRIs are a significant improvement on older classes of antidepressants, they lack efficacy for many patients and can cause distressing side effects. Combined with the high risk of SSRI discontinuation syndrome following treatment cessation, there are still issues with SSRIs as therapies for MDD and anxiety disorders.

5 5-HT and anxiety

Anxiety is one of the most common symptoms of SSRI discontinuation syndrome (see section 2.2). Moreover, extensive preclinical evidence has linked anxiety-like behaviours to altered 5-HT transmission (discussed below). This thesis therefore used anxiety as a correlate of the human discontinuation syndrome to probe the behavioural and neurobiological effects of SSRI discontinuation in mice.

5.1 Definition of anxiety

Anxiety is an emotion characterised by feelings of tension, worry, and unease, with additional physical symptoms including elevated blood pressure and heart rate (Kazdin, 2000). This can manifest behaviourally as increased vigilance or arousal, and inhibition of ongoing behaviours. Such behavioural inhibition prevents an individual from entering potentially dangerous situations. When such reactions occur in non-threatening situations, however, anxiety can be maladaptive and have a detrimental impact on an individual's mental health (Penninx et al., 2021).

According to the defensive hierarchies hypothesis, anxiety is a response to a threat with a greater 'defensive distance' than fear. These are more diffuse, less predictable, or potential future threats (Blanchard & Blanchard, 1990; Lezak et al., 2017). They therefore produce uncertainty on how best to respond. This is exemplified by approach-avoidance conflict, which occurs when there is tension between the possible reward of exploring a situation and the threats to which one might be exposed (Miller, 1944; McNaughton & Gray, 2000). By comparison, fear is evoked by definite and impending, but escapable, threats (Penninx et al., 2021), whereas panic is a response to unavoidable threats. In

contrast, depression is thought to develop when an unavoidable threat occurs repeatedly over time, leading to behaviours such as helplessness and despair (Blanchard et al., 2001).

5.2 Measuring anxiety-like behaviour in mice

Approach-avoidance conflict is also a component of rodent anxiety and presents as increased vigilance, behavioural inhibition and hypoactivity (Lezak et al., 2017). These unconditioned, ethologically relevant behaviours can be used as putative measures of anxiety in laboratory tests. Rodents innately prefer dark and enclosed spaces as they carry a lower risk of predation; yet their natural exploratory drive pushes them to enter new places in search of food, shelter, or mates. High anxiety animals are therefore less likely to explore exposed or threatening spaces than low anxiety controls (Buccafusco, 2000; Lezak et al., 2017). In contrast, anxiolytic drugs like benzodiazepines increase the time animals spend in potentially threatening areas, an effect which has been used to validate these anxiety tests (for example, Pellow et al., 1985).

One of the most commonly used unconditioned anxiety tests is the elevated plus maze (EPM) (Pellow et al., 1985; Lister, 1987; Komada et al., 2008). The elevated, cross-shaped maze consists of two opposing enclosed arms and two open arms. The open arms are brighter and more exposed than the closed arms and thus are aversive to mice. Spending less time in, making fewer entries to, and taking longer to first enter the open arms are considered evidence of heightened anxiety-like behaviour in rodents (Pellow & File, 1986). In contrast, anxiolytics such as diazepam increased the time rats spent in, and the number of entries to, the open arms of the EPM compared to vehicle-treated controls (Pellow et al., 1985; Pellow & File, 1986; Rodgers & Dalvi, 1997).

Other tests of approach-avoidance conflict in mice include the open field (OF) and light/dark box (LDB). The OF consists of a large circular or square arena with high walls. Rodents show an aversion to the open, bright, and unprotected central region (Choleris et al., 2001); thus, increased time in the central zone is considered evidence of lower anxiety (Prut & Belzung, 2003). The LDB also takes advantage of the aversion of mice for bright, open spaces (Crawley & Goodwin, 1980; Bourin & Hascoët, 2003). The test consists of a box with two compartments: one enclosed and painted black, and the other white and brightly lit from above. A doorway between the compartments allows mice placed in the dark region to choose to explore the light area. Increased light zone exploration therefore indicates lower anxiety-like behaviours (Crawley & Goodwin, 1980; Crawley, 1981).

NSF, or novelty-induced hyponeophagia (NIH), is another measure of approach-avoidance conflict (Deacon, 2011). In the NSF test, mice are placed in a novel environment and the latency to start eating recorded (Buccafusco, 2000). Variants of the paradigm use unfamiliar food and/or a novel context (Dulawa & Hen, 2005). Anxiolytic drugs reduce the latency to start eating compared to vehicle-treated controls (e.g. Holick et al., 2008). Unlike the EPM, however, these tests have the additional motivational demand of hunger to drive exploration, meaning performance can also be affected by appetite or reward-sensitivity (Merali et al., 2003).

Notably, the aforementioned tests can be confounded by changes in locomotor activity (LMA) (Lister, 1987; Rodgers et al., 1992; Handley & McBlane, 1993; Weiss et al., 1998). For instance, a mouse with a motor deficit or receiving a sedative drug may be unable to explore an apparatus to the same extent as a wildtype or vehicle-treated control. This may reduce the time it spends in the exposed part of the test and thus be misinterpreted as increased anxiety-like behaviour. Locomotor readouts from within the

test, for example the total distance the animal travelled, must therefore be considered when interpreting effects of drug treatment. Spontaneous LMA in a neutral environment should also be assessed to confirm that performance is not confounded by changes in LMA (Lister, 1990). Naturalistic behaviours such as digging can also be used to measure exploratory drive (de Brouwer et al., 2019; Pond et al., 2021).

5.3 Role of 5-HT in anxiety

There are several lines of evidence that suggest that increased 5-HT transmission increases anxiety (Gray, 1982; Iversen, 1984). For example, intra-DRN administration of 8-OH-DPAT reduced 5-HT neuron firing and was associated with increased social interaction and increased punished responding in a Vogel conflict test in rats, suggesting an anxiolytic effect (Higgins et al., 1988). Systemic administration of the $5HT_{1A}$ receptor agonist buspirone also reduced anxiety-like behaviours in rats (Cole & Rodgers, 1994).

Anatomical evidence also suggests that 5-HT may regulate anxiety, as projections from the raphe nuclei innervate key anxiety-promoting brain regions, including the amygdala, VH and mPFC (discussed further in section 5.4). For instance, electrophysiological recordings showed aversive stimuli activated 5-HT neurons (Schweimer et al., 2011; Paquelet et al., 2022). Furthermore, optogenetic and pharmacological activation of the MRN reduced the time mice spent in the open arms of the EPM compared to controls (Vicente et al., 2008; Ohmura et al., 2014; Teissier et al., 2015; Ohmura et al., 2020). This effect was abolished in mice lacking $5\text{-}HT_{2C}$ receptors (Ohmura et al., 2020), and inhibiting the MRN reduced anxiety-like behaviour in rats and mice compared to sham-operated controls (Briley et al., 1990; De Almeida et al., 1998; Andrade & Graeff, 2001; Konno et al., 2007). Thus, the activation of 5-HT-containing

projections from the MRN appear to promote anxiety-like behaviour in rodents.

Additionally, anxiety is common at the start of SSRI treatment in humans (Browning et al., 2007; NICE, 2009; Gollan et al., 2012) and in rodents (Bodnoff et al., 1989; Handley & McBlane, 1993; Griebel et al., 1995; Belzung et al., 2001; Thoeringer et al., 2010; Hestermann et al., 2014). This is likely due to 5-HTT blockade leading to increased extracellular 5-HT (Kreiss & Lucki, 1994; Mombereau et al., 2010), further suggesting that increased 5-HT transmission produces anxiety.

There is also evidence that genetic variation in key components of the 5-HT system can predispose an individual to an anxiety disorder. For example, a polymorphism in the promoter region of the 5-HTT gene (5-HTTLPR) has been linked to personality traits such as neuroticism and anxiety in humans (Lesch et al., 1996). This polymorphism consists of two alleles – long (l) and short (s). The s allele reduces 5-HTT transcription, which is hypothesised to increase extracellular 5-HT in humans. The s allele has also been linked to anxiety and increased amygdala reactivity in humans (Hariri et al., 2002). Notably, a prospective longitudinal study also found an interaction between 5-HTTLPR, early life stress and the probability of developing depression, which is often comorbid with anxiety (Caspi et al., 2003).

Experiments in transgenic mice have also linked reduced 5-HTT expression with increased anxiety-like behaviour. 5-HTT KO mice exhibited increased anxiety-like behaviour on the EPM, NSF and LDB compared to wildtype littermates (Holmes & Rodgers, 2003; Line et al., 2011). On the other hand, overexpressing 5-HTT reduced anxiety-like behaviour (Line et al., 2011; Barkus et al., 2014). 5-HT_{1A} receptor KO mice, which have reduced feedback suppression of 5-HT neurons and thus increased transmission, also have high anxiety phenotypes (Heisler et al., 1998; Gross et al., 2002).

Nonetheless, there are several caveats to this theory. Firstly, the region of the brain in which 5-HT is elevated, or the origin of that 5-HT, appears crucial in determining its role in anxiety. For example, activation of the MRN increased anxiety-like behaviour in mice, but activation of the DRN had no such effect (Ohmura et al., 2014; Ohmura et al., 2020). Moreover, selective DRN lesions did not alter anxiety-like behaviour on the EPM (Critchley et al., 1992; Konno et al., 2007). Furthermore, different subregions of the DRN project to different functional systems (Lowry et al., 2005), and have been implicated in controlling a range of emotional behaviours, such as coping, escape responses and resistance to stress (Rasmussen et al., 1995; Graeff et al., 1996). Thus, increasing 5-HT by activating raphe nuclei does not necessarily increase anxiety.

When 5-HT neurotransmission is altered is also important. For instance, inhibiting 5-HTT activity with fluoxetine during the first three postnatal weeks increased anxiety in adult mice on the OF (Ansorge et al., 2004). This replicated the high-anxiety phenotype of global 5-HTT KO mice, suggesting this anxiety was generated by reduced 5-HTT activity during development. 5-HT_{1A} receptor KO during adolescence also increased anxiety-like behaviour in adult mice, which could not be rescued by re-expressing 5-HT_{1A} receptors. In contrast, 5-HT_{1A} receptor KO in adulthood increased anxiety but could be rescued by 5-HT_{1A} re-expression (Richardson-Jones et al., 2011). Thus, changes in 5-HT-related gene expression likely assert their effects by altering anxiety circuitry development rather than by continuing to increase 5-HT transmission in adulthood.

Further evidence complicating the role of 5-HT in anxiety is the paradox that repeated SSRI treatment is used to treat anxiety disorders despite persistently elevated 5-HT. The link between the 5-HTT polymorphism, life stress and anxiety has also not been replicated (Culverhouse et al., 2018), and neither has its association with increased

amygdala reactivity (Murphy et al., 2013). Thus, the genetic influence of 5-HT on anxiety may be less robust than previously thought (Flint & Kendler, 2014).

There is also evidence that low 5-HT can cause anxiety. For instance, knocking out PET-1, a transcription factor that drives 5-HT neuron differentiation, produced more anxious mice (Hendricks et al., 2003). Raphe KO of Freud-1, a repressor of 5-HT_{1A} receptor expression, also reduced 5-HT output yet was associated with reduced time in the open arms of the EPM compared to wildtype controls, suggesting increased anxiety-like behaviour (Vahid-Ansari et al., 2017). This has led to suggestions that large changes in 5-HT, whether that be increased or decreased transmission, can increase anxiety-like behaviour in rodents (Albert et al., 2014).

In summary, it is overly simplistic to say that anxiety is caused by increased 5-HT transmission. Nonetheless, under certain circumstances, acutely increasing extracellular 5-HT or neuron firing can increase anxiety-like behaviour in mice.

5.4 Other anxiety-related brain circuitry

There are several other brain regions that mediate anxiety responses. These include the amygdala, VH, and mPFC, which make up the negative affective circuit (Williams, 2016). The amygdala integrates emotionally relevant sensory information and controls the behavioural response to anxiety-provoking stimuli (Möller et al., 1994; Farb & Ratner, 2014). The amygdalar nuclei comprise the basolateral amygdala (BLA), which is considered the input nucleus, and the central amygdala (CeA), which integrates information and sends projections to other anxiety-related brain regions (Sah et al., 2003; Pelletier & Paré, 2004). In humans and rodents, increased amygdala activity is associated with anxiety and conditioned fear learning (Hariri et al., 2002; Etkin & Wager, 2007;

Wang et al., 2011; Barkus et al., 2014). Exemplifying this, optogenetic activation of the BLA terminals in the CeA had an anxiolytic effect on the EPM (Tye et al., 2011). Notably, the amygdala appears more important in conditioned rather than unconditioned anxiety responses, as amygdala lesions did not affect EPM performance in rats (Decker et al., 1995; Sommer et al., 2001; McHugh et al., 2004).

The VH is another brain region associated with anxiety-like behaviours in rodents (Bannerman et al., 2004). The hippocampus is divided into the dorsal part at the septal pole (anterior hippocampus in humans), and the ventral part in the temporal pole (posterior in humans) (Schultz & Engelhardt, 2014). Electrophysiological recordings showed that VH activity was greatest when approach-avoidance conflict was at its highest, such as when a rat was deciding whether to enter the open arms of the EPM (McHugh et al., 2011). Moreover, VH activation increased open arm aversion on the EPM in mice compared to controls (Ohmura et al., 2020).

The anxiety-promoting effect of the VH is distinct from the role of the DH. While lesion studies showed that VH damage reduced anxiety-like behaviours, DH lesions impaired spatial learning and memory (Bannerman et al., 2003; McHugh et al., 2004). Nonetheless, neurotoxic lesions also showed that contextual fear conditioning was dependent on the DH (Richmond et al., 1999). Thus, while the DH is not thought to be a major contributor to anxiety-like behaviours, it may also be activated in anxiogenic situations due to novel spatial stimuli.

The mPFC is considered the third major component of anxiety circuitry in rodents. It provides top-down suppression of anxiety-circuitry via reciprocal connectivity with the BLA, DRN and VH, and modulates autonomic anxiety responses (Jacobs & Azmitia, 1992; Morgan & LeDoux, 1995; Jinks & McGregor, 1997; Berretta et al., 2005; Parfitt et

al., 2017; Berg et al., 2019). The mPFC therefore forms a critical feedback loop that prevents excessive anxiety-like behaviours.

Increased activation of this negative affective circuit was evident in patients with GAD (Williams, 2016). On the other hand, just one week of SSRI treatment was sufficient to reduce amygdala, hippocampal, and mPFC activity in patients with anxiety, potentially corresponding with the anxiolytic properties of these drugs (Harmer et al., 2006; Tromp et al., 2012; Klumpp et al., 2015). Consequently, the interactions between the amygdala, VH and mPFC are thought to mediate anxiety-like behaviours in rodents and subjective anxiety in humans (Parfitt et al., 2017).

These anxiety-promoting brain regions are regulated by 5-HT. For example, optogenetic activation of the MRN increased extracellular 5-HT in the VH and anxiety-like behaviour on the EPM in mice (Ohmura et al., 2020). Increased serotonergic activity therefore appears to promote risk assessment behaviours (Gray, 1982; McNaughton & Gray, 2000). According to this theory, 5-HT neurons fire and recruit regions such as the VH during anticipation of potential threat, facilitating passive avoidance behaviours. As the threat becomes more certain, 5-HT activates the amygdala to produce freezing or active avoidance behaviours characteristic of fear. Notably, 5-HT also inhibits panic responses mediated by the periaqueductal grey (PAG) to stop undirected fight or flight responses (Kiser et al., 1980; Deakin & Graeff, 1991; Graeff, 2004; Hestermann et al., 2014). In this way, 5-HT can be considered as a gating mechanism that results in the most appropriate behaviour for a given threat level, including promoting anxiety-like behaviours in response to distal or uncertain threats (McNaughton & Gray, 2000).

6 Preclinical studies of SSRI discontinuation

6.1 Behavioural studies

Despite evidence that SSRI discontinuation can cause debilitating symptoms in patients, there have been only a handful of studies on the effects of SSRI treatment cessation in rodents. In one of the few preclinical studies of SSRI discontinuation, Bosker et al. (2010) reported that citalopram discontinuation increased the acoustic startle response (ASR) in rats compared to animals maintained on citalopram treatment. This effect was evident two to seven days after discontinuation. The authors interpreted this finding as evidence of increased anxiety-like behaviour (Bosker et al., 2010), mirroring the anxiety often experienced by patients. The ASR is an appealing test for researchers as it can be easily distinguished from changes in LMA and can also be measured in humans (Lezak et al., 2017). Nonetheless, this behavioural response can also be influenced by the neural mechanisms of fear and behavioural sensitisation, complicating its interpretation. Moreover, the ASR is an involuntary reflex mediated by a simple neuronal circuit in the brainstem (Koch, 1999). Consequently, this test offers a limited understanding of the effect of SSRI discontinuation on anxiety-like behaviour in rodents.

One other study has directly investigated the effects of SSRI cessation on behaviour. Bjork et al. reported that 4 h after the last dose of repeated fluoxetine treatment, rats exhibited increased LMA compared to saline treated controls. This effect had dissipated when rats were re-tested 96 h after cessation. This study suggested that there were some acute changes to activity following SSRI discontinuation, but that there were no persistent changes compared to saline controls (Bjork et al., 1998).

While other studies have investigated behaviour several weeks after SSRI cessation (discussed in Chapter 3 section 1), no other studies have directly compared the behavioural effects of SSRI discontinuation to continued treatment or related these findings to the human discontinuation syndrome. The field also lacks a systematic analysis of the effect of SSRI discontinuation on anxiety-like behaviour in mice. Moreover, it is not known if increased anxiety-like behaviour is evident on other unconditioned anxiety tests or if it generalises to other SSRIs, such as paroxetine.

Notably, two rodent studies have investigated the effects of SSRI discontinuation on sleep, relating findings to the symptoms of insomnia and sleep disturbances. Both studies report that sleep disruption during repeated paroxetine treatment rapidly reversed to saline control levels following discontinuation (Gervasoni et al., 2002; Kantor et al., 2017). This was evident within just 24 h of the final dose of paroxetine in rats (Gervasoni et al., 2002), and sleep did not differ from vehicle-treated controls two weeks after discontinuation in mice (Kantor et al., 2017). Nonetheless, it is not known if there are emergent effects of SSRI discontinuation in the two weeks between these two studies, or how sleep disturbances might be linked to changes in 5-HT transmission following SSRI discontinuation (see Chapter 7 section 1).

6.2 Neurochemical studies

There are also limited data on the effects of SSRI discontinuation on 5-HT neurochemistry. In the most detailed study to date, Trouvin et al. investigated how tissue 5-HT and 5-HIAA concentrations changed in the weeks following fluoxetine discontinuation in rats. Repeated fluoxetine treatment reduced 5-HT and 5-HIAA in the pons, hippocampus, and cortex, which reversed within a week of discontinuation.

5-HIAA also rebounded above vehicle-treated control levels later in the discontinuation period and was still elevated 28 days after discontinuation in the hippocampus (Trouvin et al., 1993). Other studies have also reported effects on tissue 5-HT and 5-HIAA following fluoxetine and citalopram discontinuation, including a rebound increase in 5-HIAA (Caccia et al., 1992; Caccia et al., 1993; Stenfors & Ross, 2002; Bosker et al., 2010) (discussed in detail in Chapter 5 section 1.2).

To our knowledge, only one study has investigated the effects of SSRI discontinuation on extracellular 5-HT. Repeated escitalopram treatment in mice was associated with elevated hippocampal 5-HT, which fell to control levels two days after discontinuation (Guiard et al., 2012). Nonetheless, it is not known if this effect generalises to other SSRIs, or if there are further changes to 5-HT transmission later in the discontinuation period.

Changes in 5-HT-related gene expression following SSRI discontinuation are also poorly understood. In two reports, discontinuation from fluoxetine treatment was associated with the reversal of an adaptive decrease in midbrain *5-HT$_{1B}$* autoreceptor and *5-HTT* mRNA expression (Neumaier et al., 1996; Anthony et al., 2000). Nonetheless, the lack of continued treatment control groups in these studies makes it difficult to assess the changes in expression after SSRI discontinuation compared to continued treatment. It is also not known if the expression and function of other genes, including the 5-HT$_{1A}$ autoreceptor and postsynaptic 5-HT receptors, change following SSRI discontinuation.

Thus, while there is some evidence of a reduction in extracellular 5-HT following SSRI discontinuation, there is also evidence that 5-HT function may rebound above pre-treatment levels. SSRI discontinuation therefore appears to be associated with adaptive changes in the 5-HT system, although the directionality and temporality of these

changes is uncertain. Moreover, there have yet to be any studies directly linking changes in 5-HT transmission to the behavioural effects of SSRI discontinuation or attempting to establish the brain circuitry activated by such behaviours.

7 The role of 5-HT in SSRI discontinuation symptoms

SSRIs selectively inhibit the 5-HTT and cause adaptive changes in 5-HT function during a course of treatment (Sharp & Cowen, 2011). Moreover, evidence suggests that tissue and extracellular 5-HT, as well as 5-HT-related gene expression, may be affected by SSRI discontinuation. Thus, it has been hypothesised that altered 5-HT transmission may underlie SSRI discontinuation symptoms.

The predominant theory for the cause of SSRI discontinuation symptoms is that a reduction in extracellular 5-HT occurs following treatment cessation (reviewed in Harvey et al., 2003, Blier & Tremblay, 2006, Warner et al., 2006, Renoir, 2013, and Harvey & Slabbert, 2014). This theory is based on the notion that following blockade of the 5-HTT, SSRI washout likely increases 5-HT reuptake and thus reduces extracellular 5-HT.

This hypothesis is further supported by suggestions that tryptophan depletion may be inducing SSRI discontinuation symptoms rather than relapse of depression, as originally thought. For instance, when the symptoms were compared, the emergent anxiety and somatic symptoms such as nausea and dizziness following tryptophan depletion more closely resembled those of SSRI discontinuation than relapse (Delgado, 2006). Moreover, tryptophan depletion has a greater effect on mood in patients taking SSRIs compared to treatment-free patients with a history of depression, or healthy treatment-naïve controls (Delgado et al., 1990; Neumeister et al., 1997; Smith et al., 1997; Delgado et al., 1999; O'Reardon et al., 2004; Delgado, 2006). Given tryptophan depletion

reduces 5-HT in the brain (Moja et al., 1989; Nishizawa et al., 1997), these experiments could suggest that reducing extracellular 5-HT could precipitate SSRI discontinuation syndrome in humans.

Nonetheless, this hypothesis does not fit with the notion that, at least acutely and in rodents, anxiety is associated with increased 5-HT transmission (section 5.3). Moreover, observations of a rebound increase in tissue 5-HIAA in the rat brain following fluoxetine discontinuation suggests that increased 5-HT function may instead be responsible for the symptoms (Trouvin et al., 1993; Harvey & Slabbert, 2014).

A model of withdrawal reactions from other psychotropic drugs called the oppositional model of tolerance further supports the idea that a rebound increase in 5-HT may contribute to SSRI discontinuation symptoms (Andrews et al., 2011). This theory describes the forces that develop when a homeostatic mechanism has been subjected to prolonged suppression. These forces oppose the pharmacological effects of the drug in an attempt to restabilise the system (Fava, 2020). In the case of repeated SSRI treatment, this is exemplified by the desensitisation of 5-HT_{1A} autoreceptors that allows the initial suppression of 5-HT neuron firing at the start of SSRI treatment to normalise with chronic administration. When treatment is discontinued, however, these oppositional forces no longer meet resistance, potentially leading to a rebound in 5-HT function that could cause the withdrawal symptoms (Fava, 2020).

Thus, there are two, apparently conflicting, hypotheses about the neurobiological origins of SSRI discontinuation syndrome. Nonetheless, there has yet to be a systematic investigation of how changes in 5-HT transmission may cause the discontinuation symptoms.

8 Aims of this thesis

The abrupt cessation of SSRI treatment can produce a debilitating discontinuation syndrome, characterised by symptoms such as anxiety and sleep disturbances. Yet, the sparsity of preclinical data on the condition means its neurobiological basis is poorly understood. Moreover, there is currently no mouse model of SSRI discontinuation syndrome with which to investigate the mechanisms underlying the symptoms. The aim of this thesis was therefore to investigate the behavioural effects of SSRI discontinuation in mice and to investigate how changes in the 5-HT system may mediate these effects.

Based on the symptoms of the syndrome and a previous preclinical study, it was hypothesised that SSRI discontinuation would be associated with increased anxiety in mice. Chapter 3 aimed to establish the effects of SSRI discontinuation on anxiety-like behaviour in mice, in particular the effects of paroxetine cessation, and to optimise the detection of such effects by modifying the treatment protocol. The activation of anxiety-promoting brain circuitry, including the raphe nuclei, was investigated in Chapter 4. The effects of paroxetine discontinuation on regional brain tissue and extracellular 5-HT and 5-HIAA, as well as on 5-HT-related gene expression, were investigated in Chapter 5. From these experiments, it was hypothesised that changes in 5-HT transmission may cause the behavioural effects of paroxetine discontinuation. Thus, Chapter 6 investigated whether changes in 5-HT transmission could trigger the discontinuation syndrome by attempting to precipitate discontinuation in mice receiving continuous paroxetine treatment. Finally, Chapter 7 investigated the effects of paroxetine discontinuation on sleep and circadian rhythms to determine if the mouse model reproduced other symptoms of the clinical syndrome.

Chapter 2
Materials and Methods

This chapter contains the details of the techniques, tests and procedures used throughout this thesis. The Experimental Methods sections of Chapters 3 to 7 contain the application of each technique for individual experiments.

1 Animals

C57BL/6J mice (Charles River) were purchased at 7 weeks old and habituated to the holding facility for one week before the start of experiments. Mice were group housed (3-6 per cage of littermates) in open-top or individually ventilated cages (IVCs; *in vivo* microdialysis studies only, see section 8) lined with sawdust bedding. The mice had *ad libitum* access to food, water and cage enrichment consisting of sizzle nest, a cardboard house, and a cardboard tunnel. Tunnel handling was used whenever possible to minimise stress (Hurst & West, 2010; Gouveia & Hurst, 2019). Mice were housed at 21^{o}C on a 12 h light/dark cycle (light ON 07:00, lights OFF 19:00). Mice were weighed daily, with a humane endpoint set to 85% of their baseline bodyweight. Experiments followed the principles of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were conducted according to the United Kingdom Animals (Scientific Procedures) Act of 1986, under project licenses P6F11BC25, P89623056 and PP1503281.

2 Drugs and reagents

Paroxetine hydrochloride (Abcam, ab120069; Apexbio, B2252-APE) was made up at 1 mg/ml in 0.9% sodium chloride (saline). Citalopram hydrobromide (Abcam, ab120133) and venlafaxine (Apexbio, A-5355-APE) were dissolved in saline at 2 mg/ml. Citalopram was also dissolved in artificial cerebrospinal fluid (ACSF, see section 8.2 for components) at 1 μM for some microdialysis experiments. (R)-(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, Sigma-Aldrich, H140-25MG) was made up at 0.01 mg/ml and 0.05 mg/ml in saline. WAY-100635 maleate (Torcis Bioscience, 4380/10) was made up at 0.5 mg/ml in saline.

3 Repeated SSRI treatment

Mice were weighed for two to three days before the start of experiments to establish a baseline weight and to habituate them to handling. Mice received once- or twice-daily subcutaneous (*s.c.*) injections (27 G 1/2" needles) of either 10 mg/kg SSRI or the equivalent volume of saline (administered at 10 ml/kg), then treatment was either continued (saline and continuation groups) or swapped to saline injections (discontinuation group) for a further two to thirteen days, when testing was conducted. Subcutaneous (*s.c.*) injections were chosen because they are the most refined method of injection, as mice need to be minimally restrained and the risk of injury is low (Morton et al., 2001). The site of injection was rotated to reduce the chance of skin inflammation and irritation. The order mice were injected was varied each day, and at least one mouse of each treatment group was housed in each cage.

An *a priori* power calculation was conducted using GPower (v.3.1) with an effect size of 50% based on data in Bosker et al., (2010). This specified that 10 mice were needed per treatment group. A second power calculation was performed using preliminary data from the twice-daily citalopram experiment (power=0.8, α=0.05, expected effect size=0.55, based on analysis with one-way ANOVA). This specified that n=12/group was needed for further behavioural experiments. Smaller group sizes were used in the neurochemical experiments – immunohistochemical experiments (section 6) used at least 6 mice per group (as in Singewald & Sharp, 2000), regional brain tissue analysis (section 7) used 6-9 mice per group (Trouvin et al., 1993), and *in vivo* microdialysis experiments (section 8) used 8 mice per group (at least 6 per group used in Guiard et al., 2012).

The table below summarises the mice used in each SSRI discontinuation behavioural experiment and how the brains were analysed (*Table 2.1*). This does not include pilot experiments or *in vivo* microdialysis experiments – these are detailed within the Experimental Methods sections of Chapters 3 to 7.

Table 2.1 Mice included in SSRI discontinuation experiments. Shown in order of appearance in this thesis. SAL, saline; CON, continuation; DIS, discontinuation; DPAT, 8-OH-DPAT; WAY, WAY-100635. Elevated plus maze, EPM; Homecage assessment, HCA; Serotonin syndrome assessment, SSA; novelty-induced hyponeophagia, NIH; novelty-suppressed feeding, NSF; locomotor activity, LMA; aversive open field, AOF; light/dark box, LDB. N/A, not analysed.

Experiment	**Groups, sex, and group size**	**Behavioural testing**		***Ex vivo* analysis**	
		Details	**Results section**	**Details**	**Results section**
12 days once-daily paroxetine (pilot)	SAL, CON, DIS Males n=6, Females n=6	Behavioural tests on discontinuation days 2-5 (EPM, SSA, NIH, NSF, LMA, AOF, LDB)	Chapter 3 section 3.1	N=3/group males used in 5 day regional brain tissue analysis	Chapter 5 section 3.2
12 days once-daily paroxetine (2 days discontinuation)	SAL, CON, DIS Males n=8	EPM and HCA on discontinuation day 2	Combined with pilot experiment in Chapter 3 section 3.2	Left hemispheres of n=8/group used in 2 day regional brain tissue analysis	Chapter 5 section 3.1
				Right hemispheres of n=8/group used in 2 day qPCR analysis	Chapter 5 section 3.11
12 days once-daily paroxetine (5 days discontinuation)	SAL, CON, DIS Males n=12	EPM and HCA on discontinuation day 5	Chapter 3 section 3.3 N=6/group also shown in Chapter 4 section 3.4	N=6/group used in 5 day immunohistochemical experiment	Chapter 4 sections 3.5-3.6
				Left hemispheres of n=/6 group used in 5 day regional brain tissue analysis, combined with pilot experiment	Chapter 5 section 3.2
				Right hemispheres of n=6/group used in 5 day qPCR analysis	Chapter 5 section 3.11
7 days once-daily paroxetine	SAL, CON, DIS Males n=12	EPM and HCA on discontinuation day 5	Chapter 3 section 3.4	N/A	
28 days once-daily paroxetine	SAL, CON, DIS Males n=12	Behavioural tests on discontinuation days 2-5 (EPM, HCA, NSF, NIH, LMA, AOF, LDB)	Chapter 3 section 3.5	N/A	

12 days twice-daily paroxetine	SAL, CON, DIS Males n=12	Behavioural tests on discontinuation days 2-3 (EPM, LMA, OF), culled on day 5	Chapter 3 section 3.6	Left hemispheres of n=6/group used in 5 day regional brain tissue analysis	Chapter 5 section 3.3
12 days twice-daily citalopram	SAL, CON, DIS Males n=10	Behavioural tests on discontinuation days 2-3 (EPM, LMA, OF), culled on day 5	Chapter 3 section 3.7	N/A	
	SAL, CON, DIS Males n=8	EPM on discontinuation day 2	Combined with other citalopram-treated mice in Chapter 3 section 3.7	Left hemispheres of n=8/group used in regional brain tissue analysis	Chapter 5 section 3.5
12 days twice-daily venlafaxine	SAL, CON, DIS Males n=12	EPM on discontinuation day 2	Chapter 3 section 3.8	N/A	
12 days once-daily paroxetine (6 h after final injection)	SAL, CON, DIS Males n=8	N/A		Right hemispheres of n=6/group used in qPCR analysis	Chapter 5 section 3.12
0.1 mg/kg 8-OH-DPAT precipitation experiment	SAL+SAL, CON+SAL, CON+0.1 DPAT, DIS+SAL Males n=12	EPM on discontinuation day 2, SAL+SAL, CON+SAL and DIS+SAL groups received acute saline injection 60 min before the EPM, CON+0.1 DPAT received 0.1 mg/kg 8-OH-DPAT 60 min before testing	Chapter 6 section 3.2	N=6-7/group used in immunohistochemical experiment	Chapter 6 section 3.2
			N=6-7/group of SAL+SAL, CON+SAL and DIS+SAL groups also shown in Chapter 4 section 3.1	N=6-7/group of SAL+SAL, CON+SAL and DIS+SAL groups also shown in 2 day immunohistochemical analysis	Chapter 4 sections 3.2-3.3

0.5 mg/kg 8-OH-DPAT and WAY-100635 precipitation experiment	SAL+SAL, CON+SAL, CON+0.5 DPAT, CON+WAY Males n=12	EPM on equivalent of discontinuation day 2, SAL+SAL, CON+SAL received acute saline injection 60 min before the EPM, CON+0.5 DPAT received 0.5 mg/kg 8-OH-DPAT before testing, CON+WAY received 1.0 mg/kg WAY-100635 before testing	Chapter 6 section 3.7	N=6/group used in immunohistochemical experiment	Chapter 6 section 3.7
Homecage monitoring experiment (7 days discontinuation)	Saline, Paroxetine group Males n=6	Continuous homecage activity monitoring throughout dosing (12 days) and discontinuation period (7 days)	Chapter 7 (combined with 13 days discontinuation experiment)	N/A	
Homecage monitoring experiment (13 days discontinuation)	Saline, Paroxetine group Males n=6	Continuous homecage activity monitoring throughout dosing (12 days) and discontinuation period (13 days)	Chapter 7 (combined with 7 days discontinuation experiment)	N/A	

4 Behavioural tests

Mice were housed in open top cages for all behavioural experiments. For three days before the start of behavioural experiments, mice were habituated to the short-term holding room in which they would be held immediately before testing.

4.1 EPM

The EPM was conducted using a cross-shaped maze elevated 50 cm above the ground and comprising two open arms (35 x 6 cm) perpendicular to two closed arms (35 x 6 cm, 20 cm walls), and a central region (6 x 6 cm), in a dimly-lit room (Line et al., 2011). A miniature video camera was placed above the maze and the output fed to ANY-maze (Stoelting Co.) tracking software. Mice were placed facing the walls of the closed arm (counterbalanced between the two closed arms), and location automatically tracked for 300 s. The apparatus was cleaned with 0.5% Anistel (Tristel) spray before each animal to remove residual odours.

4.2 Aversive OF (AOF)

Mice were placed in a brightly lit white cylindrical chamber with a 30 cm radius. A miniature video camera was placed above the maze and the output was fed to ANY-maze software. A 10 cm diameter centre zone was designated using ANY-maze. Mice were placed against the wall in the extreme outer zone at the start of the session and their location was tracked for 5 or 10 min (Pritchett et al., 2015). The apparatus was cleaned with 0.5% Anistel spray before each animal to remove odours.

4.3 LDB

The LDB arena consisted of a black covered compartment (21 x 16 cm, 16 cm walls), with a small doorway into the light area, and a brightly lit open compartment (21 x 16 cm, 16 cm walls), all within a photobeam frame (Med Associates Inc, USA) inside a sound-attenuating cubicle. Mice were placed into the bottom-right corner of the dark compartment and their paths tracked for 10 min via infrared beam breaks using Activity Monitor Software (Med Associates Inc) (Pritchett et al., 2015).

4.4 Spontaneous LMA

Mice were placed into the novel but neutral environment of an individual plastic cage (42 x 22 cm, 20 cm walls). The cage was covered with a perforated plexiglass lid and lined with sawdust, set within a photobeam frame (San Diego Instruments) in a dimly-lit room (Line et al., 2011). A fresh cage was used for each animal to prevent the transfer of odours. Infrared beams crossed the cage horizontally and vertically, and the number of beam breaks was recorded in 5 min bins for 60 min using San Diego Instruments photobeam frame software.

4.5 NIH test

Mice were food deprived overnight (from 18:00) and weighed before the start of the experiment to assess weight loss. Condensed milk was diluted 1:1 with drinking water and placed in a small metal well on a 20 x 20 cm white plastic arena. Mice were placed onto the arena and covered with a transparent plastic measuring jug, with the well under the spout. Mice underwent 2 min trials where the latency to start continuously eating was manually recorded. If the mouse ate, the trial would be stopped and the mouse would be

returned to its homecage; if it did not eat during the first trial, it would be placed in a separate cage for at least 10 min before repeating. Mice underwent a maximum of three trials (6 min total), and if they did not eat this was recorded as a latency of 360 s (Line et al., 2011).

4.6 NSF test

Mice were food deprived overnight and weighed before the start of the experiment. Mice were placed in a white plastic box (27 x 50 cm, 10 cm walls) lined with sawdust. A 4 x 4 cm plastic tray was placed at one end of the cage and filled with pieces of familiar food pellet. Mice were placed at the opposite end of the box and their latency to start eating manually recorded (Santarelli et al., 2003). Mice underwent a 5 min trial. If the mouse ate, the trial would be stopped and the mouse would be returned to its homecage; if it did not eat during the first trial, it would be placed in a separate cage for at least 10 min before repeating. Mice underwent a maximum of two trials (10 min total), and if they did not eat this was recorded as a latency of 600 s.

4.7 Homecage assessment (HCA)

Mice were assessed in a familiar but clean homecage lined with sawdust bedding (10 x 35 cm, 15 cm walls) in a dimly lit room for 180 s. Their activity was recorded via a camera facing the front of the cage. The total distance travelled was recorded using ANY-maze and the number of digging events manually counted.

4.8 Handling assessment

Several parameters were assessed while mice received their daily injections to monitor stress throughout the experiment. The following behaviours were measured

(adapted from Stuart & Robinson, 2015): audible vocalisation, struggling during restraint for injection, attempts to bite the experimenter or needle, defecation, and urination. Mice were given a score of 1 if the behaviour was observed, and 0 if not. The total score per mouse was calculated per day.

4.9 Coat state assessment

Dirty coats often represent low self-care behaviours in mice, for example unwillingness to self-clean (Planchez et al., 2019), hence coat state was used as a measure of mouse wellbeing during behavioural experiments. Coat state was assessed on eight different body regions: head, neck, dorsal coat, ventral coat, tail, forelimbs, hindlimbs, genital region. Any areas of dirty or abnormal coat were given a score of 1, or 0 if clean. The total score per mouse was calculated per day.

4.10 Serotonin syndrome assessment (SSA)

Behaviours associated with the rodent serotonin syndrome were recorded for five, 1 min time bins in a clean homecage (Izumi et al., 2006; Haberzettl et al., 2013). The following behaviours were monitored: head weaving, forepaw treading and backward movement (intermittent behaviours, scored on a scale of 0 to 4; 0, absent; 1, present once; 2, present several times; 3, present frequently; 4, present continuously); hind limb abduction, Straub tail, tremor and low body posture (continuous behaviours, scored on a scale of 0 to 4; 0, absent; 1, perceptible; 2, weak; 3, medium; 4, maximal). The scores from the five periods were summed to calculate the total score per mouse.

5 Tissue collection

5.1 Perfusion fixation

Mice were deeply anaesthetized with an overdose of sodium pentobarbital (5 mg/kg intraperitoneally, *i.p.*) and transcardially perfused with phosphate buffered saline (PBS, Sigma-Alrich) followed by a fixative solution of 4% paraformaldehyde (PFA, Sigma Aldrich) in PBS. Brains were stored at 4^{o}C in 4% PFA for 48 h, then stored long-term in cryoprotective 30% sucrose (Sigma-Aldrich) in PBS (sucrose solution) at 4^{o}C.

5.2 Frozen tissue

Mice were killed by cervical dislocation and decapitation. Brains were removed and immediately frozen in dry ice-cold isopentane (Sigma-Aldrich), then stored at -80^{o}C.

6 Immunohistochemistry

6.1 Brain slicing

Before sectioning, brains were coated in Cryo-M-Bed embedding compound (Bright) and stored at -80^{o}C for 45 min. Brains were sectioned in the coronal plane into 30 μm sections at -20^{o}C using the Bright LOFT cryostat. The resulting free-floating sections were stored in antifreeze solution (40% PBS, 30% ethylene glycol, 30% bidistilled glycerol, all Sigma-Aldrich) at -20^{o}C.

6.2 Immunohistochemistry

Slices were selected based on their distance from bregma to contain the following regions: DRN, MRN, and dorsolateral PAG (dl-PAG) [AP: -4.7 to 4.8 mm]; VH [AP: -3.0 mm]; DH and BLA [AP: -1.8 mm]; and prelimbic cortex (PLC) [AP: +1.6 mm] (Franklin & Paxinos, 1997). Slices were washed 3 x 10 min in 500 µl PBS, 10 min in 500 µl ammonium chloride and 3 x 10 min in 500 µl PBS with 0.3% TWEEN® 80 (Sigma-Aldrich; PBS-T). Slices were blocked in PBS-T with 10% donkey serum (Sigma-Aldrich) for 1 h at room temperature. Slices were incubated in primary antibodies (*Table 2.2*) made up in PBS-T with 2% donkey serum for 1 h at room temperature then overnight at 4^{o}C.

Table 2.2 Antibodies used for immunohistochemistry. NeuN, neuronal nuclei; AF, Alexa Fluor.

Target	Host	Conjugate	Concentration	Company	Ref. number and lot
Primary antibodies					
c-FOS	Rabbit	N/A	1:1000	Abcam	Ab214672 Lots GR3283706-2, GR3428387-4
TPH2	Goat	N/A	1:1000	Abcam	Ab121013 Lots GR176206-24, GR176206-62
NeuN	Mouse	N/A	1:1000	Abcam	Ab104224 Lots GR3279940-1, GR3421759-1
Secondary antibodies					
Rabbit IgG (H+L)	Donkey	AF488	1:1000	Invitrogen	A21206 Lot 802706
Goat IgG (H+L)	Donkey	AF568	1:1000	Invitrogen	A11057 Lot 400504
Mouse IgG (H+L)	Donkey	AF647	1:1000	Invitrogen	A21202 Lot 204533

Slices were washed 2 x 10 min in 500 µl PBS-T before incubating with secondary antibodies (*Table 2.2*) made up in 500 µl PBS-T with 2% donkey serum for 2 h at room temperature. Slices were washed 2 x 10 min in 500 µl PBS-T then for 10 min in PBS.

Slices were mounted onto glass slides with Vectashield Antifade Mounting Medium (Vector Laboratories) and covered with a glass coverslip. Slides were stored at 4^{o}C.

6.3 Imaging and analysis

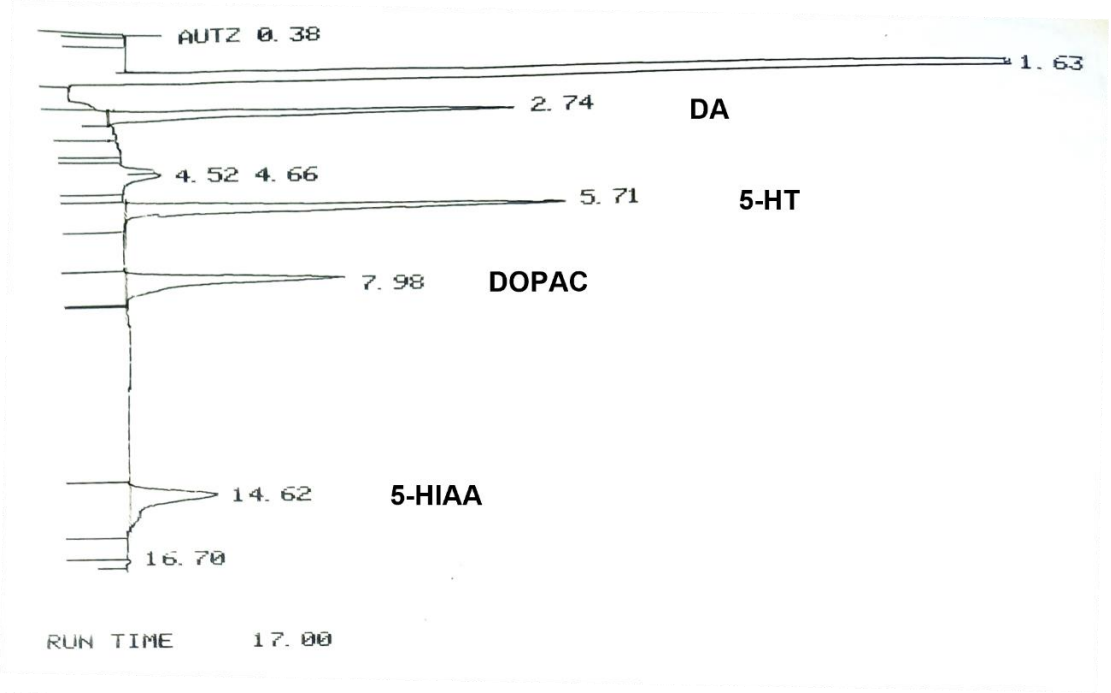
Slices were imaged on the Olympus Epifluorescence Microscope BX40 with ImageJ Micromanager v1.4 with 500 ms exposure. Images were taken at 10x and 20x magnification. Before imaging, each slice was visually inspected to confirm staining in the relevant subregions. Neurons were defined as NeuN-positive (+) nuclei. c-FOS+ neurons were defined as neurons with c-FOS immunoreactivity that co-localised with NeuN immunoreactivity. c-FOS+ TPH2+ double-labelled neurons were defined as neurons that co-localised c-FOS in TPH2+ cells.

c-FOS+, TPH2+ and NeuN+ neurons were counted in the dorsal subnucleus of the DRN [AP: -4.8, ML: 0.0, DV: -3.0 mm] and MRN [AP: -4.7, ML: 0.0, DV: -4.0 mm] at 20x magnification. c-FOS+ and NeuN+ neurons were counted in the BLA [AP: -1.8; ML: ±3.0, DV: -4.7 mm], dl-PAG [AP: -4.7, ML: ±0.5, DV: -2.0 mm] and PLC [AP: -4.7, ML: ±0.5, DV: -2.0 mm] at 20x magnification. c-FOS+ neurons were counted per slice in the dentate gyri (DG) of the VH [AP: -3.0, ML: ±2.0, DV: -2.2 mm] and DH [AP: -1.8, ML: ±0.2, DV: -2.5 mm] at 10x magnification. Images were taken from three slices per region then averaged per mouse.

7 High Performance Liquid Chromatography (HPLC) with Electrochemical Detection (ED)

7.1 Procedure

Concentrations of 5-HT, 5-HIAA, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using HPLC-ED. The HPLC-ED system consisted of a 150 × 4.6 mm Waters Spherisorb column (ODS2, 3 μm) using a mobile phase comprising 12.5% (v/v) methanol, 0.85 mM EDTA (Sigma-Aldrich), 0.13 M NaH_2PO_4 (Sigma), 0.025 mM 1-octane sulfonic acid (Fisher Scientific). The mobile phase was adjusted to pH 3.3 using phosphoric acid (Acros Organics), filtered before use (with Merck MF-Millipore^{TM} 0.22 μm MCE membranes, and a vacuum pump), and run at 1.0 mL/min flow rate. ED used a Bioanalytical Systems (LC-4/C) potentiometer with a glassy carbon working electrode set at +0.7 V. Peak times and heights were plotted by a Milton Roy® CI 4000 Computing Integrator. An external standard method was used for quantification, using standard concentrations of each neurotransmitter made up in 0.09 M perchloric acid (PCA, Fisher Scientific). A representative chromatogram of a standard solution comprising of 5 pmol of 5-HT (Sigma), 5-HIAA (Sigma-Aldrich), DA (Alfar Aesar) and DOPAC (Aldrich) is presented in *Fig. 2.1*. Prior to analysis, a standard curve for each neurotransmitter was made to verify the system could quantify the analytes linearly at the required concentration range (5 fmol/50 μl – 10 pmol/50 μl; *Fig. 2.2*).



EXTERNAL STANDARD METHOD USING HEIGHT

TIME	HEIGHT	CONC	PK NAME
2.74	39919	5.001850700	DA
5.71	42006	4.998714000	5HT
7.98	20926	4.999221400	DOPAC
14.62	8480	4.999808000	5HIAA
TOTAL	111331	19.99959410	

Figure 2.1 Representative chromatogram for a standard solution. Peak height in AU, concentration in pM. Numbers next to peaks indicate retention time (min).

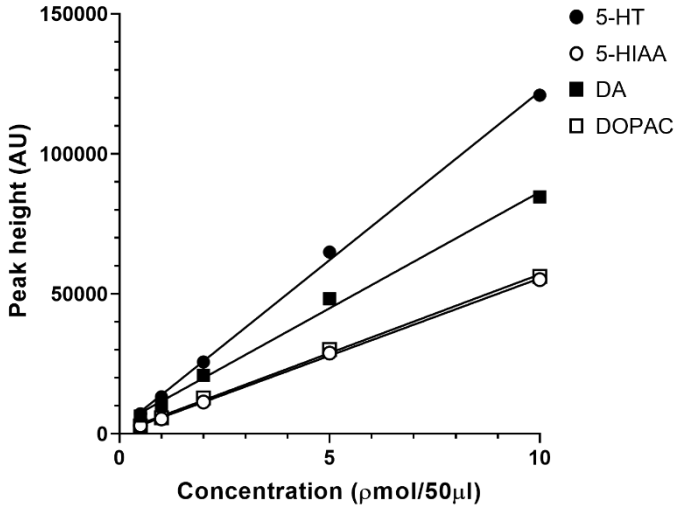


Figure 2.2 Standard calibration. Linear relationship between concentration of neurotransmitter and HPLC-ED chromatogram peak height. Points represent mean ± standard error of the mean (SEM, error bars too small to be seen) for concentrations between the range of 0.5 and 10 pmol/50µl sample. N=3/concentration.

7.2 Dissection and regional brain tissue analysis

Frozen brains were cut between the hemispheres using a scalpel and the left hemisphere of each brain was dissected to isolate the midbrain, hippocampus, striatum, and frontal cortex on ice. Samples were placed into Eppendorf tubes containing 500µl of 0.09 M PCA and weighed. Samples were sonicated and centrifuged at 13,000 rpm for 15 min at 4^{o}C. The supernatants were analysed with HPLC-ED to calculate the concentration of 5-HT, 5-HIAA, DA and DOPAC, expressed as pmol/mg wet tissue. 5-HT turnover was calculated as the ratio of 5-HIAA to 5-HT concentration (5-HIAA/5-HT ratio). Example chromatograms are presented in *Fig. 2.3*.

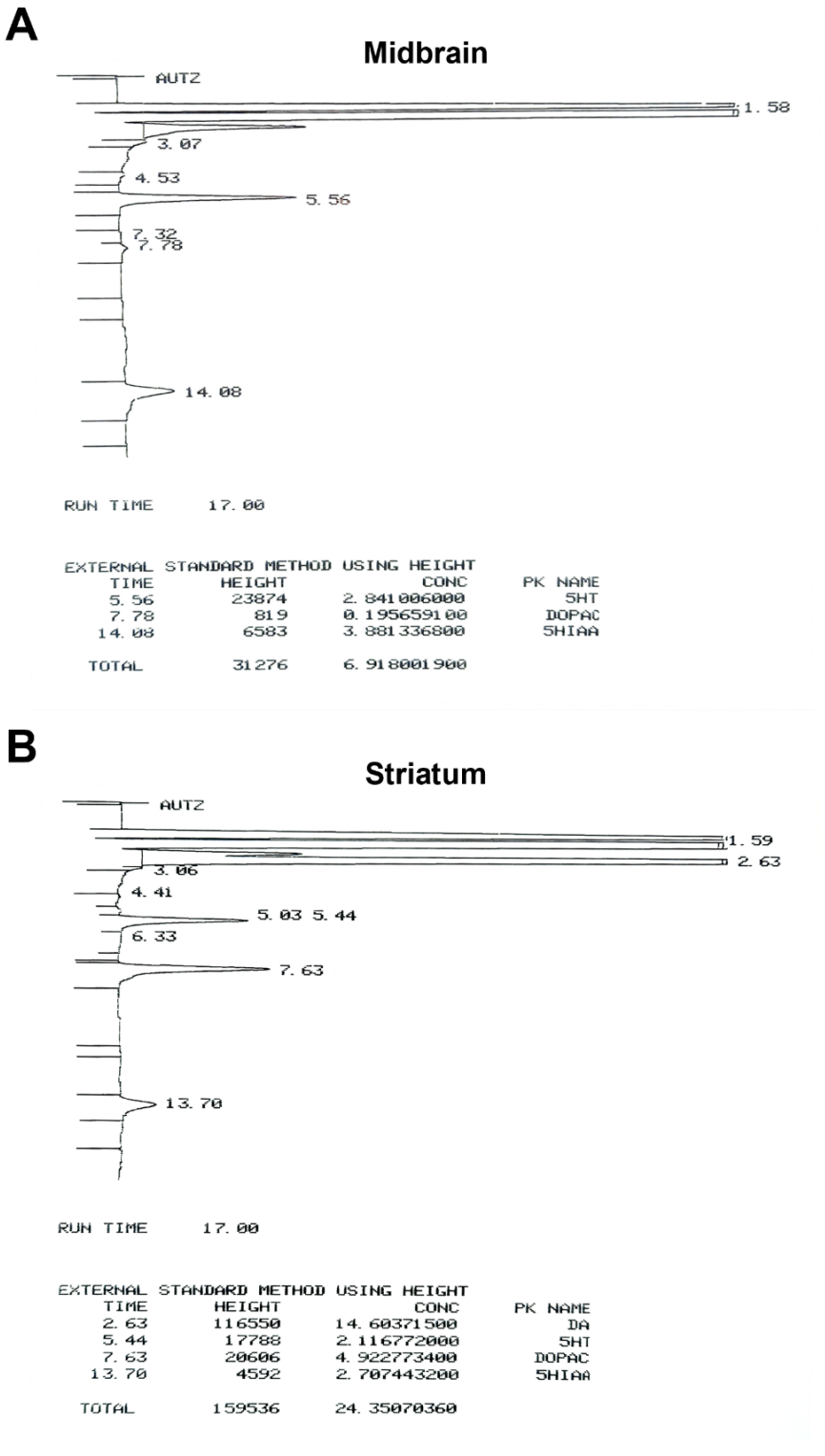


Figure 2.3 Representative chromatogram for tissue samples. Example chromatograms for midbrain (A) and striatum (B) samples. Peak height in AU, concentration in pM. Numbers next to peaks indicate retention time (min).

8 *In vivo* microdialysis

8.1 Procedure

Mice were housed in IVCs for all microdialysis experiments. Mice were anaesthetised using *i.p.* 1.3 mg/kg urethane (Sigma, made up in PBS) and maintained at a surgical plane of anaesthesia using top-up *s.c.* injections (up to a maximum cumulative dose of 1.5 mg/kg). Mice were given *s.c.* injections of 5% glucosaline (Aquapharm® No. 3, AnimalCare Ltd) throughout the recording. The skin above the skull was cleaned using chlorhexidine and 0.025% bupivacaine hydrochloride (Marcaine, AstraZeneca) administered *s.c.* 10 min before the incision. A craniotomy was performed above the VH [AP: -3.0 mm, ML: ±2.9 mm] and the dura removed.

Microdialysis probes (2 mm polyethersulphone membrane, Microbiotech) were stereotaxically implanted into the VH [AP: -3.0 mm, ML: ±2.9 mm, DV: -4.0 mm from bregma and the dura surface] (Franklin & Paxinos, 1997). Unless otherwise stated, probes were continuously perfused with ACSF (components in section 8.2) at a rate of 2 µl/min using a Carnegie Medicin Microinjection Pump CMA/100 and a 1 ml plastic syringe via fine bore polythene tubing. In all experiments, mice underwent a 60 min post-implantation recovery period to allow neurotransmitter levels to stabilise. Samples were then collected every 20 min into Eppendorf tubes containing 10 µl 0.09 M PCA and analysed for 5-HT, 5-HIAA, and DOPAC using HPLC-ED (DA was undetectable in the microdialysate). An external standard method was used for quantification, and standard calibration curves for 5-HT and 5-HIAA are presented in *Fig. 2.4*.

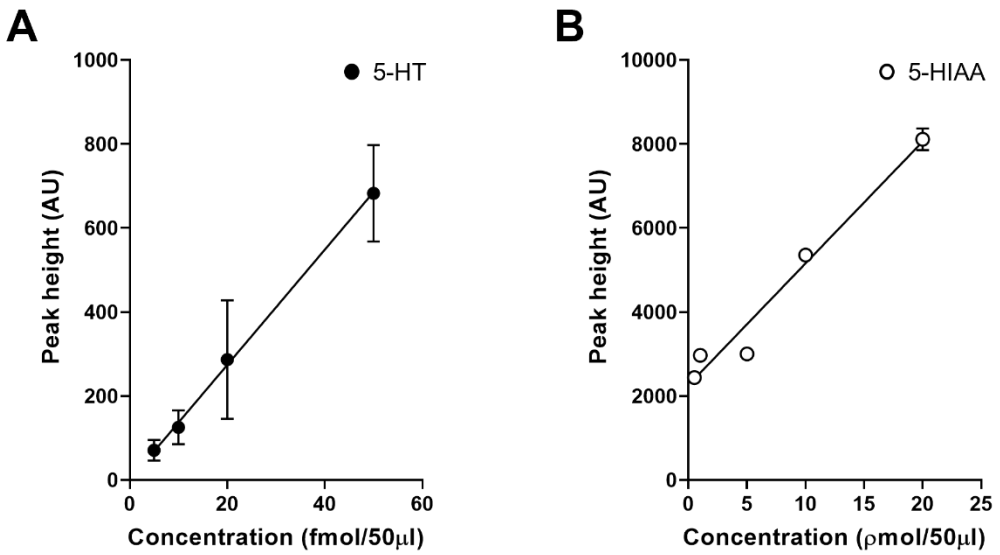


Figure 2.4 Standard calibration of 5-HT and 5-HIAA for microdialysis experiments. Linear relationship between concentration of neurotransmitter and HPLC-ED chromatogram peak height. Points represent mean ± SEM (some error bars too small to be seen) for concentrations between the range of 5 to 50 fmol/50µl samples for 5-HT (A) and 0.5 to 20 pmol/50µl sample for 5-HIAA (B). N=3/concentration.

8.2 ACSF components

For baseline recordings, regular ACSF was used (in mM): 140 NaCl (Sigma), 4 KCl (BDH Analytical Chemicals), 1.2 Na_2HPO_4 (Sigma), 0.27 NaH_2PO_4 (Sigma), 1 $MgCl_2$ (BDH), 2.4 $CaCl_2$ (BDH) and 7.2 glucose (BDH). ACSF with higher concentrations of potassium was also used to depolarise neuronal terminals (Auerbach et al., 1989; Sharp & Foster, 1989). 56 mM potassium (in mM): 87 NaCl, 56 KCl, 1.2 Na_2HPO_4, 0.27 NaH_2PO_4, 1 $MgCl_2$, 2.4 $CaCl_2$ and 7.2 glucose. 100 mM potassium (in mM): 4 NaCl, 100 KCl, 1.2 Na_2HPO_4, 0.27 NaH_2PO_4, 1 $MgCl_2$, 2.4 $CaCl_2$ and 7.2 glucose. Glucose and $CaCl_2$ were added each morning before use.

8.3 Probe localisation

At the end of recordings, brains were fixed by perfusion fixation (as in section 5.1). Brains were sliced into 30 µm sections at -20°C using the Bright LOFT cryostat to visually

identify the probe tract. If the probe were not in the VH, data from this mouse would be excluded from analysis. To image the probe tracts, slices were washed 3 x 10 min in PBS, 10 min in ammonium chloride, then 3 x 10 min in PBS-T. Slices were incubated for 10 min in 0.1% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) to stain neuronal DNA. Slices were washed 2 x 10 min in PBS-T then 10 min in PBS. Slices were mounted onto glass slides with Vectashield Antifade Mounting Medium and covered with a glass coverslip. Slices were imaged using the Olympus Epifluorescence Microscope BX40 with ImageJ Micromanager v1.4 with exposure at 500 ms at 4x and 10x magnification. An example probe location is presented in *Fig. 2.5*.

8.4 Microdialysis analysis

Example microdialysate chromatograms are presented in *Fig. 2.6*. The percentage change in neurotransmitter levels was calculated compared to the mean of the two samples before high potassium ACSF perfusion (Chapter 5). Changes in neurotransmitter levels following 8-OH-DPAT or WAY-100635 injection were also calculated as a change from the mean of the three samples preceding the injection (current concentration minus mean baseline concentration before the injection, Δ5-HT and Δ5-HIAA; Chapter 6).

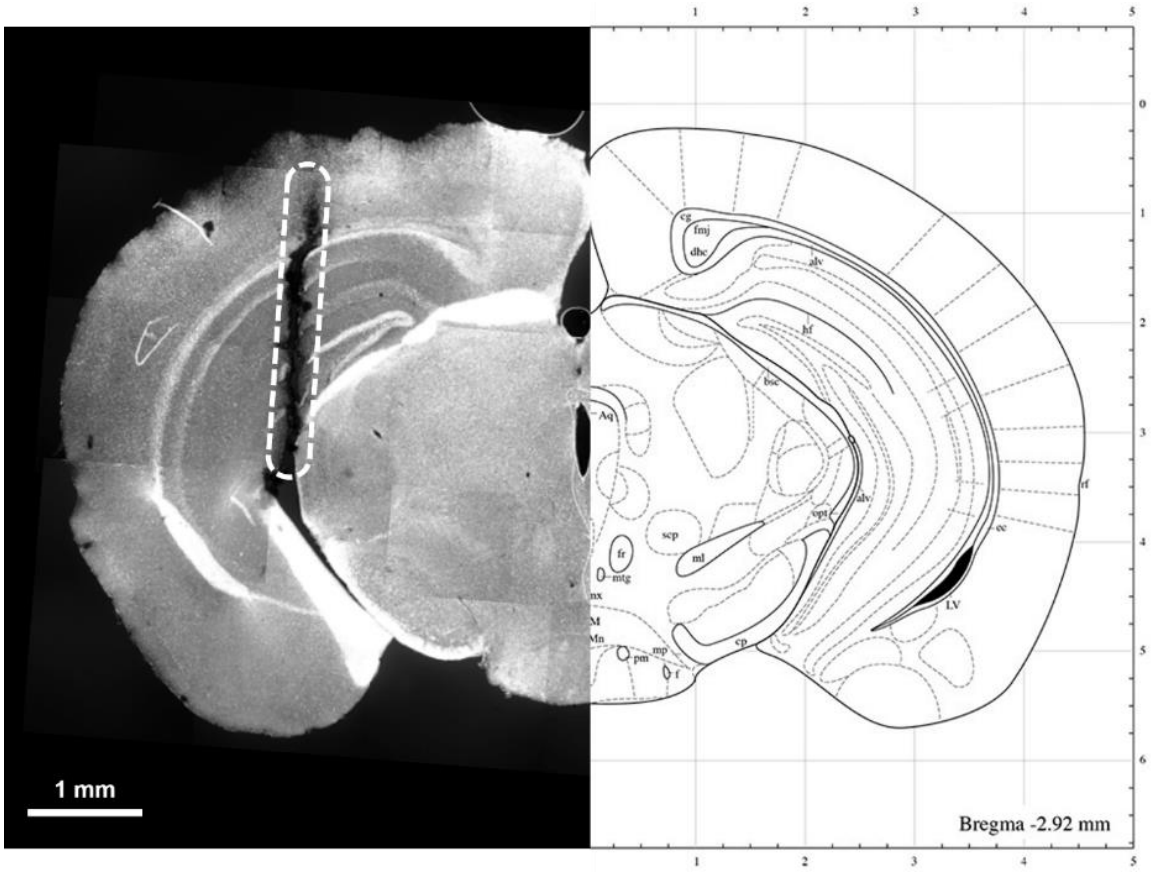


Figure 2.5 Representative image of microdialysis probe localisation in the VH. Mouse brain atlas images of the VH from Paxinos & Franklin (2007). Dotted outline represents damage caused by probe placement. Line represents 1 mm.

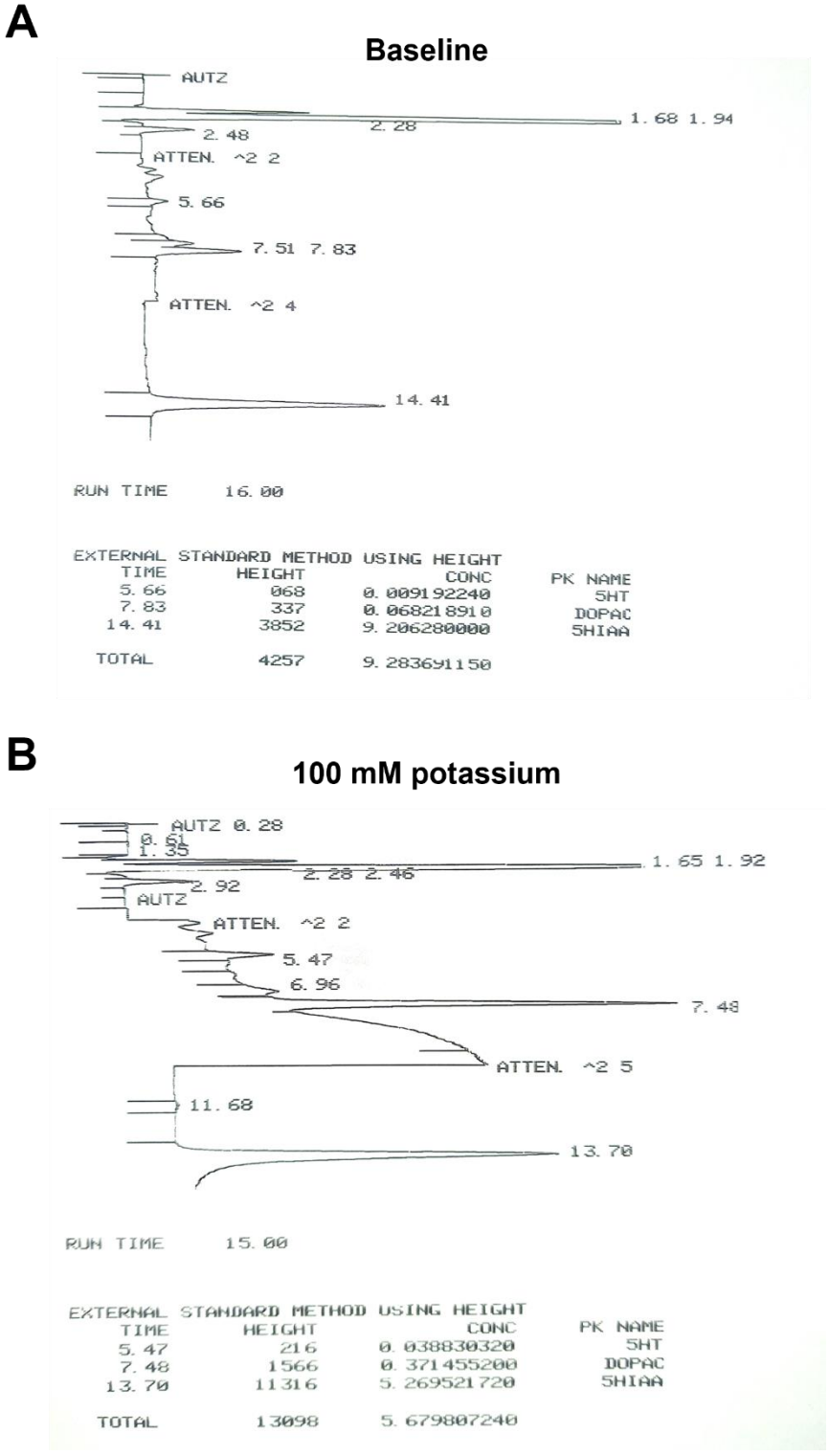


Figure 2.6 Representative chromatogram for microdialysate samples. Example chromatograms for microdialysis samples at baseline (A) and 20 min after infusion of 100 mM potassium (B) from saline injected control mice. Attenuation was lower in panel B than in standard chromatograms to better visualise peaks. Peak height in AU, concentration in pM. Numbers next to peaks indicate retention time (min).

9 Quantitative real-time polymerase chain reaction (qPCR)

9.1 Tissue dissection

Frozen brain tissue was cut into 1 mm coronal slices using a stainless steel adult mouse brain slicer (Zivic Instruments) and stainless steel fine edge blades at -20ºC. A scalpel was used to isolate the raphe nuclei (RN), hippocampus, and prefrontal cortex (PFC), based on their anatomical locations (Franklin & Paxinos, 1997). Samples were placed into Eppendorf tubes on dry ice and stored at -80ºC until RNA extraction.

9.2 RNA extraction, cDNA conversion and qPCR

RNA extraction, cDNA conversion and qPCR were conducted as in Radford-Smith et al. (2022). RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Ltd.) and β-mercaptoethanol (Sigma) as per the manufacturer's instructions. RNA was eluted into RNase-free water (20 µl for RN samples, 25 µl for hippocampus and PFC samples) and both total RNA and RNA purity immediately measured using a NanoDrop 1000 (ThermoFisher). If the 260/280 nm ratio (indicating the purity of the RNA, as opposed to DNA contamination) was between 1.8 and 2.2, the sample was deemed sufficiently pure to be used for cDNA conversion. RNA was stored at -80ºC until cDNA conversion.

RNA was converted to cDNA using the High-capacity cDNA Reverse Transcription Kit (Life Technologies) as per the manufacturer's instructions, using a T100 Thermocycler (Bio-Rad) for 10 min at 25ºC, 120 min at 37ºC and 5 min at 85ºC. 600 ng of RNA was used for hippocampus samples or 200 ng for RN and PFC samples. cDNA was stored at -20ºC until qPCR analysis.

qPCR was performed using a LightCycler® 480 instrument (Roche Diagnostics). *Table 2.3* details the primer sequences and sources. Optimal primer pairs were determined according to ThermoFisher recommendations (ThermoFisher Scientific, 2019): crossing at least one exon-exon boundary, having 18-24 bases, producing a product of 70-200 bases, containing 40-60% GC content, having annealing temperatures of between 65-75°C and within 5°C of each other, having no self-dimers, little to no secondary structures and no off-target binding. Primer details were confirmed using the National Center of Biotechnology Information Primer-BLAST tool (Ye et al., 2012) and murine gene of interest (GOI) sequences., Eurofins Genomics Oligo Analysis Tool (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/) and the Sigma-Aldrich OligoEvaluator™ (http://www.oligoevaluator.com/LoginServlet).

Table 2.3 Sequences of primers for qPCR analysis.

GOI	Forward Sequence (5' to 3')	Reverse sequence (5' to 3')	Sequence source
5-HT$_{1A}$	GACAGGCGGCAACGA TACT	CCAAGGAGCCGATGAGA TAGTT	Gorlova et al. (2020)
5-HT$_{1B}$	CCCATCAGCACCATGT ACAC	GACTTGGTTCACGTACAC AG	Guilloux et al. (2011a)
5-HT$_{2A}$	CAGGCAAGTCACAGGA TAGC	TTAAGCAGAAAGAAAA TCCCACAG	Couch et al. (2015)
5-HT$_{4}$	CCTCACAGCAACTTCTC CTT	TCCCCTGACTTCCTCAA ATA	Vogelgesang et al. (2017)
5-HTT	TGCCTTTTATATCGCCT CCTAC	CAGTTGCCAGTGTTCCA AGA	Couch et al. (2015)
Tph2	CAGGGTCGAGTACACA GAAG	CTTTCAGAAACATGGA GACG	Vogelgesang et al. (2017)
β-actin	CATTGCTGACAGGATG CAGAAGG	TGCTGGAAGGTGGACA GTGAGG	Yu et al. (2019)

Primers were used at 300 nM, with a total of 10 µl reaction volume used per well in a 384 well plate, including 5 µl Precision®PLUS qPCR Master Mix with SYBRgreen (Primer Design). 25 ng cDNA was used for each reaction, made up in RNase-free water. The following cycle was used for the PCR reaction: enzyme activation for 2 min at 95°C,

then 40 cycles of 10 s at 95ºC and 1 min at 60ºC, then held at 4ºC. The generation of a melt curve was included in each qPCR experiment to confirm only one PCR product was generated by each reaction. Samples were run in duplicate, including the genes of interest (GOI) and β-actin as a housekeeping gene (HKG).

9.3 qPCR analysis

The ΔCt method was used to analyse qPCR data using Microsoft Excel. Duplicates were averaged to give a Ct (threshold cycle) value for each GOI and the HKG per animal (failed cycles or large outliers were not included in the Ct values). For each GOI, the Ct of the housekeeping gene was subtracted from the Ct of the GOI ($\Delta Ct = Ct_{GOI} - Ct_{HKG}$). The mean ΔCt for the saline control group was then subtracted from each GOI ΔCt value (ΔΔCt). Fold changes in gene expression relative to the saline control were calculated per animal and GOI as $2^{-\Delta\Delta Ct}$. Outliers were identified in the $2^{-\Delta\Delta Ct}$ values using ROUT analysis (GraphPad Prism), and these mice excluded from the raw data for that GOI. The ΔCt method was then reapplied on the cleaned dataset to recalculate fold changes.

10 Passive infrared (PIR) activity and sleep monitoring

10.1 Activity monitoring system

The PIR activity monitoring system was set up as in Brown et al. (2016). Briefly, the equipment consisted of individually ventilated light tight chambers (LTC) with six infrared sensors mounted on the ceiling of the box and cool-white light-emitting diodes, providing 200 lux light. The software to collect infrared sensor data was written in Java

(Brown et al., 2016), and a serial message containing all activity data was sent to a PC connected via USB, and stored against a timestamp.

Mice were housed in LTCs on a 12:12 light-dark cycle (light ON 05:00, lights OFF 17:00) in a room maintained at 21^{o}C. Mice were single housed in large opaque open top cages (45 × 28 cm, 13 cm walls) positioned under each infrared sensor. Cages were lined with sawdust and mice had *ad libitum* access to food and water. Perspex blocks were placed under the wire food hoppers incorporated into the wire cage bars to prevent the infrared radiation from the mice being blocked from the sensor. A small amount of sizzle nest was provided as bedding in each cage, but no additional cage enrichment was used to prevent the sensor being obscured.

Total cage movement every 100 ms was measured and recorded as a binary movement/no movement signal. Data were binned into 10 s epochs, meaning each datapoint represented the percentage activation of the sensor during that time bin. Data were collected continuously throughout the experiment, although the recording was stopped and immediately restarted every seven days to save the data.

10.2 Activity and circadian rhythm analysis

Individual data files were combined in Microsoft Excel and converted into .csv files. Data were processed with scripts written in MATLAB R2021b, based on examples in Python freely available on GitHub (https://github.com/LozRiviera/COMPASS_paper)

Analysis methods were based on those outlined in Brown et al. (2019). Data were binned into 1 h epochs to calculate mean activity per hour. Data were plotted over 24 h or averaged over the entire day, dark period, or light period. Actograms were generated

using a freely available function from the raw activity data in 10 s time bins (https://github.com/bradmonk/actograph).

To measure disturbances in daily activity/rest cycles, measures of circadian disruption were used (Brown et al., 2019). The interday stability (IS) was calculated for each mouse, as well as IS of each treatment period (baseline, dosing, and discontinuation). IS is a value based on the variability in activity of a 24 h period compared to the total variance of the whole experiment. IS was calculated as:

$$IS = \frac{N \sum_{h=1}^{p} (\bar{X}_h - \bar{X})^2}{p \sum_{i=1}^{N} (X_i - \bar{X})^2}$$

where N is the number of data points, p is the number of data points per day (24 h of data was binned into 1 h epochs), $\bar{X}$ is the mean of all the data for that mouse, $\bar{X}_h$ are the hourly means and X_i are the individual data points (Brown et al., 2019). The output value is between 0 (no reproducibility across days) and 1 (perfect reproducibility). For calculating the IS value for each mouse in each treatment period, the days within that period were compared only to the variability of that treatment period (i.e., not to the variability across the whole experiment).

The intraday variability (IV) was calculated for each mouse, as well as IV of each treatment period. IV is a value based on the stability of a mouse's sleep and wake periods, representing the variability in how many sleep-wake transitions it does in a 24 h period. IV was calculated as:

$$IV = \frac{N \sum_{i=2}^{N} (X_i - X_{i-1})^2}{(N-1) \sum_{i=1}^{N} (\bar{X} - X_i)^2}$$

where N is the number of data points, X_i are the individual data points, and $\bar{X}$ is the mean of all the data for that mouse (Brown et al., 2019). IV is not constrained to values between 0 and 1, but a higher number represents greater variability in sleep-wake transitions.

Behaviourally-defined sleep was defined as ≥40 s of immobility, which correlates with previous EEG measurements under similar conditions (Pack et al., 2007; Fisher et al., 2012; Brown et al., 2016). Sleep was calculated from the activity data as the rolling sum over the last four 10 s bins and expressed as a binary sequence of awake (0) or asleep (1). Sleep data were binned into 1 h epochs to calculate the percentage of time asleep per hour averaged over the entire day, dark period, or light period. The number of sleep episodes per day, and in each dark and light period were also calculated. The duration of each sleep episode was calculated and grouped into sleep bout duration bins of <1 min, 1-10 min, 10-60 min or >60 min. Sleep latency was calculated as the time between the lights turning on at 05:00 and the first sleep episode. If mice were already asleep when the lights turned on, sleep latency was recorded as 0 s.

11 Statistical analysis

The normality of data was assessed with D'Agostino Pearson's test. Details of the statistical tests typically used, depending on whether the data were parametrically or non-parametrically distributed and the number of independent variables, are presented in *Table 2.4*. In general, one way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) or Kruskal Wallis test with post-hoc Uncorrected Dunn's test were used to compare between treatment groups. A mixed effect model with Geisser-Greenhouse multiple comparisons was also used instead of repeated measures ANOVA when some data were missing (Chapter 7).

Table 2.4 Statistical analysis.

Type of data	Parametric	Non-parametric
Two experimental groups, one independent variable	Unpaired Student's t-test	Unpaired Mann Whitney U test
Three or more experimental groups, one independent variable	One-way ANOVA with Fisher's LSD	Kruskal Wallis with Uncorrected Dunn's test
Two or more experimental variables, two independent variables	Two-way ANOVA with Tukey's test for multiple comparisons	N/A
Two or more variables, repeated measures over time	Repeated measures ANOVA with Bonferroni's multiple comparisons test	N/A

A meta-analysis of the effects of repeated SSRI administration on the time spent in the open arms of the EPM in wild-type adult mice was performed following unexpected results in Chapter 3 (methods detailed in Appendix 2).

Data were analysed with GraphPad Prism (v8) and IBM Statistical Product and Service Solutions (SPSS) Statistics. Data are presented as mean ± standard error of the mean (SEM) value for parametric data and median ± interquartile range (IQR) for non-parametric data. P values of $p<0.05$ were considered statistically significant throughout.

Chapter 3
Effect of SSRI discontinuation on anxiety-like behaviour in mice

Parts of the following chapter are published in Collins et al., *J. Psychopharmacol*., 2022; 36(7): 794-805.

1 Introduction

Anxiety is one of the most common symptoms of SSRI discontinuation syndrome, often appearing within just two days of drug cessation and lasting for several weeks. Nonetheless, the preclinical literature on the effects of SSRI discontinuation syndrome on rodent behaviour is extremely limited, with previous reports restricted to single behavioural tests. Moreover, there is currently no preclinical model of the condition and the behavioural effects of SSRI discontinuation in mice have yet to be systematically investigated.

1.1 Effect of SSRI discontinuation on anxiety-like behaviours in rodents

To our knowledge, there is only one previous study that directly investigated the immediate behavioural effects of SSRI discontinuation in rodents. Bosker and colleagues reported increased ASRs in rats two days after discontinuation from two weeks of citalopram administration, compared to continued treatment controls. This effect persisted at least seven days after citalopram discontinuation (Bosker et al., 2010). The authors interpreted these findings as evidence of increased anxiety-like behaviour immediately following citalopram discontinuation.

While the ASR is an interesting paradigm as it can also be used in patients to assess anxiety (Knudson & Melcher, 2016), in rodents the ASR can be influenced by several factors. These include the nature of the auditory stimulus as well as the species, sex, strain, and age of the animal being studied (Lauer et al., 2017). The ASR is also used to assess fear learning, making it difficult to differentiate conditioned fear from anxiety.

As the ASR was the only measure of anxiety-like behaviour studied by Bosker et al., the effect of SSRI discontinuation on approach-avoidance conflict is not yet known. Moreover, this study only investigated the effects of discontinuation from a single SSRI, at a single dose for one treatment duration, in rats. Consequently, there has yet to be a systematic analysis of the effects of SSRI discontinuation in mice. Moreover, the optimal treatment procedure for detecting such effects is unknown.

Other studies have reported changes to anxiety-like behaviour following cessation of SSRI treatment, although they did not attempt to model the clinical syndrome. For example, increased anxiety-like behaviour was detected on the EPM 24 h after paroxetine discontinuation compared to mice still receiving paroxetine (Goeldner et al., 2005). In contrast, other studies have found limited evidence of behavioural changes 24 h after paroxetine and citalopram discontinuation relative to vehicle-treated controls (Popik et al., 2000; Matsuzawa-Yanagida et al., 2008).

Other studies have looked at anxiety-like behaviour later in the discontinuation period. Two weeks after cessation of repeated paroxetine treatment, there were no differences in the time mice spent in the light zone of the LDB or the percentage of time spent in the open arms of the EPM compared to vehicle-treated controls (Elizalde et al., 2008). This lack of effect was also observed two weeks after fluoxetine discontinuation in mice (Popa et al., 2010), and one week after its discontinuation in mice (McAllister et

al., 2015). Together, these studies suggest that SSRI discontinuation is anxiogenic in rodents but that this effect is short-lived. Precisely when these effects appear and dissipate, however, has yet to be established.

1.2 Effect of SSRI discontinuation on LMA in rodents

SSRI discontinuation is not generally associated with changes in spontaneous LMA in rodents. Although rats discontinued from one week of escalating doses of fluoxetine had increased LMA 4 h after the first 'missed dose', there were no differences in LMA in the subsequent 96 h washout compared to saline controls (Bjork et al., 1998). This lack of effect on LMA was also observed 24 h after discontinuation from fluoxetine (Goeldner et al., 2005; Iñiguez et al., 2010), paroxetine (Matsuzawa-Yanagida et al., 2008) and sertraline (Mouri et al., 2012). No effects were detected one week after fluoxetine discontinuation in rats (Bouet et al., 2012), or two weeks after discontinuation from paroxetine (Elizalde et al., 2008) and fluoxetine (Norcross et al., 2008; McAllister et al., 2015), or over the four weeks following fluoxetine (Greene-Schloesser et al., 2011) and citalopram (Strekalova et al., 2013) discontinuation in mice. Therefore, while there is some evidence of increased anxiety following SSRI discontinuation, available evidence suggests that altered LMA is not characteristic of SSRI discontinuation in rodents.

1.3 Effect of SSRI discontinuation on stress-related and coping behaviours

Notably, how the other behavioural effects of repeated SSRI administration, for example on stress-related and coping behaviours, persist after treatment cessation have been better characterised. For example, rats exhibited increased time to become immobile in the forced swim test (FST) 24 h after the last dose of fluoxetine (Iñiguez et al., 2010), as well as two days after discontinuation from 14 days of sertraline treatment (Zhao et al.,

2009), suggesting the effects of repeated SSRI treatment were still evident immediately following cessation. Moreover, the coat state of rats discontinuing citalopram did not differ from that of vehicle controls, suggesting that SSRI discontinuation did not produce an acute depressive-like phenotype (Moret & Briley, 1990). Thus, although the FST has been criticised for lacking predictive validity regarding the effects of acute and chronic SSRI treatment on helplessness behaviours (for example, Kara et al., 2018), these studies suggest there was no immediate increase in stress-like behaviours following SSRI discontinuation in rats.

Stress and coping behaviours have also been studied later in the discontinuation period. One week after discontinuation from repeated fluoxetine treatment, there were no differences in the time rats spent immobile in the FST compared to saline controls (Bouet et al., 2012). Similar effects were seen 21 days after discontinuation (Iñiguez et al., 2010). Two weeks after paroxetine discontinuation, there was also no effect on FST immobility or sucrose preference (Elizalde et al., 2008). Similarly, no effects were seen six weeks after discontinuation from escitalopram treatment in mice on the sucrose preference test, tail suspension test (TST) or FST (Popa et al., 2010).

There are also no immediate or long-term effects of SSRI discontinuation on other naturalistic behaviours. For example, the time mice spent in social interactions did not differ from that of saline-treated controls 24 h after cessation of repeated sertraline treatment (Mouri et al., 2012). Moreover, while repeated paroxetine treatment reduced marble burying, two weeks after its discontinuation it did not differ from saline controls (Elizalde et al., 2008). Three weeks after discontinuation from fluoxetine treatment mice also showed no differences in conditioned fear recall or extinction compared to vehicle-

treated controls (Norcross et al., 2008). Thus, these studies suggest that there were overt changes to other naturalistic behaviours following SSRI discontinuation in rodents.

1.4 Hypothesis and aims

The aims of this chapter were to establish the behavioural effects of SSRI discontinuation in mice, to optimise the detection of such effects by altering SSRI treatment and frequency, and to determine if these are common across several SSRIs. Paroxetine was initially chosen as it is most likely to cause SSRI discontinuation syndrome in patients (Price et al., 1996; Gastaldon et al., 2022). A range of behavioural tests were conducted in the first week following discontinuation to assess the time course and generalisability of the effects of SSRI discontinuation in mice.

2 Experimental Methods

2.1 Animals

C57BL/6J male and female mice (Charles River, 7 weeks old) were group housed (3 per cage) in open-top cages with their littermates for at least one week before the start of treatment.

2.2 Drugs and reagents

Paroxetine, citalopram, and venlafaxine were dissolved in saline and administered *s.c*. Paroxetine was chosen as it is the SSRI most likely to cause discontinuation symptoms in patients (Price et al., 1996; Gastaldon et al., 2022), and administered at 10 mg/kg in accordance with previous preclinical studies (Sillaber et al., 2008; Elizalde et al., 2008; Thoeringer et al., 2010). Citalopram and venalafaxine were also administered at 10 mg/kg to mirror other reports of their behavioural effects in mice (Karlsson et al., 2011; Venzala et al., 2012). Although these doses are considerably higher than the equivalent daily dosage in humans (Taylor et al., 2021), they account for the faster metabolism of SSRIs in rodents (Overø, 1982; Howell et al., 1994; Kreilgaard et al., 2008). The frequency of treatment was also increased to twice-daily in some experiments to account for the rapid clearance. A treatment duration of 12 days was initially chosen as 10-14 days is regularly used to test the effects of 'repeated' SSRI treatment in mice (Kobayashi et al., 2008; Baek et al., 2015; Turcotte-Cardin et al., 2019). Later experiments extended dosing to 28 days, as in Santarelli et al. (2003). *S.c.* injections were used throughout to ensure the exact timing of the washout period – for instance, drinking

water administration would not ensure that mice received their final dose, or in fact the same amount, of the SSRI precisely 48 h before behavioural testing began.

2.3 Experimental design

Mice were allocated to one of three experimental groups by stratified randomisation: i) saline group (SAL): repeated saline + saline continuation; ii) continuation group (CON): repeated SSRI + SSRI continuation; iii) discontinuation group (DIS): repeated SSRI + saline. Mice were weighed for two days before the start of the experiment to establish a baseline body weight and to habituate them to handling, and then daily during dosing and discontinuation. Mice received once- or twice-daily *s.c.* injections of 10 mg/kg paroxetine, citalopram or venlafaxine, or the equivalent volume of saline for 7, 12 or 28 days, then initial treatment was either continued (saline and continuation groups) or swapped to saline injections (discontinuation group) for a further two or five days, when the behavioural tests were conducted (*Fig 3.1*). Individual experiments are summarised in *Table 3.1*.

Table 3.1 Experimental design. All SSRIs given at 10 mg/kg per injection. Elevated plus maze, EPM; Homecage assessment, HCA; Serotonin syndrome assessment, SSA; novelty-induced hyponeophagia, NIH; novelty-suppressed feeding, NSF; locomotor activity, LMA; aversive open field, AOF; light/dark box, LDB. † One female mouse excluded from the continuation group due to receiving the wrong injection on discontinuation day one. †† Initial experiment involving n=10/group (EPM, AOF and LMA), groups sizes were later increased by n=8/group for the EPM.

SSRI	Dosing period duration (days)	Injection schedule	Group sex and size	Behavioural tests (discontinuation day of test)
Paroxetine (pilot exp.)	12	Once-daily	Males n=6, females n=5-6†	EPM, SSA (day 2); NIH, NSF (day 3); LMA, AOF (day 4); LDB (day 5)
Paroxetine	12	Once-daily	Males n=14 (males from pilot exp., n=8 additional)	EPM, HCA (day 2)
Paroxetine	12	Once-daily	Males n=12	EPM, HCA (day 5)
Paroxetine	7	Once-daily	Males n=12	EPM, HCA (day 2)
Paroxetine	28	Once-daily	Males n=12	EPM, HCA (day 2); NSF, NIH (day 3); LMA, AOF (day 4); LDB (day 5)
Paroxetine	12	Twice-daily	Males n=12	EPM (day 2), LMA, AOF (day 3)
Citalopram	12	Twice-daily	Males n=18††	EPM (day 2), LMA, AOF (day 3)
Venlafaxine	12	Twice-daily	Males n=12	EPM, HCA (day 2)

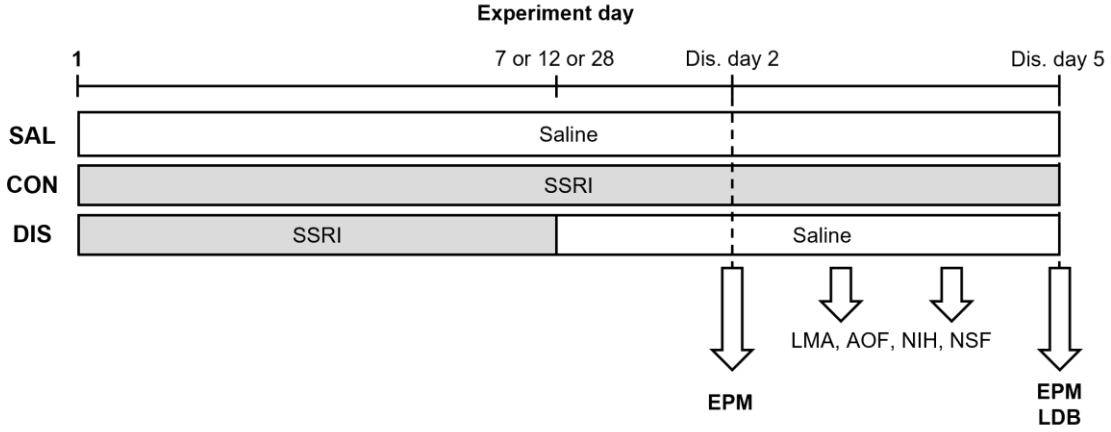


Figure 3.1 Experimental design. Saline (SAL), continuation (CON), discontinuation (DIS). Note, the tests used and the order in which they were conducted varied according to the experiment but were constant between animals within an experiment (see *Table 3.1* for details).

2.4 Behavioural tests

Mice were habituated to a temporary holding room adjacent to the testing room for at least 1 h before testing and on the three days preceding behavioural testing. All testing was conducted in the light phase (10:00 – 17:00) and blind to treatment.

Behavioural tests were conducted as in Chapter 2 section 4. Briefly, in the afternoon of discontinuation day two or five, anxiety-like behaviour was assessed using the EPM for 5 min. LMA and digging events were also tracked in a clean homecage for 3 min at least 1 h before testing on the EPM. Spontaneous LMA was assessed using infrared beam break boxes for 60 min on discontinuation day three for the 12 and 28 once-daily paroxetine experiments, and day four for the twice-daily paroxetine and citalopram experiments. NIH (max. 6 min) and NSF (max. 10 min) were applied following overnight food deprivation on discontinuation day three after 12 and 28 days of paroxetine treatment. In the 12 days once-daily paroxetine experiment, NIH was performed in the morning then NSF at least 1 h later. In the 28 days paroxetine experiment, NSF was performed in the morning then NIH at least 1 h later. Anxiety-like behaviour was also measured using the AOF (5 or 10 min) and LDB (10 min) on discontinuation day three to five. Weight data are included in Appendix 1 unless otherwise stated.

2.5 Refinements

Throughout the experiments, mouse stress levels were assessed using a scale adapted from Stuart and Robinson (2015). Coat state was also assessed at each injection to detect any deterioration in animal wellbeing, or physical injury or irritation, arising from repeated injections. Due to limited evidence of stress or coat state deterioration in any of the mice, these data were not analysed statistically.

2.6 Statistical analysis

D'Agostino-Pearson's test for normality was applied to all data sets. If data were normally distributed, then one-way ANOVA with post-hoc Fisher's LSD was used to compare between treatment conditions. If the data were non-parametric, or sample sizes were small ($n \leq 6$ per group as in the pilot study), then Kruskal Wallis with Uncorrected Dunn's test was used. Repeated measures ANOVA with post-hoc Bonferroni's test was used to analyse body weight over time and horizontal LMA. Weight change during the discontinuation period was calculated as the numerical change in weight from discontinuation day two or five and the final day of paroxetine treatment (day 12). Data were analysed with GraphPad Prism (v8), except for the weight and horizontal LMA data, which were analysed in IBM SPSS Statistics. In the 12 day once-daily paroxetine pilot experiment, sex was also assessed as a co-factor using IBM SPSS Statistics. Parametric data are presented as mean ± SEM values, and non-parametric data are presented as median ± IQR. P values of $p<0.05$ were considered statistically significant. All analyses were performed blind to treatment.

3 Results

3.1 Effect of discontinuation from 12 days of once-daily paroxetine treatment in male and female mice

An exploratory pilot experiment (6 males, 5-6 females per group) tested the effect of discontinuation from 12 days of once-daily paroxetine treatment on anxiety-like behaviour in mice. Two-way ANOVA revealed a main effect of treatment for the time mice spent in the open arms of the EPM on discontinuation day two ($F(2,29)=9.050$, $p=0.0009$; *Fig. 3.2A*). There was also a significant sex effect ($F(1,29)=16.85$, $p=0.0003$) and a treatment*sex interaction ($F(2,29)=6.357$, $p=0.0051$), so henceforth analyses were conducted on male and female mice separately.

In male mice on discontinuation day two, paroxetine discontinuation increased anxiety-like behaviour on the EPM compared to saline controls but not continued paroxetine treatment. Paroxetine continuation and discontinuation reduced the time spent in and the number of entries to the open arms compared to saline controls (time: $H(2)=9.789$, $p=0.0027$, post-hoc SAL vs CON $p=0.0309$, SAL vs DIS $p=0.0030$; *Fig 3.2A;* entries: $H(2)=11.55$, $p=0.0005$, post-hoc SAL vs CON $p=0.0285$, SAL vs DIS $p=0.0009$; *Fig 3.2B*). Paroxetine discontinuation, but not continuation, increased the latency to enter the open arms compared to saline controls ($H(2)=6.327$, $p=0.0360$, SAL vs DIS $p=0.0157$; *Fig. 3.2C*). Paroxetine continuation and discontinuation reduced the number of head dips compared to saline controls ($H(2)=10.56$, $p=0.0013$, post-hoc SAL vs CON $p=0.0481$, SAL vs DIS $p=0.0013$; *Fig 3.2D*). Paroxetine discontinuation, but not continuation, reduced the distance travelled on the EPM compared to saline controls ($H(2)=10.96$, $p=0.0009$, post-hoc SAL vs DIS $p=0.0001$; *Fig 3.2E*).

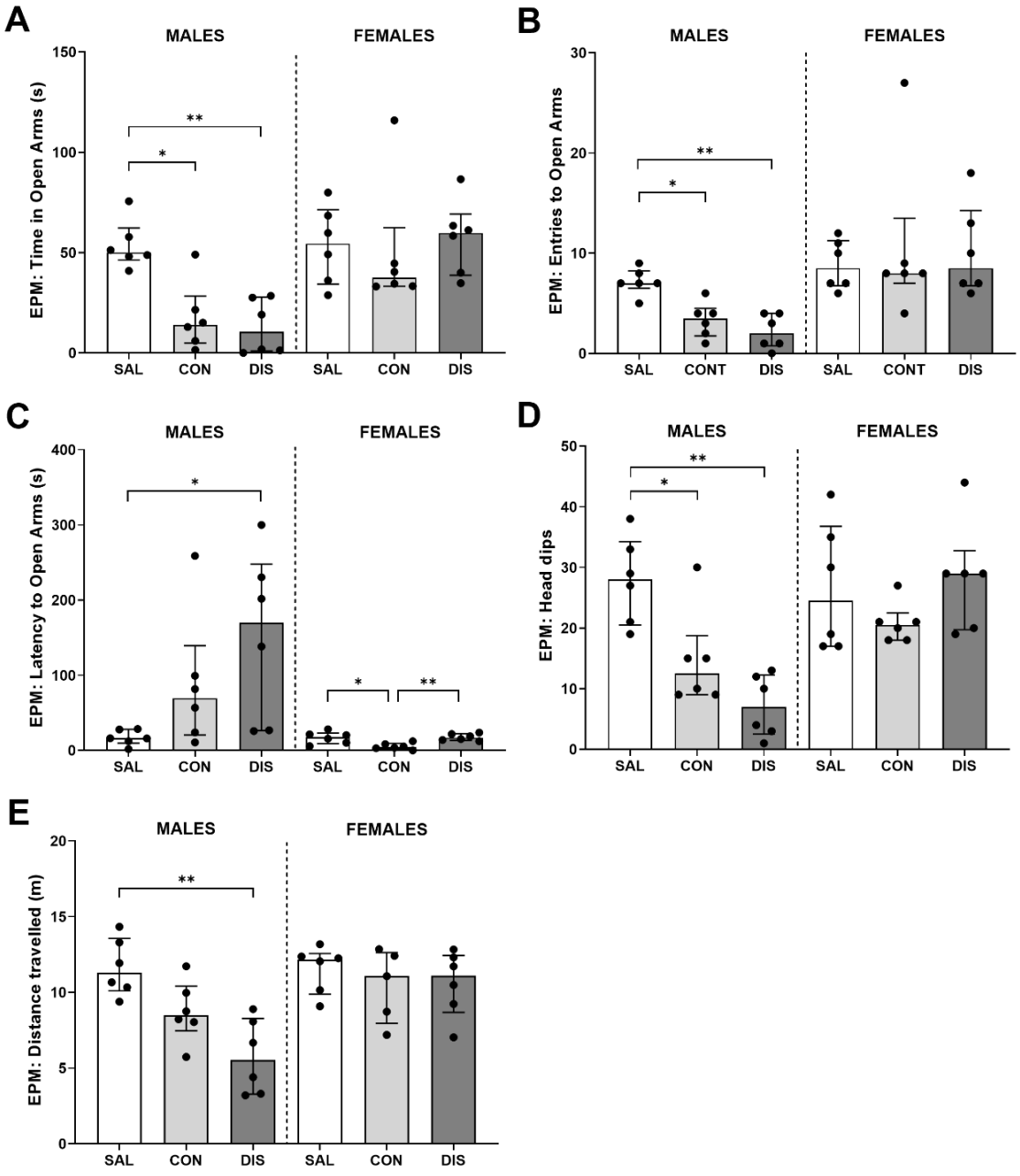


Figure 3.2 Effect of two days discontinuation from 12 days of once-daily paroxetine treatment in male and female mice on the EPM. Bars represent the median ± IQR values for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (males n=6, females n=6), CON (males n=6, females n=5), DIS (males n=6, females n=6). Individual values are indicated by dots. Kruskal-Wallis with post-hoc Uncorrected Dunn's test, * $p<0.05$, ** $p<0.01$.

In female mice, however, paroxetine discontinuation was not associated with changes in anxiety-like behaviour. There were no effects of treatment on time spent in or

the number of entries to the open arms (time: H(2)=4.171, p=0.1218; *Fig. 3.2A*; entries: H(2)=0.4613, p=0.8097; *Fig. 3.2B*). Paroxetine continuation reduced the latency to enter the open arms compared to saline controls (H(2)=6.264, p=0.0341, post-hoc SAL vs CON p=0.0355), which reversed to saline levels following discontinuation (CON vs DIS p=0.0189; *Fig. 3.2C*). There was no effect of treatment on the number of head dips or the distance travelled on the EPM (head dips: H(2)=1.921, p=0.4045; *Fig. 3.2D*; distance: H(2)=0.5150, p=0.7926; *Fig. 3.2E*).

On discontinuation day two, there was no evidence of the behaviours characteristic of serotonin syndrome in either male or female mice, hence data were not analysed.

On discontinuation day three, paroxetine continuation increased the latency of male mice to start eating in the NIH test, which reversed to saline levels following discontinuation (*Table 3.2*; post-hoc SAL vs CON p=0.0350, CON vs DIS p=0.0350).

Table 3.2 Effect of three days discontinuation from 12 days once-daily paroxetine treatment in male and female mice on the NIH and NSF. Max. 360 s for NIH, 600 s for NSF. Mice were food deprived and weighed on the morning of testing. Each value is a median and IQR [25th percentile, 75th percentile]. Bold values indicate significant effects of treatment. * p<0.05 SAL vs CON, # p<0.05 CON vs DIS, n=5-6/group.

	MALES				FEMALES			
	Group (median [25th,75th])			Kruskal	Group (median [25th,75th])			Kruskal
Parameter	**SAL**	**CON**	**DIS**	**Wallis**	**SAL**	**CON**	**DIS**	**Wallis**
NIH								
Δ Weight %	-3.6 [-5.4, -18]	-2.8 [-4.5, -1.2]	-3.5 [-5.5, -1.2]	H(2)=0.8889 p=0.6644	-1.2 [-6.9, 2.6]	-2.8 [-5.0, -0.9]	-1.8 [-6.0, 0.1]	H(2)=0.9240 p=0.6522
Latency to eat (s)	48.9 [11.9, 138.8]	180.0 [139.9, 323.3] *	70.3 [26.9, 175.7] #	**H(2)=7.731 p=0.0139**	195.9 [105.2, 240.4]	131.9 [75.9, 215.3]	122.2 [30.3, 201.3]	H(2)=0.2924 p=0.8808
NSF								
Latency to eat (s)	274.1 [175.4, 330.6]	307.0 [193.6,322.3]	293.5 [93.1, 419.7]	H(2)=0.0818 p=0.9821	251.6 [111.3, 350.7]	243.7 [83.4, 507.9]	223.4 [88.9, 341.2]	H(2)=0.1053 p=0.9549

There was no effect of treatment on the latency to start eating on the NSF test in male mice, and no effects on either test in female mice (*Table 3.2*). There was no effect

of treatment on overnight weight loss due to food deprivation in either male or female mice (*Table 3.2*).

In the morning of discontinuation day four, neither paroxetine continuation nor discontinuation altered spontaneous LMA in male or female mice. There was no effect of treatment on the number of horizontal beam breaks in either sex (male: $F(2,15)=1.692$, 0.217; *Fig 3.3A*; female: $F(2,14)=2.669$, $p=0.102$; *Fig 3.3B*). The number of horizontal beam breaks decreased over time in both sexes (effect of time, males: $F(11,165)=21.114$, $p<0.001$; females: $F(11,154)=17.876$, $p<0.001$), but there were no treatment*time interactions (males: $F(22,165)=0.998$, $p=0.469$; females: $F(22,154)=1.232$, $p=0.228$). There were also no effects of treatment on the number of rearings (vertical beam breaks) in male or female mice (*Table 3.3*).

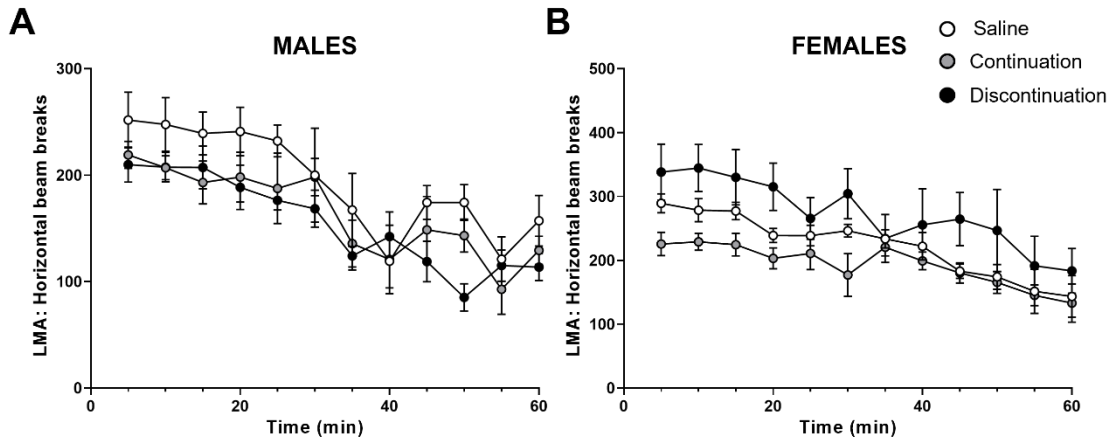


Figure 3.3 Effect of four days discontinuation from 12 days of once-daily paroxetine treatment in male and female mice on LMA. Points represent the mean ± SEM values for the number of horizontal beam breaks over time in male (A) and female (B) mice. 60 min LMA assessment. Saline (males n=6, females n=6), Continuation (males n=6, females n=5), Discontinuation (males n=6, females n=6). Repeated measures ANOVA with post-hoc Bonferroni's test.

Table 3.3 Effect of four days discontinuation from 12 days once-daily paroxetine treatment in male and female mice on the number of vertical beam breaks in LMA testing. 60 min LMA assessment. Each value is a median and IQR [25th percentile, 75th percentile]. N=5-6/group.

	MALES				FEMALES			
	Group (median [25th,75th])			Kruskal	Group (median [25th,75th])			Kruskal
Parameter	**SAL**	**CON**	**DIS**	**Wallis**	**SAL**	**CON**	**DIS**	**Wallis**
Vertical beam breaks	181.5 [148.0, 204.5]	170.5 [146.0, 180.5]	199.0 [180.0, 200.0]	H(2)=2.8538 p=0.2507	171.5 [124.8, 190.0]	181.0 [156.8,204.5]	202.0 [163.0, 233.8]	H(2)=1.556 p=0.4842

In the afternoon of discontinuation day four, neither paroxetine continuation nor discontinuation altered anxiety-like behaviour on the AOF. There were no effects of treatment on centre zone time or distance travelled in male or female mice (*Table 3.4*).

Table 3.4 Effect of four days discontinuation from 12 days once-daily paroxetine treatment in male and female mice on the AOF. 10 min testing on the AOF. Each value is a median and IQR [25th percentile, 75th percentile]. N=5-6/group.

	MALES				FEMALES			
	Group (median [25th,75th])			Kruskal	Group (median [25th,75th])			Kruskal
Parameter	**SAL**	**CON**	**DIS**	**Wallis**	**SAL**	**CON**	**DIS**	**Wallis**
Time in centre (s)	14.7 [7.4, 19.4]	18.6 [4.4, 46.4]	10.0 [5.1, 16.3]	H(2)=0.7368 p=0.7150	9.9 [8.1, 14.6]	9.2 [7.6, 23.7]	13.8 [8.4, 43.9]	H(2)=0.9240 p=0.6522
Distance travelled (m)	46.4 [41.6, 61.2]	37.0 [32.3, 64.0]	45.5 [36.1, 53.0]	H(2)=3.591 p=0.1699	58.1 [50.1, 67.7]	53.0 [39.4, 66.0]	67.0 [38.1, 107.7]	H(2)=0.9468 p=0.6401

On discontinuation day five, paroxetine continuation and discontinuation increased anxiety-like behaviour on the LDB in male mice, but paroxetine discontinuation produced a small anxiolytic effect in female mice. In male mice, paroxetine continuation and discontinuation reduced time spent in the light zone and distance travelled in the LDB compared to saline controls (time: H(2)=8.667, p=0.0069, post-hoc SAL vs CON p=0.0058, SAL vs DIS p=0.0231; *Fig. 3.4A*; distance: H(2)=10.15, p=0.0021, post-hoc SAL vs CON p=0.0069, SAL vs DIS p=0.0049; *Fig. 3.4B*). In female mice, paroxetine discontinuation increased time spent in the light zone compared to paroxetine

continuation but neither paroxetine continuation nor discontinuation was significantly different from saline controls (H(2)=6.538, p=0.0304, post-hoc CON vs DIS p=0.0011), (*Fig. 3.4A*). There was no effect of treatment on the distance travelled in the LDB by female mice (H(2)=1.064, p=0.6226; *Fig. 3.4B*).

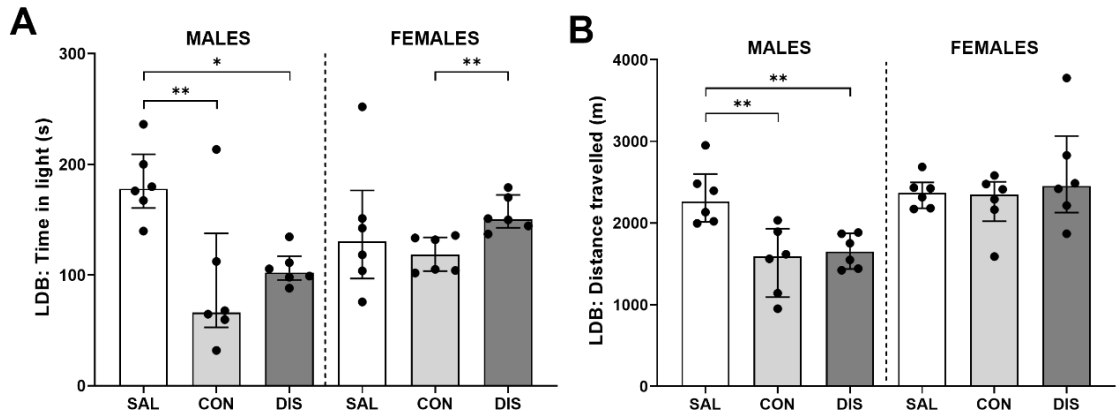


Figure 3.4 Effect of five days discontinuation from 12 days of once-daily paroxetine treatment in male and female mice on the LDB. Bars represent the median ± IQR values for time spent in the light zone (A) and distance travelled on the LDB (B). 10 min testing on the LDB. SAL (males n=6, females n=6), CON (males n=6, females n=5), DIS (males n=6, females n=6). Individual values are indicated by dots. Kruskal-Wallis with post-hoc Uncorrected Dunn's test, * $p<0.05$, ** $p<0.01$.

Neither paroxetine continuation nor discontinuation significantly altered the weight of male or female mice during the experiment (*Suppl. Fig. 1.1A-B*).

In summary, this pilot study found that two days of discontinuation from 12 days of once-daily paroxetine treatment increased anxiety-like behaviour on the EPM in male mice compared to saline controls. Continued paroxetine treatment also increased anxiety-like behaviour. These effects were not present in female mice, and there was no effect of discontinuation from 12 days of once-daily paroxetine on spontaneous LMA in either sex. There were also no effects of paroxetine discontinuation on anxiety tests performed later in the discontinuation period.

3.2 Effect of two days discontinuation from 12 days of once-daily paroxetine treatment in male mice

To increase the power of the exploratory study, the EPM was repeated in a separate group of male mice (n=8/group additional mice to generate n=14/group total). The combined data confirmed that two days of paroxetine discontinuation increased anxiety-like behaviour on the EPM. Paroxetine continuation and discontinuation both reduced the time spent in the open arms compared to saline controls ($F(2,38)=6.830$, $p=0.0029$, post-hoc SAL vs CON $p=0.0101$, SAL vs DIS $p=0.0011$; *Fig. 3.5A*). Paroxetine discontinuation reduced the number of entries to the open arms compared to continued paroxetine and saline controls ($F(2,38)=13.94$, $p<0.0001$, post-hoc SAL vs DIS $p<0.0001$, CON vs DIS $p=0.0349$; *Fig. 3.5B*). Paroxetine continuation also reduced open arm entries compared to saline controls ($p=0.0046$; *Fig. 3.5B*). Paroxetine discontinuation increased the latency to enter the open arms and reduced the number of head dips compared to continued paroxetine and saline controls (latency: $F(2,38)=7.790$, $p=0.0015$, post-hoc SAL vs DIS $p=0.0004$, CON vs DIS $p=0.0201$; *Fig. 3.5C*; head dips: $F(2,38)=3.827$, $p=0.0306$, post-hoc SAL vs DIS $p=0.0033$, CON vs DIS $p=0.0242$; *Fig. 3.5D*). Paroxetine discontinuation reduced the distance travelled on the EPM compared to continued paroxetine and saline controls ($F(2,38)=17.96$, $p<0.0001$, post-hoc SAL vs DIS $p<0.0001$, CON vs DIS $p=0.0006$; *Fig. 3.5E*). Paroxetine continuation also reduced distance travelled compared to saline controls ($p=0.0494$; *Fig. 3.5E*).

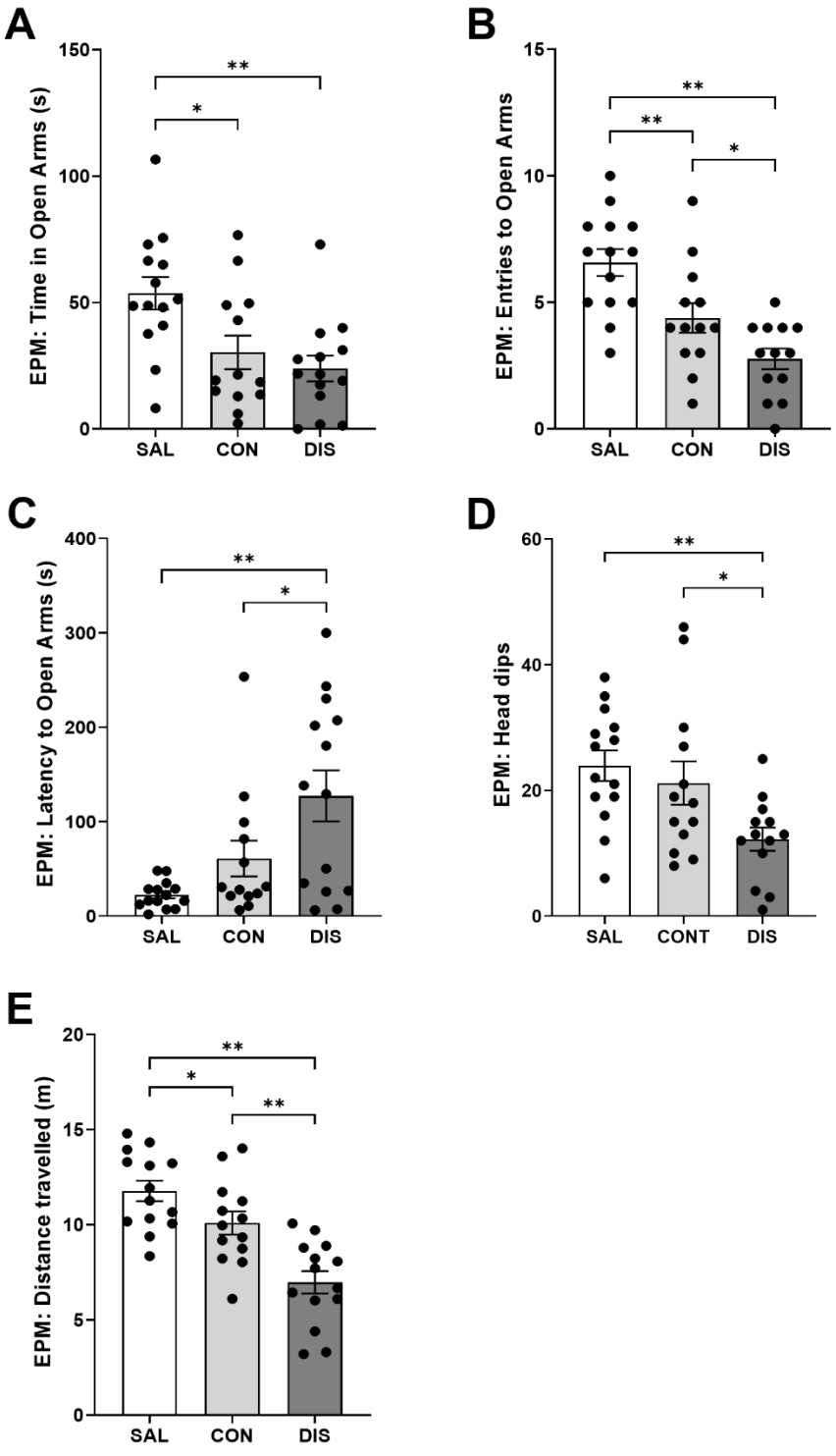


Figure 3.5 Effect of two days discontinuation from 12 days of once-daily paroxetine treatment in male mice on the EPM. Bars represent the mean ± SEM values for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=14), CON (n=13, one mouse excluded due to receiving excessive aggression from another mouse throughout the dosing period), DIS (n=14). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In a clean homecage, paroxetine continuation and discontinuation reduced the distance travelled compared to saline controls (*Table 3.5*; post-hoc SAL vs CON p=0.0052, SAL vs DIS p=0.0125). Paroxetine continuation reduced the number of digging events, but paroxetine discontinuation reversed digging back to saline control levels (*Table 3.*5; post-hoc CON vs DIS p=0.0053).

Table 3.5 Effect of two days discontinuation from 12 days once-daily paroxetine treatment on LMA and digging in a clean homecage. 3 min testing in a homecage. Each value is a mean ± SEM. Bold values indicate significant effects of treatment. * p<0.01 SAL vs CON. # p<0.05 SAL vs DIS, † p<0.01 CON vs DIS. N=11-12/group (one mouse from the continuation group excluded due to receiving excessive aggression from another mouse throughout the dosing period).

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Distance travelled (m)	5.0 ± 0.3	3.4 ± 0.4 *	3.6 ± 0.4 #	**F(2,20)=5.241 p=0.0148**
Number of digging events	9.0 ± 2.9	1.8 ± 0.8	14.9 ± 4.2 †	**F(2,20)=4.243 p=0.0291**

Neither paroxetine continuation nor discontinuation significantly altered the weight of the mice. There was no significant effect of treatment, time, or a treatment*time interaction (treatment: F(2,38)=0.433, p=0.652; time: F(14,532)=67.94, p<0.001, interaction: F(28,532)=0.470, p=0.991; *Fig. 3.6A*). There was also no significant effect of treatment on weight change between the end of repeated paroxetine treatment (day 12) and discontinuation day two (day 14; F(2,38)=0.0129, p=0.9872; *Fig. 3.6B*).

In summary, this combined dataset showed that two days of discontinuation from 12 days of once-daily paroxetine treatment increased anxiety-like behaviour on the EPM in male mice, which was distinct from the anxiogenic effect of continued paroxetine. Although LMA was decreased in the clean homecage during continued and discontinued paroxetine, the differences in EPM performance demonstrated additional behavioural effects of paroxetine discontinuation compared to continued paroxetine treatment.

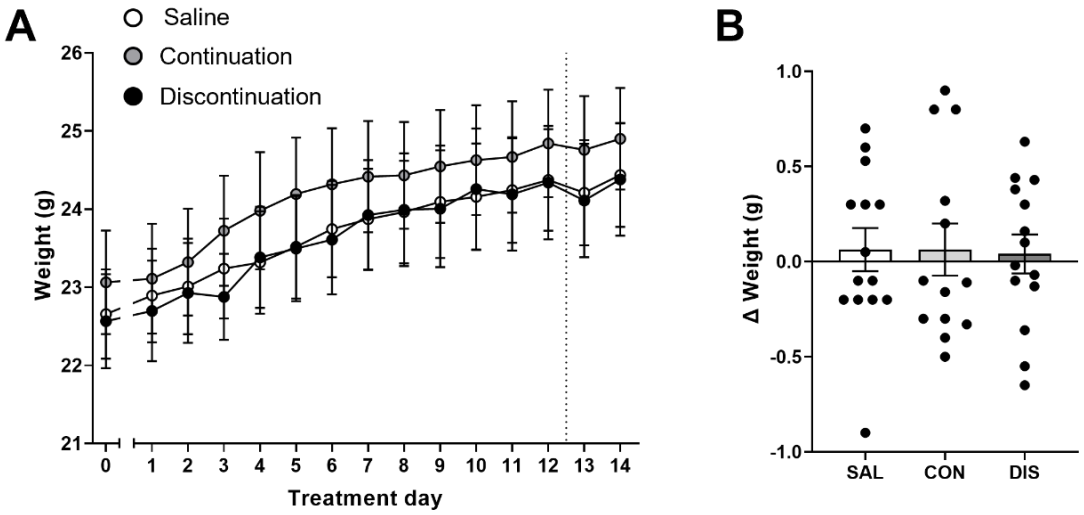


Figure 3.6 Effect of two days discontinuation from 12 days of once-daily paroxetine treatment on the weight of male mice. Points represent the mean ± SEM values for body weight each day (A) and weight change between treatment day 12 and 14 (B). Individual values are indicated by dots, dotted line represents discontinuation. SAL (n=14), CON (n=13), DIS (n=14). Repeated measures ANOVA with post-hoc Bonferroni's test (A) or one-way ANOVA with post-hoc Fisher's LSD (B).

3.3 Effect of five days discontinuation from 12 days of once-daily paroxetine

Despite increased anxiety-like behaviours evident on the EPM on discontinuation day two, the pilot study failed to detect a difference compared to continued paroxetine on the AOF and LDB when they were performed later in the discontinuation period. To determine whether the behavioural effects of paroxetine cessation persisted after day two, male mice were tested on the EPM five days after discontinuation.

On discontinuation day five, there was no evidence of increased anxiety-like behaviour. There were no effects of discontinuation on any EPM parameter (time: $F(2,33)=1.451$, $p=0.2490$; *Fig. 3.7A*; entries: $F(2,33)=1.100$, $p=0.3449$; *Fig. 3.7B*; latency: $H(2)=1.365$, $p=0.5052$; *Fig. 3.7C*; head dips: $F(2,33)=2.422$, $p=0.1043$; *Fig. 3.7D*; distance travelled; $F(2,33)=2.588$, $p=0.0908$; *Fig. 3.7E*). Paroxetine continuation also had no effect on EPM performance compared to saline controls (*Fig. 3.7A-E*).

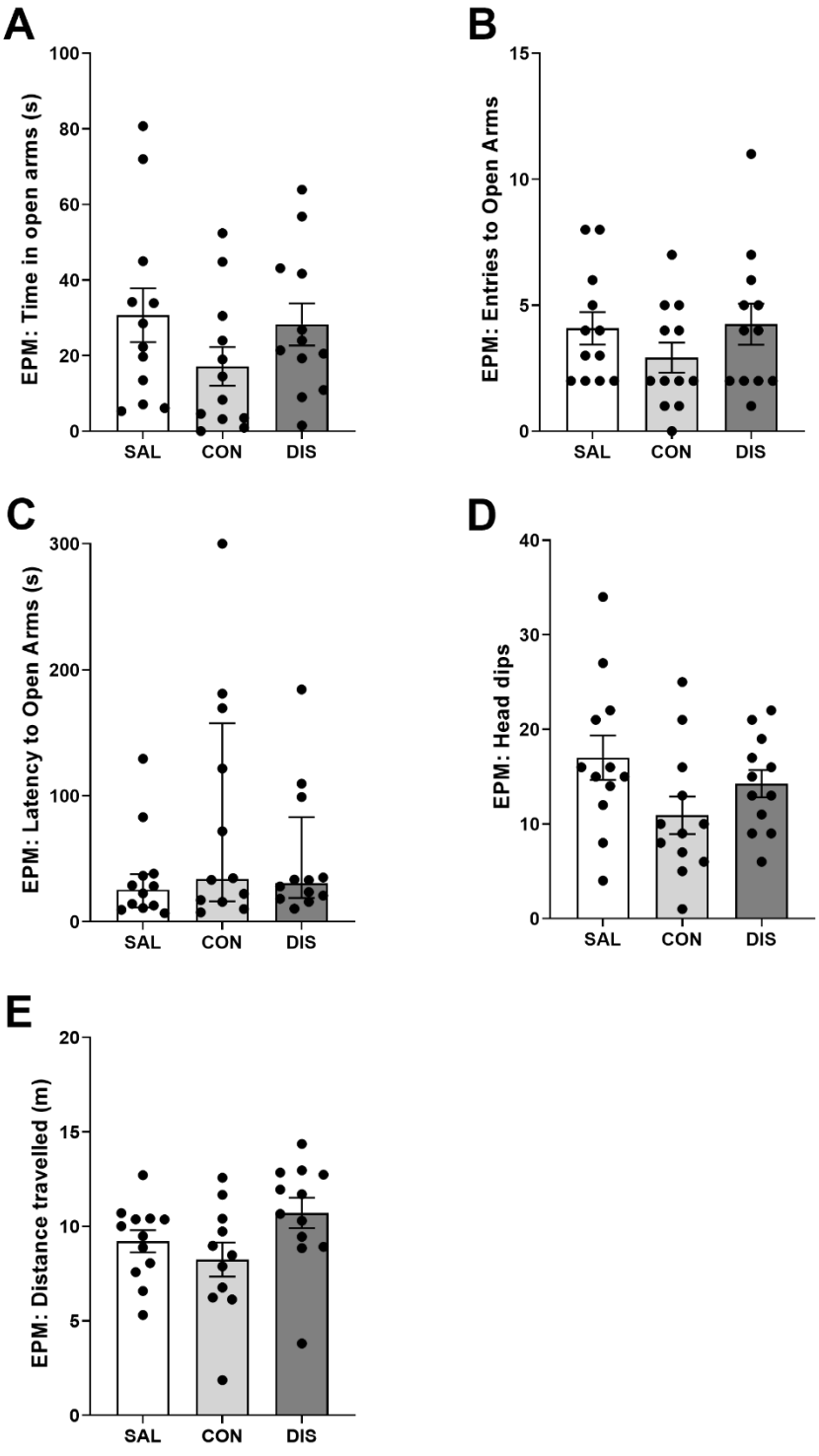


Figure 3.7 Effect of five days discontinuation from 12 days of once-daily paroxetine treatment in male mice on the EPM. 5 min testing on the EPM. Bars represent the mean ± SEM values (A-B, D-E) or median ± IQR (C) for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). SAL (n=12), CON (n=12), DIS (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD (A-B, D-E), Kruskal Wallis with post-hoc Uncorrected Dunn's test (C).

In a clean homecage, there was a trend towards an effect of treatment on distance travelled (*Table 3.6*). Paroxetine continuation reduced distance travelled, but paroxetine discontinuation did not significantly alter LMA compared to continued paroxetine or saline controls (post-hoc SAL vs CON p=0.0263). Paroxetine discontinuation increased the number of digging events compared to continued paroxetine but was not significantly different from saline controls (*Table 3.6*; post-hoc SAL vs DIS p=0.0163).

Table 3.6 Effect of five days discontinuation from 12 days once-daily paroxetine treatment on LMA and digging in a clean homecage. 3 min testing in the homecage. Each value is a mean ± SEM. Bold values indicate significant effects of treatment. * p<0.05 SAL vs CON, † p<0.05 CON vs DIS. N=12/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Distance travelled (m)	3.7 ± 0.2	3.1 ± 0.2 *	3.6 ± 0.1	F(2,33)=3.117 p=0.0576
Number of digging events	9.3 ± 2.8	2.8 ± 1.0	11.8 ± 2.8 †	**F(2,33)=3.369 p=0.0474**

Paroxetine continuation increased weight gain compared to saline controls, whereas paroxetine discontinuation reduced weight gain. There was a trend towards an effect of treatment (F(2,33)=3.035, p=0.0616), and a significant effect of time and a treatment*time interaction (time: F(17,561)=220.4, p<0.001; interaction F(34,561)=3.676, p<0.001; *Fig. 3.8A*). Between-subject pairwise comparisons showed that paroxetine treatment increased weight compared to saline controls from treatment days 10 to 17. This was also significantly greater than paroxetine discontinuation on treatment days 15 to 17 (*Fig. 3.8BA*). Paroxetine discontinuation reduced weight gain from the end of repeated paroxetine treatment to discontinuation day five (day 17) compared to continued paroxetine and saline controls (F(2,33)=10.01, p=0.0004, post-hoc SAL vs DIS p=0.0011, CON vs DIS p=0.0002; *Fig. 3.8B*).

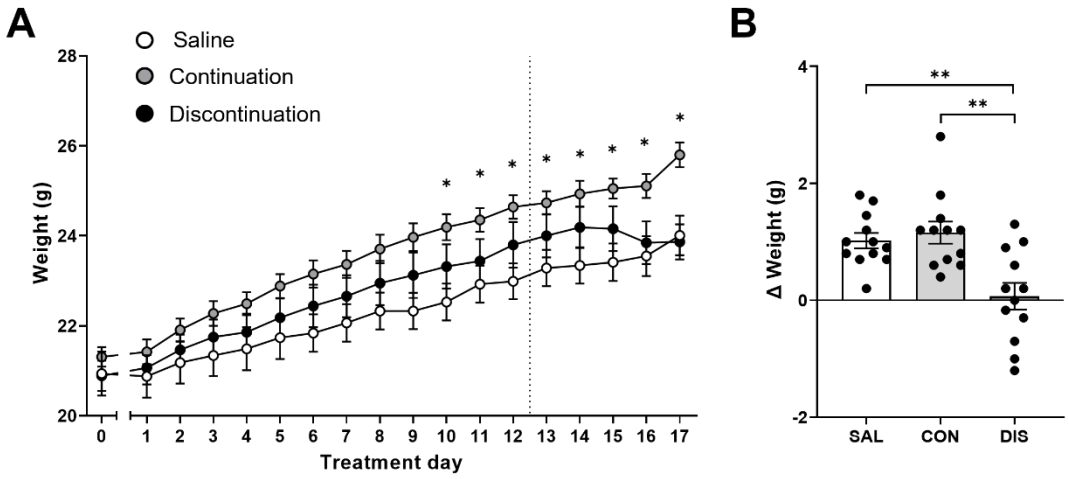


Figure 3.8 Effect of five days discontinuation from 12 days of once-daily paroxetine treatment on the weight of male mice. Points represent the mean ± SEM values for body weight each day (A) and weight change between treatment day 12 and 17 (B). Individual values are indicated by dots, dotted line represents discontinuation. SAL (n=12), CON (n=12), DIS (n=12). Repeated measures ANOVA with post-hoc Bonferroni's test (A) or one-way ANOVA with post-hoc Fisher's LSD (B), * $p<0.05$, ** $p<0.01$.

In summary, five days of discontinuation from 12 days of once-daily treatment paroxetine was not associated with increased anxiety-like behaviour on the EPM.

3.4 Effect of discontinuation from seven days of once-daily paroxetine

To investigate the duration of treatment required to produce discontinuation effects, mice received once-daily injections of paroxetine for just seven days.

On discontinuation day two, paroxetine discontinuation was not associated with increased anxiety-like behaviour on the EPM. There were no effects of discontinuation on any EPM parameter (time: $F(2,33)=0.1256$, $p=0.8824$; *Fig. 3.9A*; entries: $F(2,33)=0.6936$, $p=0.5069$; *Fig. 3.9B*; latency: $H(2)=1.610$, $p=0.4471$; *Fig. 3.9C*; head dips: $F(2,33)=0.0048$, $p=0.9952$; *Fig. 3.9D*; distance travelled: $F(2,33)=1.262$, $p=0.2964$; *Fig. 3.9E*). There were also no effects of paroxetine continuation on EPM performance (*Fig. 3.9A-E*).

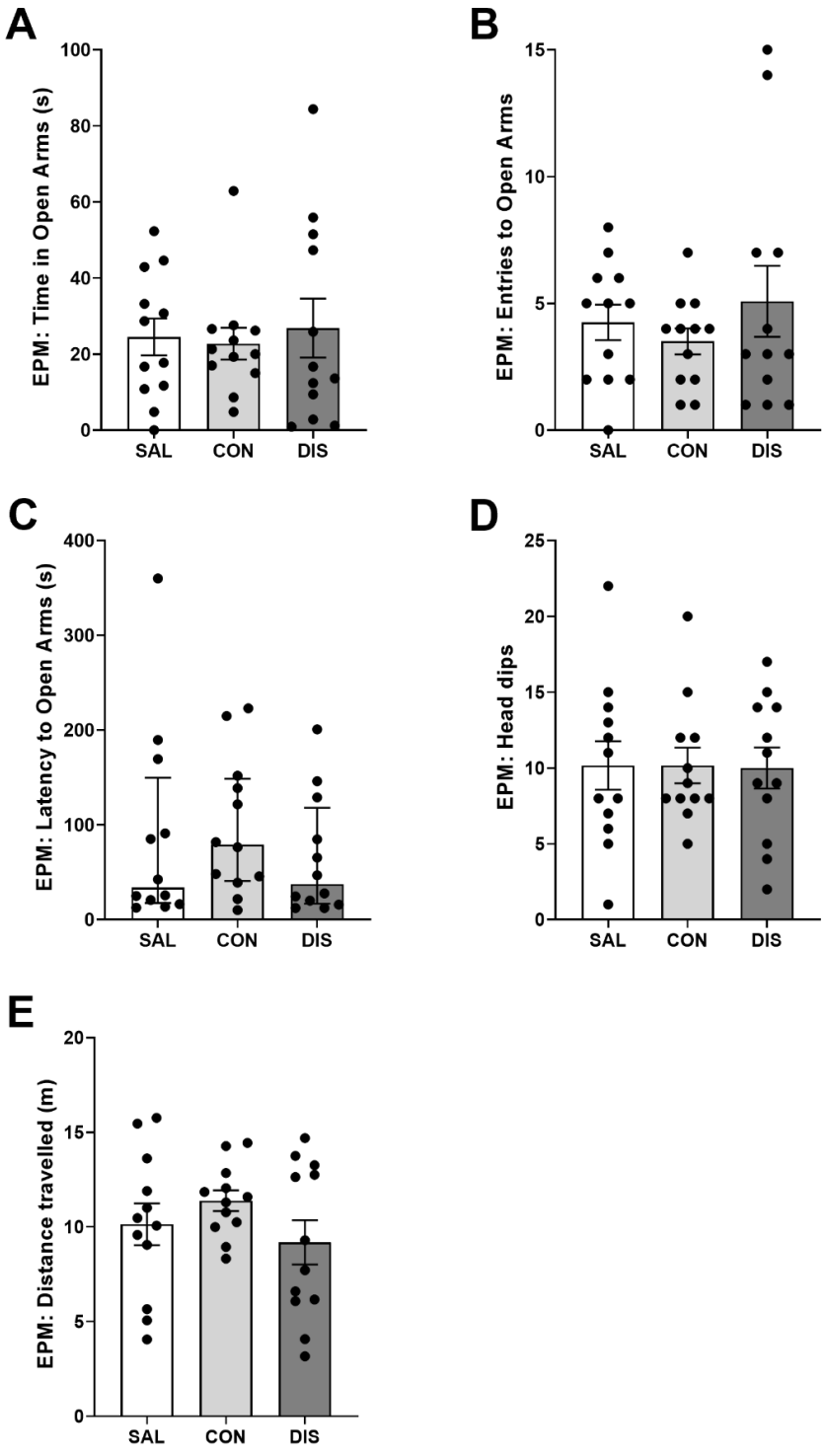


Figure 3.9 Effect of two days discontinuation from seven days of once-daily paroxetine treatment on the EPM. Bars represent the mean ± SEM values (A-B, D-E) or median ± IQR (C) for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=12), CON (n=12), DIS (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD (A-B,D-E) or Kruskal Wallis with post-hoc Uncorrected Dunn's test (C).

There were also no effects of paroxetine continuation or discontinuation on LMA or digging events in a clean homecage (*Table 3.7*). Neither treatment significantly altered weight gain (*Suppl. Fig. 1.2*).

Table 3.7 Effect of two days discontinuation from 7 days paroxetine treatment on LMA and digging in a clean homecage. 3 min testing in a homecage. Each value is a mean ± SEM, N=12/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Distance travelled (m)	4.2 ± 0.3	3.9 ± 0.2	3.2 ± 0.3	F(2,33)=2.063 p=0.1602
Number of digging events	8.4 ± 2.7	8.3 ± 2.1	4.5 ± 2.0	F(2,33)=0.9901 p=0.3823

In summary, neither seven days of paroxetine treatment nor its discontinuation increased anxiety-like behaviour in mice.

3.5 Effect of discontinuation from 28 days of once-daily paroxetine

To determine whether a longer period of treatment produced a greater discontinuation effect, male mice received once-daily injections of paroxetine for 28 days before discontinuation.

On discontinuation day two, paroxetine discontinuation increased anxiety-like behaviour on the EPM but this was only to a slightly greater extent than continued paroxetine treatment. Paroxetine discontinuation reduced time spent in the open arms compared to paroxetine continuation and saline controls ($F(2,29)=6.649$, $p=0.0042$, post-hoc SAL vs DIS $p=0.0011$, CON vs DIS $p=0.0495$; *Fig. 3.10A*). Paroxetine continuation and discontinuation reduced the number of entries to the open arms, increased the latency to enter the open arms, reduced the number of head dips and reduced distance travelled compared to saline controls but were not significantly different from each other (time: $F(2,29)=11.19$, $p=0.0002$, post-hoc SAL vs CON $p=0.0016$, SAL vs DIS $p<0.0001$; *Fig.*

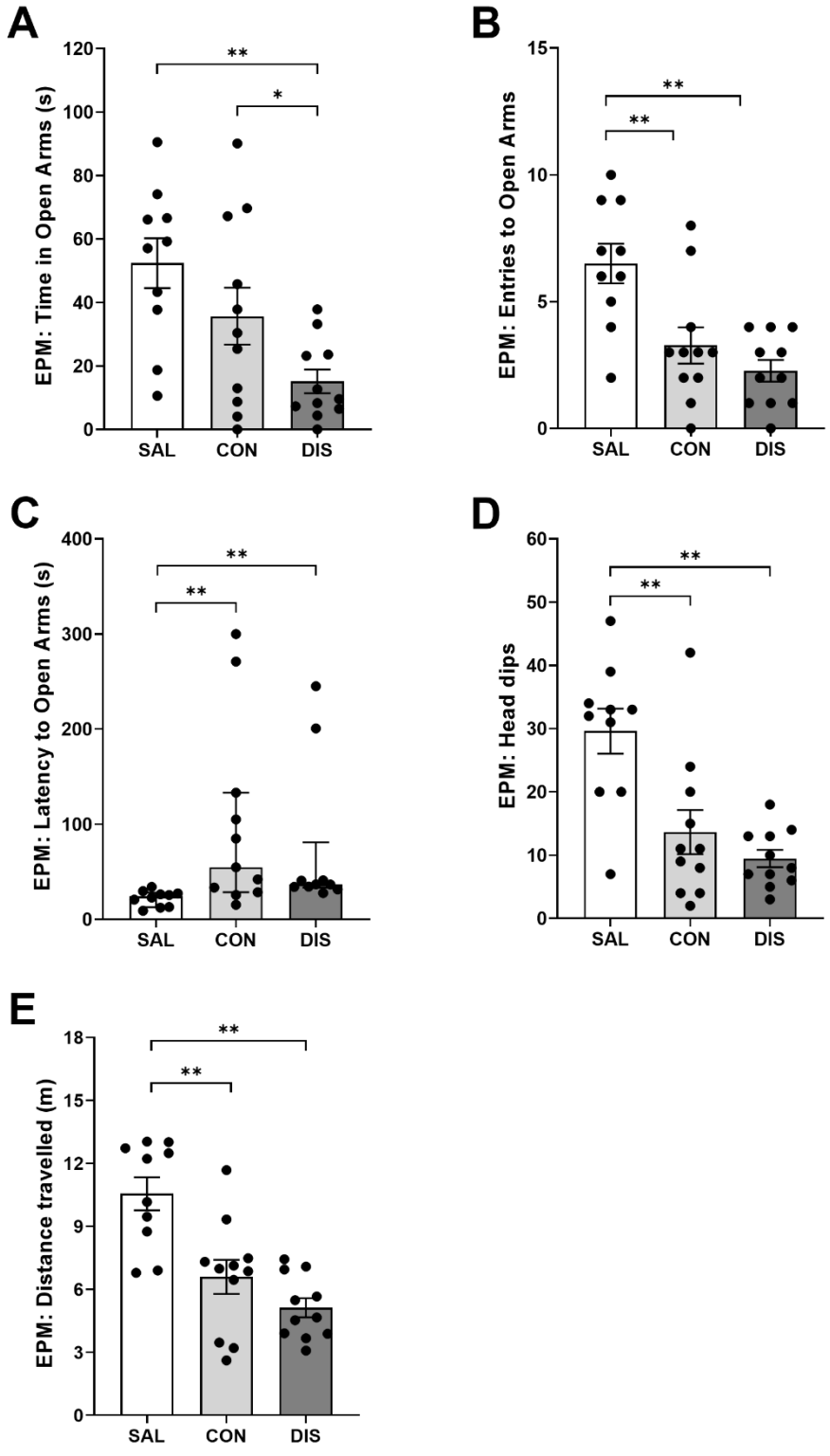


Figure 3.10 Effect of two days discontinuation from 28 days of once-daily paroxetine treatment on the EPM. Bars represent the mean ± SEM values (A-B, D-E) or median ± IQR (C) for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=10, one mouse excluded

due to lighting issues during testing, data from another mouse was lost during recording), CON (n=11, one mouse excluded due to lighting issues during testing), DIS (n=11, one mouse excluded due to lighting issues during testing). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD (A-B, D-E) or Kruskal Wallis with post-hoc Uncorrected Dunn's test (C), * p<0.05, ** p<0.01.

3.10B; entries: F(2,29)=13.38, p=0.0012, post-hoc SAL vs CON p=0.0011, SAL vs DIS p=0.0021; *Fig. 3.10C*; latency: H(2)=13.38, p=0.0012, post-hoc SAL vs CON p=0.0011, SAL vs DIS p=0.0021; head dips: F(2,29)=12.62, p=0.0001, post-hoc SAL vs CON p=0.0007, SAL vs DIS p<0.0001; *Fig. 3.10D*; distance travelled: F(2,29)=15.73, p<0.0001, post-hoc SAL vs CON p=0.0004, SAL vs DIS p<0.0001; *Fig. 3.10E*).

In a clean homecage, both paroxetine continuation and discontinuation reduced distance travelled compared to saline controls (*Table 3.8*; post-hoc SAL vs CON p<0.0001, SAL vs DIS p<0.0001). Paroxetine continuation reduced the number of digging events, which reversed to saline control levels after discontinuation (*Table 3.8*; post-hoc SAL vs CON p=0.0081).

Table 3.8 Effect of two days discontinuation from 28 days paroxetine treatment on LMA and digging in a clean homecage. 3 min testing in a homecage. Each value is a mean ± SEM or median and IQR [25th percentile, 75th percentile]. Bold values indicate significant effects of treatment. * p<0.01 SAL vs CON, † p<0.01 CON vs DIS. N=12/group.

	Group (mean ± SEM, median [25th,75th])			**ANOVA/**
Parameter	**SAL**	**CON**	**DIS**	**Kruskal-Wallis**
Distance travelled (m)	4.6 ± 0.2	3.0 ± 0.3 *	2.5 ± 0.2 #	**F(2,33)=23.93 p<0.0001**
Number of digging events	12.5 [4.8,16.0]	2.0 [0.3,5.3] *	5.5 [1.3,8.8]	**H(2)=7.181 p=0.0276**

On discontinuation day three, paroxetine continuation increased the latency to start eating on the NSF compared to saline controls (*Table 3.9*; post-hoc SAL vs CON p=0.0014), but paroxetine discontinuation did not differ from continued paroxetine or

saline controls (*Table 3.9*). There was no effect of treatment on NIH performance or on overnight weight loss due to food deprivation (*Table 3.9*).

Table 3.9 Effect of three days discontinuation from 28 days once-daily paroxetine treatment on the NSF and NIH. Max. 360 s for NIH, 600 s for NSF. Mice were food deprived overnight and weighed on the morning of testing. Each value is a mean ± SEM (weight) or median and IQR [25th percentile, 75th percentile]. Bold values indicate significant effects of treatment. * p<0.05 SAL vs CON. N=12/group.

	Group (mean ± SEM, median [25th,75th])			**ANOVA/**
Parameter	**SAL**	**CON**	**DIS**	**Kruskal-Wallis**
NSF				
Δ Weight %	-1.3 ± 0.2	-1.2 ± 0.1	-1.1 ± 0.1	F(2,33)=0.5788 p=0.5663
Latency to eat (s)	185.6 [117.6, 292.4]	321.1 [303.0, 483.2]*	311.7 [238.9, 323.1]	**H(2)=10.36 p=0.0056**
NIH				
Latency to eat (s)	20.1 [12.35, 253.5]	24.2 [11.93, 360.0]	122.3 [14.40, 146.7]	H(2)=0.2967 p=0.8621

On the morning of discontinuation day four, paroxetine continuation and discontinuation reduced spontaneous LMA compared to saline controls, but they were not significantly different from each other. Paroxetine continuation and discontinuation reduced the number of horizontal beam breaks compared to saline controls (treatment: F(2,32)=3.872, p=0.032, post-hoc SAL vs CON p=0.025, SAL vs DIS p=0.021). There was a significant decrease in the number of horizontal beam breaks over time (F(11,341)=35.59, p<0.001) but no treatment*time interaction (F(22,341)=0.794, p=0.733; *Fig. 3.11*). There was no effect of treatment on the number of rearings (vertical beam breaks) (*Table 3.10*).

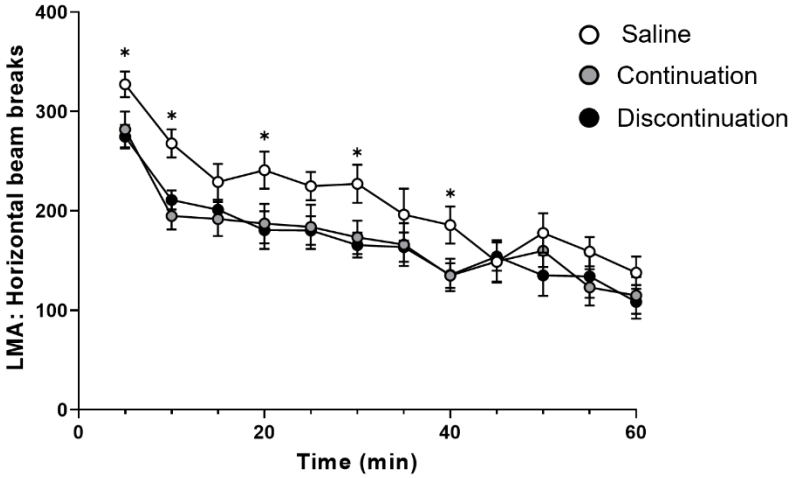


Figure 3.11 Effect of four days discontinuation from 28 days of once-daily paroxetine treatment on LMA. Points represent the mean ± SEM values for the number of horizontal beam breaks over time. Post-hoc analysis showed LMA was significantly lower in the paroxetine continuation group compared to saline controls at 5 ($p=0.032$), 10 ($p<0.001$), 30 ($p=0.028$) and 40 min ($p=0.033$). LMA was also significantly lower in the paroxetine discontinuation group compared to controls at 5 ($p=0.014$), 10 ($p=0.003$), 20 ($p=0.033$), and 30 ($p=0.013$) min. 60 min LMA assessment. Saline (n=12), Continuation (n=11, one mouse excluded due to issues during testing), Discontinuation (n=12). Repeated measures ANOVA with post-hoc Bonferroni's test, * $p<0.05$ between-subject comparisons.

Table 3.10 Effect of four days discontinuation from 28 days paroxetine treatment on the number of vertical beam breaks in LMA testing. 60 min LMA assessment. Each value is a mean ± SEM. N=11-12/group (one mouse excluded from continuation group due to issues during testing).

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Vertical beam breaks	167.2 ± 14.5	181.5 ± 8.7	169.3 ± 15.5	F(2,32)=0.3360 p=0.7172

In the afternoon of discontinuation day four, neither paroxetine continuation nor discontinuation altered centre zone time in the AOF (*Table 3.11*). Nonetheless, both reduced the distance travelled compared to saline controls (*Table 3.11*; post-hoc SAL vs CON $p<0.0001$, SAL vs DIS $p=0.0131$) but they were not significantly different from each other on either parameter.

Table 3.11 Effect of four days discontinuation from 28 days once-daily paroxetine treatment on the AOF. 10 min testing on the AOF. Each value is a mean ± SEM. Bold values indicate significant effects of treatment. * p<0.01 SAL vs CON. # p<0.05 SAL vs DIS. N=12/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Time in centre (s)	11.9 ± 1.0	8.5 ± 1.6	7.8 ± 1.7	F(2,33)=2.327 p=0.1140
Distance travelled (m)	64.6 ± 4.7	40.1 ± 3.0 *	49.8 ± 4.0 #	**F(2,33)=10.01 p<0.0004**

On discontinuation day five, paroxetine continuation and discontinuation increased anxiety-like behaviour in the LDB compared to saline controls but were not significantly different from each other. Paroxetine continuation and discontinuation reduced time in the light zone and the distance travelled in the LDB compared to saline controls (time: F(2,29)=7.468, p=0.0024, post-hoc SAL vs CON p=0.0045, SAL vs DIS p=0.0014; *Fig. 3.12A*; distance travelled: F(2,29)=9.782, p=0.0006, post-hoc SAL vs CON p=0.0007, SAL vs DIS p=0.0006; *Fig. 3.12B*).

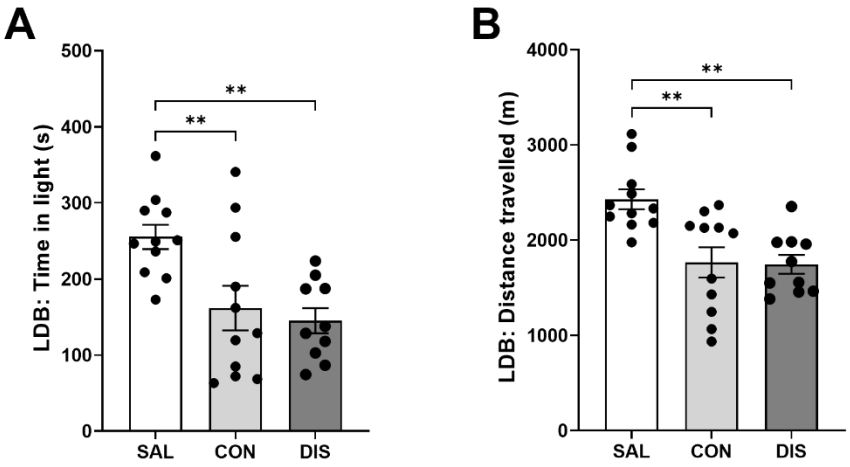


Figure 3.12 Effect of five days discontinuation from 28 days of once-daily paroxetine treatment on the LDB. Bars represent the mean ± SEM values for time spent in the light zone (A) and distance travelled on the LDB (B). 10 min testing on the LDB. SAL (n=11, one mouse excluded due to lighting issues during testing), CON (n=11, one mouse excluded due to lighting issues during testing), DIS (n=10, two mice excluded due to lighting issues during testing). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, ** p<0.01.

Repeated paroxetine treatment increased weight gain during the experiment but paroxetine discontinuation did not differ from saline controls (*Suppl. Fig. 1.3*).

In summary, 28 days of repeated paroxetine treatment and its discontinuation increased anxiety-like behaviours in male mice. Although there was a reduction in the time spent in the open arms of the EPM two days after paroxetine discontinuation compared to continued treatment, this was the only significant difference found between the treatments across all behavioural tests. Moreover, the detection of increased anxiety-like behaviour following paroxetine discontinuation was confounded by a reduction in general LMA. Thus, discontinuation from 28 days of paroxetine treatment did not produce greater anxiogenic effects that discontinuation from 12 days of treatment.

3.6 Effect of discontinuation from 12 days of twice-daily paroxetine

To test whether increasing the frequency of paroxetine treatment (hence increasing overall daily dose) would produce a more pronounced discontinuation effect, mice received twice-daily injections of paroxetine for 12 days.

On discontinuation day two, paroxetine discontinuation increased anxiety-like behaviour on the EPM. Paroxetine continuation and discontinuation reduced time in the open arms compared to saline controls ($F(2,33)=10.83$, $p=0.0002$, post-hoc SAL vs CON $p=0.0143$, SAL vs DIS $p<0.0001$; *Fig. 3.13A*). Paroxetine discontinuation also reduced the number of entries to the open arms compared to saline controls ($F(2,33)=3.482$, $p=0.0424$, post-hoc SAL vs DIS $p=0.0141$; *Fig. 3.13B*). There was no effect of treatment on the latency to enter the open arms ($F(2,33)=2.174$, $p=0.3373$; *Fig 3.13C*). Paroxetine discontinuation reduced the number of head dips and the distance travelled compared to continued paroxetine and saline controls (head dips: $F(2,33)=8.815$, $p=0.0009$, post-hoc

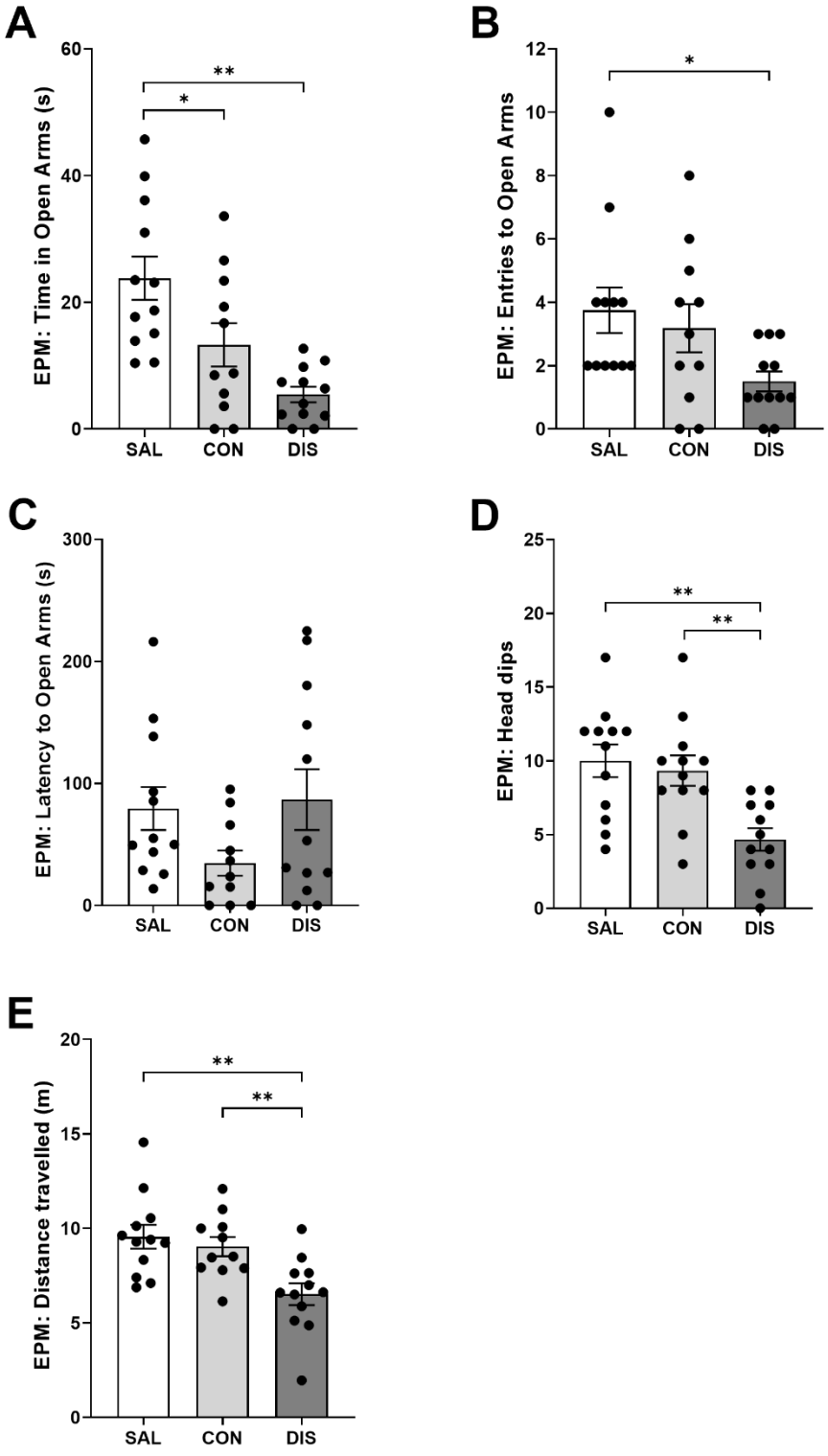


Figure 3.13 Effect of two days discontinuation from 12 days of twice-daily paroxetine treatment on the EPM. Bars represent the mean ± SEM values for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=12), CON (n=12), DIS (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

SAL vs DIS p=0.0005, CON vs DIS p=0.0019; *Fig. 3.13D*; distance travelled: F(2,33)=7.469, p=0.0021, post-hoc SAL vs DIS p=0.0008, CON vs DIS p=0.0086; *Fig. 3.13E*).

In the morning of discontinuation day three, neither paroxetine continuation nor discontinuation altered spontaneous LMA. There was no effect of treatment on horizontal beam breaks (F(2,33)=0.073, p=0.930; *Fig. 3.14*). LMA reduced over time (F(11,363)=54.01, p<0.001), but there was no time*treatment interaction (F(22,363)=1.305, p=0.163). There was no effect of treatment on the number of rearings (vertical beam breaks) (*Table 3.12*).

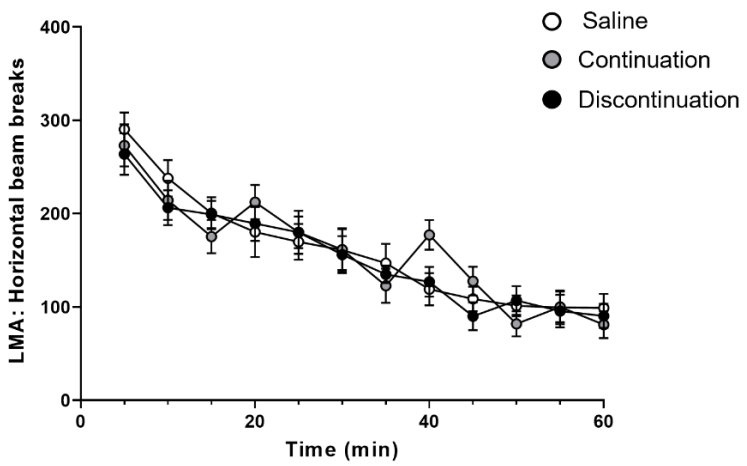


Figure 3.14 Effect of three days discontinuation from 12 days of twice-daily paroxetine treatment on LMA. Points represent the mean ± SEM values for the number of horizontal beam breaks over time. 60 min LMA assessment. Saline (n=12), Continuation (n=12), Discontinuation (n=12). Repeated measures ANOVA with post-hoc Bonferroni's test.

Table 3.12 Effect of three days discontinuation from 12 days twice-daily paroxetine treatment on the number of vertical beam breaks in LMA testing. 60 min LMA assessment. Each value is a mean ± SEM. N=12/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Vertical beam breaks	190.4 ± 10.4	170 ± 10.4	173.1 ± 6.3	F(2,33)=1.293 p=0.2881

In the afternoon of discontinuation day four, paroxetine continuation and discontinuation reduced distance travelled compared to saline controls on the AOF (*Table 3.13*; post-hoc SAL vs CON p=0.0074, SAL vs DIS p=0.0062), but there was no effect of treatment on centre zone time (*Table 3.13*).

Table 3.13 Effect of three days discontinuation from 12 days twice-daily paroxetine treatment on the AOF. 5 min testing on the AOF. Each value is a mean ± SEM. Bold values indicate significant effects of treatment. * p<0.01 SAL vs CON. # p<0.01 SAL vs DIS. N=12/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Time in centre (s)	6.6 ± 0.9	3.8 ± 1.0	5.7 ± 1.0	F(2,33)=2.167 p=0.0797
Distance travelled (m)	28.3 ± 1.8	21.6 ± 1.7 *	21.4 ± 1.5 #	**F(2,33)=5.560 p=0.0083**

Neither paroxetine continuation nor discontinuation significantly altered weight gain during the experiment (*Suppl. Fig. 1.4*).

In summary, two days of discontinuation from 12 days of twice-daily paroxetine treatment increased anxiety-like behaviour on the EPM compared to continued paroxetine and saline controls. Nonetheless, this effect was mainly evident as a decrease in the number of head dips and reduced distance travelled and was not distinct from the anxiogenic effect of continue paroxetine treatment on open arm exploration. Thus, the anxiogenic effect of discontinuation from 12 days of twice-daily paroxetine was not greater than following discontinuation from once-daily treatment.

3.7 Effect of discontinuation from 12 days of twice-daily citalopram

To test the generalisability of the behavioural effects of paroxetine discontinuation to other SSRIs, mice received twice-daily injections of citalopram for 12 days.

On discontinuation day two, citalopram discontinuation increased anxiety-like behaviour in the EPM but only compared to saline controls. There were no effects of treatment on the time spent in or the number of entries to the open arms (time: F(2,51)=0.5349, p=0.5890; *Fig. 3.15A*; entries: F(2,51)=1.751, p=0.1839; *Fig. 3.15B*). Citalopram discontinuation increased the latency to enter the open arms compared to saline controls (H(2)=7.220, p=0.0271, post-hoc SAL vs DIS p=0.0078; *Fig. 3.15C*). There was no treatment effect on the number of head dips, or the distance travelled on the EPM (head dips: F(2,21)=0.2985, p=0.7451; *Fig. 3.15D*; distance travelled: F(2,51)=1.949, p=0.1529; *Fig. 3.15E*). Citalopram continuation did not alter EPM performance compared to saline controls (*Fig. 3.15A-E*).

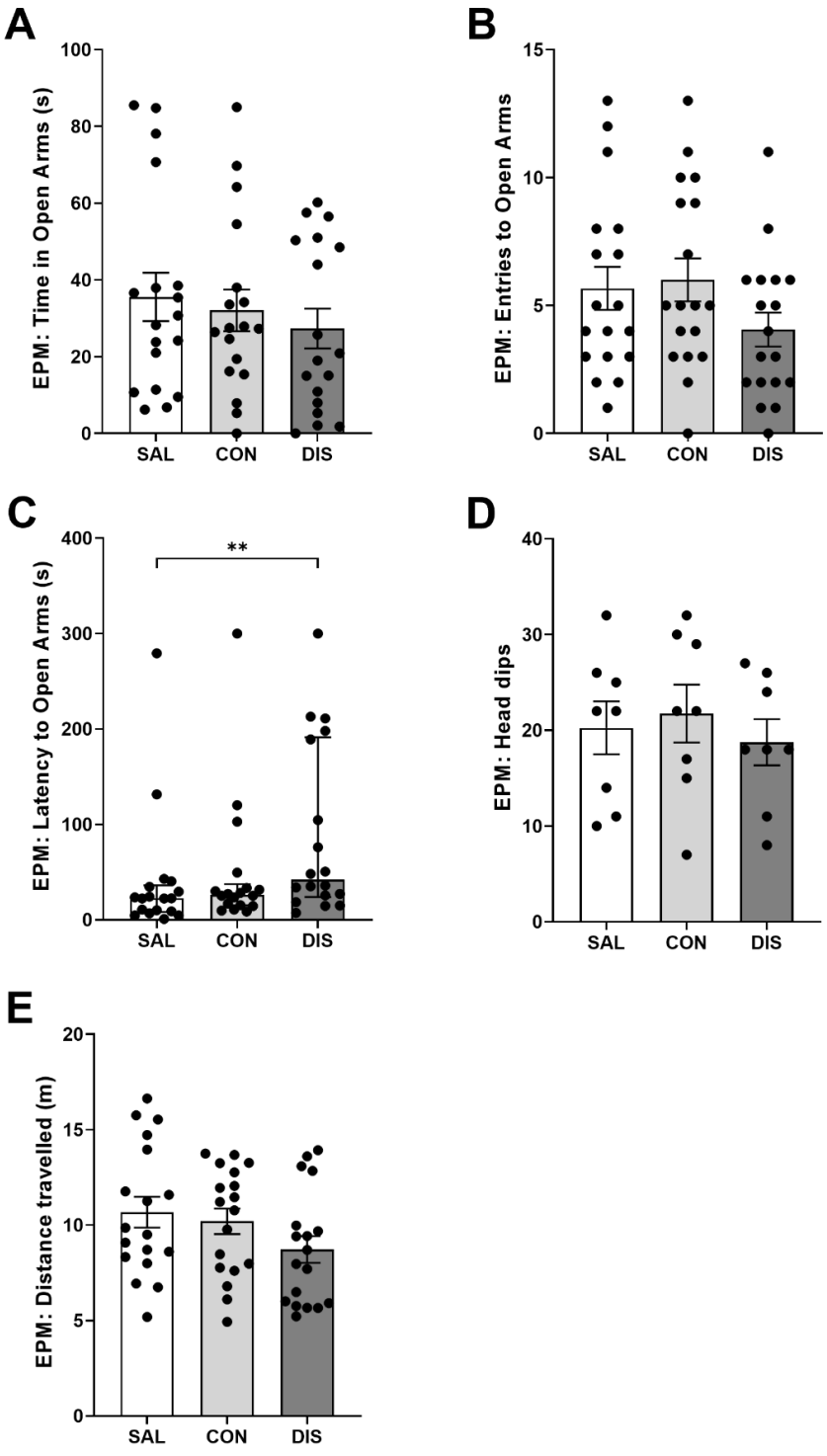


Figure 3.15 Effect of two days discontinuation from 12 days of twice-daily citalopram treatment on the EPM. Bars represent the mean ± SEM values (A-B, D-E) or median ± IQR (C) for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=18), CON (n=18), DIS (n=18), n=8/group for number of head dips (D). Individual values are indicated by dots. One-way ANOVA

with post-hoc Fisher's LSD (A-B, D-E), Kruskal Wallis with post-hoc Uncorrected Dunn's test (C), ** p<0.01.

In the morning of discontinuation day three, neither citalopram continuation nor discontinuation altered spontaneous LMA. There was no effect of treatment on the number of horizontal beam breaks (F(2,27)=0.458, p=0.638; *Fig. 3.16*). LMA reduced over time (F(11,297)=79.21, p<0.001), and there was a time*treatment interaction (F(22,297)=1.1914, p=0.009), but there were no significant differences between the groups at any specific time point. Citalopram continuation reduced the number of rearings (vertical beam breaks) compared to saline controls, but there was no effect of citalopram discontinuation (*Table 3.14*; post-hoc SAL vs CON p<0.0001).

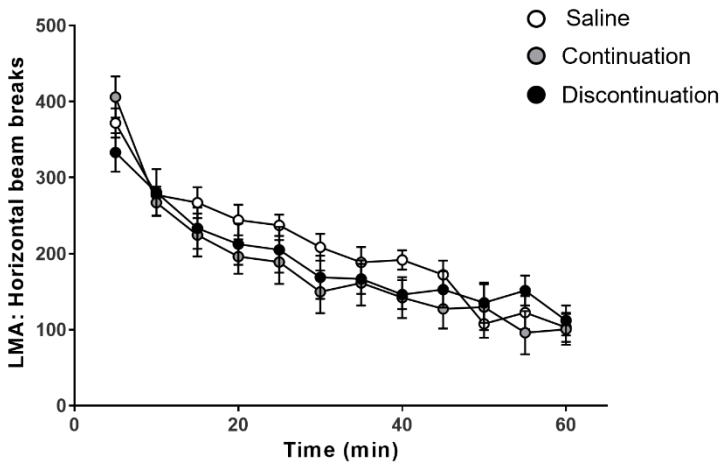


Figure 3.16 Effect of three days discontinuation from 12 days of twice-daily citalopram treatment on LMA. Points represent the mean ± SEM values for the number of horizontal beam breaks over time. 60 min LMA assessment. Saline (n=10), Continuation (n=10), Discontinuation (n=10). Repeated measures ANOVA with post-hoc Bonferroni's test.

Table 3.14 Effect of three days discontinuation from 12 days twice-daily citalopram treatment on the number of vertical beam breaks in LMA testing. 60 min LMA assessment. Each value is a mean ± SEM. Bold values indicate significant effects of treatment. * p<0.001 SAL vs CON. N=10/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Vertical beam breaks	202.3 ± 6.6	156.1 ± 16.2 *	171.4 ± 10.0	**F(2,27)=4.111 p=0.0278**

In the afternoon of discontinuation day three, there were no effects of treatment on the time in the centre zone or distance travelled on the AOF (*Table 3.15*). Neither citalopram continuation nor discontinuation significantly altered the weight of the mice (*Suppl. Fig 1.5*).

Table 3.15 Effect of three days discontinuation from 12 days twice-daily citalopram treatment on the AOF. 5 min testing on the AOF. Each value is a mean ± SEM. N=10/group.

	Group (mean ± SEM)			
Parameter	**SAL**	**CON**	**DIS**	**ANOVA**
Time in centre (s)	9.1 ± 1.2	9.3 ± 1.0	8.9 ± 1.0	F(2,27)=0.4738 p=0.6279
Distance travelled (m)	37.4 ± 3.0	32.3 ± 2.3	32.3 ± 3.1	F(2,27)=0.6227 p=0.5440

In summary, two days of discontinuation from citalopram treatment weakly increased anxiety-like behaviour on the EPM compared to saline controls but was not significantly different from continued treatment. There were no effects on LMA.

3.8 Effect of discontinuation from 12 days of twice-daily venlafaxine

To further test the generalisability of the behavioural effects of paroxetine discontinuation to other antidepressants, mice received twice-daily injections the SNRI venlafaxine for 12 days. On discontinuation day two, venlafaxine discontinuation increased anxiety-like behaviour on the EPM compared to saline controls. Nonetheless, this was indistinguishable from the effect of venlafaxine continuation, which also increased anxiety-like behaviour. Venlafaxine continuation and discontinuation reduced the time spent in the open arms compared to saline controls ($F(2,33)=5.196$, $p=0.0111$, post-hoc SAL vs CON $p=0.0128$, SAL vs DIS $p=0.0064$; *Fig. 3.17A*). There were no significant effects of treatment on the number of entries to or the latency to enter the open arms (entries: $F(2,33)=2.856$, $p=0.0718$; *Fig. 3.17B*; latency: $H(2)=0.4250$, $p=0.8086$;

Fig. 3.17C). Venlafaxine continuation and discontinuation both reduced the number of head dips compared to saline controls (F(2,33)=5.373, p=0.0.0095, post-hoc SAL vs CON p=0.0278, SAL vs DIS p=0.0033; *Fig. 3.17D*), but there was no effect of treatment on the distance travelled (F(2,33)=2.452, p=0.1016; *Fig 3.17E*). Neither venlafaxine continuation nor its discontinuation significantly altered the weight of the mice (*Suppl. Fig. 1.6*).

In summary, two days of discontinuation from 12 days of twice-daily venlafaxine treatment weakly increased anxiety-like behaviour in mice compared to saline controls but this was not distinguishable from the effect of continued venlafaxine administration.

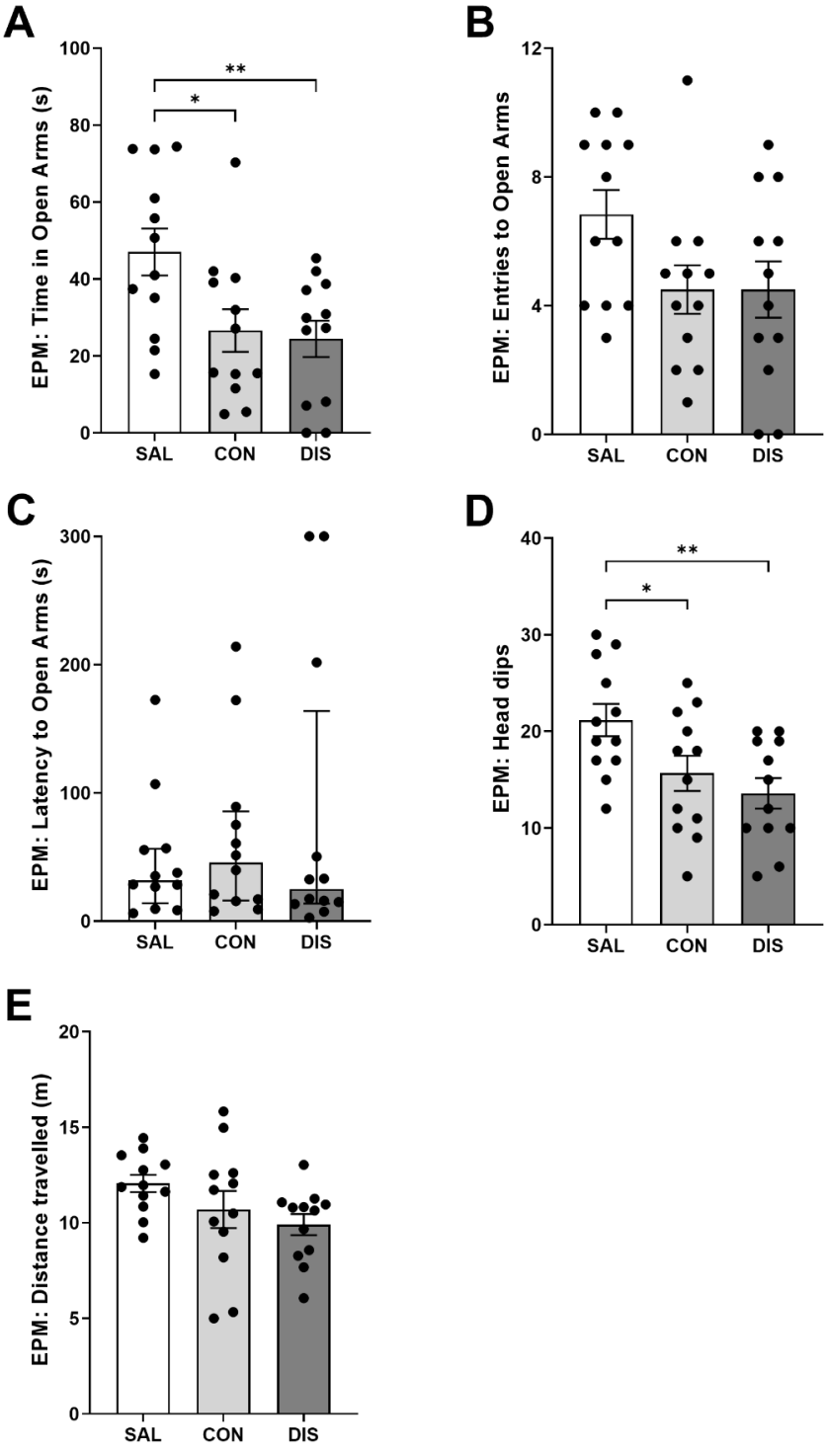


Figure 3.17 Effect of two days discontinuation from 12 days of twice-daily venlafaxine treatment on the EPM. Bars represent the mean ± SEM values (A-B, D-E) or median ± IQR (C) for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL (n=12), CON (n=12), DIS (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD (A-B, D-E), Kruskal Wallis with post-hoc Uncorrected Dunn's test (C), * $p<0.05$, ** $p<0.01$.

4 Discussion

SSRI discontinuation syndrome is typified by symptoms such as heightened anxiety that appear in the first few days after treatment cessation (Davies & Read, 2019). The current experiments investigated the behavioural effects of SSRI discontinuation in mice, and it was hypothesised that SSRI discontinuation would increase anxiety-like behaviour compared to mice receiving continued SSRI treatment.

In a pilot study, male mice exhibited heightened anxiety-like behaviour two days after discontinuation from 12 days of once-daily paroxetine treatment compared to saline-treated controls, although this was not readily distinguishable from continued paroxetine administration. In contrast, female mice showed no evidence of an anxiogenic effect of discontinuation. Increasing the power of the experiment showed that two days after paroxetine discontinuation, male mice exhibited increased anxiety-like behaviour on the EPM compared to continued paroxetine and saline controls. Hypoactivity was also detected on the EPM, but there was no effect of discontinuation on spontaneous LMA in a separate assessment. The anxiogenic effect of paroxetine discontinuation had dissipated after five days, and there were no consistent effects of paroxetine discontinuation on the AOF, NIH or NSF. Increasing the duration of treatment to 28 days, or the frequency of injections to twice daily, did not enhance the behavioural effects of discontinuation. On the other hand, discontinuation after just seven days of paroxetine treatment did not alter anxiety-like behaviour. Citalopram and venlafaxine discontinuation also increased anxiety-like behaviours on some EPM parameters but were generally difficult to distinguish from continued SSRI treatment.

4.1 Paroxetine discontinuation increased anxiety-like behaviour on the EPM

In a pilot study, two days of paroxetine discontinuation increased anxiety-like behaviour in male mice on the EPM. This was evident as reduced time spent in and number of entries to the open arms, increased latency to enter the open arms, reduced head dips, and decreased distance travelled on the maze, compared to saline controls. With only six mice per group, however, this experiment was underpowered and mice undergoing paroxetine discontinuation were not significantly different from those receiving continued paroxetine treatment. When additional male mice were added to the experiment, paroxetine discontinuation produced a robust increase in anxiety-like behaviour on the EPM, indicated by decreased open arm entries, increased latency to first entry, reduced head dips and reduced distance travelled. By comparison, performance on the EPM did not differ from saline controls on discontinuation day five, suggesting the behavioural effects of discontinuation had already resolved.

Increasing the duration of paroxetine treatment from 12 to 28 days did not increase the behavioural effect of discontinuation; instead, it suppressed spontaneous LMA during both continued and discontinued paroxetine. Increasing the frequency of paroxetine injections from once- to twice-daily increased anxiety measures following discontinuation compared to continued treatment, but again this was not to a greater extent than 12 days once-daily treatment.

Evidence of increased anxiety-like behaviour two days after paroxetine cessation is consistent with a previous report showing that citalopram discontinuation increased the ASR in rats (Bosker et al., 2010). Fluoxetine discontinuation also reduced the time spent in the light of the LDB 24 h after its cessation in rats (Matsuzawa-Yanagida et al., 2008), similar to the present findings.

The rapid emergence of these behavioural effects is consistent with the short half-life of paroxetine in rodents ($t_{1/2}$=6.3 h, Kreilgaard et al., 2008). Although serum drug levels were not measured in the current experiment, paroxetine has previously been shown to fall below therapeutic levels within 48 h and to be undetectable in blood plasma 96 h after treatment cessation in rats (el Mansari et al., 1995; Benmansour et al., 1999; Hajós-Korcsok et al., 2000; Karanges et al., 2011). Moreover, citalopram is undetectable in the plasma within 24 h of discontinuation from repeated administration (Ceglia et al., 2004). The current data are also in line with clinical observations that patients can experience discontinuation symptoms within just two days of placebo substitution from paroxetine treatment (Rosenbaum et al., 1998; Michelson et al., 2000; Fava et al., 2015).

Nonetheless, unlike patients who may experience SSRI discontinuation symptoms for several weeks (Fava et al., 2015; Davies & Read, 2019), the anxiety-like effects of paroxetine discontinuation in mice had dissipated after five days. This also differs from the findings of Bosker et al., which reported increased startle responses seven days after citalopram cessation (Bosker et al., 2010). One reason for this discrepancy could be the different SSRIs used. Citalopram has a shorter half-life than paroxetine in rodents ($t_{1/2}$=1.5 h; Overø, 1982), meaning its washout was likely faster, potentially triggering more robust and thus persistent behavioural effects. The route of drug administration may also have affected SSRI washout. Bosker et al. administered citalopram via osmotic minipumps, meaning plasma levels were likely more stable during dosing than the daily bolus injections used here (Cremers et al., 2000a; Bosker et al., 2010). This potentially induced longer lasting effects of discontinuation. In a study more closely resembling the current experiments, two weeks after discontinuation from repeated 10 mg/kg paroxetine injections, C57BL/6 mice did not show evidence of persistent anxiety on the EPM or

LDB (Elizalde et al., 2008), supporting the notion that the anxiogenic effect of paroxetine discontinuation is short-lived in mice.

4.2 Paroxetine discontinuation reduced locomotion on the EPM but not spontaneous LMA

Increased anxiety-like behaviours following paroxetine discontinuation were evident on the EPM as fewer open arm entries and/or increased latency to enter the open arms, as well as reduced distance travelled. Classically, the time spent in the open arms and the proportion of entries to the open arms are considered the purest measures of anxiety, based on the effects of anxiolytic agents such as benzodiazepines (Pellow et al., 1985; Rodgers et al., 1992; Dawson et al., 1995). Yet, the detection of anxiety-like behaviours on the EPM can be confounded by changes in LMA (Lister, 1987, 1990; Handley & McBlane, 1993; Weiss et al., 1998). Consequently, reduced distance travelled on the EPM may result in fewer arm entries in total rather than the specific avoidance of the open arms. If the EPM is considered in isolation, reduced distance travelled following discontinuation could be interpreted as hypoactivity rather than increased anxiety *per se*.

Nonetheless, paroxetine discontinuation was not associated with reduced spontaneous LMA compared to continued treatment when tested in a neutral environment (except for the 28 day experiment, see below). Although there was some evidence of reduced LMA in a clean homecage following continued and discontinued paroxetine, there were no differences between these treatment conditions in the homecage or in separate LMA testing. There was also no effect of citalopram discontinuation on LMA in the current experiments, and other studies of SSRI discontinuation have also not detected changes in general LMA in rodents (Bjork et al., 1998; Goeldner et al., 2005; Matsuzawa-

Yanagida et al., 2008; Iñiguez et al., 2010; Mouri et al., 2012). Thus, the hypoactivity exhibited on the EPM following paroxetine discontinuation was likely context-specific and evidence of behavioural inhibition and passive avoidance resulting from the anxiogenic environment (Gray, 1982; McNaughton & Gray, 2000) rather than a global suppression of activity or motor dysfunction.

In contrast, discontinuation from 28 days of paroxetine treatment reduced spontaneous LMA compared to saline controls, which could have contributed to EPM performance. Hence, 12 days of treatment was used for future experiments as it avoided the locomotor confound of longer treatment.

Nevertheless, the performance of mice on the EPM after discontinuation from 12 days of treatment may not reflect anxiety *per se*. Without further investigation, the possibility that the behavioural effects could be linked to other unpleasant experiences of discontinuation, such as dizziness, and sensory and GI disturbances that feature in the clinical syndrome, cannot be excluded.

4.3 The effects of SSRI discontinuation on other anxiety tests were inconclusive

A battery of anxiety tests was applied to characterise the duration and generalisability of the discontinuation effects following 12 and 28 days of paroxetine treatment. This experimental design was chosen because unconditioned anxiety tests such as these can be confounded by habituation and learning if repeated (File, 2001). Aside from the EPM, however, there were no clear effects of paroxetine discontinuation relative to both continued treatment and saline controls on the other behavioural tests.

Repeated paroxetine reduced the number of digging events when tested in a clean homecage, and this effect had reversed within two days of paroxetine discontinuation.

Digging is a naturalistic behaviour in mice to avoid pain or noxious stimuli, seek food or shelter, or simply represents exploratory activity (Pond et al., 2021). Hence, it can be used as a general measure of wellbeing, motor function, stress, and neurological dysfunction (Dudek et al., 1983; Deacon et al., 2001; Latham & Mason, 2004; Deacon, 2006; Jirkof, 2014). The lack of effect of paroxetine discontinuation compared to saline controls on digging suggests the suppression of exploratory activity resulting from continued paroxetine treatment reversed rapidly following treatment cessation.

Continued paroxetine increased the latency to start eating in the NIH and NSF tests, and this effect reversed to saline control levels three days after paroxetine discontinuation. These findings further support the notion that the behavioural effects of paroxetine discontinuation were short-lasting in mice. Nonetheless, although these tests also assess approach-avoidance conflict, appetite is an additional motivational demand in the NSF and NIH tests. In the current experiments, there was no effect of paroxetine treatment or discontinuation on the weight lost due to overnight food deprivation, suggesting that appetite did not vary between the groups. Nonetheless, conclusions on the effect of appetite on test behaviour cannot be drawn because homecage food consumption was not tested. Moreover, the different test orders between the 12 and 28 day experiments make the results of the NIH and NSF difficult to interpret and compare between experiments.

When performed on discontinuation days three or four, the AOF did not detect effects of paroxetine discontinuation, with performance indistinguishable from that of continued treatment. Moreover, both continued and discontinued paroxetine decreased time in the light zone of the LDB on discontinuation day five. The lack of difference between continued and discontinued paroxetine at this time is consistent with the notion that the behavioural effects of paroxetine cessation dissipated rapidly after

discontinuation day two. This effect was confirmed in test-naïve mice assessed on the EPM on discontinuation day five. Thus, the increased anxiety-like behaviour displayed by mice undergoing paroxetine continuation and discontinuation may have arisen from the stress of previous behavioural testing rather than persistent effects of discontinuation.

An alternative explanation for the lack of clear effects of paroxetine discontinuation on the other behavioural tests is that the EPM is particularly sensitive to the effects of CNS drug cessation, as it is also used in studies of cocaine and amphetamine withdrawal (Vuong et al., 2010; El Hage et al., 2012; Perez & De Biasi, 2015). Although all the tests used in the battery assess approach-avoidance conflict, they have different sensorimotor and motivational demands, and the EPM may simply be the most sensitive to the specific effects of SSRI discontinuation. Further experiments using the AOF and LDB on discontinuation days two to five in test-naïve mice would be needed to determine if discontinuation effects are only detectable by the EPM.

4.4 Prolonged treatment with paroxetine was needed to produce behavioural effects upon discontinuation

Mice discontinued from 12 and 28 days of repeated paroxetine treatment exhibited heightened anxiety-like behaviour, but this was not seen after just seven days of treatment. Other data in mice have shown that the antidepressant effects on the FST emerge between days five and 11 of SSRI treatment (Santarelli et al., 2003). This suggests that neuroadaptive processes may occur in this critical time window.

The timing of the development of discontinuation effects also agrees with clinical findings that one week of SSRI administration is not sufficient to produce discontinuation symptoms (Yonkers et al., 2015). Nonetheless, the syndrome can emerge after just five

to six weeks of treatment and is reliably detected after eight weeks (Robert et al., 1995; Markowitz et al., 2000; Montgomery et al., 2004b; Sir et al., 2005; Baldwin et al., 2006a). Together, these studies suggest that neuroadaptive processes that occur during prolonged SSRI administration are necessary for the later emergence of discontinuation effects.

4.5 Citalopram and venlafaxine discontinuation increased anxiety-like behaviours relative to saline controls

Citalopram and venlafaxine discontinuation also increased anxiety-like behaviours on the EPM compared to saline controls. In the case of citalopram discontinuation, this was distinguishable from continued citalopram treatment, which did not alter EPM performance. The behavioural effects of venlafaxine discontinuation, however, were confounded by an anxiogenic effect of repeated venlafaxine treatment. Overall, discontinuation effects were more apparent following discontinuation from paroxetine than citalopram or venlafaxine.

To some extent, these results mirror clinical observations that paroxetine is the SSRI most likely to cause discontinuation symptoms (Price et al., 1996; Rosenbaum et al., 1998; Hindmarch et al., 2000; Tint et al., 2008; Read et al., 2018). This is likely due to its short half-life in humans (21 h, Bourin et al. (2001), compared to 35 h for citalopram Marken and Munro, 2000), resulting in its rapid clearance from the body (Rosenbaum et al., 1998; Fava et al., 2015). Comparatively, fluoxetine has a half-life of four to six days and an active metabolite (Baldo & Rose, 2019), so it is unlikely to cause the discontinuation syndrome (Price et al., 1996). Hence, fluoxetine was not tested in the current experiments, although it could provide a valuable negative control for the current model of SSRI discontinuation.

In contrast to humans, paroxetine has a longer half-life than citalopram in rodents, and yet citalopram discontinuation did not produce more robust anxiety-like behaviours. This suggests the propensity of paroxetine to cause discontinuation symptoms may arise from other factors including its higher affinity for NET and M1 receptors (Sánchez & Meier, 1997; Blier & Tremblay, 2006; Renoir, 2013; Sanchez et al., 2014). Furthermore, the C57BL/6 mice used here are reported to show dose-dependent responses to paroxetine but not citalopram on the FST and TST, suggesting this strain is particularly sensitive to paroxetine, potentially contributing to its greater effects (Jin et al., 2017).

Unique among the drugs tested is the SNRI venlafaxine. Venlafaxine has a very short half-life in humans (3-4 h, Schweizer et al., 1997), and so like paroxetine it causes problems upon its discontinuation (Montgomery et al., 2004b; Sir et al., 2005; Read et al., 2018). The half-life of venlafaxine is also short in mice (1 h, Howell et al., 1994), so it was predicted to produce similar effects to paroxetine discontinuation. The minimal effects seen here may therefore reflect its weaker inhibition of 5-HTT than paroxetine and citalopram (Renoir, 2013), with other studies failing to show effects on anxiety-like behaviours when administered at 10 mg/kg (Karlsson et al., 2011; Venzala et al., 2012). It is possible, however, that the effects of venlafaxine discontinuation were obscured by the anxiogenic effects of repeated venlafaxine treatment, resembling the effects seen in the first paroxetine pilot experiment. Thus, increasing the power of this experiment and further optimisation of the dosing protocol (for example varying the dose, frequency, and duration of treatment) are needed to determine if venlafaxine discontinuation also produces the same anxiety-like behaviours as paroxetine discontinuation in mice.

4.6 Female mice did not display behavioural effects of paroxetine discontinuation

While paroxetine discontinuation increased anxiety-like behaviour in male mice, no such effects were seen in female mice. Paroxetine discontinuation had no effect on the time or entries to the open arms of the EPM or on distance travelled or general LMA. Paroxetine continuation increased latency to enter the open arms in female mice, but this simply reversed to saline control levels following paroxetine discontinuation.

This is the first report of SSRI discontinuation in female rodents, so the cause of this sexual dimorphism is unknown. Nonetheless, sex differences in the 5-HT system could contribute to this variation. For example, lower 5-HTT mRNA expression was reported in the raphe nuclei of female mice compared to males (Renoir et al., 2011). 5-HTT transporter density has also been shown to positively correlate with the anxiolytic effects of antidepressant drugs in mice (Mirza et al., 2007), potentially explaining why female mice were less sensitive to paroxetine treatment. Moreover, female mice are thought to metabolise fluoxetine more effectively than males due to greater cytochrome P450 activity (Hodes et al., 2010; Renoir et al., 2011). The steady state of plasma paroxetine in females may therefore have been lower or less stable, thus discontinuation did not produce behavioural effects. Further experiments using higher or more frequent doses of paroxetine would help to elucidate the role of 5-HTT expression and drug metabolism in female mice.

Alternatively, variation in the oestrous stage of the mice at the time of testing may have obscured any effects. For instance, anxiolytic effects of fluoxetine treatment were only detected during dioestrus (Yohn et al., 2020a). This factor should therefore be considered in future investigations of paroxetine discontinuation in female mice.

4.7 Repeated paroxetine treatment was itself anxiogenic

An unexpected finding was that repeated paroxetine treatment was anxiogenic in male mice, observed across several experiments on the EPM, AOF, NIH, NSF and LDB. Continued paroxetine also reduced spontaneous LMA following 28 days of treatment, and consistently reduced exploratory digging.

The preclinical literature is surprisingly inconsistent on the effect of repeated SSRI administration on anxiety-like behaviour in mice, with anxiogenic, anxiolytic and no effects frequently reported (Santarelli et al., 2003; Elizalde et al., 2008; David et al., 2009; Baek et al., 2015; Turcotte-Cardin et al., 2019), including with paroxetine (Goeldner et al., 2005; Thoeringer et al., 2010; Guilloux et al., 2011b). In fact, 28 days of paroxetine treatment increased the time spent in the light zone of the LDB (Sillaber et al., 2008), directly contradicting the current findings.

One explanation for the anxiogenic effect of repeated SSRI treatment is that it increases extracellular 5-HT concentrations. Numerous preclinical studies have reported that increased 5-HT transmission, either by knocking out 5-HTT or through activation of 5-HT neurons, increases anxiety-like behaviour in rodents (Chapter 1 section 5.3).

Nonetheless, as inhibitors of the 5-HTT, all three of the drugs tested here will have increased extracellular 5-HT, yet only paroxetine and venlafaxine treatment increased anxiety-like behaviour. The propensity of paroxetine and venlafaxine to increase anxiety may therefore reflect their affinity for the anxiety-provoking noradrenergic system. For instance, paroxetine increased noradrenergic transmission in the frontal cortex in mice (David et al., 2003), and as an SNRI venlafaxine also inhibits NET and increases extracellular NA, potentially explaining why they increase anxiety-like behaviours.

An alternative explanation for these anxiogenic effects is that mice were experiencing discontinuation effects even on continued treatment. In experiments using once-daily injections, behaviour was assessed 18 h after the final injection, thus paroxetine may have been sufficiently clear to cause discontinuation effects. Nonetheless, similar anxiogenic effects were seen following twice-daily injections, when mice were tested just 6 h after a paroxetine injection. This suggests that heightened anxiety-like behaviour was a distinct effect of repeated paroxetine treatment and not early evidence of discontinuation effects.

The anxiogenic effects of paroxetine and venlafaxine could also have arisen from the ability of SSRIs to enhance emotional learning, by heightening the salience of negative environmental stimuli, such as the stress of daily injections, to produce more anxious mice (Harmer, 2008; Harmer et al., 2017). However, there was no evidence of coat state deterioration or increased typical stress-like behaviours (such as vocalisation and urination during injections) in any of the mice as the experiment progressed, suggesting repeated injections were minimally stressful. Additionally, the lack of anxiogenic effect of repeated citalopram treatment further suggests the anxiogenic effects of paroxetine and venlafaxine are not generic effects of repeated SSRI treatment; instead, they are likely due to the specific properties of paroxetine and venlafaxine.

4.8 A meta-analysis of the effects of chronic SSRI treatment on EPM performance in mice

To address the apparent inconsistencies in the literature and the variable effects of different SSRIs in the current experiments, a systematic review and meta-analysis was performed on the effects of repeated SSRI treatment on anxiety-like behaviour on the

EPM in unstressed and wild-type adult mice (Appendix 2). Overall, there was no significant effect of SSRI treatment on the time spent in the open arms of the EPM, suggesting repeated SSRI treatment does not consistently alter anxiety-like behaviour.

Nonetheless, there was substantial variation between the effect sizes reported in the literature, with responses varying greatly from robust anxiolytic effects (Vahid-Ansari et al., 2017) to anxiogenic effects (Venzala et al., 2012; Turcotte-Cardin et al., 2019). Even within the current experiments, there was variation in the extent to which repeated paroxetine treatment was anxiogenic. Although there were no significant influences of variables such as the sex, mouse strain, treatment duration, or route of administration on the overall response to SSRI treatment, variation in experimental design likely contributed to the inconsistencies between studies (Carobrez & Bertoglio, 2005). For instance, repeated paroxetine injections decreased the time spent in the open arms of the EPM (Elizalde et al., 2008), whereas repeated fluoxetine injections were anxiolytic on the NSF (Santarelli et al., 2003). These findings suggest the SSRI and the test conducted may affect the response detected. Moreover, the dose of SSRI used may also alter its effects, with increased anxiety-like behaviours evident on the EPM after repeated 8 mg/kg fluvoxamine treatment but not after 2 or 4 mg/kg (Rodgers et al., 1997). Different experimental variables can therefore produce vastly different behavioural effects.

Critically, repeated SSRI treatment did not consistently produce an anxiolytic effect in mice. This finding was unexpected given the clinical use of SSRIs as treatments for anxiety disorders (Allgulander, 2009)[3] and could suggest that preclinical protocols do not

[3] Nonetheless, like the antidepressant effects of SSRI therapy, the efficacy of SSRIs for anxiety disorders also varies. For instance, a meta-analysis of clinical trials found that fluoxetine produced remission in only 61% of patients, and escitalopram in only 26% (Baldwin et al., 2011). Thus, the anxiolytic effects of SSRIs in patients are also heterogenous, casting further doubt on the assumption that repeated SSRI treatment in rodents should be anxiolytic.

model the effects of SSRIs in patients. This could be due to the short treatment durations – SSRIs are acutely anxiogenic and it can take several weeks for patients to experience subjective improvements in anxiety (Baldwin et al., 2005; Kavan et al., 2009). The four to six week treatment durations regularly used in rodent studies may not be sufficient to produce anxiolytic effects. These findings could also suggest that the EPM is a poor model for detecting clinically-relevant effects of SSRIs.

Alternatively, the lack of anxiolytic effect could arise from the administration of SSRIs to wildtype or non-stressed mice. In humans, SSRIs are taken by patients with existing mood disorders and may act to reverse neurological deficits underlying these disorders (Chapter 1 section 4.3) that wildtype mice do not have. Supporting this notion, SSRIs have more robust anxiolytic effects when administered to stressed mice, such as those subjected to chronic mild unpredictable stress or corticosterone administration, than controls (for example, David et al., 2009, Christiansen et al., 2011, Robinson et al., 2016, Yohn et al., 2020b, and Cordner et al., 2021). Consequently, using non-stressed mice may have limited the ability of these experiments to detect effects of SSRI treatment that resemble those seen in patients. Overall, repeated SSRI treatment had no consistent effect on EPM performance wildtype in mice, yet there was substantial heterogeneity between studies. These results demonstrate that assumptions about the anxiolytic effects of SSRI treatment in patients do not readily translate to mice, highlighting the difficulty in modelling psychiatric disorders in preclinical experiments (discussed further in Chapter 8 section 3.3).

4.9 Conclusions

Overall, these data provide evidence of a short-lasting behavioural effect of SSRI

discontinuation in mice, characterised by increased anxiety-like behaviour and anxiety-provoked hypoactivity on the EPM. The detection of this effect on the second day of discontinuation correlates with findings of increased anxiety in patients within days of stopping SSRI treatment. Discontinuation effects were evident in male but not female mice and more pronounced following discontinuation of paroxetine than citalopram or venlafaxine. The discontinuation effects were dependent on prolonged paroxetine treatment, suggesting neuroadaptive changes were necessary for their generation. Although this behavioural paradigm cannot claim to fully recapitulate SSRI discontinuation syndrome, it can be used as a model to investigate the neurobiological mechanisms involved in the symptoms, for example by assessing changes in neuronal activation or 5-HT neurochemistry.

Chapter 4
Effect of paroxetine discontinuation on the activation of anxiety circuitry

Parts of the following chapter are published in Collins et al., *Br J Pharmacol*, 2022; doi: 10.1111/bph.15944; and Collins et al., *Neuroscience Applied*, 2022; 1, Suppl. 2: 100652.

1 Introduction

Experiments in the previous chapter found that two days of paroxetine discontinuation increased anxiety-like behaviour and produced task-specific hypoactivity compared to continued paroxetine and saline-treated control mice. Here, the activity-dependent immediate early gene *c-Fos* was used to investigate the activation of neural circuits associated with the behavioural effects of paroxetine discontinuation.

1.1 c-FOS as a marker of neuronal activation

c-FOS is a rapid and transiently inducible transcription factor, encoded by the *Fos* gene. *c-Fos* is part of a larger family of immediate early genes that convert extracellular signals into gene expression changes (Sheng & Greenberg, 1990; Morgan & Curran, 1991; Herdegen & Leah, 1998).

Neuronal activation triggers c-FOS expression (Morgan et al., 1987). This can be caused by endogenous neurotransmitters and growth factors activating excitatory receptors, increased intracellular calcium concentrations, increased neuronal firing rates, increased intracellular cAMP or drugs binding to membrane receptors (Morgan & Curran, 1986; Ghosh et al., 1994; Cohen & Greenberg, 2008). Following neuronal activation,

calcium and cAMP activate intracellular signalling cascades leading to *c-Fos* mRNA transcription (Chaudhuri et al., 2000; Squire et al., 2012). *c-Fos* mRNA is translocated into the cytoplasm where the protein in synthesised, before re-entering the nucleus to bind to enhancer and promoter regions on DNA to increase activity-dependent gene expression (Sambucetti & Curran, 1986; Möller et al., 1994).

c-Fos is the most transient member of the *Fos* family. Its mRNA is only detectable for 20 to 90 min after stimulation (Morgan et al., 1987; Dragunow & Faull, 1989; Hu et al., 1994; Torres et al., 1998; Day et al., 2008), with protein expression peaking after 90 to 120 min (Kovács, 2008). The short-lived and stimulus-evoked expression of c-FOS makes it a useful experimental tool to measure recent neural activity. Co-localisation of c-FOS immunoreactivity with a marker of neuronal nuclei, such as the transcription factor NeuN, demonstrates its expression and putative activity in the nucleus (Bullitt, 1990). Cell-type specific c-FOS expression can also be determined with additional immunohistochemical stains for key marker proteins, such as TPH2 for 5-HT soma.

c-FOS expression is low under unstimulated conditions, thus providing a high signal-to-noise ratio when it is activated. Nonetheless, any neuronal activation can trigger c-FOS expression, meaning it is not inherently linked to an experimental manipulation. For example, c-FOS expression can be induced by the stress of animal handling and injections before and after a behavioural test (Sharp et al., 1991; Duncan et al., 1996). Its detection can therefore lack temporal and stimulus-specificity. Careful controls for the overall experience of the animal are therefore needed to identify the causal trigger of c-FOS expression (Dragunow & Faull, 1989).

1.2 c-FOS as a measure of activation of anxiety circuitry

Anxiety-provoking environments such as the EPM increase global c-FOS expression in the rodent brain, including in anxiety-promoting brain regions (Duncan et al., 1996; Hinks et al., 1996; Ábrahám & Kovács, 2000; Linden et al., 2004; Salomé et al., 2004). For instance, c-FOS can be used as a marker of amygdala activation – increased *c-Fos* mRNA was first detected in the rat amygdala just 15 min after exposure to the EPM (Graeff et al., 1993; Silveira et al., 1993; Hinks et al., 1996). c-FOS immunoreactivity was also increased in the amygdala following OF exposure compared to homecage controls (Boguszewski & Zagrodzka, 2005), as well as in response to acute restraint stress (Senba et al., 1993; Salchner & Singewald, 2002).

c-FOS expression was also specifically increased in the rat BLA following EPM exposure, immobilisation stress and the administration of anxiogenic drugs (Duncan et al., 1996; Singewald et al., 2003; Salomé et al., 2004; Muigg et al., 2009; Kovács et al., 2018). The number of c-FOS immunoreactive cells in the BLA was also negatively correlated with EPM open arm entries, suggesting that greater c-FOS expression in the BLA was associated with elevated anxiety-like behaviour in rats (Muigg et al., 2009).

c-FOS immunohistochemistry has been used to demonstrate VH activation in anxiogenic environments. For instance, mice confined to the open arms of the EPM had a greater number of c-FOS+ neurons in the VH than mice restricted to the closed arms (Gomes et al., 2005). In fact, simply placing a mouse in a novel environment was sufficient to induce c-FOS expression in the VH (Procaccini et al., 2011).

There is also evidence of increased c-FOS expression in the DH following anxiety-like behaviours. For instance, c-FOS expression was increased in the DG of mice and rats following EPM exposure (Linden et al., 2004; Muigg et al., 2009), as well as in

the CA1 and CA3 subfields (Hinks et al., 1996; Linden et al., 2004; Salomé et al., 2004; Gomes et al., 2005). This could suggest that anxiety also activates the DH. Yet, given the role of the DH in spatial memory (Kjelstrup et al., 2002; Bannerman et al., 2003; McHugh et al., 2004), this activation is more likely to reflect the novelty or spatial inputs of the task rather than anxiety *per se*.

The mPFC is also activated during anxiety, evident as increased c-FOS expression following EPM exposure in mice (Duncan et al., 1996; Linden et al., 2004) and OF exposure in rats (Hale et al., 2008) compared to test-naive controls. Anxiogenic drugs also increased c-FOS expression in the mPFC (Singewald et al., 2003; Ait Bali et al., 2022). Specific subregions of the mPFC were also activated during EPM exposure, with c-FOS immunoreactivity increased in the prelimbic cortex (PLC) (Muigg et al., 2009; Peng et al., 2019), infralimbic cortex (IL) (Graeff et al., 1993; Berg et al., 2019) and anterior cingulate cortex (ACC) (Salomé et al., 2004).

Although not typically considered part of the anxiety-promoting circuitry, anxiogenic situations can also induce c-FOS expression in the rodent PAG (Silveira et al., 1993; Linden et al., 2004; Gomes et al., 2005). c-FOS expression was specifically increased in the dl-PAG, which is associated with 'fight or flight' responses in rodents (Muigg et al., 2009). Exposure to the aversive context after contextual fear conditioning (a common readout of conditioned anxiety) also increased c-FOS immunoreactivity in the rat dl-PAG compared to homecage controls (Zienowicz et al., 2007), further linking its activation to anxiety-like behaviours.

c-FOS immunoreactivity is therefore a sensitive molecular marker of anxiety circuitry activation. It has been used to identify key brain regions including the amygdala,

VH and mPFC, as well as other regions that regulate emotional behaviours, such as the DH and PAG.

1.3 Heightened anxiety-like behaviour is associated with greater c-FOS expression

c-FOS expression is also thought to increase 'dose-dependently' with elevated anxiety; that is, rodents bred to exhibit heightened anxiety-like behaviours have more c-FOS+ neurons in anxiety-promoting brain regions than low anxiety animals (Linden et al., 2003; Linden et al., 2004; Salomé et al., 2004; Muigg et al., 2009; Peng et al., 2019). Rats that froze more in a contextual fear conditioning paradigm also had greater c-FOS immunoreactivity in the BLA compared to low freezers (Lehner et al., 2009). Moreover, increased anxiety-like behaviour on the EPM and OF was positively correlated with c-FOS expression of the hippocampus (Salomé et al., 2004). Interestingly, while high-anxiety rodents exhibited greater c-FOS immunoreactivity after behavioural testing, at baseline c-FOS expression did not differ between high- and low-anxiety animals (Salomé et al., 2004; Lehner et al., 2009). These findings suggest that increased anxiety-like behaviour is associated with greater c-FOS expression in anxiety-provoking brain regions in response to potentially dangerous situations, but not under baseline conditions.

1.4 Effect of anxiety-like behaviour on c-FOS expression in 5-HT neurons

The serotonergic system is considered a key mediator of anxiety in rodents, with increased 5-HT transmission typically associated with increased anxiety-like behaviour (as discussed in Chapter 1 section 5.3). Increased c-FOS immunoreactivity has also been detected in the DRN following anxiety-provoking situations, for example after EPM exposure (Silveira et al., 1993; Gomes et al., 2005; Muigg et al., 2009; Berg et al., 2019), acute restraint stress (Senba et al., 1993) and acute intruder stress (Tanaka et al., 2017).

Anxiogenic T-maze exposure also increased c-FOS expression in TPH2+ neurons in the DRN (Zangrossi & Graeff, 1997; Spiacci et al., 2012). c-FOS expression in the mouse MRN was also induced by EPM exposure (Gomes et al., 2005), further implicating 5-HT neurons in the regulation of anxiety-like behaviours.

c-FOS immunohistochemistry has also been used to elucidate the mechanisms by which 5-HT regulates anxiety-like behaviours, principally via its interactions with other brain regions. For example, a systemic injection of 5-HT increased c-FOS in the amygdala and PLC, which was associated with reduced time in the open arms of the EPM (Sanders et al., 2019). Data also suggest that increased 5-HT transmission supresses PAG activation, evident as reduced c-FOS in the dl-PAG following acute escitalopram treatment (Hestermann et al., 2014). Increased 5-HT activity is therefore thought to promote risk assessment behaviours but suppress panic responses (Graeff et al., 1996; McNaughton & Gray, 2000), effects which can be mapped with c-FOS immunoreactivity.

1.5 Neural circuitry associated with psychotropic drug withdrawal states

c-FOS expression has also been used to map the neural circuitry activated by the anxiogenic effects of withdrawal from CNS drugs. For instance, spontaneous and precipitated morphine withdrawal increased c-FOS immunoreactivity in the raphe nuclei, hippocampus, cortex, amygdala, and PAG (Hayward et al., 1990; Bhat et al., 1992; Stornetta et al., 1993; Beckmann et al., 1995; Chieng et al., 1995; Rasmussen et al., 1995; Georges et al., 2000; Hamdy et al., 2001). Similar activation was seen in the mouse DRN following withdrawal from amphetamine (Tanaka et al., 2017).

These observations suggest that discontinuation from other psychotropic drugs can induce c-FOS expression in anxiety-related brain circuitry, including the raphe nuclei.

Given the lack of data on SSRI discontinuation, comparisons can be drawn from these other withdrawal states to hypothesise that SSRI discontinuation may be associated with increased c-FOS expression in the 5-HT system, as well as in other anxiety-promoting brain regions.

1.6 Hypothesis and aims

Anxiety-like behaviour, including withdrawal reactions induced by discontinuation from other psychotropic drugs, is thought to be associated with increased c-FOS expression in anxiety-promoting brain regions. Moreover, increased c-FOS expression in 5-HT neurons in the DRN and MRN is common across several environmental and drug-induced anxiety states. Hence, the aim of this chapter was to use c-FOS immunohistochemistry to identify the components of the mouse anxiety circuitry that were activated by paroxetine discontinuation. Based on behavioural data in the previous chapter, it was hypothesised that two days of paroxetine discontinuation would be associated with increased c-FOS immunoreactivity in anxiety-related brain regions, such as the BLA, VH and mPFC, as well as in 5-HT neurons in the DRN and MRN. These effects were predicted to have reversed five days after discontinuation, when there was no longer evidence of increased anxiety-like behaviour on the EPM.

2 Experimental Methods

2.1 Animals

C57BL/6J male mice (Charles River, 7 weeks old) were group housed (3-4 per cage) in open-top cages for at least one week before the start of treatment.

2.2 Drugs and reagents

Paroxetine was dissolved in saline and administered *s.c.*

2.3 Experimental design

Mice were allocated to one of three experimental groups by stratified randomisation: i) saline group (SAL): repeated saline + saline continuation; ii) continuation group (CON): repeated paroxetine + paroxetine continuation; iii) discontinuation group (DIS): repeated paroxetine + saline. Mice were weighed for two days before the start of the experiment to establish a baseline weight and to habituate them to handling, then daily throughout the experiment. Mice received once daily *s.c.* injections of 10 mg/kg paroxetine, or the equivalent volume of saline, for 12 days, then treatment was either continued (saline and continuation groups) or swapped to saline injections (discontinuation group) for a further two or five days (*Fig. 4 .1*).

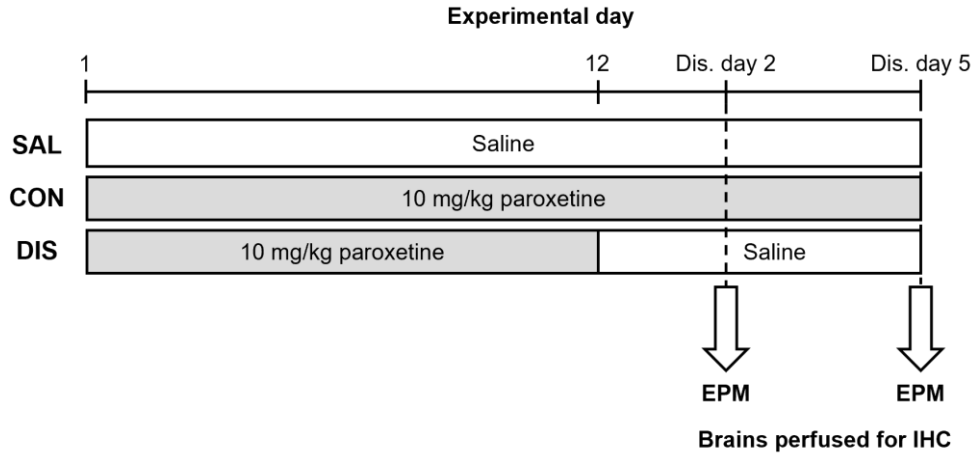


Figure 4.1 Experimental design. Two cohorts of mice assessed on the EPM on discontinuation days two and five. IHC, immunohistochemistry.

All mice were assessed on the EPM. Mice were habituated to a temporary holding room adjacent to the testing room for at least 1 h before testing and on the three days preceding behavioural testing. Mice were tested on the EPM for 5 min in the light phase (13:00 – 16:00 h). Mice were tested in two cohorts (*Table 4.1*): in the first cohort, mice were assessed on the equivalent of discontinuation day two; in the second cohort, mice were tested on discontinuation day five. All experiments were conducted with the experimenter blind to treatment.

Table 4.1 Experimental design. A subgroup of mice from each experiment were perfused for c-FOS immunohistochemistry experiments, EPM experiments had n=12/group in total. † All mice in the two-day experiment received a *s.c.* injection of 10 ml/kg saline 60 min before the EPM as part of a later experiment.

Discontinuation day	Group sex and size	Relevant behavioural experiment
2	Males SAL n=6, CON n=6, DIS n=7	Chapter 6 section 3.2 †
5	Males n=6/group	Chapter 3 section 3.3

A subset of mice from each experiment were transcardially perfused with PBS then 4% PFA 90 min after the start of behavioural testing (150 min after the injection, as in Chapter 2 section 5.1). Brains were stored at 4^{o}C in 30% sucrose solution.

2.4 Immunohistochemistry and c-FOS quantification

Immunohistochemistry experiments were performed as in Chapter 2 section 6. Briefly, coronal brain slices containing the DRN and MRN (*Fig. 4.2A-B*), DH and VH (*Fig. 4.3A-B*), BLA, dl-PAG and PLC (*Fig. 4.4A-C*) were immunohistochemically stained for c-FOS and NeuN. DRN and MRN slices were also stained for TPH2 to identify 5-HT neurons. Images were taken using epifluorescence microscopy (two images at 10x magnification for DH and VH, 20x magnification for other regions) and c-FOS immunoreactivity quantified using Image J.

In the DRN, MRN, BLA, dl-PAG and PLC, c-FOS+ neurons were defined as cells with immunoreactivity co-localised with NeuN+ nuclei, rather than in the cytoplasm of the cell. In the DH and VH, however, c-FOS+ neurons were simply defined as nuclear-shaped staining, as the image resolution was not high enough to identify individual NeuN+ nuclei due to the density of cell packing.

In the dorsal subregion of the DRN and in the MRN (boxes in *Fig 4.2* indicate the region of interest, ROI), the total number of c-FOS+ neurons, as well as the number of c-FOS+ TPH2+ and c-FOS+ TPH2-negative (−) neurons, were counted. The number of TPH2+ neurons and NeuN+ neurons in each image were also quantified (*Fig. 4.2C*). In the DH and VH, the number of c-FOS+ neurons in the whole DG of that slice were counted (*Fig. 4.3C*). In the BLA, dl-PAG and PLC, the number of c-FOS+ and NeuN+ neurons were counted (*Fig. 4.4D*). Images were analysed from three slices per region per mouse, then data were averaged per mouse. *Table 4.2* summarises the analysis of c-FOS expression in each brain region. Slice staining, imaging, and counting were conducted with the experimenter blind to treatment.

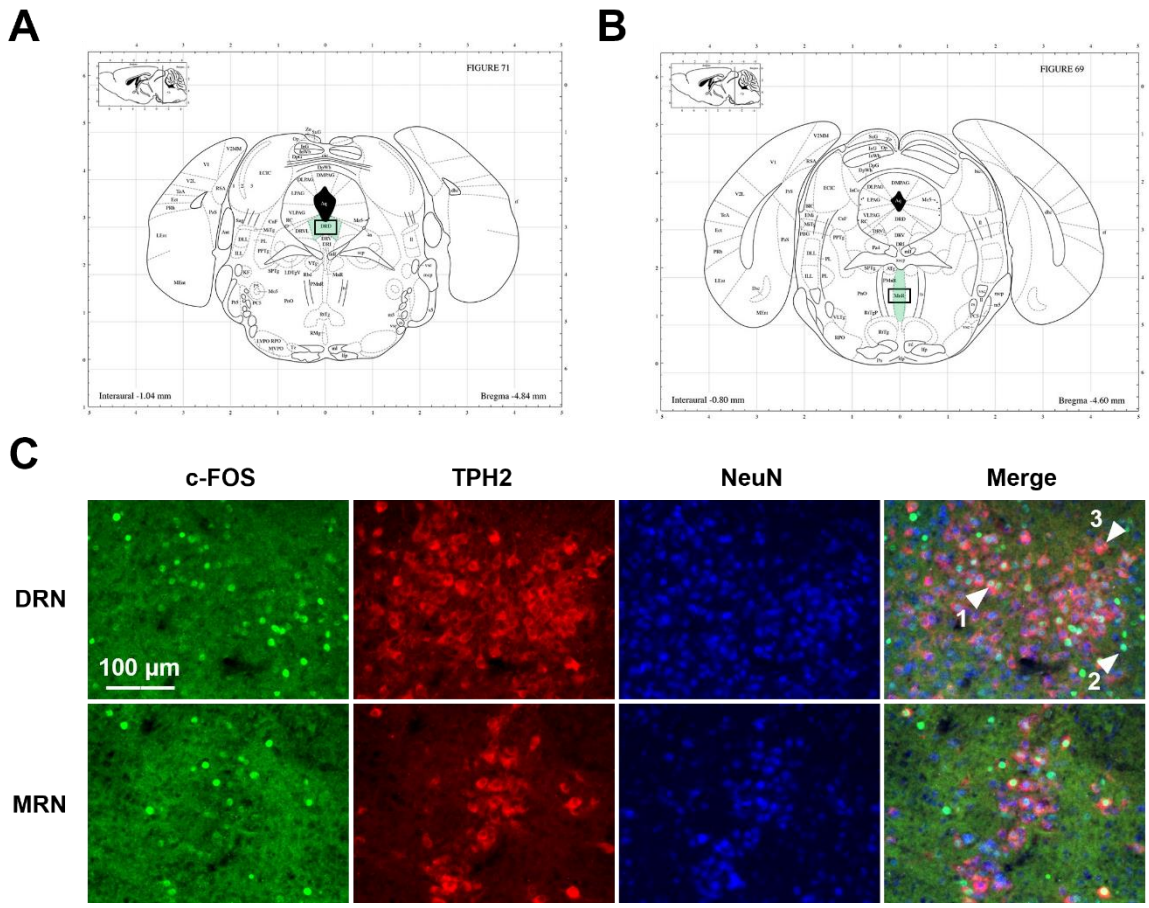


Figure 4.2 Representative images of immunohistochemical staining of the dorsal subregion of the DRN and the median raphe nucleus MRN. Mouse brain atlas images of the DRN (A) and MRN (B) from Paxinos & Franklin (2007). Green area highlights brain region, black box represents where images were taken for quantification (ROI). Representative images of c-FOS, TPH2, NeuN, and the images merged (C). Line represents 100 µm. Arrow 1: c-FOS+ TPH2+ neuron; arrow 2: c-FOS+ TPH2− neuron; arrow 3: TPH2+ neuron.

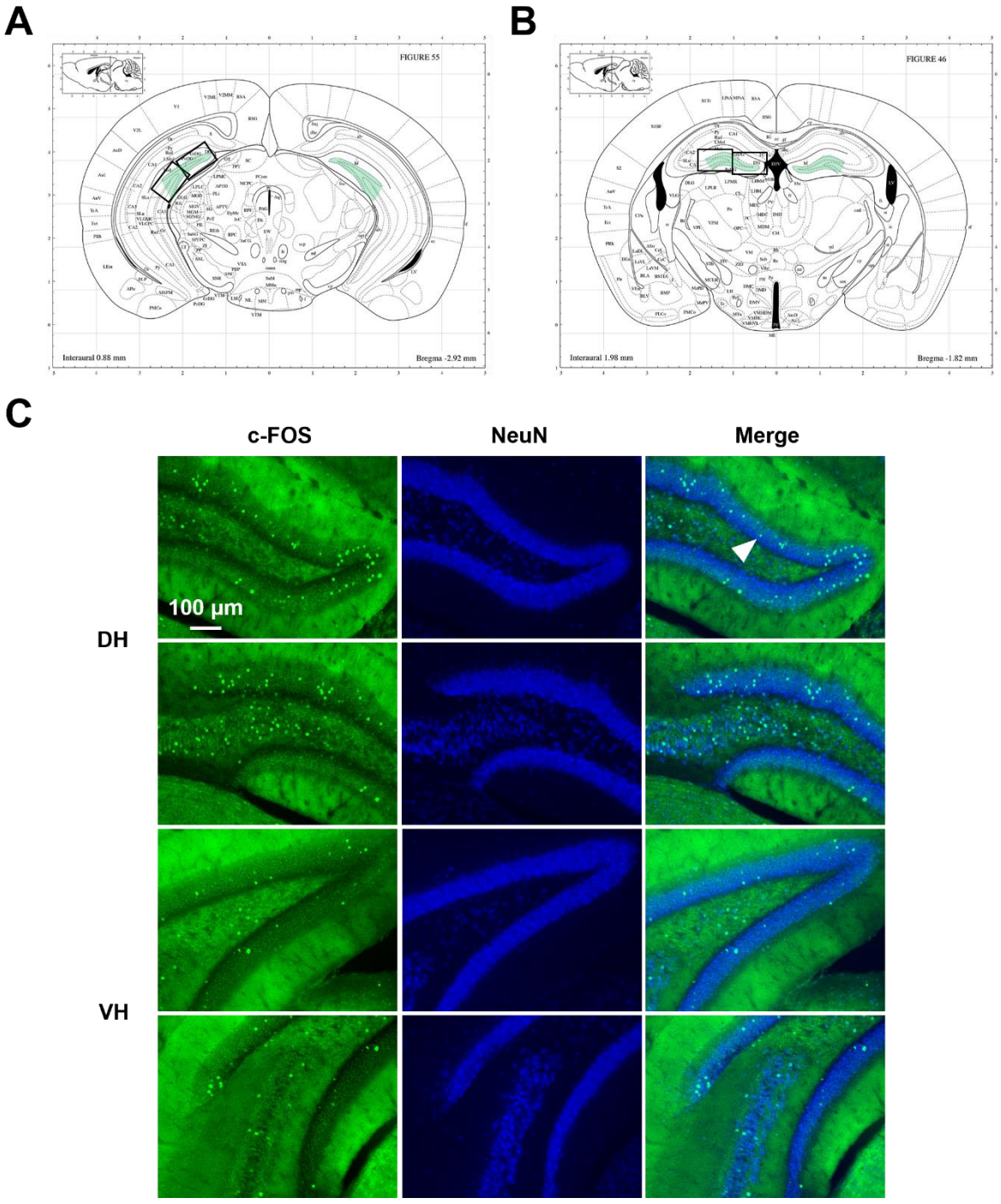


Figure 4.3 Representative images of immunohistochemical staining of the hippocampus. Mouse brain atlas images of the DH (A) and VH (B). Green area highlights brain region, black box represents the two images taken per slice to quantify the whole DG (ROI). Representative images of c-FOS, NeuN and the images merged (C). Line represents 100 µm. Arrow: c-FOS+ neuron.

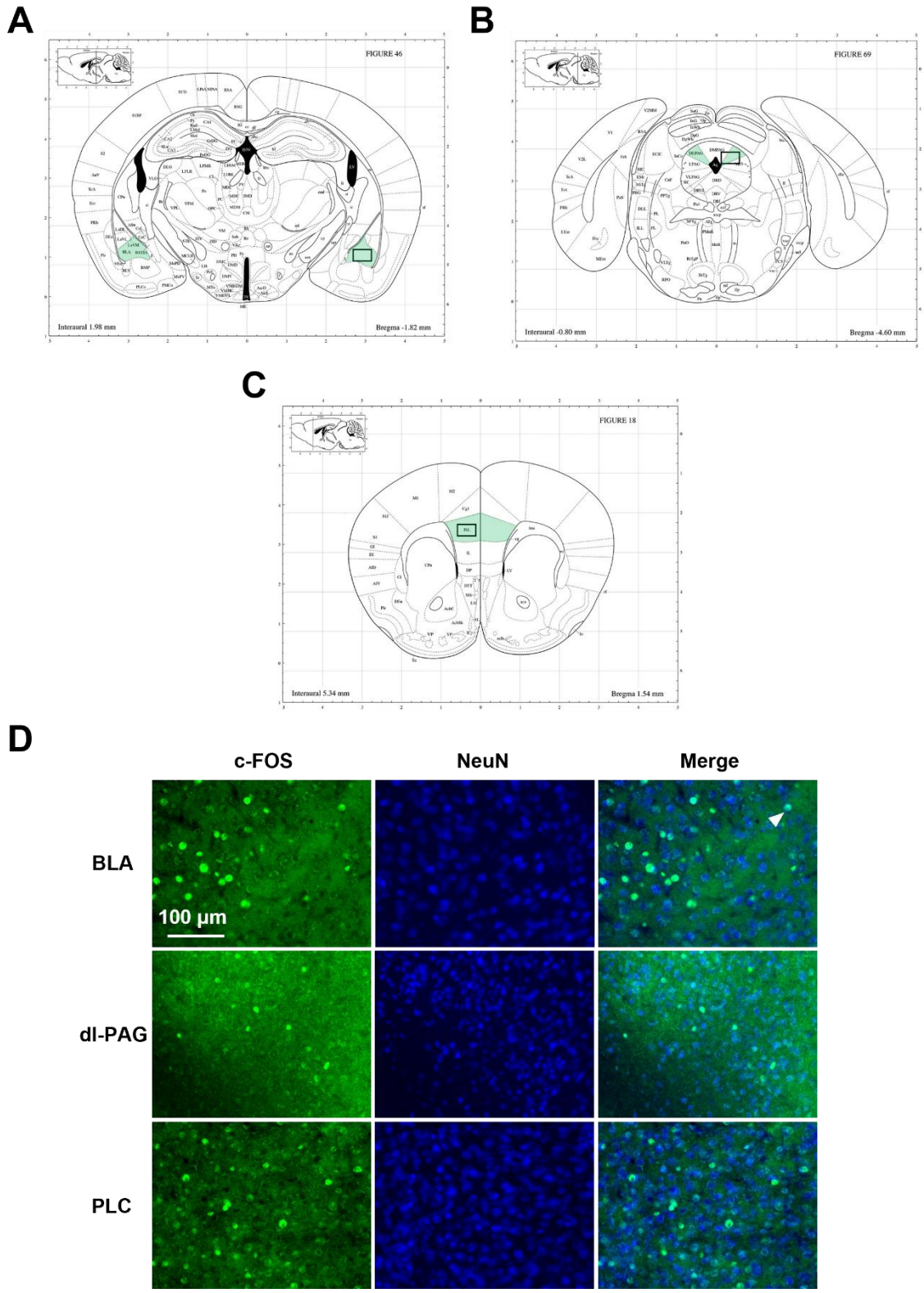


Figure 4.4 Representative images of immunohistochemical staining of anxiety-related brain regions. Mouse brain atlas images of the BLA (A), d-PAG (B) and the PLC (C). Green area highlights brain region, black box represents where images were taken for quantification (ROI). Representative images of c-FOS, NeuN, and the images merged (C). Line represents 100 µm. Arrow: c-FOS+ neuron.

Table 4.2 Analysis of c-FOS immunoreactivity.

Immunolabelling	Definition of parameter	Regions
c-FOS+ neurons (%)	c-FOS+ / NeuN+	DRN, MRN, BLA, dl-PAG, PLC
c-FOS+ TPH2+ neurons (%)	c-FOS+ TPH2+ / TPH2+	DRN, MRN
c-FOS+ TPH2− neurons (%)	c-FOS+ TPH2− / (NeuN+ – TPH2+)	DRN, MRN
TPH2+ neurons (%)	TPH2+ / NeuN+	DRN, MRN
Number of c-FOS+ neurons per brain slice	Total c-FOS+	DH, VH

2.5 Statistical analysis

D'Agostino-Pearson's test for normality was applied to all data sets. If data were determined to be normally distributed, one-way ANOVA with Fisher's LSD was used to compare between treatment conditions. If the data were determined to be non-parametric, then Kruskal Wallis with Uncorrected Dunn's test was used. The EPM data of the mice included in c-FOS immunoreactivity experiments were analysed using the above statistical approach, with data summarised in the current chapter. To further clarify the effect of continued paroxetine on c-FOS expression, c-FOS data were also normalised to the mean saline value of c-FOS expression per region for that experiment, then the two normalised datasets were combined. Analysis was conducted using GraphPad Prism (v8). Data are presented as mean ± SEM values for parametric data and median ± IQR for non-parametric data. P values of $p<0.05$ were considered statistically significant. All analyses were performed blind to treatment.

3 Results

3.1 Effect of two days discontinuation from paroxetine treatment on anxiety-like behaviour on the EPM

As seen in the previous chapter, two days of paroxetine discontinuation increased anxiety-like behaviour compared to continued paroxetine and saline controls. Paroxetine discontinuation reduced the time spent in the open arms, the number of entries to the open arms, the number of head dips, and the distance travelled on the EPM compared to continued paroxetine treatment, and there were trends towards reductions compared to saline controls (*Table 4.3*; post-hoc time: SAL vs DIS p=0.0574, CON vs DIS p=0.0007; entries: CON vs DIS p=0.0051; head dips: SAL vs DIS p=0.0684, CON vs DIS p=0.0036; distance: SAL vs DIS p=0.0191, CON vs DIS p=0.0556). There was no effect of treatment on the latency to enter the open arms (*Table 4.3*). Paroxetine continuation did not alter EPM performance.

Table 4.3 Effect of two days discontinuation from paroxetine treatment on the EPM. 5 min testing on the EPM. Each value is a mean ± SEM or median and IQR [25th percentile, 75th percentile]. Bold values indicate significant effects of treatment, * p<0.05 SAL vs DIS, # p<0.01 CON vs DIS. N=6-7/group.

	Group (mean ± SEM, median [25th,75th])			**ANOVA/**
Parameter	**SAL**	**CON**	**DIS**	**Kruskal Wallis**
Time in open arms (s)	26.8 ± 4.9	44.4 ± 9.4	9.6 ± 2.3 #	**F(2,16)=8.640 p=0.0029**
Entries to open arms	4.8 ± 0.8	7.0 ± 1.3	3.0 ± 0.5 #	**F(2,16)=5.246 p=0.0177**
Latency to enter open arms (s)	30.0 [21.0, 90.6]	14.7 [7.3, 33.3]	45.6 [7.1, 155.2]	H(2)=2.708 p=0.2687
Head dips	13.5 ± 1.8	18.0 ± 3.5	7.4 ± 0.9 #	**F(2,16)=5.879 p=0.0122**
Distance travelled (m)	11.8 ± 0.7	11.3 ± 0.5	9.4 ± 0.7 *	**F(2,16)=3.868 p=0.0426**

3.2 Effect of two days discontinuation from paroxetine treatment on c-FOS expression in the DRN and MRN

Sections were first examined to verify the anatomical location of TPH2 and NeuN staining, as well as to confirm that c-FOS immunoreactivity was evident following EPM exposure. TPH2+ cells were clearly stained in all subregions of the DRN and in the MRN, as in Lowry et al. (2005).

The dorsal subregion of the DRN was located immediately ventral to the cerebral aqueduct (*Fig. 4.2*). In the ROI in the dorsal DRN, there were on average 113 TPH2+ and 244 NeuN+ neurons, meaning 44% of neurons were TPH2+. c-FOS immunoreactivity was evident in all subregions of the DRN. Across all images taken, 25% of neurons in the ROI of the dorsal DRN were c-FOS+. c-FOS was expressed in both TPH2+ and TPH2− negative neurons: 42% of c-FOS+ neurons were TPH2+ and 58% were TPH2−.

In the DRN, paroxetine continuation reduced the percentage of c-FOS+ neurons compared to saline controls ($F(2,16)=9.089$, $p=0.0023$, post-hoc SAL vs CON $p=0.0426$). In comparison, paroxetine discontinuation reversed the percentage of c-FOS+ neurons back to saline control levels (CON vs DIS $p=0.0006$; *Fig 4.5A*). Paroxetine discontinuation increased the percentage of c-FOS+ TPH2+ neurons compared to continued paroxetine and saline controls ($F(2,16)=10.85$, $p=0.0011$, post-hoc SAL vs DIS $p=0.0082$, CON vs DIS $p=0.0003$; *Fig. 4.5B*). There were no effects of treatment on the percentage of c-FOS+ TPH2− neurons ($F(2,16)=2.547$, $p=0.1095$; *Fig. 4.5C*), or the percentage of neurons that were TPH2+ ($F(2,16)=0.2801$, $p=0.7593$; *Fig. 4.5D*).

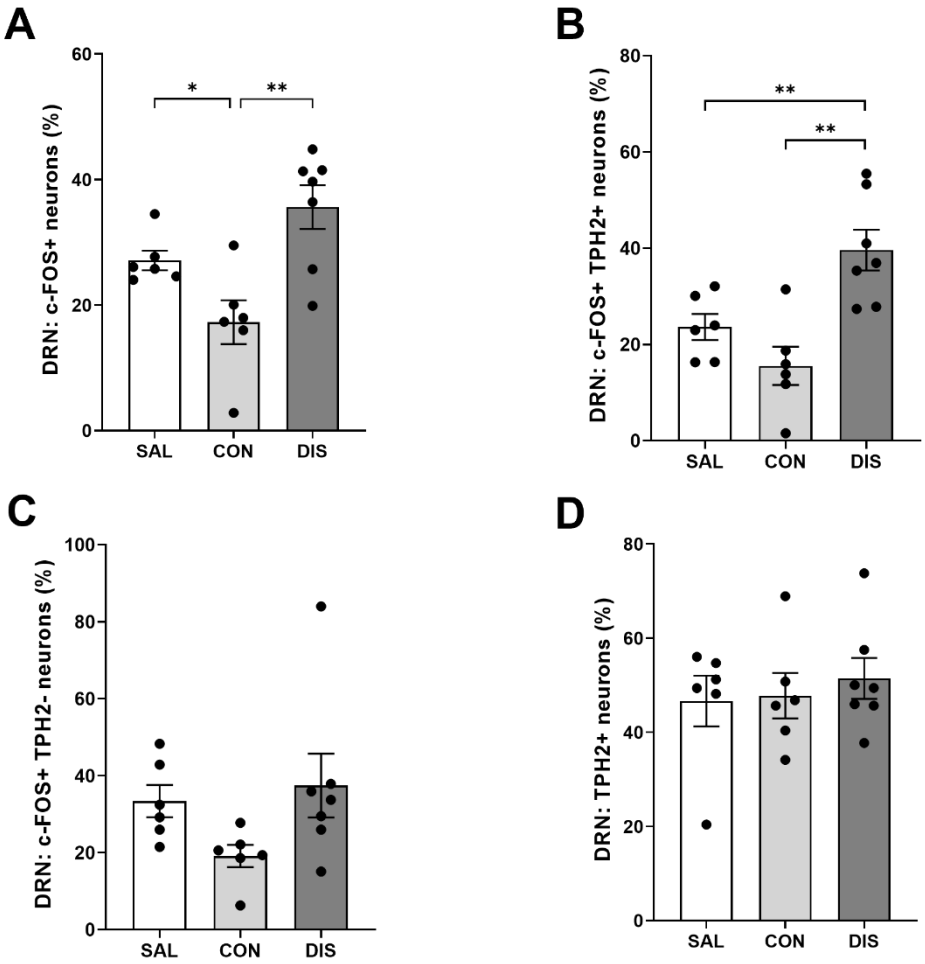


Figure 4.5 Effect of two days discontinuation from paroxetine treatment on c-FOS expression in the dorsal subregion of the DRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D). SAL (n=6), CON (n=6), DIS (n=7). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

The MRN was located ventral to the DRN with TPH2+ neurons aligned along the midline of the slice (*Fig. 4.2*). In the ROI, there were on average 46 TPH2+ and 217 NeuN+ neurons, meaning 22% of neurons were TPH2+. Across all images taken, 18% of neurons in the ROI in the MRN were c-FOS+. c-FOS was expressed in both TPH2+ and TPH2− neurons: 36% of c-FOS+ neurons were TPH2+ and 64% were TPH2−.

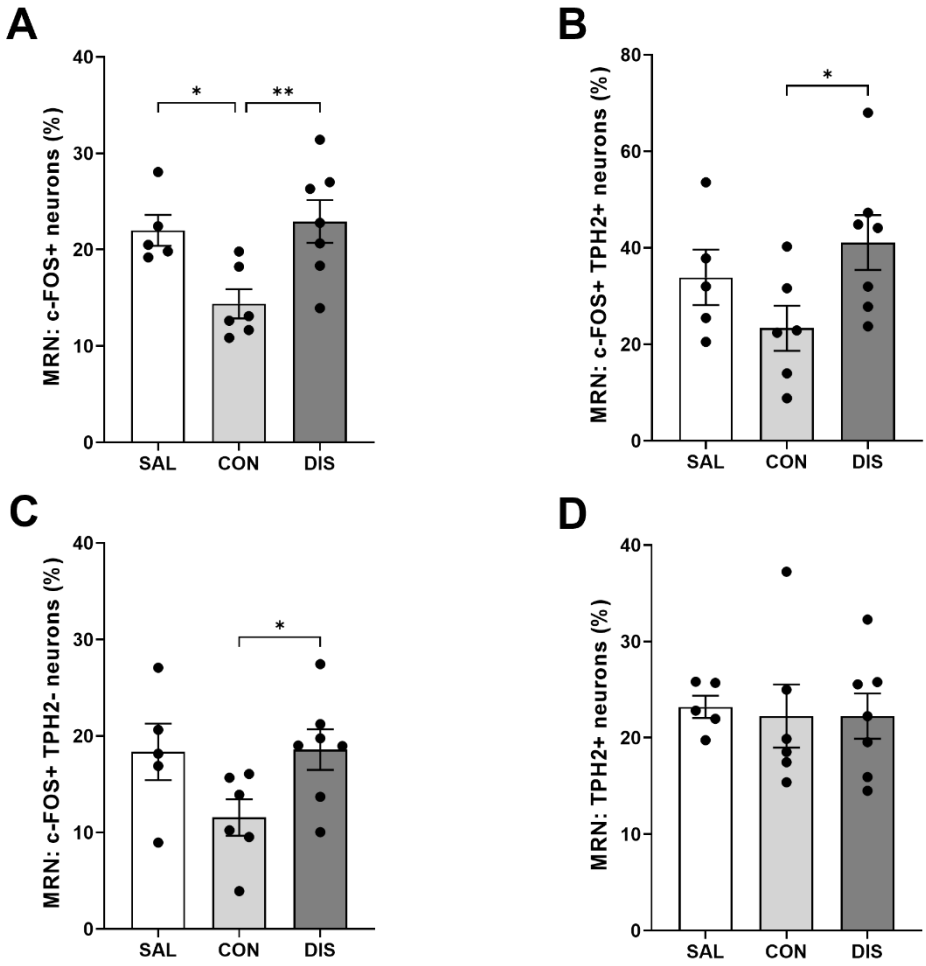


Figure 4.6 Effect of two days discontinuation from paroxetine treatment on c-FOS expression in the MRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D). SAL (n=5, one brain excluded due to tissue damage during slicing), CON (n=6), DIS (n=7). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In the MRN, paroxetine continuation reduced the percentage of c-FOS+ neurons compared to saline controls, which reversed following paroxetine discontinuation ($F(2,15)=6.148$, $p=0.0112$, post-hoc SAL vs CON $p=0.0167$, CON vs DIS $p=0.0050$; *Fig. 4.6A*). There was a trend towards an effect of treatment on the percentage of c-FOS+ TPH2+ neurons ($F(2,15)=2.888$, $p=0.0869$). Post-hoc tests showed that paroxetine discontinuation increased the percentage of c-FOS+ TPH2+ neurons compared to continued paroxetine, although this was not significant from saline controls (post-hoc

CON vs DIS p=0.0299; *Fig 4.6B*). There was also a trend towards an effect of treatment on the percentage of c-FOS+ TPH2− neurons, and post-hoc tests again showed that paroxetine discontinuation increased c-FOS compared to continued paroxetine, but this did not differ from saline controls (F(2,15)=3.116, p=0.0738, post-hoc CON vs DIS p=0.0380; *Fig 4.6C*). There was no effect of treatment on the percentage of neurons that were TPH2+ (F(2,15)=0.04277, p=0.9582; *Fig. 4.6D*).

In summary, paroxetine continuation reduced c-FOS expression in the DRN and MRN. Two days of paroxetine discontinuation produced a rebound increase in the percentage of c-FOS+ TPH2+ neurons in the DRN. Paroxetine discontinuation also increased the percentage of c-FOS+ neurons in the MRN, but this reflected a reversal back to saline control levels. The overall proportion of neurons that were TPH2+ did not differ in either region.

3.3 Effect of two days discontinuation from paroxetine treatment on c-FOS expression in other anxiety-related brain regions

The DG of the hippocampus was identified by dense NeuN immunostaining. There were on average 63 c-FOS+ neurons in the DG of the DH and 41 in the VH (*Fig. 4.3*). In the DH, paroxetine continuation reduced the number of c-FOS+ neurons, which reversed to saline control levels following discontinuation (F(2,16)=5.585, p=0.0145, post-hoc SAL vs CON p=0.0263, CON vs DIS p=0.0054; *Fig. 4.7A*). In the VH, paroxetine discontinuation increased the number of c-FOS+ neurons compared to saline controls, and there was a trend towards an increase compared to continued paroxetine (F(2,16)=7.280, p=0.0056, post-hoc SAL vs DIS p=0.0016, CON vs DIS p=0.0539; *Fig. 4.7B*).

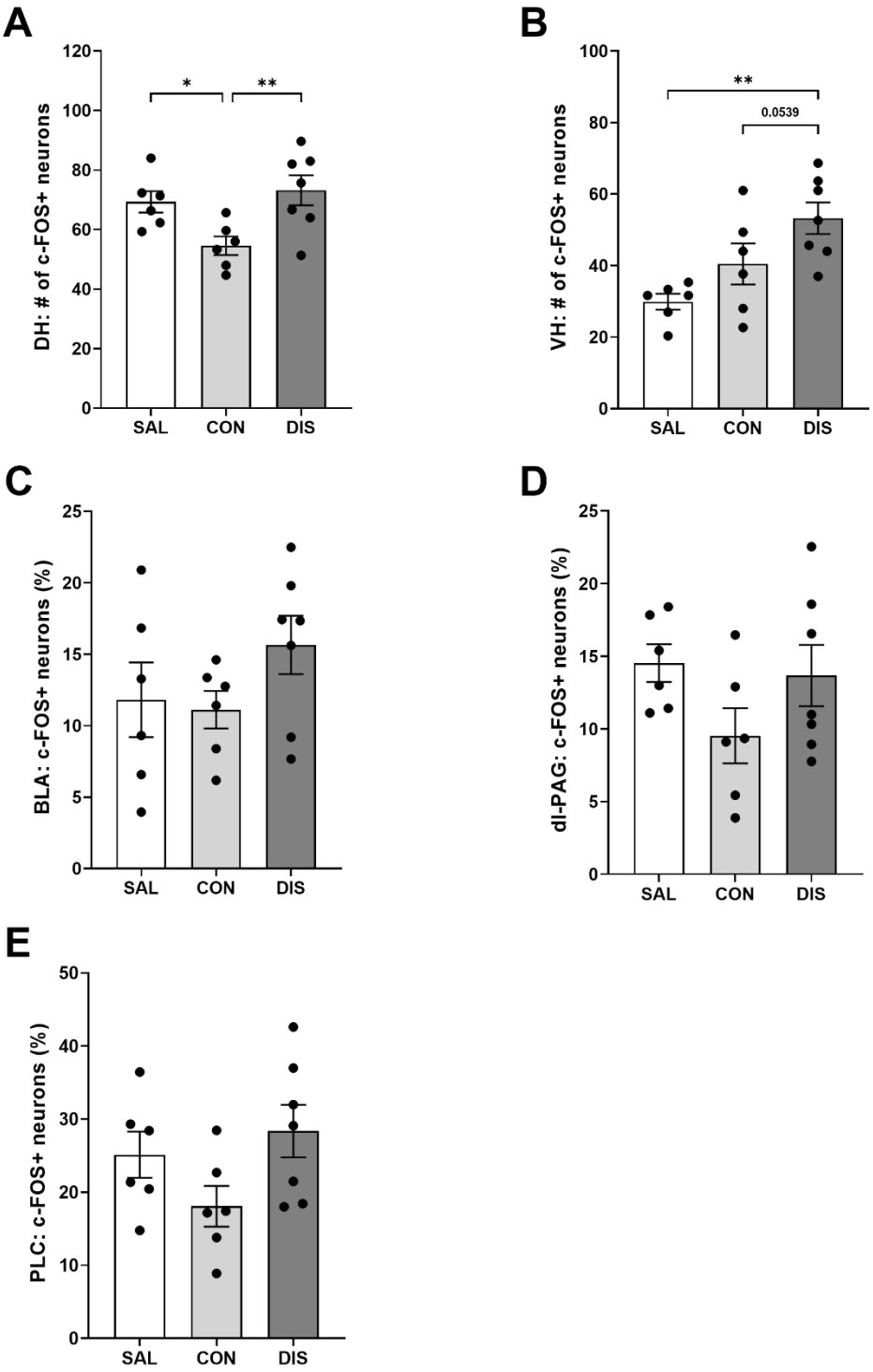


Figure 4.7 Effect of two days discontinuation from paroxetine treatment on c-FOS expression in other anxiety-related brain regions. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the DH (A), number of c-FOS+ neurons in the VH (B), percentage of c-FOS+ neurons in the BLA (C), percentage of c-FOS+ neurons in the dl-PAG (D) and percentage of c-FOS+ neurons in the PLC (E). SAL (n=6), CON (n=6), DIS (n=7). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

The BLA was identified between the ventral ends of the amygdalar capsule and the external capsule of the corpus callosum (*Fig. 4.4*). There were on average 214 NeuN+ neurons in the ROI, and 14% of the neurons were c-FOS+. There was no effect of treatment on the percentage of c-FOS+ neurons in the BLA ($F(2,16)=1.458$, $p=0.2621$; *Fig. 4.7C*). The dl-PAG was identified as dorsolateral to the cerebral aqueduct (*Fig. 4.4*). There were on average 274 NeuN+ neurons in the ROI, and 13% of the neurons were c-FOS+. There was no effect of treatment on the percentage of c-FOS+ neurons in dl-PAG ($F(2,16)=2.016$, $p=0.1656$; *Fig. 4.7D*). The PLC was identified as lateral to the midline in the mPFC, just dorsal to the corpus callosum (*Fig. 4.4*). There were on average 285 NeuN+ neurons in the ROI, and 24% of the neurons were c-FOS+. There was also no effect of treatment on the percentage of c-FOS+ neurons in the PLC ($F(2,16)=2.613$, $p=0.1042$; *Fig 4.7E*).

In summary, two days of paroxetine discontinuation increased c-FOS immunoreactivity in the VH and reversed the paroxetine-induced decrease in c-FOS expression in the DH. There were no effects of paroxetine discontinuation on c-FOS immunoreactivity in the BLA, dl-PAG or PLC. Thus, increased anxiety-like behaviour on discontinuation day two was associated with the activation of 5-HT neurons in the raphe nuclei, and greater VH activation.

3.4 Effect of five days discontinuation from 12 days paroxetine on anxiety-like behaviour on the EPM

Previous experiments found no evidence of increased anxiety-like behaviour on the EPM in mice five days after paroxetine discontinuation compared to continued paroxetine or saline controls (Chapter 3 section 3.3). Even in the subset of mice used here for

immunohistochemical analysis, there were no significant effects of five days of paroxetine discontinuation on any EPM parameter (*Table 4.4*). There were also no effects of paroxetine continuation.

Table 4.4 Effect of five days discontinuation from paroxetine treatment on the EPM. 5 min testing on the EPM. Each value is a mean ± SEM or median and IQR [25th percentile, 75th percentile]. N=6/group.

	Group (mean ± SEM, median [25th,75th])			ANOVA/
Parameter	**SAL**	**CON**	**DIS**	**Kruskal Wallis**
Time in open arms (s)	24.1 ± 4.3	15.0 ± 8.1	22.8 ± 6.8	F(2,15)=0.5585 p=0.5835
Entries to open arms	3.8 ± 0.6	2.0 ± 0.7	2.8 ± 0.7	F(2,15)=1.820 p=0.1960
Latency to enter open arms (s)	20.9 [9.8, 59.8]	78.1 [9.4, 202.1]	66.1 [25.0, 128.3]	H(2)=1.485 p=0.5022
Head dips	15.7 ± 1.2	9.3 ± 3.4	13.5 ± 2.1	F(2,15)=1.768 p=0.2015
Distance travelled (m)	8.2 ± 0.4	8.4 ± 2.7	8.1 ± 1.1	F(2,15)=0.0089 p=0.9911

3.5 Effect of five days discontinuation from paroxetine treatment on c-FOS expression in the DRN and MRN

The location and extent of c-FOS, TPH2 and NeuN immunoreactivity were broadly similar to the first experiment. Notably, though, the proportion of c-FOS+ neurons in the DRN, and the number of c-FOS+ neurons in the VH, were half those of the previous experiment across all three treatment groups. This may reflect batch-to-batch variation in immunohistochemical staining.

Since increased anxiety-like behaviour was not evident five days after paroxetine discontinuation, it was hypothesised that c-FOS expression in anxiety-related brain regions would not be significantly different from saline controls. Yet, in the DRN, paroxetine discontinuation still increased the percentage of c-FOS+ neurons, and c-FOS+ TPH2+ neurons, compared to continued paroxetine and saline controls (c-FOS+:

F(2,14)=8.765, p=0.0030, post-hoc SAL vs DIS p=0.0009, CON vs DIS p=0.0153; *Fig 4.8A;* c-FOS+ TPH2+: F(2,14)=12.40, p=0.0001, post-hoc SAL vs DIS p=0.0035, CON vs DIS p=0.0003; *Fig. 4.8B*). There were no effects of treatment on the percentage of c-FOS+ TPH2− neurons (F(2,14)=2.870, p=0.0903; *Fig. 4.8C*) or the percentage of neurons that were TPH2+ (F(2,14)=1.580, p=0.2383; *Fig. 4.8D*).

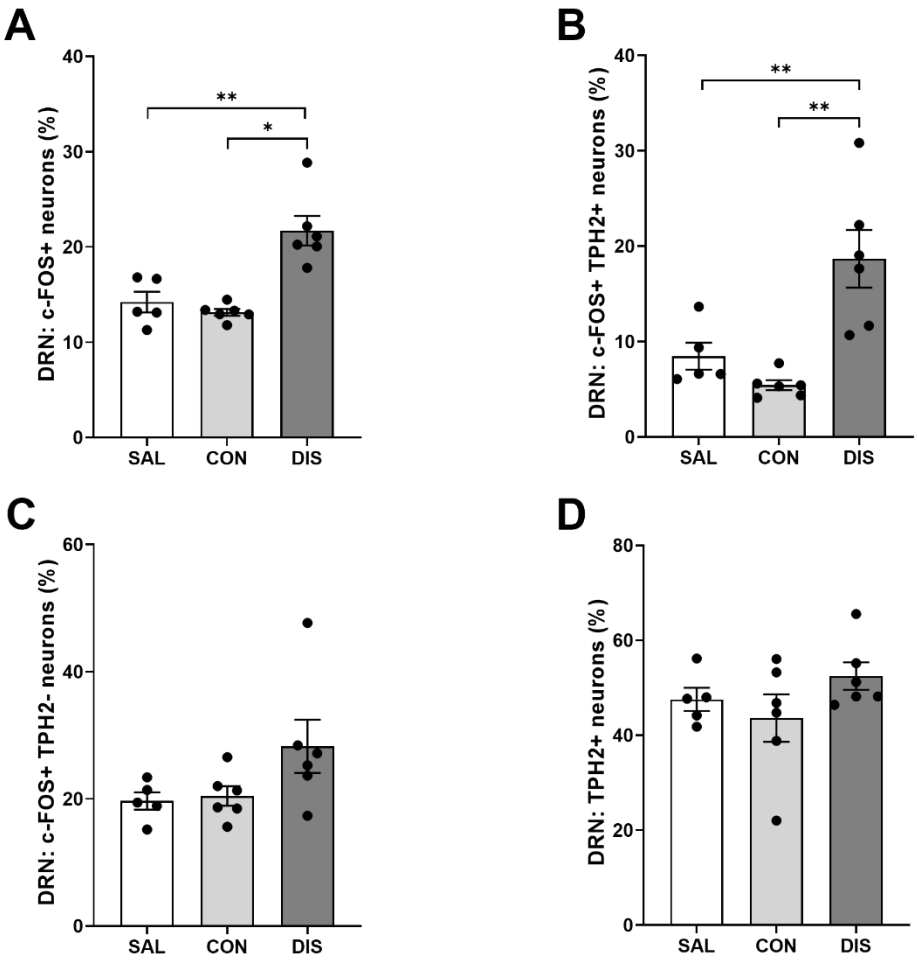


Figure 4.8 Effect of five days discontinuation from paroxetine treatment on c-FOS expression in the dorsal subregion of the DRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D). SAL (n=5, one brain damaged during slicing), CON (n=6), DIS (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In the MRN, there was no effect of treatment on the overall percentage of c-FOS+ neurons (F(2,15)=0.9501, p=0.4088; *Fig 4.9A*). Nonetheless, paroxetine continuation

reduced the percentage of c-FOS+ TPH2+ neurons, which reversed back to saline control levels following paroxetine discontinuation (F(2,15)=5.415, p=0.0170, post-hoc SAL vs CON p=0.0482, CON vs DIS p=0.0056; *Fig. 4.9B*). There were no effects of treatment on the percentage of c-FOS+ TPH2− neurons (F(2,15)=0.8937, p=0.4299; *Fig 4.9C*) or the percentage of neurons that were TPH2+ (F(2,15)=0.5693, p=0.5777; *Fig 4.9D*).

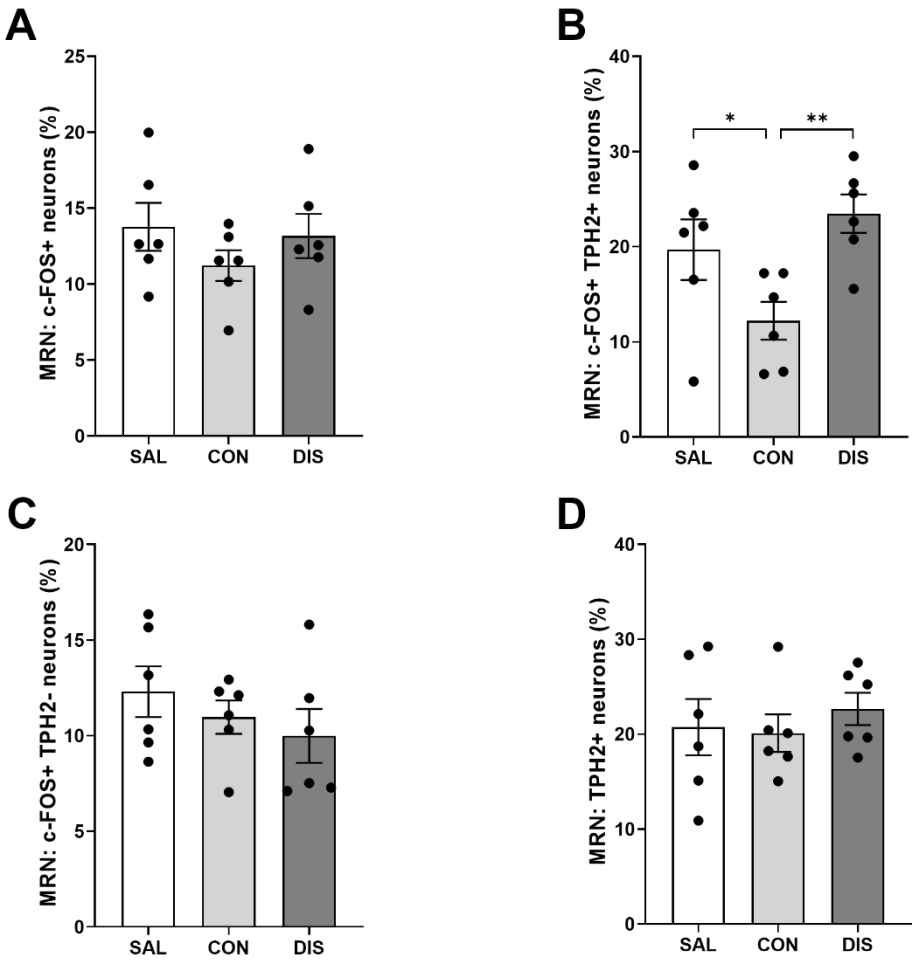


Figure 4.9 Effect of five days discontinuation from paroxetine treatment on c-FOS expression in the MRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D). SAL (n=6), CON (n=6), DIS (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In summary, the percentage of c-FOS+ TPH2+ neurons in the DRN was still elevated relative to controls five days after paroxetine discontinuation, despite no

evidence of increased anxiety-like behaviour at this time. Similar to following two days after discontinuation, the percentage of c-FOS+ TPH2+ neurons in the MRN was at saline control levels.

3.6 Effect of five days discontinuation from 12 days paroxetine on c-FOS expression in other anxiety-related brain regions

There were no effects of treatment on the number of c-FOS+ neurons in the DH ($F(2,15)=0.6556$, $p=0.5334$; *Fig. 4.10A*), VH ($F(2,15)=0.5693$, $p=0.5777$; *Fig. 4.10B*), BLA ($F(2,15)=0.0578$, $p=0.9440$; *Fig. 4.10C*), dl-PAG ($F(2,15)=0.1639$, $p=0.8503$; *Fig. 4.10D*) or PLC ($F(2,15)=1.084$, $p=0.3633$; *Fig. 4.10E*). Thus, five days of paroxetine discontinuation was no longer associated with increased c-FOS expression in the VH. This lack of effect resembled the absence of an anxiogenic effect five days after paroxetine discontinuation.

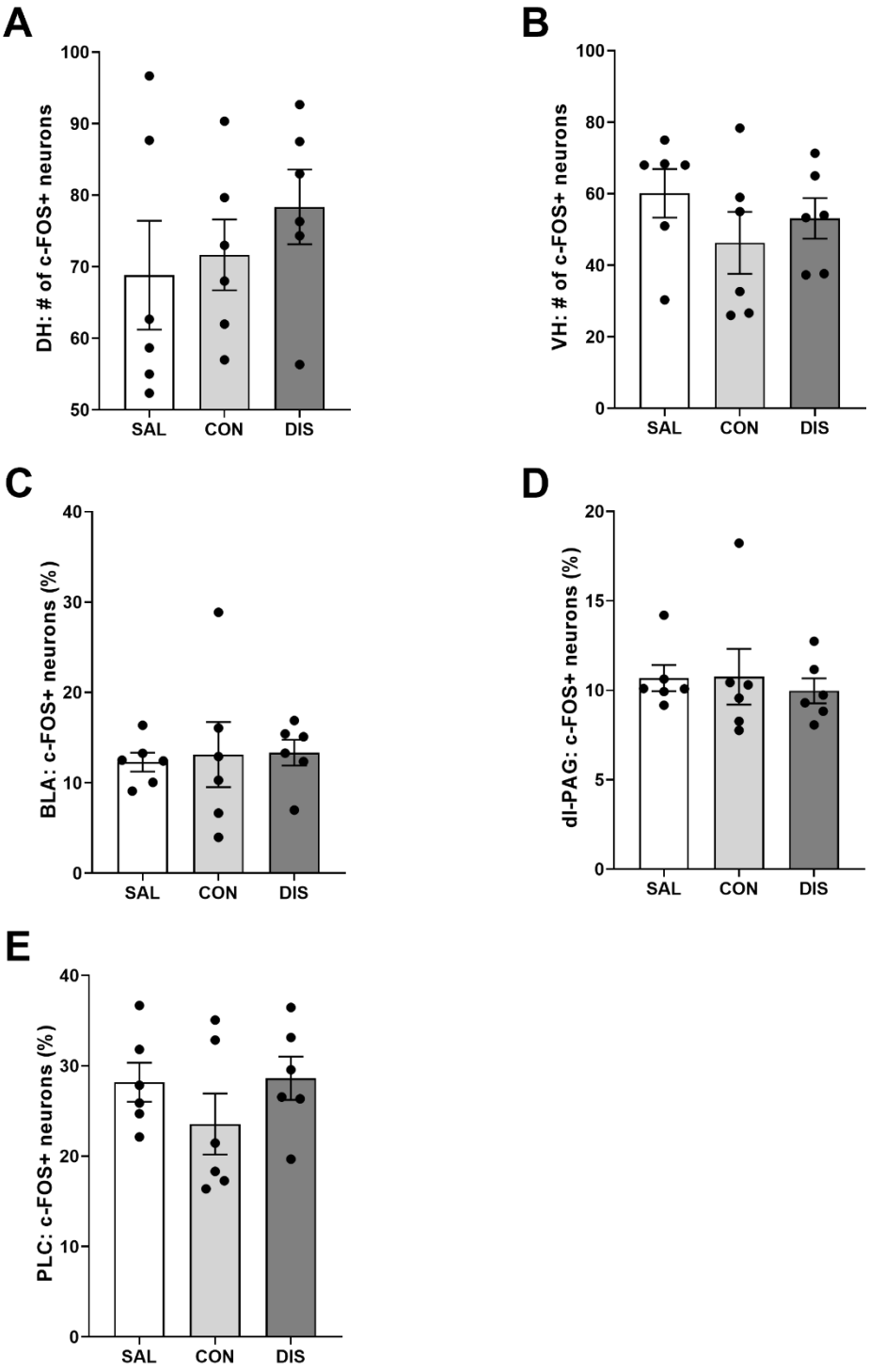


Figure 4.10 Effect of five days discontinuation from paroxetine treatment on c-FOS expression in other brain regions. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the DH (A), number of c-FOS+ neurons in the VH (B), percentage of c-FOS+ neurons in the BLA (C), percentage of c-FOS+ neurons in the dl-PAG (D) and percentage of c-FOS+ neurons in the PLC (E). SAL (n=6), CON (n=6), DIS (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD.

3.7 Combined effects of paroxetine continuation on c-FOS expression

In the individual experiments, the effect of continued paroxetine treatment was inconsistent, both in the raphe nuclei and downstream structures. To clarify its effect on c-FOS expression, data from the two experiments were normalised to the mean saline value for each experiment and then combined.

In the DRN, paroxetine continuation reduced the percentage of c-FOS+ neurons and c-FOS+ TPH2+ neurons compared to saline controls (c-FOS+: $F(3,32)=13.77$, $p<0.0001$, post-hoc SAL vs CON $p=0.0345$; *Fig. 4.11A*; c-FOS+ TPH2+: $F(3,32)=9.878$, $p<0.0001$, post-hoc SAL vs CON $p=0.0044$; *Fig. 4.11B*). As in the individual experiments, two and five days of paroxetine discontinuation increased the percentage of c-FOS+ neurons, and produced a rebound increase in the percentage of c-FOS+ TPH2+ neurons in the DRN (c-FOS+: SAL vs DIS 2 $p=0.0034$, SAL vs DIS 5 $p=0.0020$, CON vs DIS 2 $p<0.0001$, CON vs DIS 5 $p<0.0001$; *Fig. 4.11A;* c-FOS+ TPH2+: SAL vs DIS 2 $p=0.0177$, SAL vs DIS 5 $p=0.0374$, CON vs DIS 2 $p<0.0001$, CON vs DIS 5 $p=0.0002$; *Fig. 4.11B*). There were no significant differences between paroxetine continuation and saline controls in terms of the percentage of c-FOS+ TPH2− neurons, although five days of paroxetine discontinuation increased the percentage of c-FOS+ TPH2− neurons compared to continued paroxetine ($F(3,32)=3.123$, $p=0.0394$, post-hoc CON vs DIS 5 $p=0.0055$; *Fig. 4.11C*). There were no significant effects of paroxetine continuation or discontinuation on the percentage of neurons that were TPH2+ ($F(3,32)=0.8019$, $p=0.5020$; *Fig. 4.11D*).

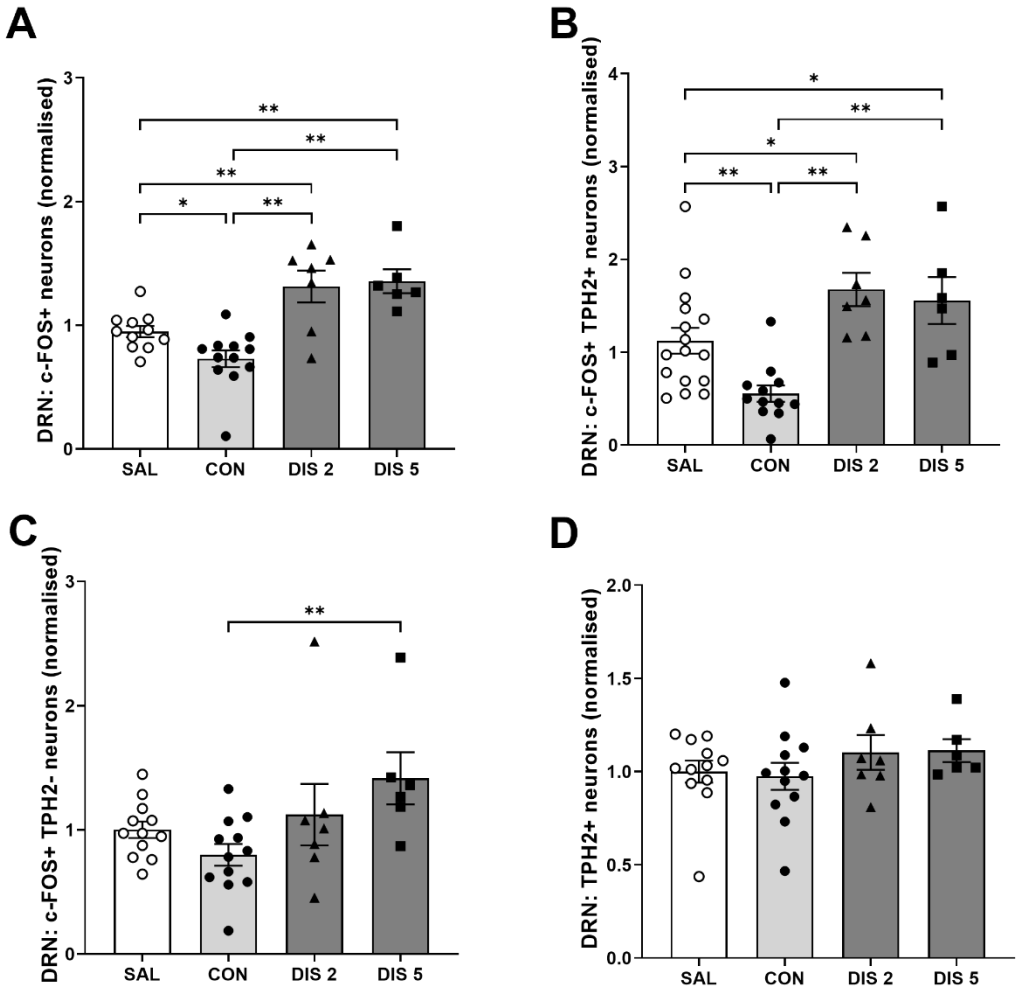


Figure 4.11 Combined effects of paroxetine continuation and discontinuation on c-FOS expression in the dorsal subregion of the DRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D) relative to saline controls. SAL (n=11), CON (n=12), DIS 2 (n=7), DIS 5 (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In the MRN, paroxetine continuation reduced the percentage of c-FOS+ neurons and c-FOS+ TPH2+ neurons compared to saline controls (c-FOS+: $F(3,32)=3.895$, $p=0.0183$, post-hoc SAL vs CON $p=0.0084$; *Fig. 4.12A*; c-FOS+ TPH2+: $F(3,32)=5.449$, $p=0.0038$, post-hoc SAL vs CON $p=0.0212$; *Fig. 4.12B*). Two but not five days of paroxetine discontinuation increased the percentage of c-FOS+ neurons compared to continued paroxetine (CON vs DIS 2 $p=0.0075$), and both two and five days of paroxetine

discontinuation increased the percentage of c-FOS+ TPH2+ neurons compared to continued paroxetine (CON vs DIS 2 p=0.0016, CON vs DIS 5 p=0.0036), but neither day was significantly different from saline controls. There were no effects of paroxetine continuation or discontinuation on the percentage of c-FOS+ TPH2− neurons (F(3,32)=2.053, p=0.1261; *Fig. 4.12C*) or the percentage of neurons that were TPH2+ (F(3,32)=0.3837, p=0.7654; *Fig. 4.12D*).

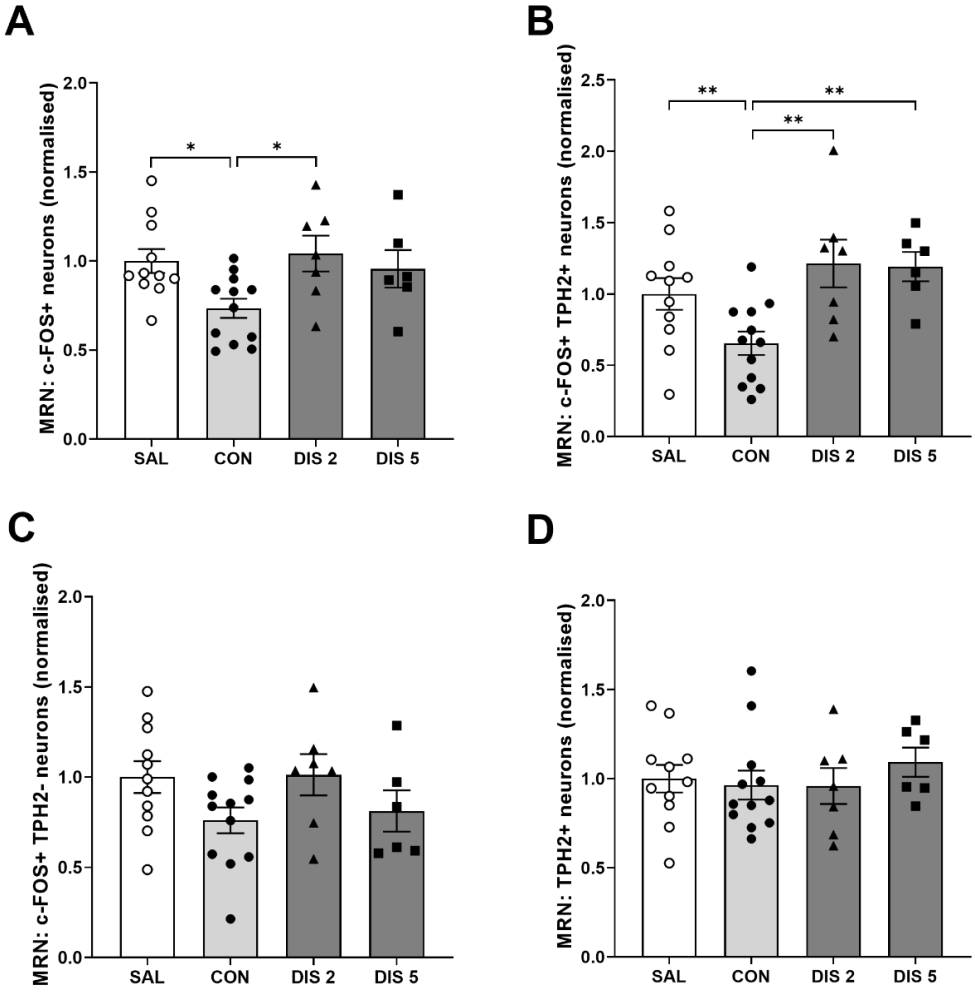


Figure 4.12 Combined effects of paroxetine continuation and discontinuation on c-FOS expression in the MRN. Bars represent the mean ± SEM values for the percentage of c-FOS+ neurons (A), percentage of c-FOS+ TPH2+ neurons (B), percentage of c-FOS+ TPH2− neurons (C) and percentage of TPH2+ neurons (D) relative to saline controls. SAL (n=11), CON (n=12), DIS 2 (n=7), DIS 5 (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * p<0.05, ** p<0.01.

There were no effects of paroxetine continuation or discontinuation on the number of c-FOS+ neurons in the DH ($F(3,33)=1.977$, $p=0.1366$; *Fig. 4.13A*). In the VH, two but not five days of paroxetine discontinuation increased the number of c-FOS+ neurons compared to continued paroxetine and saline controls ($F(3,33)=8.790$, $p=0.0002$, post-hoc SAL vs DIS 2 $p<0.0001$, CON vs DIS 2 $p=0.0002$, DIS 2 vs DIS 5 $p=0.0001$; *Fig. 4.13B*). There were no effects of treatment on the percentage of c-FOS+ neurons in the BLA ($F(3,33)=0.9753$, $p=0.4161$; *Fig. 4.13C*), dl-PAG ($F(3,33)=0.6607$, $p=0.5821$; *Fig. 4.13D*) or PLC ($F(3,33)=2.722$, $p=0.0601$; *Fig. 4.13E*).

In summary, the combined analysis confirmed that paroxetine continuation reduced the percentage of c-FOS+ neurons, and specifically the percentage of c-FOS+ TPH2+ neurons in the DRN and MRN. By comparison, there was no reduction in c-FOS immunoreactivity following continued paroxetine treatment in other regions.

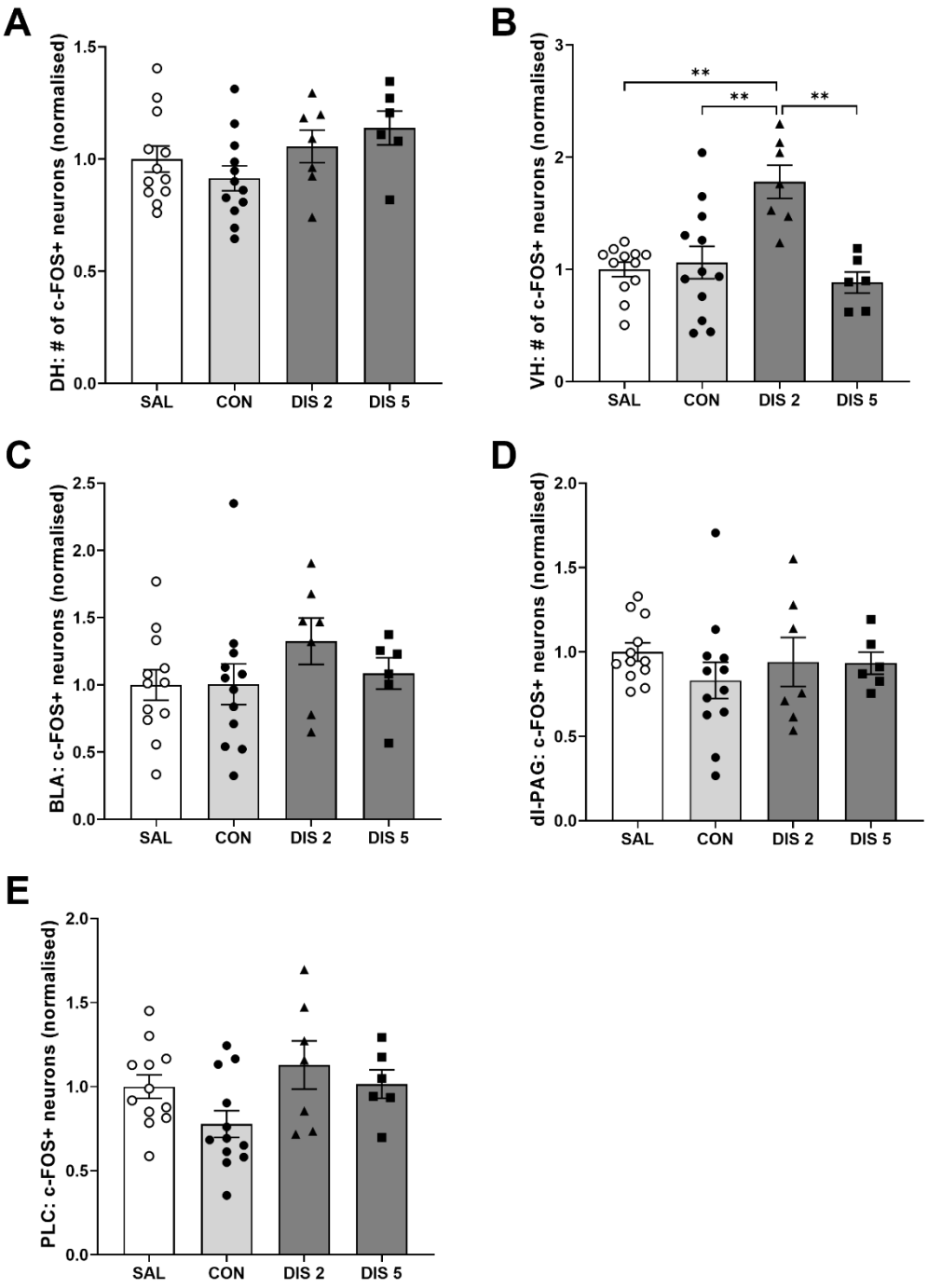


Figure 4.13 Combined effects of paroxetine continuation and discontinuation on c-FOS expression in other brain regions. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the DH (A), number of c-FOS+ neurons in the VH (B), percentage of c-FOS+ neurons in the BLA (C), percentage of c-FOS+ neurons in the dl-PAG (D) and percentage of c-FOS+ neurons in the PLC (E) relative to saline controls. SAL (n=12), CON (n=12), DIS 2 (n=7), DIS 5 (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, ** $p<0.01$.

4 Discussion

Experiments in the previous chapter found that two days of paroxetine discontinuation was associated with increased anxiety-like behaviour and behavioural inhibition on the EPM. Here, c-FOS immunohistochemistry was used to investigate the effect of paroxetine discontinuation on the activation of anxiety-related neural circuitry.

Two days of paroxetine discontinuation was again shown to increase anxiety-like behaviour on the EPM compared to controls. This effect was associated with greater c-FOS immunoreactivity in TPH2+ neurons in the DRN and MRN compared to continued paroxetine treatment, which even rebound above saline controls in the DRN. These results suggest that paroxetine discontinuation was associated with the activation of 5-HT neurons. Two days of paroxetine discontinuation was also associated with increased c-FOS expression in the VH compared to controls, suggesting that increased anxiety-like behaviour could also be linked to hippocampal activation.

By comparison, there was no evidence of increased anxiety-like behaviour five days after paroxetine discontinuation. Nonetheless, c-FOS expression was still elevated in TPH2+ neurons in the DRN and MRN compared to continued paroxetine treatment, and again rebounded above saline controls in the DRN. On the other hand, there were no effects of five days of paroxetine discontinuation on VH c-FOS expression.

4.1 Two days of paroxetine discontinuation was associated with increased c-FOS immunoreactivity in 5-HT neurons and in the VH

Two days after paroxetine discontinuation, c-FOS immunoreactivity was increased in TPH2+ neurons in the dorsal subregion of the DRN compared to both continued paroxetine and saline controls, implying there was evidence of a rebound activation of

5-HT neurons. There was also an increased percentage of c-FOS+ TPH2+ neurons in the MRN relative to continued paroxetine treatment, although this simply represented a return to saline control levels. In contrast, there was little evidence of an increase in the percentage of c-FOS+ TPH2− neurons in either region, suggesting the specific activation of 5-HT neurons. Importantly, there were no effects of treatment on the percentage of TPH2+ neurons, meaning the absolute number of 5-HT neurons did not change.

These findings are consistent with other reports that anxiety activates 5-HT neurons. For example, EPM exposure induced c-FOS expression in the DRN of rodents (Jacobs & Fornal, 1991; Silveira et al., 1993; Gomes et al., 2005; Berg et al., 2019). Elevated c-FOS expression in the DRN also correlated with increased anxiety-like and avoidance behaviours in rats (Salomé et al., 2004; Maier & Watkins, 2005; Muigg et al., 2009; Popa et al., 2010). This activation includes the dorsal subregion of the DRN – for example, mice exhibiting heightened inhibitory avoidance behaviours on the elevated T-maze (spending more time in the closed arms) had greater c-FOS expression in TPH2+ neurons in the dorsal DRN compared to lower-anxiety mice (Spiacci et al., 2012). Other studies have also detected increased c-FOS expression in the MRN of mice following exposure to the EPM (Gomes et al., 2005).

Moreover, evidence suggests that 5-HT neuron activation can promote anxiety-like behaviour. For instance, optogenetic stimulation of 5-HT neurons in the dorsal DRN reduced the time mice spent in the centre zone of the OF compared to controls (Ren et al., 2018). Similarly, activation of the MRN reduced the time mice spent in the open arms of the EPM (Ohmura et al., 2014; Ohmura et al., 2020) and increased anxiety-related defensive behaviours in rats (Vicente et al., 2008). Thus, anxiety can be both a cause and a consequence of 5-HT neuron activation. In the case of paroxetine discontinuation,

increased c-FOS immunoreactivity in 5-HT neurons two days after paroxetine cessation could be interpreted as evidence that the aversive nature of paroxetine discontinuation activated anxiety circuitry. Alternatively, it could suggest that increased 5-HT transmission promoted anxiety-like behaviour on the EPM.

Two days of paroxetine discontinuation also increased c-FOS immunoreactivity in the VH compared to continued paroxetine and saline controls. Other studies support this finding, in that anxiety-provoking situations such as the EPM and OF were associated with increased c-FOS expression in the VH (Hale et al., 2008; Peng et al., 2019; Vo et al., 2021). Moreover, activation of the VH reduced the time mice spent in the open arms of the EPM (Vo et al. 2021), whereas lesioning or optogenetic inhibition of the VH was anxiolytic across different unconditioned test of anxiety (Kjelstrup et al., 2002; Bannerman et al., 2003; McHugh et al., 2004; Padilla-Coreano et al., 2016). These studies agree with the notion that VH activation is linked to increased anxiety-like behaviour.

Paroxetine discontinuation was also associated with behavioural inhibition on the EPM, which has been linked to increased serotonergic transmission and VH activity (McNaughton & Gray, 2000). For instance, increased c-FOS expression in the dorsal DRN was correlated with reduced motor output in rats (Abrams et al., 2005; Lowry et al., 2005). Optogenetic and chemogenetic activation of the DRN also suppressed LMA in anxiety tests in mice (Teissier et al., 2015; Whitney et al., 2016; Correia et al., 2017; Ren et al., 2018). Furthermore, rats with neurotoxic lesions to the VH were hyperactive on the successive alleys test (an alternative to the EPM) compared to sham-operated controls (Maren, 1999; Richmond et al., 1999; Kjelstrup et al., 2002; Bannerman et al., 2004). Thus, there is evidence that VH and 5-HT neuron activation may be linked to reduced exploratory drive in potentially threatening situations.

From the current data, it could be speculated that a serotonergic projection to the VH may be responsible for the behavioural effects seen on the EPM. 5-HT is thought to regulate the responses of broader neural networks to aversive stimuli, including via projections to the hippocampus (Deakin & Graeff, 1991; McNaughton & Gray, 2000; Sengupta & Holmes, 2019). The MRN is typically thought of as the predominant serotonergic mediator of anxiety-like behaviour, and most 5-HT inputs to the VH arise from the MRN. For example, neurotoxic MRN lesions reduced anxiety-like behaviour on the EPM, but there were no such effects of lesions to the DRN (Critchley et al., 1992; Adams et al., 2008). Moreover, activation of the MRN increased anxiety-like behaviour on the EPM, via 5-HT release in the VH, whereas DRN activation did not produce these behavioural effects (Ohmura et al., 2014; Teissier et al., 2015; Ohmura et al., 2020; Chaves et al., 2022). This could suggest that the activation of 5-HT projections from the MRN to the VH could have mediated the anxiety-like behaviours evident two days after paroxetine discontinuation. Although MRN somatic activation did not increase above saline levels (like in the DRN), it is possible that 5-HT released from MRN projections acted on more sensitive postsynaptic receptors. Thus, the net effect of 5-HT in downstream regions could have been potentiated and thus driven anxiety.

Nonetheless, there is emerging evidence that 5-HT neurons originating in the DRN can promote anxiety-like behaviours. A subpopulation of neurons in the caudal DRN also project to the VH via the medial forebrain bundle (Azmitia & Segal, 1978; Sharp et al., 1990; McQuade & Sharp, 1997; Abrams et al., 2005; Lowry et al., 2008). It has recently been demonstrated that the optogenetic activation of this subpopulation increased anxiety-like behaviour in mice on the EPM via synchronised DRN and VH activity (Fortin-Houde et al., 2022; unpublished work, personal communication with the Amilhon

lab). The developing links between the DRN and VH suggest a hitherto unappreciated role of the DRN in anxiety responses.

It is therefore plausible that the greater activation of the DRN and/or the MRN could be mediating the increased anxiety-like behaviour two days after paroxetine discontinuation. The rebound activation of the DRN above saline control levels, compared to the return to baseline levels of activation in the MRN, could be evidence that DRN activity is the predominant driver of this anxiety. Nonetheless, from c-FOS data alone it is not possible to determine the relative importance of the DRN and MRN in the behavioural effects observed on EPM. Moreover, without knowing the effect of this activation on downstream structures, it cannot be concluded which, if either, of the raphe nuclei is driving anxiety-like behaviour, or role of the VH in mediating this effect.

4.2 There was a dissociation between c-FOS expression in 5-HT neurons and anxiety-like behaviour on discontinuation day five

As reported in Chapter 3, five days after paroxetine discontinuation mice no longer exhibited evidence of increased anxiety-like behaviour compared to controls; yet c-FOS expression in 5-HT neurons in the DRN remained elevated. The reason for the dissociation between the behavioural and neurobiological effects of paroxetine discontinuation is not clear but could reflect several possibilities. Firstly, increased 5-HT neuron activation was not sufficient to elicit anxiety-like behaviour on discontinuation day five, suggesting it may not have been causal on discontinuation day two either. For instance, other brain structures could have generated the anxiety-like effects independent of the DRN or MRN. The lack of effect of five days of paroxetine discontinuation on c-FOS expression in the VH further suggests that other brain structures may be more

closely linked to the behavioural effects than raphe activity. Increased 5-HT neuron activation may therefore have been a consequence of paroxetine discontinuation and not causal in the generation of anxiety-like behaviours. Alternatively, the ongoing activation could reflect other behavioural responses, such as the inhibition of panic or coping strategies (Deakin & Graeff, 1991; Ren et al., 2018), that were not measured here.

It is also possible that on discontinuation day two, increased 5-HT neuron activation promoted anxiety-like behaviours on the EPM. This activation persisted on discontinuation day five; however, neuroadaptive changes in postsynaptic structures may have occurred that reduced the response to 5-HT signalling. Consequently, mice no longer displayed evidence of increased anxiety-like behaviour. For instance, inputs from other brain regions could have interacted with, suppressed, or overridden the effects of 5-HT on anxiety-promoting brain circuitry. Changes in the sensitivity of postsynaptic receptors later in the discontinuation period may also have altered the downstream response to 5-HT signalling, independently of somatic activation.

Hence, there are likely to be complex neuroadaptive changes in 5-HT transmission and receptor sensitivity in the week following paroxetine discontinuation that have not yet been explored. It is possible that increased serotonergic drive could have increased anxiety-like behaviour two days after discontinuation but not produced such effect on discontinuation day five. Nonetheless, the causal role of these circuits in the behavioural effects of paroxetine discontinuation cannot be determined from these correlative data. Further work is therefore needed to elucidate if the rebound activation of the DRN, or increased activity of the MRN and VH, mediated the anxiety-like behaviour on discontinuation day two.

4.3 Paroxetine discontinuation did not alter c-FOS expression in other anxiety-promoting brain regions

Neither two nor five days of paroxetine discontinuation increased c-FOS immunoreactivity in other emotion-related brain regions. The lack of effect of paroxetine discontinuation on c-FOS expression in the BLA was unexpected, as anxiogenic environments have previously been shown to induce c-FOS expression in this region (Duncan et al., 1996; Linden et al., 2003; Salomé et al., 2004; Ait Bali et al., 2022). The BLA also receives inputs from the hippocampus and DRN (Bocchio et al., 2015; Sengupta et al., 2017; Yang & Wang, 2017), which were activated by paroxetine discontinuation. The lack of a downstream effect on BLA activation was therefore surprising.

However, this could be explained by the contributions of each brain region to different anxiety-like behaviours. The VH is thought to mediate passive avoidance behaviours, such as making fewer entries to the open arms of the EPM. On the other hand, the amygdala is implicated in active avoidance, such as directed escape responses, which are not assessed on the EPM (Graeff et al., 1993; McNaughton & Gray, 2000). Moreover, neither amygdalar lesions nor optogenetic activation of 5-HT terminals in the BLA altered EPM performance (Decker et al., 1995; Sommer et al., 2001; Kjelstrup et al., 2002; Bannerman et al., 2004; McHugh et al., 2004; Yu et al., 2022). Thus, while the amygdala appears to coordinate conditioned or learned anxiety, the VH may be more influential in unconditioned anxiety responses, including following paroxetine discontinuation.

There were also no changes in c-FOS immunoreactivity in the PLC. Generally, increased anxiety-like behaviour in rodents has been associated with increased c-FOS expression in the PLC, such as following anxiogenic drugs or EPM exposure (Hinks et al., 1996; Linden et al., 2003; Singewald et al., 2003; Peng et al., 2019; Ait Bali et al.,

2022). This activation may reflect the role of the mPFC in top-down feedback suppression of other anxiety-promoting brain regions to prevent excessive anxiety-like behaviour (Lacroix et al., 2000; Amat et al., 2005). Thus, the lack of effect of paroxetine discontinuation the PLC could suggest that this inhibitory influence was not present following SSRI cessation, hence mice exhibited greater anxiety-like behaviours.

Alternatively, like the amygdala, the mPFC may not be critical in anxiety-like behaviours in unconditioned tests of anxiety. The mPFC provides cortical control of behaviour based on prior knowledge and experiences (Jacobs & Moghaddam, 2021). In the case of exposure to the novel EPM, the mice did not have any existing knowledge of the environment and so the input from the mPFC was probably minimal. Exemplifying this, lesioning the ACC and the orbitofrontal cortex, other mPFC subregions, had no effects on unconditioned anxiety test performance in test-naïve mice (Rudebeck et al., 2007; Mariano et al., 2009). This could explain why the PLC was not activated by the EPM during paroxetine discontinuation.

Paroxetine discontinuation did not alter c-FOS expression in the dl-PAG, a brain region associated with panic-like responses. Greater c-FOS expression has previously been reported in the dl-PAG of high-anxiety mice compared to low-anxiety controls (Muigg et al., 2009), as well as following panicogenic yohimbine administration (Singewald & Sharp, 2000). However, the behaviour of mice undergoing paroxetine discontinuation was more consistent with behavioural inhibition than panic responses, suggesting dl-PAG activation does not mediate these effects. In contrast, the ventrolateral PAG (vl-PAG) is the subregion implicated in behavioural inhibition (Bernard & Bandler, 1998; Singewald & Sharp, 2000). Future experiments could quantify c-FOS expression in the vl-PAG to examine its activation during paroxetine discontinuation.

Unlike the VH, paroxetine discontinuation did not alter c-FOS expression in the DH. Although there was some evidence of a reversal of the paroxetine-induced decrease in the number of c-FOS+ neurons on discontinuation day two, analysis of the combined datasets found no overall effects. The literature lacks consistency on the effects of anxiety on DH c-FOS expression – some studies have reported increased DH c-FOS expression following EPM exposure (Linden et al., 2004; Muigg et al., 2009) whereas others have not detected changes (Linden et al., 2003). This variation could reflect the poor controls for the novelty of the test environment or sensory stimulation in these studies, which often compare c-FOS expression after anxiety tests to test-naïve controls (Duncan et al., 1996; Linden et al., 2004; Hale et al., 2008). It is therefore unclear whether anxiety is driving c-FOS expression, or if it simply arises from the novel environment. Nonetheless, the current data suggest that paroxetine discontinuation was not associated with increased c-FOS expression in the DH.

Overall, the lack of effect of paroxetine discontinuation on c-FOS expression in the BLA, PLC, dl-PAG and DH suggests that these regions are not activated following treatment cessation.

4.4 Paroxetine continuation reduced c-FOS expression in 5-HT neurons

Continued paroxetine treatment reduced c-FOS expression in the 5-HT neurons of the DRN and MRN relative to saline controls. This effect was clearest when the data from the two experiments were combined. On the other hand, continued SSRI treatment did not consistently alter c-FOS expression in other brain regions.[4]

[4] A caveat of this, however, is that in the current experiments the mice receiving continued paroxetine treatment did not exhibit increased anxiety-like behaviour compared to saline controls. How c-FOS expression was affected in mice that did demonstrate increased anxiety-like behaviour while receiving paroxetine, such as in Chapter 3, may not have been the same but has not yet been assessed.

Previously, it was shown that chronic SSRI treatment did not alter c-FOS expression in the raphe nuclei (Veening et al., 1998; Lino-de-Oliveira et al., 2001; Fraga et al., 2005; Payet et al., 2018). Acute SSRI treatment, however, is known to suppress 5-HT neurons via 5-HT_{1A} autoreceptor activation (Hajós et al., 1995). Although the 5-HT_{1A} autoreceptors are thought to desensitise with repeated SSRI treatment (Albert et al., 1996), the firing rate of 5-HT neurons in the DRN was still suppressed after two weeks of escitalopram treatment (Guiard et al., 2012). Reduced c-FOS expression following repeated paroxetine treatment may therefore reflect this residual autoreceptor sensitivity and thus suppression of the 5-HT neurons.

These findings therefore provide evidence that paroxetine discontinuation was associated with a rebound increase in 5-HT neuron activation following a prolonged period of inhibition. Moreover, this was specific to 5-HT neurons as repeated paroxetine treatment did not alter the activation of neurons in other anxiety-related brain regions.

4.5 Comparisons to other withdrawal states

As there are no other studies of the effects of SSRI discontinuation on c-FOS expression, comparisons with other withdrawal states may help to elucidate common effects of psychiatric drug discontinuation on anxiety circuitry activation. For example, morphine and amphetamine withdrawal also increased the number of c-FOS+ neurons in the DRN and hippocampus (Hayward et al., 1990; Bhat et al., 1992; Stornetta et al., 1993; Beckmann et al., 1995; Georges et al., 2000; Tanaka et al., 2017). Similar anxiety-promoting neural circuits may therefore be activated in all three withdrawal states.

Another similarity is the rebound activation of monoaminergic neurotransmitters following discontinuation. In the current study, paroxetine discontinuation was associated

with the rebound activation of 5-HT neurons in the DRN. In the case of TCA withdrawal, increased cholinergic drive has been observed (Dilsaver et al., 1987). Moreover, noradrenergic rebound was evident in rats in the days following morphine withdrawal (Zhu & Zhou, 2001), including increased *c-Fos* mRNA expression in the LC following naloxone-precipitated and spontaneous withdrawal compared to on-drug controls (Hayward et al., 1990). Thus, the rebound activation of monoaminergic systems appears to be a common effect of psychiatric drug withdrawal.

Nonetheless, caution must be taken when drawing comparisons to other withdrawal states as the brain circuits and receptors involved are very different to those of SSRIs. For example, the effects of opioids on the dopaminergic system are thought to contribute to the cravings and drug-seeking behaviours characteristic of morphine withdrawal (Kosten & George, 2002). These symptoms are not seen following SSRI discontinuation (Chapter 1 section 2.8). Thus, while it is interesting to compare to other withdrawal states, the mechanisms underlying SSRI discontinuation syndrome cannot be directly implied from such studies.

4.6 Limitations of c-FOS immunohistochemistry

c-FOS immunohistochemistry provided evidence that the DRN, MRN and VH were activated during paroxetine discontinuation. However, these experiments could not distinguish between increased spontaneous activity of neurons and an increased response to anxiogenic test exposure. c-FOS expression is thought to be low under unstimulated conditions, even in high-anxiety animals (Salomé et al., 2004). It is therefore likely that increased c-FOS expression following paroxetine discontinuation arose from the greater anxiogenic experience of the mice on the EPM rather than baseline changes in neuronal

activity. Nonetheless, without quantifying c-FOS expression in homecage (untested) controls, the possibility of baseline changes to neuronal activation cannot be excluded.

c-FOS quantification cannot determine the nature of the activation, whether it be increased calcium entry, higher firing rates or changes to firing pattern – it is simply an indication that plasticity-related gene expression has been induced. Electrophysiological recordings of the DRN, MRN and VH are therefore needed to directly measure the effect of SSRI discontinuation on neuronal firing rates. The balance between tonic and burst firing, both at baseline and in mice on the EPM, could also be explored. Even then, tissue or extracellular levels of 5-HT and other neurotransmitters in downstream structures need to be investigated to determine the functional output of this increased neuronal activation.

4.7 Conclusions

Overall, paroxetine discontinuation was associated with the persistent and specific activation of 5-HT neurons in the DRN and MRN. In the DRN, this represented a rebound above saline controls. This effect was associated with increased anxiety-like behaviour on the EPM two days after paroxetine discontinuation but was dissociated from behaviour on discontinuation day five. This suggests that changes in somatic activation alone do not account for the behavioural effects of SSRI discontinuation. Increased c-FOS expression in the VH also suggests anxiety-like behaviour is associated with VH activity. Nonetheless, the effect of this neuronal activation on 5-HT neurochemistry is unknown. Experiments in the next chapter investigated the effect of paroxetine discontinuation on tissue and extracellular 5-HT, as well as 5-HT-related gene expression.

Chapter 5
Effect of SSRI discontinuation on *ex vivo* and *in vivo* 5-HT neurochemistry

Parts of the following chapter are published in Collins et al., *Eur Neuropsychopharmacol*, 2021; 53, Suppl. 1: S407-S408.

1 Introduction

Immunohistochemistry experiments in the previous chapter found that paroxetine discontinuation was associated with 5-HT neuronal activation. Here, *ex vivo* and *in vivo* measurement of 5-HT neurochemistry, as well as 5-HT-related gene expression, were used to further investigate the effect of SSRI discontinuation on the 5-HT system.

1.1 Effects of SSRI treatment on 5-HT neurochemistry

The effects of acute and repeated SSRI treatment on 5-HT neurochemistry are well established but must first be considered to understand what happens following SSRI cessation. SSRIs increase extracellular 5-HT due to blockade of the 5-HTT, which is evident in the raphe nuclei (Malagié et al., 1995; Hajós-Korcsok et al., 2000) and downstream projection regions such as the hippocampus (Bosker et al., 2001). In the raphe nuclei, increased extracellular 5-HT activates 5-HT_{1A} autoreceptors, which suppress 5-HT neuron firing (Aghajanian & Lakoski, 1984; Chaput et al., 1986; Gartside et al., 1995; Hajós et al., 1995; Cremers et al., 2000b; Czachura & Rasmussen, 2000; Hajós-Korcsok et al., 2000). This feedback inhibition also occurs in 5-HT terminals via 5-HT_{1B} autoreceptors to suppress 5-HT release (Engel et al., 1986; Fink & Göthert, 2007).

Despite the drop in 5-HT neuron firing, extracellular 5-HT levels remain elevated, likely due to the continued blockade of the 5-HTT (Malagié et al., 1995; Arborelius et al., 1996; Bel & Artigas, 1999; Hajós-Korcsok et al., 2000; Malagié et al., 2000; Ceglia et al., 2004; Popa et al., 2010). Moreover, although 5-HT_{1A} and 5-HT_{1B} autoreceptors are reported to lose sensitivity over time (Albert et al., 1996; Cremers et al., 2000a; Bosker et al., 2001), this desensitisation may not be complete. For instance, electrophysiological recordings in the mouse DRN showed that even after two weeks of escitalopram treatment, 5-HT neuron firing was still reduced compared to pre-treatment rates. This suggests that the 5-HT_{1A} autoreceptors were persistently activated and thus continued to suppress 5-HT neuron activity (Guiard et al., 2012). Reduced c-FOS immunoreactivity in 5-HT neurons during repeated paroxetine treatment (Chapter 4) is further evidence that the autoreceptors continue to suppress neuronal activation throughout SSRI administration.

This continual suppression of 5-HT neuron activity is also evident as reduced 5-HT synthesis (Esteban et al., 1999), due to the activity-dependent properties of TPH2 (Hrdina, 1987; Ehret et al., 1989). Thus, numerous studies have reported reduced regional tissue levels of 5-HT and 5-HIAA during repeated treatment with an SSRI in rodents (Hrdina, 1987; Caccia et al., 1992; Caccia et al., 1993; Trouvin et al., 1993; Bosker et al., 2010).

1.2 Effect of SSRI discontinuation on *ex vivo* 5-HT neurochemistry

There have been very few studies of the effects of SSRI discontinuation on 5-HT neurochemistry. In the first study to investigate 5-HT neurochemistry following SSRI cessation, Trouvin et al. showed that low regional brain tissue levels of 5-HT and 5-HIAA recovered to saline control levels within a week of fluoxetine discontinuation in the rat

midbrain, hippocampus, and cortex. 5-HT concentrations remained at saline control levels two weeks after discontinuation. 5-HIAA, on the other hand, rebounded above control levels in the hippocampus and midbrain one week after fluoxetine discontinuation, and remained elevated in the hippocampus for at least 28 days. These results suggest that SSRI discontinuation may be associated with a persistent upregulation of the 5-HT system.

Similar effects were reported by subsequent studies. For instance, 5-HT was not different from saline control levels seven days after paroxetine and sertraline discontinuation in the rat cortex and hippocampus (Caccia et al., 1993), or two days after citalopram discontinuation in the cortex and midbrain (Bosker et al., 2010). Other data also support the rebound increase in hippocampal 5-HIAA one week after fluoxetine discontinuation in rats (Caccia et al., 1993).

SSRI discontinuation has also been shown to alter the 5-HIAA/5-HT ratio, a measure of 5-HT turnover. Hippocampal 5-HIAA/5-HT ratio rebounded above control levels one week after fluoxetine discontinuation and remained elevated 28 days after cessation (Trouvin et al., 1993). Citalopram cessation also increased the 5-HIAA/5-HT ratio in the VH compared to continued treatment and vehicle-treated controls, which was already evident two days after discontinuation (Bosker et al., 2010). These studies further suggest that the 5-HT system is upregulated following SSRI discontinuation.

Overall, the existing data suggest that SSRI discontinuation is associated with a rebound increase in 5-HT neurochemistry, consistent with evidence of increased 5-HT neuron activity in Chapter 4. Nonetheless, data from other SSRIs are limited and there has yet to be an analysis of how regional 5-HT and 5-HIAA change over time following paroxetine discontinuation in mice.

1.3 Effect of SSRI discontinuation on *in vivo* brain extracellular 5-HT and 5-HIAA

Despite evidence of a rebound increase in 5-HT transmission, several reviews have speculated that the discontinuation syndrome may be caused by a rapid fall in extracellular 5-HT. This may arise due to the recovery of 5-HT reuptake following SSRI cessation (Blier & Tremblay, 2006; Delgado, 2006; Renoir,2013). Yet, there is limited experimental evidence to support this hypothesis. In one of the few examples (Guiard et al., 2012), *in vivo* microdialysis in the VH of anaesthetised mice demonstrated that extracellular 5-HT increased during repeated escitalopram administration, then fell to saline control levels within two days of discontinuation. Other rodent microdialysis studies have also suggested that extracellular 5-HT did not differ from that of saline controls in the two weeks following SSRI cessation (Gardier et al., 1994; Arborelius et al., 1996; Moret & Briley, 1996; Cremers et al., 2000a; Gardier et al., 2003; Ceglia et al., 2004; Bosker et al., 2010; Popa et al., 2010; Klomp et al., 2014).

Thus, there is some evidence that SSRI discontinuation may be associated with a reduction in extracellular 5-HT back to baseline levels. Nonetheless, how extracellular 5-HT changes in response to neuronal stimulation during SSRI discontinuation has not yet been studied.

1.4 Effect of SSRI discontinuation on 5-HT-related gene expression

Continued treatment with an SSRI is thought to be associated with adaptive changes in 5-HT receptor and, possibly, 5-HTT expression (Cowen et al., 2014). Yet, there is minimal data available regarding the effects of SSRI discontinuation on 5-HT-related gene expression. Of particular interest are the 5-HT_{1A} and 5-HT_{1B} autoreceptors due to their regulatory influence on the 5-HT system. *In situ* hybridisation showed that *5-HT_{1B}*

receptor mRNA in the DRN was reduced compared to vehicle-treated controls immediately after repeated paroxetine or sertraline treatment, and it returned to baseline levels during the discontinuation period (Anthony et al., 2000). There are, however, no similar studies on the effect of SSRI discontinuation on 5-HT_{1A} autoreceptor expression.

There is also some evidence of changes to 5-HTT expression following SSRI discontinuation. Repeated SSRI treatment has been shown to reduce 5-HTT expression in the DRN (Lesch et al., 1993; Benmansour et al., 1999). One week after fluoxetine cessation, however, *in situ* hybridisation showed that 5-HTT mRNA in the DRN was not different to vehicle controls (Neumaier et al., 1996). There were no further changes to *5-HTT* expression later in the discontinuation period (Sawyer et al., 2012).

Overall, *5-HTT* and *5-HT_{1B}* expression appear to be reduced by chronic SSRI treatment but return to baseline levels following discontinuation, although the effect on the 5-HT_{1A} autoreceptor is unknown. Moreover, there are currently no studies on the effect of SSRI discontinuation on the expression of postsynaptic 5-HT receptors.

1.5 Hypothesis and aims

The aim of this chapter was to investigate the effects of paroxetine discontinuation on 5-HT neurochemistry. These studies used *ex vivo* measurement of regional brain tissue 5-HT and 5-HIAA two and five days after paroxetine discontinuation, as well as two days after citalopram discontinuation. *In vivo* microdialysis in anaesthetised mice was also used to assess baseline and evoked extracellular 5-HT following paroxetine cessation. These experiments were supplemented with qPCR analysis of the mRNA expression of several 5-HT receptors, including 5-HT_{1A} and 5-HT_{1B} autoreceptors and 5-HTT. Analysis

of DA neurochemistry was also included in these studies as a comparator to demonstrate the specificity of the effects of SSRI discontinuation to the 5-HT system.

Based on evidence of a rebound increase in 5-HT neuron activation in the previous chapter, and the existing literature on 5-HT neurochemistry, it was hypothesised that paroxetine discontinuation would be associated with increased 5-HT function. Moreover, based on the, albeit limited, reports in the literature, it was hypothesised that gene expression changes during repeated SSRI treatment would reverse following discontinuation.

2 Experimental Methods

2.1 Animals

C57BL/6J male mice (Charles River, 7 weeks old) were group housed (3-6 per cage) in open-top cages or IVCs for at least one week before the start of treatment.

2.2 Drugs and reagents

Paroxetine and citalopram were dissolved in saline and administered *s.c.*

2.3 *Ex vivo* neurochemistry experimental design

Mice were allocated to one of three experimental groups by stratified randomisation: i) saline group (SAL): repeated saline + saline continuation; ii) continuation group (CON): repeated SSRI + SSRI continuation; iii) discontinuation group (DIS): repeated SSRI + saline. Mice received once- or twice-daily *s.c.* injections of 10 mg/kg SSRI, or the equivalent volume of saline for 12 days, then treatment was either continued (SAL and CON groups) or swapped to saline injections (DIS group) for a further two or five days (*Fig. 5.1*)

All mice were tested on the EPM. A subset of mice from each experiment were killed by cervical dislocation 90 min after testing. Brains were immediately frozen in dry ice-cold isopentane and stored at -80^{o}C. Individual experiments are summarised in *Table 5.1*.

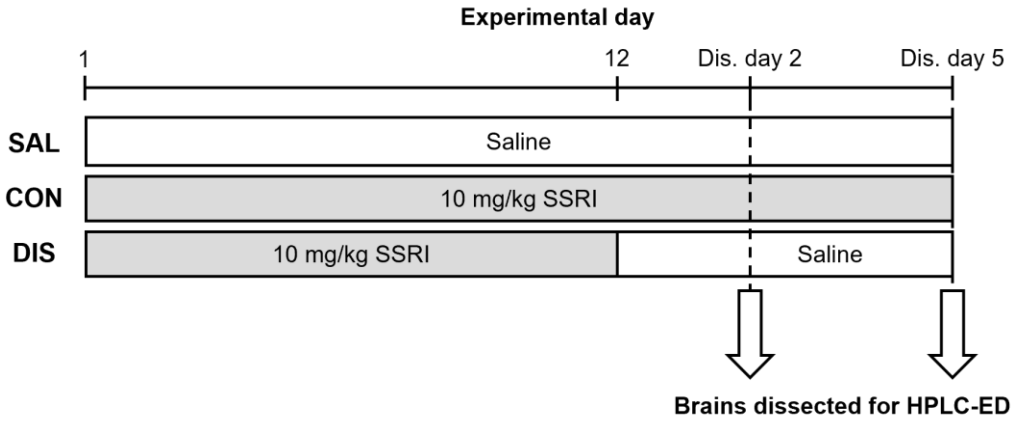


Figure 5.1 *Ex vivo* neurochemistry experimental design. Separate cohorts of mice tested on the EPM on discontinuation days two and five before analysing brains with HPLC-ED.

Table 5.1 *Ex vivo* neurochemistry experimental design.

SSRI	Dosing period and frequency	Disc. day	Time since final injection of paroxetine	Group sex and size	Relevant behavioural experiment
Paroxetine	12 days once-daily	2	CON: 18 h DIS: 48 h	Males n=8	Chapter 3 section 3.2
Paroxetine	12 days once-daily	5	CON: 18 h DIS: 120 h	Males n=9	Chapter 3 section 3.1 and Chapter 3 section 3.3
Paroxetine	12 days twice-daily	5	CON: 6 h DIS: 120 h	Males n=6	Chapter 3 section 3.6
Citalopram	12 days twice-daily	2	CON: 6 h DIS: 48 h	Males n=8	Chapter 3 section 3.7

Tissue samples were analysed with HPLC-ED as in Chapter 2 section 7. On the day of tissue analysis, the midbrain, hippocampus, striatum, and frontal cortex were dissected from the left hemispheres of the brains on ice and weighed in Eppendorf tubes containing 0.09 M PCA. Samples were sonicated, centrifuged, then stored on ice. Samples were analysed for 5-HT, 5-HIAA, DA and DOPAC using HPLC-ED. Samples were analysed by an experimenter blind to treatment groups.

2.4 *In vivo* microdialysis experimental design

Microdialysis procedure. Microdialysis experiments were performed as in Chapter 2 section 8. Briefly, mice were anaesthetised with urethane and a microdialysis probe was stereotaxically implanted in the VH [AP: -3.0 mm, ML: ±2.9 mm, DV: -4.0 mm]. Probes were continuously perfused (2.0 µL/min) with standard ACSF or ACSF containing 56 mM or 100 mM potassium (Auerbach et al., 1989; Sharp & Foster, 1989). Microdialysate samples were collected every 20 min into Eppendorf tubes containing 10 µl 0.09 M PCA and immediately analysed with HPLC-ED. Mice were transcardially perfused with PBS and 4% PFA and brains were stored at 4ºC in 30% sucrose solution. Coronal brain slices containing the VH were stained with DAPI to identify the probe tracts (*Fig. 2.5*). All brains showed probe tracts in the VH, so no animals were excluded.

Pilot experiments. Initial experiments tested the effect of perfusing the probes with 56 mM or 100 mM potassium on extracellular 5-HT and 5-HIAA. After a 120 min baseline recording, microdialysis probes were infused with high potassium ACSF for 20 min, then returned to standard ACSF for a further 60 min. Ten min were allowed after the start of high potassium ACSF infusion before starting to collect the first microdialysate sample to account for the dead space in the tubing. In separate experiments, mice received an acute *s.c.* injection of 10 mg/kg paroxetine, then the microdialysate was sampled for a further 120 min.

Paroxetine discontinuation experiment. Mice were allocated to one of four experimental groups: i) saline group (SAL); ii) continuation group (CON); iii) discontinuation day two group (DIS 2); iv) discontinuation day five group (DIS 5). Mice received once-daily 10 mg/kg *s.c.* injections of paroxetine or the equivalent volume of saline for 12 days, then treatment was either continued (SAL and CON groups) or

swapped to saline injections (DIS group) for a further two or five days (*Fig 5.2A*). The experiment was performed in two cohorts, each of four mice per treatment group.

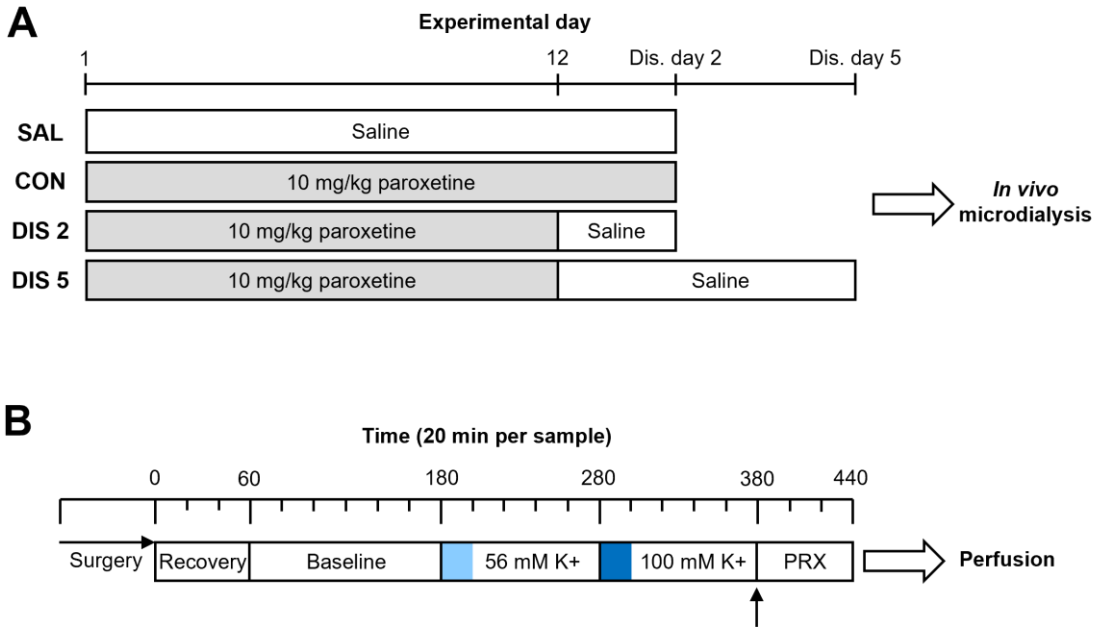


Figure 5.2 *In vivo* microdialysis experimental design. Treatment protocol (A) and microdialysis experimental design (B). Blue boxes indicate infusion of 56 or 100 mM potassium (K+) via the ACSF. PRX, paroxetine, injection indicated by arrow.

On the morning of the experiment, the microdialysis probe was implanted in the VH. After a 120 min baseline period, the probe was sequentially perfused with 56 mM and 100 mM potassium ACSF. Finally, an acute *s.c.* injection of 10 mg/kg paroxetine was administered (*Fig. 5.2B*).

2.5 Gene expression experimental design

Mice were allocated to one of three experimental groups by stratified randomisation: i) saline group (SAL); ii) continuation group (CON); iii) discontinuation group (DIS). Mice received once daily 10 mg/kg *s.c.* injections of paroxetine, or the equivalent volume of saline for 12 days, then treatment was either continued (SAL and

CON groups) or swapped to saline injections (DIS group) for a further two or five days, when tissue was collected.

Individual experiments are summarised in *Table 5.2*. In the first experiment, mice were tested on the EPM on discontinuation day two and killed by cervical dislocation 90 min after testing. In the second experiment, mice were tested on the EPM and killed on discontinuation day five. In the third experiment, mice were not tested on the EPM, but were killed 6 h after the final injection of paroxetine for the CON group and 30 h for the DIS group (*Table 5.2*). Tissue samples were immediately frozen and stored at -80ºC.

Table 5.2 Gene expression experimental design.

Disc. day	SSRI, dosing period and frequency	Time since final injection of paroxetine	Group sex and size	Relevant behavioural experiment
2	12-days once-daily paroxetine	CON: 18 h DIS: 48 h	Males n=8	Chapter 3 section 3.2
5	12-days twice-daily	CON: 18 h DIS: 120 h	Males n=6	Chapter 3 section 3.3
2	12-days once-daily paroxetine	CON: 6 h DIS: 30 h	Males n=8	N/A

The raphe nuclei (RN), hippocampus and PFC were dissected on ice (hippocampus and PFC from the right hemisphere only) and stored at -80ºC until analysis. RNA was extracted, converted to cDNA, and analysed using qPCR, as in Chapter 2 section 9. The relative mRNA expression of the following genes of interest (GOI) were analysed: *5-HT$_{1A}$*, *5-HT$_{1B}$*, *5-HTT*, and *Tph2* in the RN; and *5-HT$_{1A}$*, *5-HT$_{1B}$*, *5-HT$_{2A}$* and *5-HT$_{4}$* in the hippocampus and PFC. GOI expression was normalised to the expression of the housekeeping gene, β-actin. Samples were analysed by an experimenter blind to treatment groups.

2.6 Statistical analysis

Ex vivo neurochemistry analysis. Tissue concentrations of 5-HT, 5-HIAA, DA and DOPAC are expressed as pmol/mg wet tissue. Data were determined to be parametric using D'Agostino Pearson's test for normality and so were analysed with repeated measures ANOVA with Bonferroni's test (region as within-subject factor, treatment as between-subject factor). To assess changes in tissue neurochemistry over time, data from the 12-day paroxetine experiments were normalised to the mean saline control value for each experiment, then the data were combined. Data were expressed as the time since the final injection of paroxetine (6 or 18 h for continuation, 48 or 120 h for discontinuation). Normalised data were analysed using one-way ANOVA with Fisher's LSD to assess how 5-HT and 5-HIAA changed over time.

In vivo microdialysis analysis. 5-HT, 5-HIAA and DOPAC were analysed over time using repeated measures ANOVA with post-hoc Bonferroni's test. The peak responses to potassium-evoked depolarisation were assessed for normality using D'Agostino Pearson's test. If data were parametric, then one-way ANOVA with Fisher's LSD was used. If data were non-parametric, Kruskal Wallis with Uncorrected Dunn's test was used. To evaluate the relative response to the small potassium stimulus versus the response to larger, near-maximal stimulus, the peak 5-HT response following 56 mM potassium was expressed as a proportion of the 100 mM peak response per mouse (both values were needed for each mouse to be included in calculations). This is a similar approach to *in vitro* studies of presynaptic vesicular release using synaptophluorins (Miesenböck et al., 1998; Granseth et al., 2006; Zhao et al., 2011; Jeans et al., 2017). Data were determined to be parametric and analysed using one-way ANOVA with Fisher's LSD.

Gene expression analysis. mRNA expression for each GOI was expressed relative to saline controls using the ΔCt method. Outliers were identified using ROUT analysis and removed before ΔCt analysis. Data were analysed with one-way ANOVA with Fisher's LSD. ΔCt values for each gene are included to show the absolute amounts of each gene detected in samples (*Suppl. Fig. 3.1*).

ROUT analysis was used to remove significantly outlying datapoints from the *ex vivo* and *in vivo* data. Repeated measures ANOVAs were conducted using IBS SPSS Statistics, and one-way and two-way ANOVAs, Kruskal Wallis and ROUT analyses were conducted using GraphPad Prism. Data are presented as mean ± SEM or median ± IQR. P values of $p<0.05$ were considered statistically significant. All analyses were conducted blind to treatment.

3 Results

3.1 Effect of two days discontinuation from once-daily paroxetine on *ex vivo* neurochemistry

Paroxetine continuation (18h after last dose) reduced regional brain tissue levels of 5-HT compared to saline controls. This effect reversed within two days of paroxetine discontinuation (effect of treatment: $F(2,21)=4.481$, $p=0.014$, between-subject post-hoc SAL vs CON $p=0.042$, CON vs DIS $p=0.023$; *Fig. 5.3A*). The absolute levels of 5-HT differed between the brain regions but there was no treatment*region interaction, suggesting paroxetine continuation and discontinuation had the same effects in all four regions (effect of region: $F(3,63)=15.68$, $p<0.001$; treatment*region interaction: $F(6,63)=0.366$, $p=0.899$; *Fig. 5.3A*).

Similarly, paroxetine continuation reduced 5-HIAA compared to saline controls, which had recovered two days after paroxetine discontinuation (treatment: $F(2,21)=4.220$, $p=0.029$, post-hoc SAL vs CON $p=0.011$, CON vs DIS $p<0.001$; *Fig. 5.3B*). The absolute levels of 5-HIAA also differed between brain regions but there was also no treatment*region interaction (region: $F(3,63)=32.11$, $p<0.001$; interaction: $F(6,63)=1.592$, $p=0.165$; *Fig. 5.3B*).

The 5-HIAA/5-HT ratio differed between brain regions but there was no effect of treatment or a treatment*region interaction (treatment: $F(2,21)=2.205$, $p=0.135$; region: $F(3,63)=34.48$, $p<0.001$; interaction: $F(6,63)=1.123$, $p=0.359$; *Fig. 5.3C*).

There were no effects of treatment on DA or DOPAC levels (data are available in Appendix 3, *Suppl. Table 3.1*). In summary, repeated paroxetine treatment reduced 5-HT and 5-HIAA, and both recovered to saline control levels two days after discontinuation.

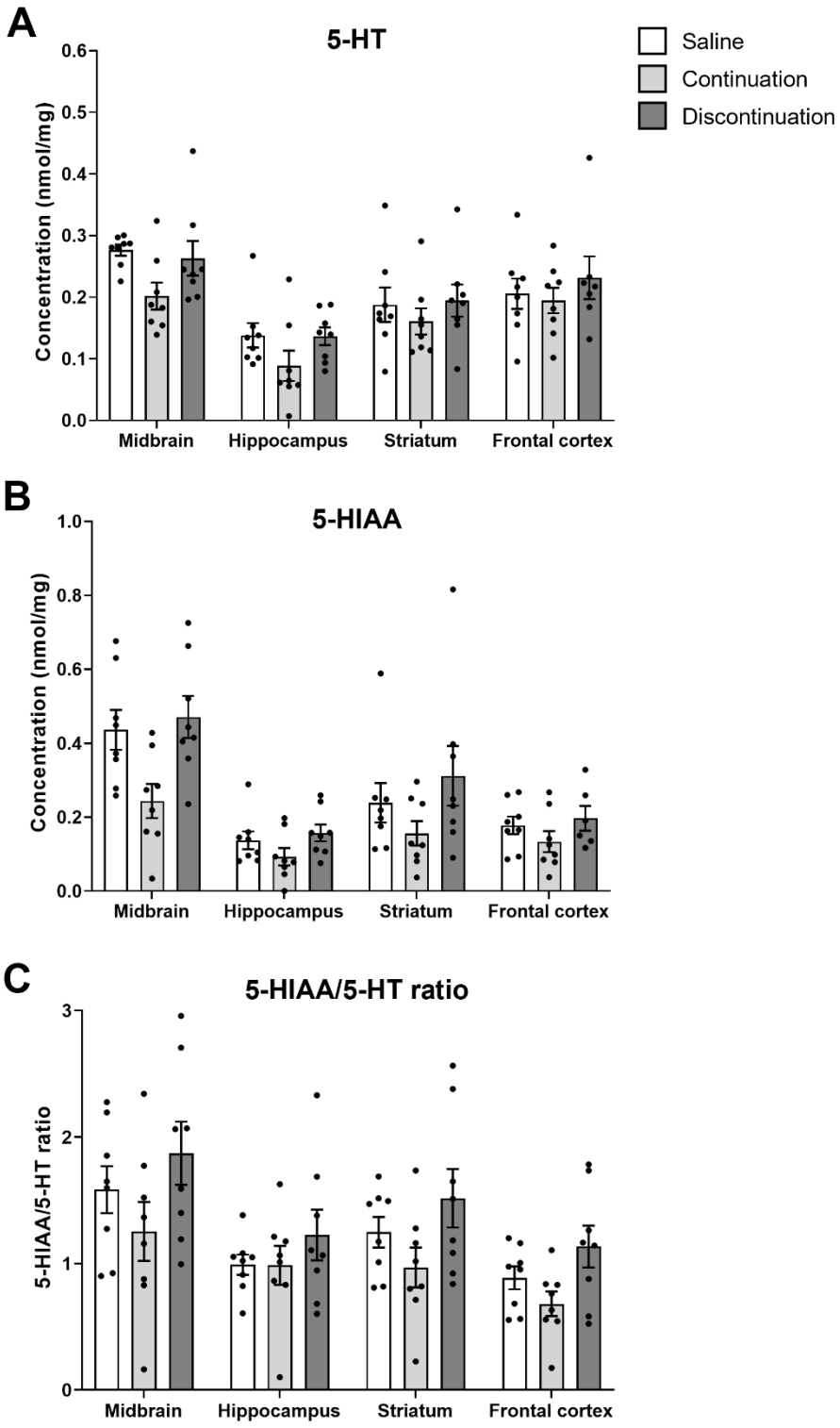


Figure 5.3 Effect of two days discontinuation from once-daily paroxetine treatment on *ex vivo* 5-HT neurochemistry. Bars represent the mean ± SEM values for 5-HT (A) and 5-HIAA (B) per mg of wet tissue, and the 5-HIAA/5-HT ratio (C). SAL (n=8), CON (n=8), DIS (n=8). Individual values are indicated by dots. Repeated measures ANOVA with post-hoc Bonferroni's test.

3.2 Effect of five days discontinuation from once-daily paroxetine on *ex vivo* neurochemistry

As in the previous experiment, paroxetine continuation (18h after last dose) reduced 5-HT compared to saline controls. Five days after discontinuation, however, 5-HT rebounded above continued paroxetine and saline control levels (treatment: $F(2,24)=9.939$, $p=0.001$, post-hoc SAL vs CON $p=0.009$, SAL vs DIS $p=0.011$, CON vs DIS $p<0.001$; *Fig. 5.4A*). There was a significant effect of region but no treatment*region interaction (region: ($F(3,72)=41.51$, $p<0.001$; interaction: $F(6,72)=0.742$, $p=0.618$; *Fig. 5.4A*).

Paroxetine continuation did not alter 5-HIAA compared to saline controls. By comparison, five days of paroxetine discontinuation increased 5-HIAA compared to continued paroxetine and saline controls (treatment: $F(2,24)=4.234$, $p=0.027$, post-hoc SAL vs DIS $p=0.027$, CON vs DIS $p<0.001$; *Fig. 5.4B*). There was a significant effect of region but no treatment*region interaction (region: $F(3,72)=46.18$, $p<0.001$; interaction: $F(6,72)=0.788$, $p=0.582$; *Fig. 5.4B*).

The 5-HIAA/5-HT ratio again differed between brain regions but there was no effect of treatment or a treatment*region interaction (treatment: $F(2,24)=0.327$, $p=0.724$; region: $F(3,72)=53.05$, $p<0.001$; interaction: $F(6,72)=1.400$, $p=0.227$; *Fig. 5.4C*). There were no effects of treatment on DA or DOPAC levels (*Suppl. Table 3.2*).

In summary, regional brain tissue levels of 5-HT and 5-HIAA rebounded above saline control levels five days after paroxetine discontinuation.

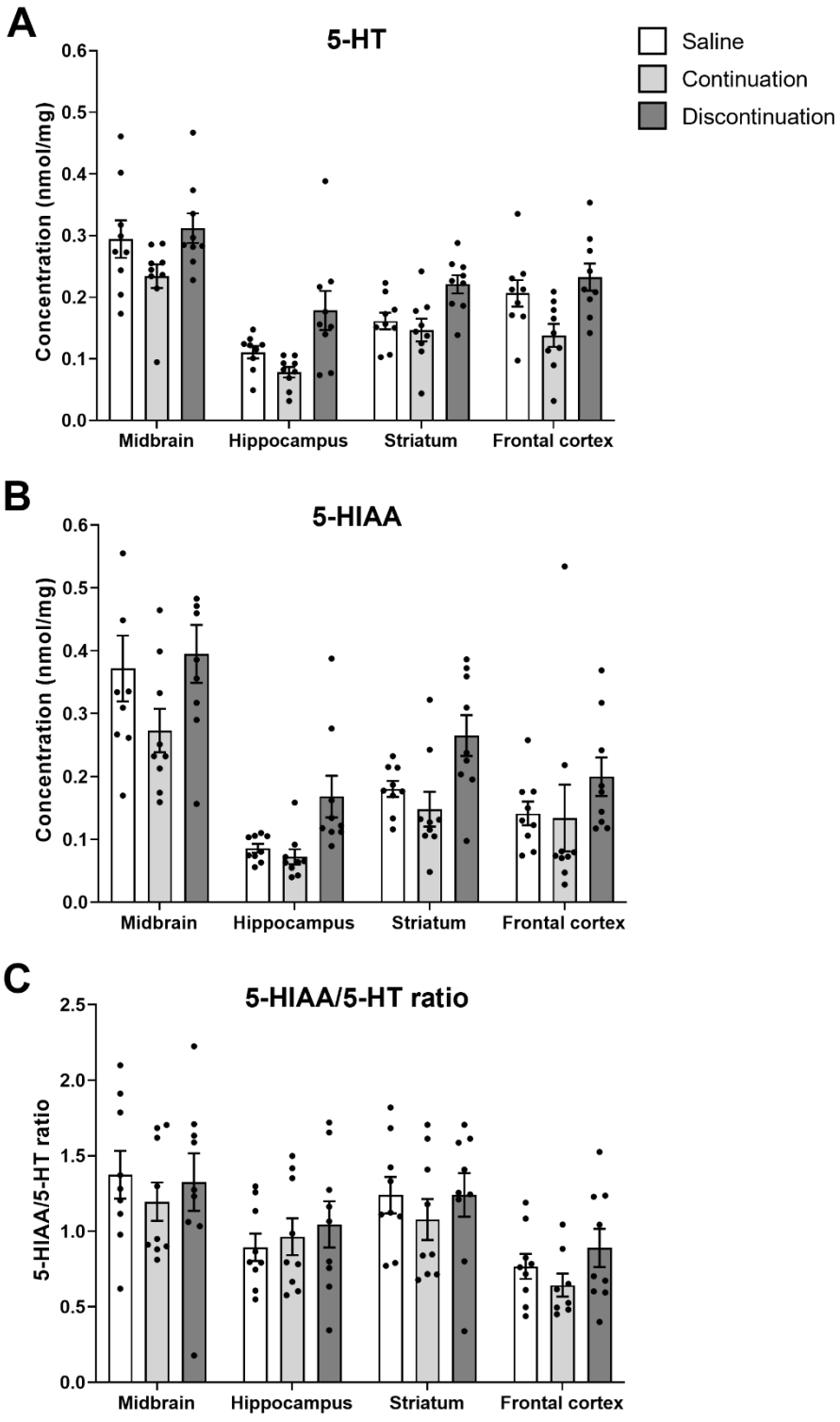


Figure 5.4 Effect of five days discontinuation from once-daily paroxetine treatment on *ex vivo* 5-HT neurochemistry. Bars represent the mean ± SEM values for 5-HT (A) and 5-HIAA (B) per mg of wet tissue, and the 5-HIAA/5-HT ratio (C). SAL (n=9), CON (n=9), DIS (n=9). Individual values are indicated by dots. Repeated measures ANOVA with post-hoc Bonferroni's test.

3.3 Effect of five days discontinuation from twice-daily paroxetine on *ex vivo* neurochemistry

Further experiments were conducted to determine if the effects of paroxetine discontinuation were greater after more frequent dosing. Paroxetine continuation (6 h after last dose) reduced 5-HT compared to saline controls, which reversed back to saline control levels five days after discontinuation (treatment: $F(2,15)=9.772$, $p=0.002$, post-hoc SAL vs CON $p=0.001$, CON vs DIS $p<0.001$; *Fig. 5.5A*). There was a significant effect of region but no treatment*region interaction (region: $F(3,45)=18.38$, $p<0.001$; interaction: $F(6,45)=1.379$, $p=0.244$; *Fig. 5.5A*).

Paroxetine continuation also reduced 5-HIAA compared to saline controls. Five days after discontinuation, 5-HIAA rebounded above continued paroxetine and saline controls (treatment: $F(2,15)=13.95$, $p<0.001$, post-hoc SAL vs CON $p=0.001$, SAL vs DIS $p=0.006$, CON vs DIS $p<0.001$; *Fig. 5.5B*). There was an effect of region and a significant treatment*region interaction. Within-region comparisons showed that paroxetine discontinuation increased 5-HIAA in all brain regions compared to continued paroxetine treatment (region: $F(3,45)=28.57$, $p<0.001$; interaction: $F(6,45)=2.846$, $p=0.020$, post-hoc midbrain: CON vs DIS $p=0.017$; hippocampus: CON vs DIS $p=0.001$; striatum: CON vs DIS $p=0.018$; frontal cortex: CON vs DIS $p=0.045$; *Fig. 5.5B*).

The 5-HIAA/5-HT ratio differed between brain regions but there was no effect of treatment or a treatment*region interaction (treatment: $F(2,15)=1.039$, $p=0.378$; region: $F(3,45)=10.44$, $p<0.001$; interaction: $F(6,45)=1.294$, $p=0.279$; *Fig. 5.5C*). There were no effects of treatment on DA or DOPAC levels (*Suppl. Table 3.3*).

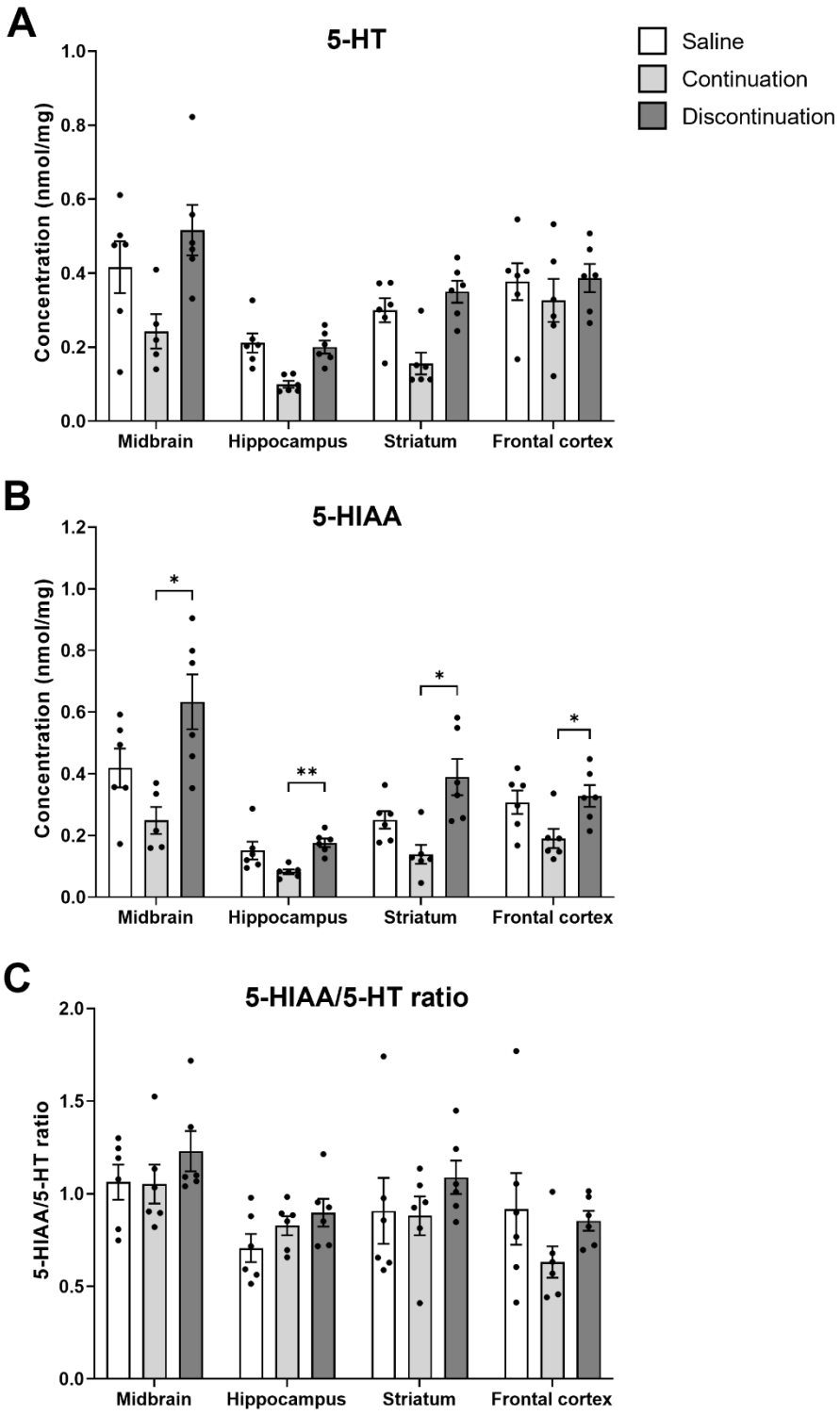


Figure 5.5 Effect of five days discontinuation from twice-daily paroxetine treatment on *ex vivo* 5-HT neurochemistry. Bars represent the mean ± SEM values for 5-HT (A) and 5-HIAA (B) per mg of wet tissue, and the 5-HIAA/5-HT ratio (C). SAL (n=6), CON (n=6), DIS (n=6). Individual values are indicated by dots. Repeated measures ANOVA with post-hoc Bonferroni's test, within-region comparisons * $p<0.05$, ** $p<0.01$.

In summary, five days of discontinuation from twice-daily paroxetine increased 5-HIAA compared to continued paroxetine treatment, like in the previous experiment. Nonetheless, this effect was not greater than following once-daily paroxetine treatment.

3.4 Effect of paroxetine discontinuation on 5-HT neurochemistry over time

To illustrate how 5-HT and 5-HIAA levels changed over time in each brain region, data were normalised to the mean saline control value for each experiment. The data were then combined and plotted against the time since the mice last received a paroxetine injection. Here, 6 and 18 h represent continued paroxetine treatment, and 48 h (two days) and 120 h (five days) represent paroxetine discontinuation.

In the midbrain, 5-HT was greater 120 h after paroxetine discontinuation than it was during continued paroxetine treatment. 5-HIAA was greater 48 and 120 h after discontinuation than during continued treatment (effect of time, 5-HT: $F(3,42)=4.445$, $p=0.0086$, post-hoc 6 h vs 120 h $p=0.0053$, 18 h vs 120 h $p=0.0037$; 5HIAA: $F(3,42)=7.080$, $p=0.0006$, post-hoc 6 h vs 48 h $p=0.0397$, 6 h vs 120 h $p=0.0049$, 18 h vs 48 h $p=0.0123$, 18 h vs 120 h $p=0.0002$; *Fig. 5.6A*).

In the hippocampus, both 5-HT and 5-HIAA were greater 48 and 120 h after paroxetine discontinuation than during continued paroxetine treatment (5-HT: $F(3,42)=8.476$, $p=0.0002$, post-hoc 6 h vs 48 h $p=0.0096$, 6 h vs 120 h $p=0.0002$, 18 h vs 48 h $p=0.0418$, 18 h vs 120 h $p=0.0002$; 5HIAA: $F(3,42)=8.562$, $p=0.0001$, 6 h vs 48 h $p=0.0474$, 6 h vs 120 h $p=0.0003$, 18 h vs 120 h $p<0.0001$; *Fig. 5.6B*).

In the striatum, 5-HT and 5-HIAA were greater 48 and 120 h after paroxetine discontinuation than during continued treatment. 5-HT also significantly increased between 6 and 18 h after the final paroxetine injection in the continuation groups (5-HT:

F(3,42)=10.03, p<0.0001, post-hoc 6 h vs 18 h p=0.0170, 6 h vs 48 h p=0.0035, 6 h vs 120 h p<0.0001, 18 h vs 120 h p=0.0006; 5-HIAA: F(3,42)=9.106, p<0.0001, post-hoc 6 h vs 48 h p=0.0080, 6 h vs 120 h p=0.0002, 18 h vs 48 h p=0.0240, 18 h vs 120 h p=0.0001; *Fig. 5.6C*).

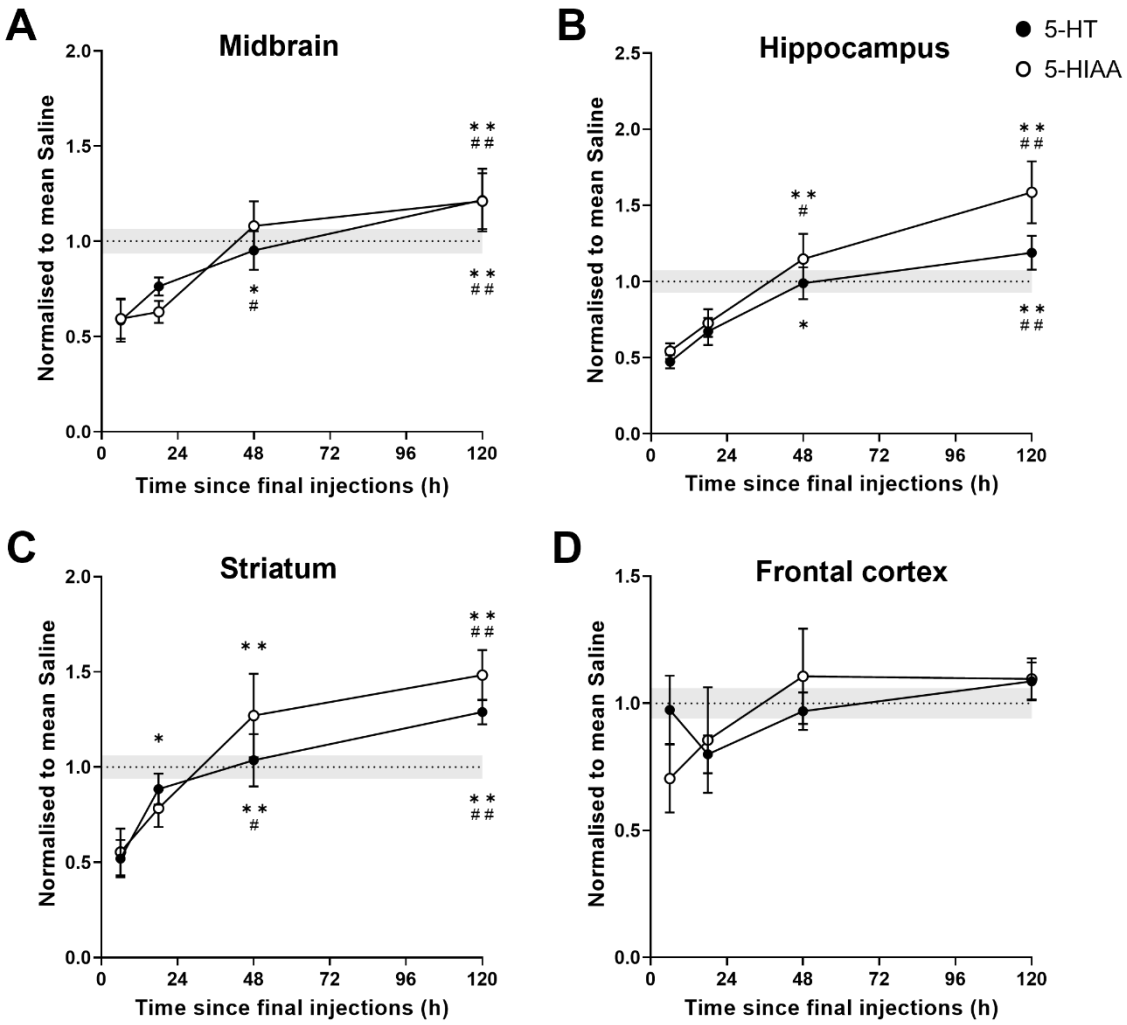


Figure 5.6 Effect of discontinuation from paroxetine treatment on *ex vivo* 5-HT neurochemistry over time. Effect of paroxetine continuation (6 and 18 h after final paroxetine injection) and paroxetine discontinuation (48 and 120 h after final paroxetine injection) on the amount of 5-HT and 5-HIAA per mg of wet tissue normalised to the mean of saline control samples in the midbrain (A), hippocampus (B), striatum (C) and frontal cortex (D). Data presented as in Trouvin et al. (1993), *Fig. 1-2*. N=6-17/time point. Dotted line and grey bar represent mean ± SEM of saline controls, points represent mean ± SEM for continuation and discontinuation. One-way ANOVA with post-hoc LSD. * p<0.05 ** p<0.01 vs 6 h, # p<0.05 ## p<0.01 vs 18 h. 5-HT statistics above dotted line, 5-HIAA below.

Neither paroxetine continuation nor discontinuation significantly altered 5-HT or 5-HIAA in the frontal cortex (effect of time, 5-HT: $F(3,42)=2.722$, $p=0.1574$; 5-HIAA: $F(3,42)=0.6984$, $p=0.5591$; *Fig. 5.6D*).

3.5 Effect of two days discontinuation from twice-daily citalopram treatment on *ex vivo* neurochemistry

To determine whether the neurochemical effects of paroxetine discontinuation generalised to other SSRIs, *ex vivo* experiments were performed two days after citalopram discontinuation. Although the absolute levels of 5-HT differed between brain regions, there was no effect of treatment or a treatment*region interaction (treatment: $F(2,21)=0.956$, $p=0.401$; region: $F(3,63)=22.91$, $p<0.001$; interaction: $F(6,63)=0.948$, $p=0.468$; *Fig. 5.7A*).

Citalopram continuation did not alter 5-HIAA compared to saline controls. Two days of citalopram discontinuation increased 5-HIAA compared to continued paroxetine, although it did not differ from saline controls (treatment: $F(2,21)=3.540$, $p=0.047$, post-hoc CON vs DIS $p=0.002$; *Fig. 5.7B*). There was a significant effect of region but no treatment*region interaction (region: $F(3,63)=35.10$, $p<0.001$; interaction: $F(6,63)=1.026$, $p=0.417$; *Fig. 5.7B*).

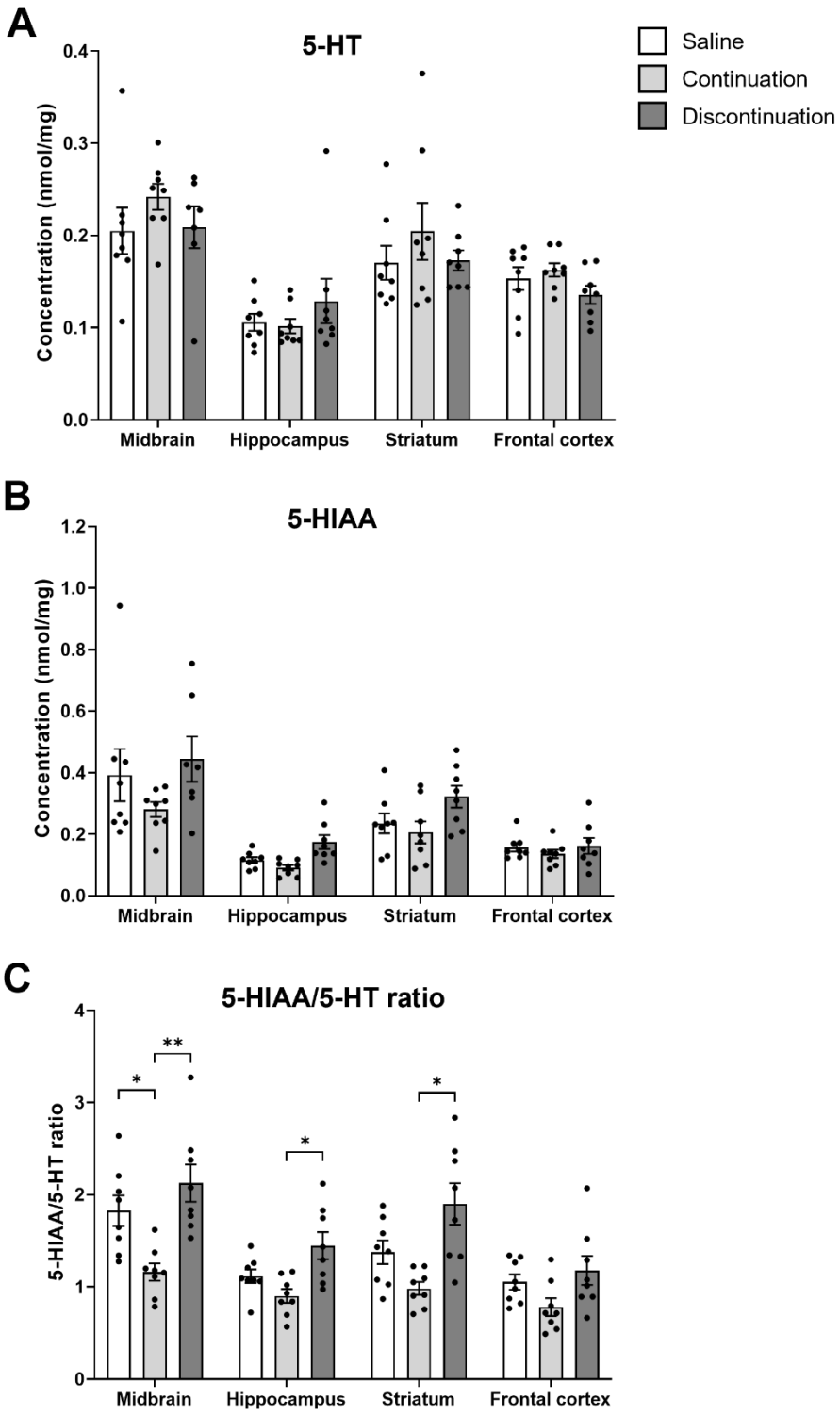


Figure 5.7 Effect of two days discontinuation from twice-daily citalopram treatment on *ex vivo* 5-HT neurochemistry. Bars represent the mean ± SEM values for 5-HT (A) and 5-HIAA (B) per mg of wet tissue, and the 5-HIAA/5-HT ratio (C). SAL (n=8), CON (n=8), DIS (n=8). Individual values are indicated by dots. Repeated measures ANOVA with post-hoc Bonferroni's test, * $p<0.05$, ** $p<0.01$.

Citalopram continuation reduced the 5-HIAA/5-HT ratio compared to saline controls. Two days after citalopram discontinuation, however, the 5-HIAA/5-HT ratio had rebounded above continued citalopram and saline control levels (treatment: $F(2,21)=8.444$, $p=0.002$, post-hoc SAL vs CON $p<0.001$, SAL vs DIS $p=0.004$, CON vs DIS $p<0.001$; *Fig. 5.7C*). There was a significant effect of region and a treatment*region interaction (region: $F(3,63)=57.95$, $p<0.001$; interaction: $F(6,63)=4.879$, $p<0.001$; *Fig. 5.7C*). In the midbrain, citalopram continuation reduced 5-HIAA, which reversed to saline control levels following discontinuation (post-hoc SAL vs CON $p=0.014$, CON vs DIS $p=0.005$). Citalopram discontinuation increased 5-HIAA in the hippocampus and striatum compared to continued paroxetine (hippocampus: CON vs DIS $p=0.022$; striatum: CON vs DIS $p=0.013$).

DA was undetectable in these samples, and there was no effect of treatment on DOPAC (*Suppl. Table 3.4*). In summary, two days of citalopram discontinuation produced a rebound increase in the 5-HIAA/5-HT ratio compared to continued paroxetine and saline controls.

3.6 Validation of the microdialysis protocol

5-HT and 5-HIAA were both detectable in basal hippocampal microdialysate samples (5-HT: 14.4 ± 2.9 fmol/40µl; 5-HIAA: 3.6 ± 0.1 pmol/40µl) and were stable during the 120 min baseline period. Local infusion of 56 mM potassium via the ACSF produced a 49% increase in 5-HT, and a 22% decrease in 5-HIAA, compared to the baseline period. The maximal responses occurred 20 min after the start of infusion, and both had returned to baseline levels 40 min after the end of the infusion (*Fig. 5.8A*). Local infusion of 100 mM potassium produced a 126% increase in 5-HT, and a 37% decrease

in 5-HIAA, compared to the baseline period. The maximal responses also occurred 20 min after the start of infusion and had returned to baseline levels 60 min after the end of infusion (*Fig. 5.8B*). An acute *s.c.* injection of 10 mg/kg paroxetine increased 5-HT in the microdialysate, peaking at a 119% increase from baseline 40 min after injection, and remained elevated 120 min after the injection (*Fig. 5.8C*). There was a 32% decrease in 5-HIAA following paroxetine challenge, which continued to decrease 120 min after injection (*Fig. 5.8C*).

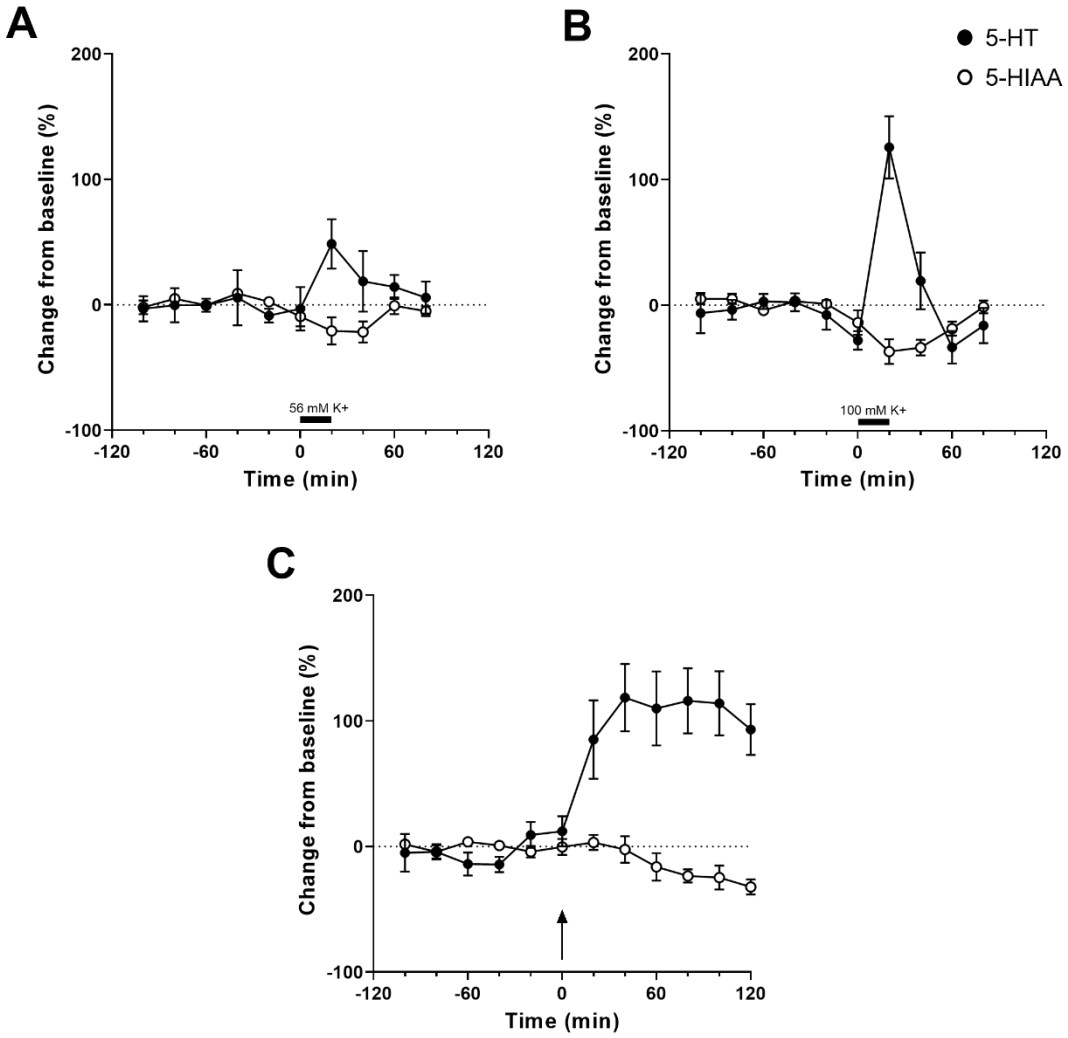


Figure 5.8 Validation of the in vivo microdialysis protocol. Dots represent mean ± SEM of the percentage change in 5-HT and 5-HIAA per 40 µl microdialysate (fmol/40µl 5-HT, pmol/40 µl 5-HIAA) following 56 mM (A) or 100 mM (B) potassium infusion (indicated by bar), or acute *s.c.* injection of 10 mg/kg paroxetine (indicated by arrow) (C). N=5 per stimulus.

3.7 Effect of paroxetine discontinuation on basal hippocampal extracellular 5-HT and 5-HIAA

Paroxetine continuation increased basal extracellular 5-HT compared to saline controls. This effect had reversed by discontinuation day two, representing a fall in extracellular 5-HT, and remained at control levels on discontinuation day five (effect of treatment: $F(3,28)=9.339$, $p<0.001$; effect of time: $F(5,140)=5.651$, $p<0.001$; time*treatment interaction: $F(15,140)=5.651$, $p<0.001$; between-subject comparisons: SAL vs CON $p=0.001$, CON vs DIS 2 $p<0.001$, CON vs DIS 5 $p<0.001$; *Fig. 5.9*).

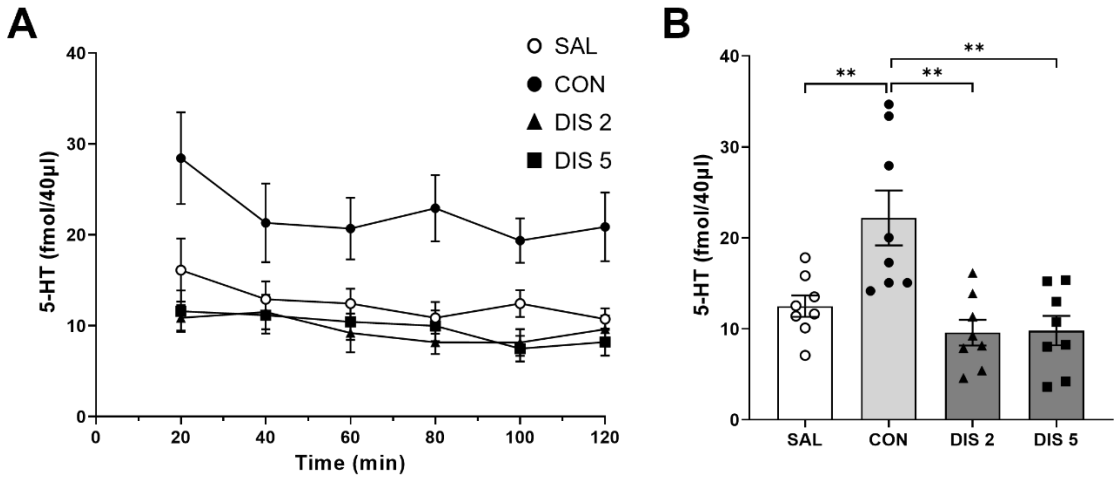


Figure 5.9 Effect of paroxetine discontinuation on basal hippocampal 5-HT. Points represent mean ± SEM of 5-HT per 40 µl microdialysate (fmol/40µl) during 120 min baseline recording (A). Bars represent ± SEM of mean basal 5-HT, individual values are indicated by dots (B). SAL (n=8), CON (n=8), DIS 2 (n=8), DIS 5 (n=8). Repeated measures ANOVA with post-hoc Bonferroni's test, effects of treatment shown in panel B, ** $p<0.01$.

5-HIAA levels were approximately two-fold higher in the first cohort of mice compared to the second cohort, irrespective of treatment group, hence cohort was added as a factor in analyses. Continued paroxetine treatment reduced basal extracellular 5-HIAA compared to saline controls. This effect had reversed two days after paroxetine discontinuation, and 5-HIAA rebounded above saline control levels on discontinuation

day five. Notably, cohort was a significant influence on the absolute levels of 5-HIAA, but there was no treatment*cohort interaction (treatment: $F(3,28)=10.165$, $p<0.001$; time: $F(5,130)=3.214$, $p=0.009$; time*treatment interaction: $F(15,130)=1.113$, $p=0.351$; between-subject comparisons: SAL vs CON $p=0.002$, SAL vs DIS 5 $p=0.040$, CON vs DIS 2 $p<0.001$, CON vs DIS 5 $p<0.001$; effect of cohort: $F(1,3)=60.856$, $p<0.001$; treatment*cohort interaction: ($F(3,24)=1.426$, $p=0.219$; *Fig. 5.10*).

Notably, there were no effects of paroxetine continuation or discontinuation on basal hippocampal extracellular DOPAC (treatment: $F(3,28)=0.290$, $p=0.829$; time: $F(5,140)=0.971$, $p=0.345$; interaction: $F(15,140)=1.262$, $p=0.245$; data not shown).

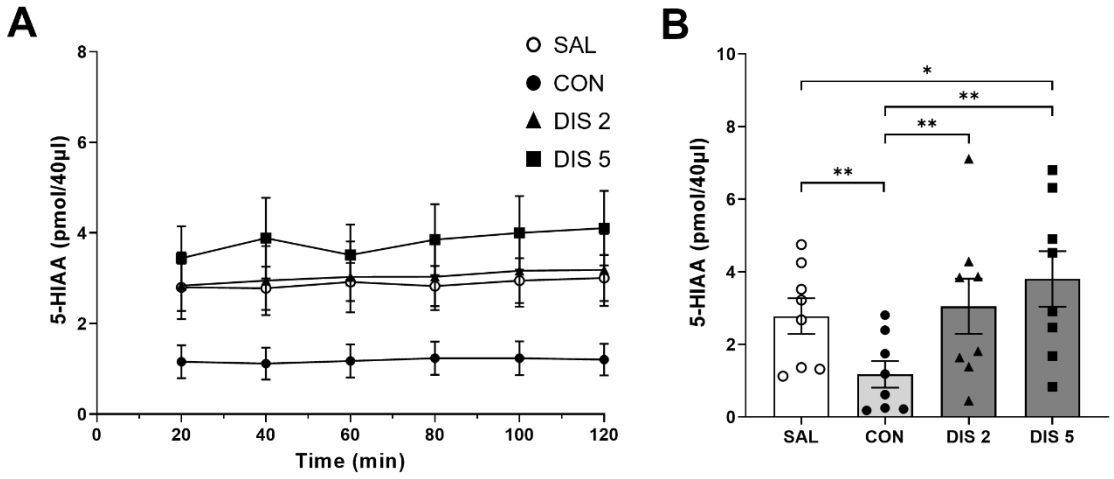


Figure 5.10 Effect of paroxetine discontinuation on basal hippocampal 5-HIAA. Points represent mean ± SEM of 5-HIAA per 40 µl microdialysate (pmol/40µl) during 120 min baseline recording (A). Bars represent ± SEM of mean basal 5-HIAA, individual values are indicated by dots (B). SAL (n=8), CON (n=8), DIS 2 (n=8), DIS 5 (n=8). Repeated measures ANOVA with post-hoc Bonferroni's test, effects of treatment shown in panel B, * $p<0.05$, ** $p<0.01$.

3.8 Effect of paroxetine discontinuation on potassium-evoked hippocampal extracellular 5-HT

Local infusion of 56 mM potassium increased 5-HT in the microdialysate of saline control mice, but this effect was attenuated in mice receiving continued paroxetine

treatment. Consequently, 5-HT was significantly lower 40 min after the start of potassium infusion compared to saline controls (treatment: $F(3,27)=4.333$, $p=0.016$; time: $F(5,135)=7.392$, $p<0.001$; interaction: $F(15,135)=2.171$, $p=0.012$; between-subject comparisons: 40 min SAL vs CON $p=0.031$; *Fig. 5.11A*). Following discontinuation, however, the response to 56 mM potassium infusion did not differ to that of saline controls. Correspondingly, paroxetine continuation reduced the peak potassium response (20 min after the start of the infusion) compared to saline controls. This effect had reversed two days after discontinuation and did not differ from saline controls five days after discontinuation ($H(3)=17.66$, $p=0.0005$, post-hoc SAL vs CON $p=0.0147$, CON vs DIS 2 $p=0.0005$, CON vs DIS 5 $p=0.0001$; *Fig. 5.11B*).

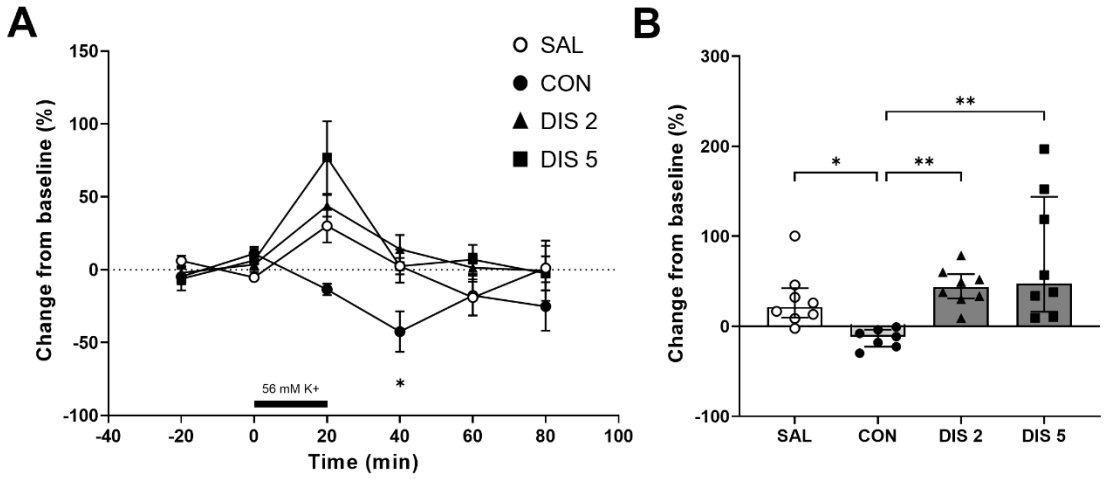


Figure 5.11 Effect of paroxetine discontinuation on hippocampal extracellular 5-HT in response to 56 mM potassium. Points represent mean ± SEM of percentage change in 5-HT per 40 µl microdialysate (fmol/40µl) following potassium infusion (A). Bar represents duration of potassium infusion. Repeated measures ANOVA with post-hoc Bonferroni's test, * $p<0.05$ between-subject pairwise comparisons. Bars represent median ± IQR of the peak change in 5-HT 20 min after start of potassium infusion (B). SAL (n=8), CON (n=7), DIS 2 (n=8), DIS 5 (n=8). Individual values are indicated by dots, Kruskal Wallis with Uncorrected Dunn's test, * $p<0.05$, ** $p<0.01$.

By comparison, local infusion of 100 mM potassium increased 5-HT in the microdialysate to the same extent in all treatment groups, and there were no significant

differences in recovery after the end of potassium infusion (treatment: F(3,25)=0.653, p=0.591; time: F(5,125)=28.174, p<0.001; interaction: F(15,125)=0.880, p=0.059). Correspondingly, there were also no significant differences in the peak potassium response (F(3,25)=0.4583, p=0.7139; *Fig. 5.12B*).

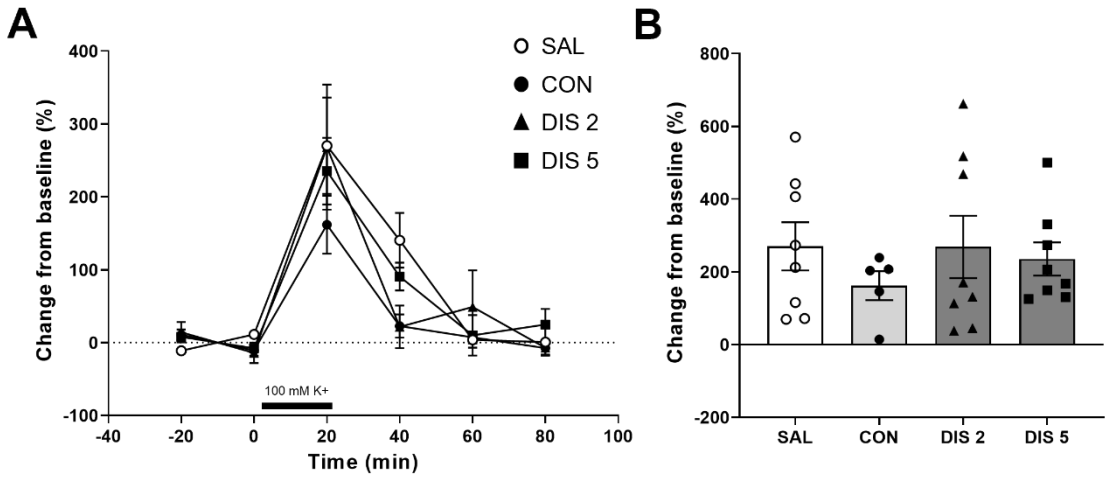


Figure 5.12 Effect of paroxetine discontinuation on hippocampal extracellular 5-HT in response to 100 mM potassium. Points represent mean ± SEM of percentage change in 5-HT per 40 µl microdialysate (fmol/40µl) following potassium infusion (A). Bar represents duration of potassium infusion. Repeated measures ANOVA with post-hoc Bonferroni's test. Bars represent ± SEM of the peak change in 5-HT 20 min after start of potassium infusion (B). SAL (n=8), CON (n=5), DIS 2 (n=8), DIS 5 (n=8). Individual values are indicated by dots, one-way ANOVA with post-hoc Fisher's LSD.

When the peak response to the 56 mM potassium infusion was expressed as a ratio of the peak response to 100 mM potassium infusion, the potassium-evoked increase in extracellular 5-HT was significantly greater both two and five days after paroxetine discontinuation compared to continued paroxetine treatment and saline controls (F(3,24)=5.387, p=0.0059, post-hoc SAL vs DIS 2 p=0.0257, CON vs DIS 2 p=0.0032, SAL vs DIS 5 p=0.0308, CON vs DIS 5 p=0.0038; *Fig. 5.13*). These results could suggest that 5-HT terminals in the VH were more sensitive to depolarising stimuli following paroxetine discontinuation.

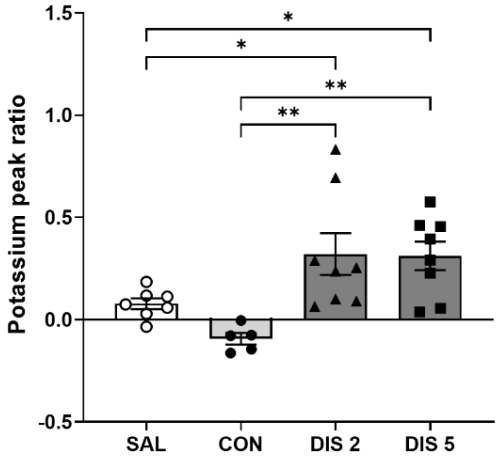


Figure 5.13 Effect of paroxetine discontinuation on the ratio of 56 mM and 100 mM potassium responses. Bars represent mean ± SEM of the ratio of the peak percentage change responses to 56 and 100 mM potassium infusions on 5-HT per 40 µl microdialysate (fmol/40µl). SAL (n=7, one outlier excluded), CON (n=5), DIS 2 (n=8), DIS 5 (n=8). Individual values are indicated by dots, one-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

3.9 Effect of paroxetine discontinuation on potassium-evoked hippocampal extracellular 5-HIAA

Local infusion of 56 mM potassium reduced 5-HIAA in the microdialysate to the same extent in all treatment groups, and there were no significant differences in recovery after the end of potassium infusion (treatment: $F(3,26)=0.584$, $p=0.631$; time: $F(5,130)=31.929$, $p<0.001$; interaction: $F(15,135)=0.584$, $p=0.631$; *Fig. 5.14A*). Local infusion of 100 mM potassium also reduced 5-HIAA in the microdialysate in all treatment groups. Although there was a significant treatment*time interaction, between-subject pairwise comparisons found no significant differences between treatment groups at any time following potassium infusion (treatment: $F(3,24)=1.437$, $p=0.257$; $F(15,120)=1.379$, $p=0.277$; $F(15,120)=2.302$, $p=0.007$; *Fig. 5.14B*).

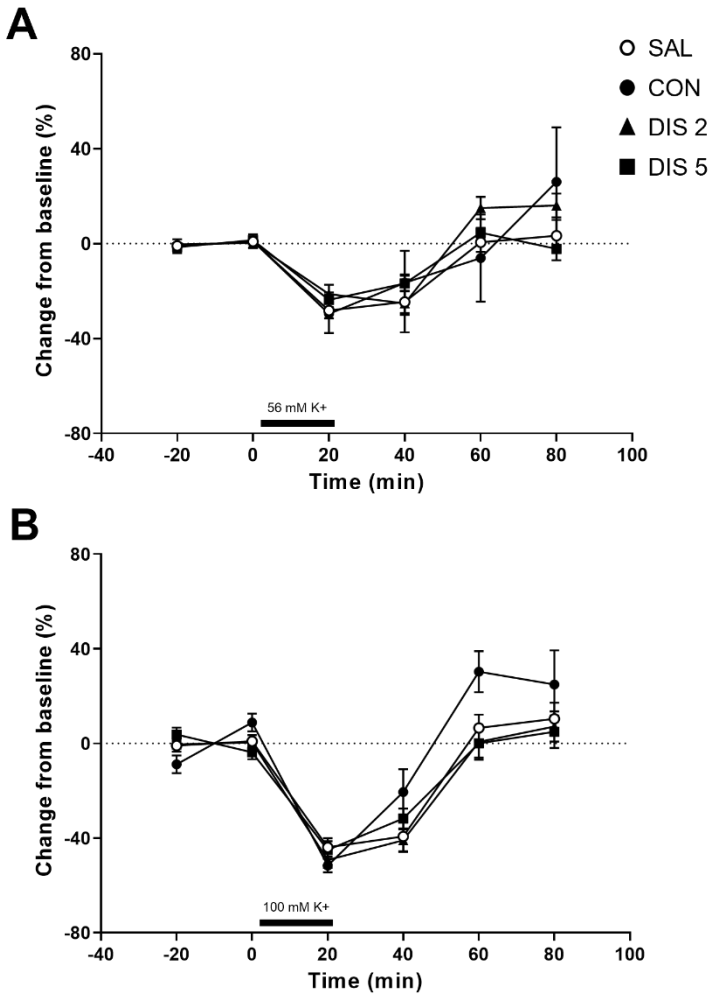


Figure 5.14 Effect of paroxetine discontinuation on hippocampal extracellular 5-HIAA in response to potassium. Points represent mean ± SEM of percentage change in 5-HIAA per 40 µl microdialysate (pmol/40µl) following potassium infusion for 56 mM potassium (A) and 100 mM potassium (B). SAL (n=8), CON (n=5-6), DIS 2 (n=8), DIS 5 (n=8). Bar represents duration of potassium infusion. Repeated measures ANOVA with post-hoc Bonferroni's test.

3.10 Effect of paroxetine discontinuation on extracellular 5-HT and 5-HIAA in response to acute paroxetine challenge

Acute paroxetine challenge increased 5-HT in the microdialysate to the same extent in all treatment groups (treatment: $F(3,23)=1.158$, $p=0.351$; time: $F(4,92)=13.188$, $p<0.001$; interaction: $F(12,92)=1.111$, $p=0.364$; *Fig. 5.15A*). Consequently, there was also no effect on the peak 5-HT response 40 min after the injection ($F(3,23)=0.9711$,

p=0.4233; *Fig. 5.15B*). Similarly, paroxetine challenge reduced 5-HIAA to the same extent across the treatment groups (treatment: F(3,23)=1.793, p=0.174; time: F(4,88)=7.778, p<0.001; interaction: F(12,88)=1.992, p=0.360; data not shown).

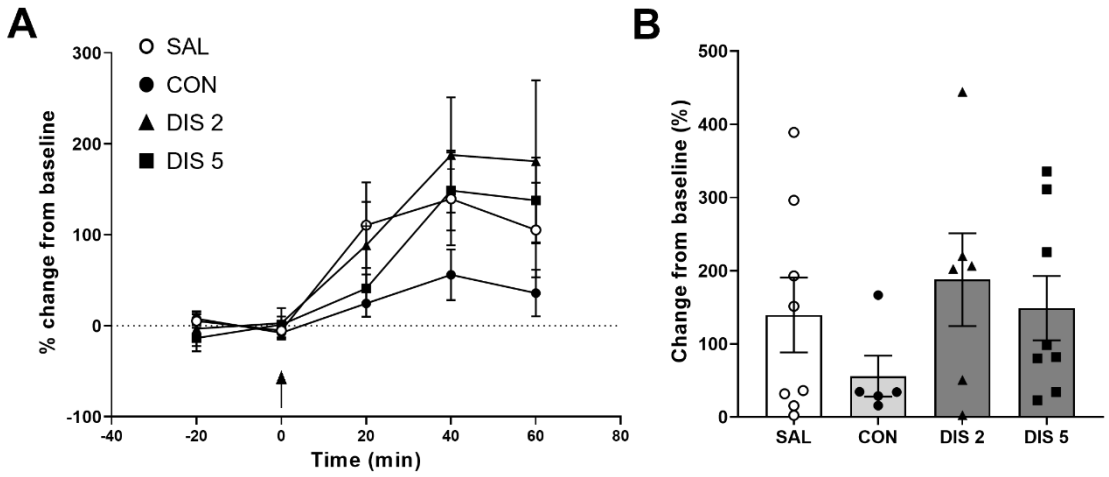


Figure 5.15 Effect of paroxetine discontinuation on hippocampal extracellular 5-HT in response to acute paroxetine challenge. Points represent mean ± SEM of percentage change in 5-HT per 40 µl microdialysate (fmol/40µl) following paroxetine challenge (A). Arrow represents acute injection. Repeated measures ANOVA with post-hoc Bonferroni's test. Bars represent ± SEM peak change in 5-HT 40 min after paroxetine injection (B). SAL (n=8), CON (n=5), DIS 2 (n=6), DIS 5 (n=8). Individual values are indicated by dots, one-way ANOVA with post-hoc Fisher's LSD.

3.11 Effect of two and five days paroxetine discontinuation on 5-HT-related gene expression

Evidence from the microdialysis experiments suggested that 5-HT terminals were more sensitive to high potassium following paroxetine discontinuation. Hence, 5-HT_{1A} and 5-HT_{1B} receptor mRNA were analysed to investigate whether this could be related to changes in autoreceptor expression. The effect of paroxetine discontinuation on 5-HT_{1A} heteroreceptor expression, and 5-HT_{1B} autoreceptor and heteroreceptor expression, were also explored in the hippocampus and PFC.

Neither two nor five days of paroxetine discontinuation were associated with

changes in *5-HT$_{1A}$* or *5-HT$_{1B}$* autoreceptor expression in the raphe nuclei, or changes in receptor expression in the hippocampus or PFC (discontinuation day two, *Table 5.3*; discontinuation day five, *Table 5.4*). There were also no effects of treatment on the expression of other 5-HT related genes, including *Tph2* and *5-HTT* in the raphe nuclei, and *5-HT$_{2A}$* and *5-HT$_{4}$* receptors in the hippocampus and PFC (*Tables 5.3-5.4*). There were also no effects of continued paroxetine treatment on gene expression. One reason for this could be that tissue samples were collected too long after the final injection of paroxetine to detect persistent effects on mRNA expression.

Table 5.3 Effect of two days paroxetine discontinuation on 5-HT-related gene expression. SAL (n=6-7), CON (n=6-8), DIS (n=7), outliers identified using ROUT analysis and removed.

	Relative mRNA (mean ± SEM)			
GOI	**SAL**	**CON**	**DIS**	**ANOVA**
Raphe nuclei				
5-HT$_{1A}$	1.04 ± 0.13	1.38 ± 0.25	0.93 ± 0.16	F(2,19)=1.546 p=0.2386
5-HT$_{1B}$	1.01 ± 0.06	1.13 ± 0.10	0.93 ± 0.06	F(2,19)=1.760 p=0.1989
5-HTT	2.04 ± 0.94	1.33 ± 0.44	2.92 ± 1.42	F(2,16)=0.5518 p=0.5865
Tph2	2.07 ± 0.96	1.19 ± 0.36	2.96 ± 1.5	F(2,16)=0.6735 p=0.5238
Hippocampus				
5-HT$_{1A}$	1.01 ± 0.05	1.16 ± 0.07	0.98 ± 0.06	F(2,21)=2.679 p=0.0920
5-HT$_{1B}$	1.03 ± 0.09	1.18 ± 0.06	1.00 ± 0.05	F(2,21)=1.886 p=0.1765
5-HT$_{2A}$	1.04 ± 0.10	1.05 ± 0.05	1.06 ± 0.06	F(2,21)=0.040 p=0.9606
5-HT$_{4}$	1.02 ± 0.07	1.20 ± 0.08	0.98 ± 0.04	F(2,21)=3.231 p=0.0589
PFC				
5-HT$_{1A}$	1.02 ± 0.08	1.06 ± 0.08	0.96 ± 0.05	F(2,21)=0.5645 p=0.5771
5-HT$_{1B}$	1.03 ± 0.09	1.13 ± 0.04	0.98 ± 0.08	F(2,21)=1.286 p=0.3021
5-HT$_{2A}$	1.04 ± 0.11	1.15 ± 0.10	1.07 ± 0.03	F(2,21)=0.4614 p=0.6367
5-HT$_{4}$	1.02 ± 0.07	1.12 ± 0.10	0.96 ± 0.07	F(2,21)=0.8986 p=0.4237

Table 5.4 Effect of five days paroxetine discontinuation on 5-HT-related gene expression. SAL (n=4-6), CON (n=6), DIS (n=5-6), outliers identified using ROUT analysis and removed.

	Relative mRNA (mean ± SEM)			
GOI	**SAL**	**CON**	**DIS**	**ANOVA**
Raphe nuclei				
5-HT$_{1A}$	1.03 ± 0.12	0.85 ± 0.06	1.07 ± 0.26	F(2,14)=1.281 p=0.3084
5-HT$_{1B}$	1.02 ± 0.08	1.10 ± 0.17	0.85 ± 0.06	F(2,15)=1.177 p=0.3352
5-HTT	1.01 ± 0.06	0.51 ± 0.10	1.43 ± 0.05	F(2,12)=2.287 p=0.1440
Tph2	1.01 ± 0.072	0.48 ± 0.10	1.25 ± 0.53	F(2,13)=1.353 p=0.2927
Hippocampus				
5-HT$_{1A}$	1.02 ± 0.09	1.08 ± 0.06	1.10 ± 0.08	F(2,15)=0.3268 p=0.7263
5-HT$_{1B}$	1.04 ± 0.14	0.87 ± 0.04	0.94 ± 0.08	F(2,15)=0.086 p=0.4640
5-HT$_{2A}$	1.02 ± 0.08	0.87 ± 0.04	0.93 ± 0.07	F(2,15)=1.234 p=0.3190
5-HT$_{4}$	1.02 ± 0.08	0.93 ± 0.05	1.04 ± 0.11	F(2,15)=0.4623 p=0.6385
PFC				
5-HT$_{1A}$	1.02 ± 0.08	1.15 ± 0.07	1.02 ± 0.08	F(2,15)=0.8851 p=0.4332
5-HT$_{1B}$	1.01 ± 0.07	1.06 ± 0.09	1.17 ± 0.17	F(2,15)=0.4864 p=0.6242
5-HT$_{2A}$	1.02 ± 0.07	1.01 ± 0.08	1.18 ± 0.16	F(2,15)=0.7104 p=0.5072
5-HT$_{4}$	1.00 ± 0.05	0.92 ± 0.07	0.86 ± 0.10	F(2,14)=0.8139 p=0.4630

3.12 Effect of two days paroxetine discontinuation on 5-HT-related gene expression (repeat experiment)

To assess if there would be a greater effect of paroxetine continuation on mRNA expression if brains were tested sooner after the final injection of paroxetine, relative mRNA expression was quantified in brains extracted 6 h after the final injection. This meant that brains were taken from mice undergoing paroxetine discontinuation on discontinuation day two (30 h after the last paroxetine injection).

In the raphe nuclei, there were no effects of paroxetine continuation, but paroxetine discontinuation reduced *5-HT$_{1A}$* autoreceptor expression compared to continued paroxetine treatment (F(2,21)=2.949, p=0.0444, post-hoc CON vs DIS p=0.0383; *Fig. 5.16A*). There were no effects of treatment on *5-HT$_{1B}$* autoreceptor expression (F(2,19)=1.153, p=0.3370; *Fig. 5.16B*), or on *5-HTT* or *Tph2* expression (*Table 5.5*).

In the hippocampus, there were no effects of paroxetine continuation or discontinuation on *5-HT$_{1A}$* or *5-HT$_{1B}$* expression (5-HT1A: F(2,15)=1.643, p=0.2263; *Fig. 5.16C*; 5-HT1B: F(2,21)=0.5439, p=0.5884; *Fig. 5.16D*). There were also no effects of treatment on the expression of postsynaptic *5-HT$_{2A}$* or *5-HT$_{4}$* receptors (*Table 5.5*).

In the PFC, paroxetine discontinuation reduced the expression of *5-HT$_{1A}$* heteroreceptors compared to continued paroxetine and saline controls (F(2,21)=4.529, p=0.0232, post-hoc SAL vs DIS p=0.0391, CON vs DIS p=0.0090; *Fig. 5.16A*). There were no effects of treatment on the expression of *5-HT$_{1B}$* receptors (F(2,21)=2.098, p=0.1477; *Fig. 5.25B*) or *5-HT$_{2A}$* or *5-HT$_{4}$* receptor expression (*Table 5.5*).

Table 5.5 Effect of 30 h paroxetine discontinuation on 5-HT-related gene expression. SAL (n=8), CON (n=8), DIS (n=8), outliers identified using ROUT analysis and removed.

	Relative mRNA (mean ± SEM)			
GOI	**SAL**	**CON**	**DIS**	**ANOVA**
Raphe nuclei				
5-HTT	1.21 ± 0.23	1.52 ± 0.24	1.18 ± 0.15	F(2,21)=0.8162 p=0.4556
Tph2	1.14 ± 0.16	1.51 ± 0.32	1.13 ± 0.11	F(2,21)=1.047 p=0.3686
Hippocampus				
5-HT$_{2A}$	1.08 ± 0.21	0.62 ± 0.14	0.84 ± 0.22	F(2,21)=1.440 p=0.2679
5-HT$_{4}$	1.15 ± 0.21	0.87 ± 0.07	2.15 ± 0.82	F(2,21)=1.668 p=0.2241
PFC				
5-HT$_{2A}$	1.01 ± 0.06	1.03 ± 0.09	0.93 ± 0.10	F(2,21)=0.3668 p=0.6973
5-HT$_{4}$	1.08 ± 0.16	0.94 ± 0.09	1.81 ± 0.64	F(2,21)=1.264 p=0.3052

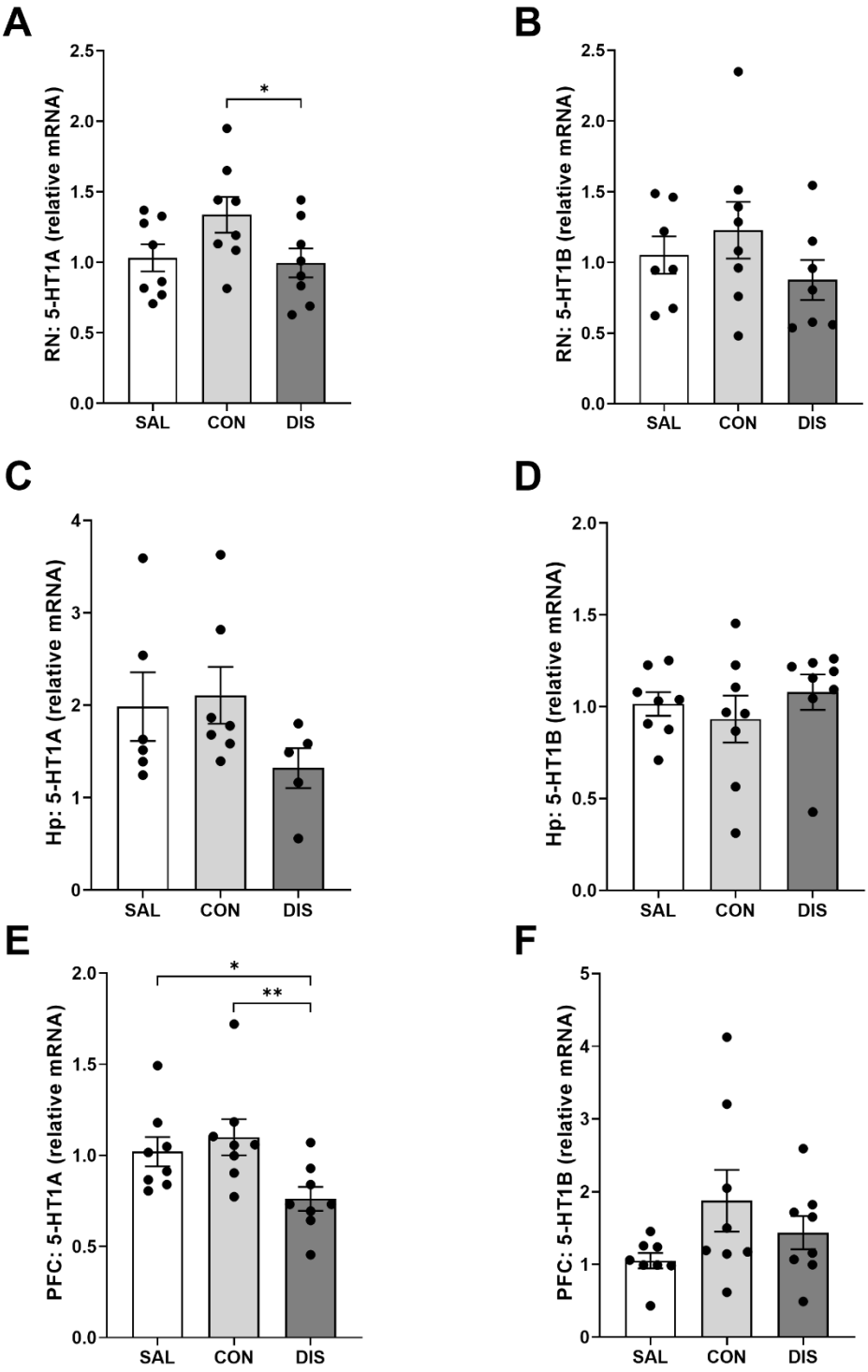


Figure 5.16 Effect of two days paroxetine discontinuation on *5-HT$_{1A}$* and *5-HT$_{1B}$* receptor expression. Bars represent the mean ± SEM values for the fold-change in the expression of *5-HT$_{1A}$* receptor (A, C, E) and *5-HT$_{1B}$* receptor (B, D, F) relative to saline controls in the raphe nuclei (RN; A-B), hippocampus (C-D) and PFC (E-F). SAL (n=8), CON (6 h after final injection, n=6-8), DIS (30 h after final injection, n=5-8), outliers identified using ROUT analysis and removed. Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

4 Discussion

Experiments in the previous chapter found evidence of increased 5-HT neuron activity two and five days after paroxetine discontinuation. Moreover, previous studies have reported changes to brain tissue levels of 5-HT following SSRI cessation (see section 1.2). In this chapter, 5-HT neurochemistry, and the expression of the 5-HT autoreceptors, were assessed in mice undergoing paroxetine discontinuation.

Analysis of *ex vivo* brain tissue found reduced concentrations of 5-HT and 5-HIAA during continued paroxetine treatment. Two days after paroxetine discontinuation, however, 5-HT and 5-HIAA had recovered to saline control levels, and there was evidence of a rebound increase above saline controls by discontinuation day five. *In vivo* microdialysis experiments also showed that continued paroxetine treatment increased basal extracellular 5-HT in the VH, which fell rapidly to saline control levels within two days of discontinuation. On the other hand, extracellular 5-HIAA rebounded above saline levels on day five. Moreover, the potassium-evoked release of 5-HT was suppressed in mice receiving continued paroxetine treatment, but this response was enhanced compared to continued paroxetine and saline controls following discontinuation. These findings suggest that 5-HT terminals were more sensitive to depolarising stimuli following paroxetine cessation. These changes were associated with a reduction in *5-HT$_{1A}$* receptor mRNA expression in the raphe nuclei and in the forebrain on the second day after paroxetine discontinuation.

Overall, these neurochemical data support the c-FOS immunohistochemical evidence that paroxetine discontinuation was associated with an increase in 5-HT neuron activation two and five days after discontinuation. They also suggest that baseline extracellular 5-HT fell to saline control levels rapidly after treatment cessation.

4.1 Continued paroxetine treatment suppressed 5-HT synthesis and metabolism but increased extracellular 5-HT

In *ex vivo* experiments, repeated paroxetine treatment depleted 5-HT and 5-HIAA regional brain concentrations compared to saline controls. These findings agree with similar studies on the effects of repeated paroxetine, fluoxetine, and citalopram treatment on 5-HT neurochemistry (Caccia et al., 1992; Caccia et al., 1993; Trouvin et al., 1993; Bosker et al., 2010; Karanges et al., 2011). They also suggest that repeated paroxetine treatment was associated with reduced 5-HT synthesis, supported by other studies showing lower concentrations of 5-HTP in the hippocampus and cortex of rats during repeated SSRI treatment (Esteban et al., 1999; Stenfors & Ross, 2002; Bosker et al., 2010). Reduced 5-HIAA also suggests that 5-HT synthesis was suppressed, as less 5-HT was available to be metabolised into 5-HIAA.

On the other hand, *in vivo* microdialysis performed in the VH showed that repeated paroxetine treatment produced a two-fold increase in basal extracellular 5-HT compared to saline controls, but a reduction in extracellular 5-HIAA. Numerous other microdialysis studies have also demonstrated elevated extracellular 5-HT following chronic SSRI treatment (Rutter et al., 1994; Malagié et al., 1995; Arborelius et al., 1996; Bel & Artigas, 1999; Ceglia et al., 2004; Popa et al., 2010; Guiard et al., 2012). This includes following repeated paroxetine treatment (Hajós-Korcsok et al., 2000; Malagié et al., 2000), and in the hippocampus of rats and mice (Hajós-Korcsok et al., 2000; Popa et al., 2010; Guiard et al., 2012). Extracellular 5-HIAA also decreased with repeated citalopram treatment in the rat frontal cortex (Arborelius et al., 1996). Thus, repeated paroxetine treatment was associated with increased extracellular 5-HT but reduced 5-HIAA.

The reduction in 5-HT synthesis and metabolism[5] was likely mediated by elevated extracellular 5-HT. In the raphe nuclei, 5-HT activates somatodendritic 5-HT_{1A} autoreceptors, which suppress 5-HT neuron firing and TPH2 activity (Chaput et al., 1986; Hrdina, 1987; Ehret et al., 1989; Boadle-Biber, 1993; Hajós-Korcsok et al., 2000). The rise in extracellular 5-HT in terminal regions may also have caused 5-HT_{1B} autoreceptor activation and thus a fall in local 5-HT synthesis (Engel et al., 1986). Notably, reduced 5-HT terminal excitability may also have been responsible for the attenuated response to local 56 mM potassium infusion in mice receiving continued paroxetine treatment.

Repeated citalopram treatment produced similar effects on 5-HT synthesis. Although there were no effects on 5-HT or 5-HIAA alone, citalopram treatment reduced the 5-HIAA/5-HT ratio, supporting the notion that 5-HT synthesis was suppressed during repeated SSRI treatment (Cremers et al., 2000a; Bosker et al., 2010). Nonetheless, the neurochemical effects of repeated citalopram treatment were less extensive than with repeated paroxetine treatment. This is likely due to the short half-life of citalopram (Overø, 1982), so its effects on 5-HT synthesis may have been more transient.

Thus, continued SSRI treatment was associated with a reduction in regional brain tissue 5-HT and 5-HIAA, as well as increased extracellular 5-HT. This was likely associated with persistent 5-HT autoreceptor activation.

[5] It is interesting to note that the concentration of 5-HIAA in the microdialysate was approximately one thousand times higher than the concentration of 5-HT. This could have arisen from the greater uptake of 5-HIAA into the microdialysis probe or the better detection of 5-HIAA by the HPLC-ED (Kumar et al., 1999). Yet, others have noted a similar relationship between extracellular DA and its metabolite DOPAC (Zetterström et al., 1989). These results suggest that the majority of 5-HT is metabolised into 5-HIAA without being involved in exocytosis for neurotransmission. This excess synthesis is thought to create capacity in the system to increase vesicular 5-HT stores if necessary. For example, at rest there is sufficient 5-HT in the readily releasable pool of vesicles that excess 5-HT can be immediately metabolised into 5-HIAA. Following prolonged neuronal stimulation and release of 5-HT, however, vesicular 5-HT must be rapidly replenished. A constant high rate of synthesis means that 5-HT can be quickly redirected into vesicular storage and away from metabolism into 5-HIAA, without having to alter the rate of synthesis (Zetterström et al., 1989).

4.2 Paroxetine discontinuation produced a rebound increase in 5-HT synthesis

Two days after paroxetine discontinuation, brain tissue 5-HT and 5-HIAA concentrations had recovered to saline control levels. By discontinuation day five, however, there was evidence of a rebound increase in both 5-HT and 5-HIAA. The 5-HIAA/5-HT ratio also rebounded above controls two days after citalopram discontinuation. These data agree with previous studies showing that regional concentrations of 5-HT increased within seven days of fluoxetine, sertraline, and paroxetine discontinuation in the rat midbrain and cortex (Caccia et al., 1992; Caccia et al., 1993; Trouvin et al., 1993). Moreover, rebound increases in 5-HIAA and the 5-HIAA/5-HT ratio were reported in the hippocampus within a week of fluoxetine discontinuation (Trouvin et al., 1993; Stenfors & Ross, 2002), and just two days after citalopram discontinuation in rats (Bosker et al., 2010). These effects likely arose due to increased 5-HT synthesis following SSRI cessation. Accordingly, hippocampal 5-HTP accumulation rebounded above vehicle control levels two days after citalopram discontinuation (Bosker et al., 2010). A similar effect was evident three days after fluoxetine discontinuation in the hippocampus and frontal cortices of rats (Stenfors & Ross, 2002). Thus, paroxetine discontinuation was likely associated with a rebound increase in 5-HT synthesis.

Notably, there was some variation in the effects of discontinuation from different SSRIs. For instance, tissue 5-HT and 5-HIAA levels recovered within two days of discontinuation in the current experiments, whereas it took at least a week following fluoxetine discontinuation (Caccia et al., 1993; Trouvin et al., 1993). Moreover, while it took five days for a rebound increase in 5-HT function to develop following paroxetine discontinuation, it was already evident as an increase in the 5-HIAA/5-HT ratio two days

after citalopram cessation (Bosker et al., 2010). Thus, the neurochemical effects of SSRI discontinuation appear to take longer to emerge after administration of SSRIs with longer half-lives, likely due to their protracted washout.

Nonetheless, these data cannot attest to the full duration of changes to 5-HT synthesis following paroxetine discontinuation. Increased hippocampal 5-HIAA was still evident 28 days after fluoxetine cessation in rats (Trouvin et al., 1993), suggesting paroxetine cessation effects may persist for longer. Further experiments are therefore needed to assess 5-HT neurochemistry for several weeks after paroxetine discontinuation.

4.3 Paroxetine discontinuation reduced basal hippocampal 5-HT to baseline levels

Another key finding from the current experiments was the rapid fall in extracellular 5-HT in the VH within two days of paroxetine discontinuation. Guiard et al. (2012) reported similar findings with escitalopram; repeated escitalopram treatment increased extracellular 5-HT in the mouse VH, but two days after its washout, 5-HT had fallen to saline control levels. Several studies have also noted that extracellular 5-HT did not differ from vehicle controls two days after discontinuation from fluvoxamine, citalopram and fluoxetine (Cremers et al., 2000a; Bosker et al., 2001; Klomp et al., 2014), including in the mouse hippocampus (Popa et al., 2010). Notably, however, these studies lacked on-drug controls. Thus, the current study was the first to demonstrate that extracellular 5-HT recovers to control levels within two days of paroxetine discontinuation.

This rapid reduction in extracellular 5-HT likely reflects the removal of 5-HTT blockade following drug withdrawal. This may also have driven the rebound increase in 5-HT synthesis evident in the *ex vivo* analysis, as lower extracellular 5-HT would have reduced the activation of 5-HT_{1A} autoreceptors and thus disinhibited TPH2 activity. The

rebound increase in basal hippocampal extracellular 5-HIAA further supports the findings from the *ex vivo* experiments that tissue 5-HIAA levels increased, again likely due to increased 5-HT synthesis.

4.4 Paroxetine discontinuation potentiated the depolarisation-evoked increase in hippocampal extracellular 5-HT

Both two and five days of paroxetine discontinuation were associated with proportionally greater responses to potassium-evoked depolarisation compared to continued paroxetine and saline controls. One explanation for this effect is that increased 5-HT synthesis following paroxetine discontinuation increased vesicular 5-HT content, hence 5-HT release was greater in response to depolarisation. Nonetheless, it is likely that this would also potentiate 5-HT release in response to 100 mM potassium infusion. Thus, a more plausible explanation for this effect is that 5-HT terminals were more sensitive to stimulation following paroxetine discontinuation, making them more likely to respond to smaller depolarising stimuli, such as 56 mM potassium. By comparison, 100 mM potassium may have been sufficient to depolarise all 5-HT terminals and release 5-HT regardless of the underlying sensitivity of the neuron. This would account for why the larger response did not differ between treatment conditions.

There have been no other *in vivo* studies of the effect of SSRI discontinuation on depolarisation-evoked 5-HT release with which to compare these results. Nonetheless, el Mansari et al. (1995) showed that in *ex vivo* guinea pig frontal cortex slices taken two days after paroxetine discontinuation (and preloaded with radiolabelled 5-HT), the electrical stimulation of 5-HT terminals produced a greater increase in extracellular 5-HT compared to slices taken from saline-treated animals. The authors interpreted this finding

as evidence of increased 5-HT release due to the ongoing desensitisation of 5-HT_{1B} autoreceptors during repeated paroxetine treatment. Nonetheless, due to the washout of paroxetine before the experiment, this may in fact reflect enhanced sensitivity to stimulation following discontinuation.

Notably, paroxetine discontinuation did not alter the 5-HT response to acute paroxetine challenge. Other reports have also failed to detect sensitisation to acute SSRI administration during the discontinuation period (Invernizzi et al., 1995; Cremers et al., 2000a). Nonetheless, in the current experiments mice had already been exposed to two lengthy potassium infusions, so the response to acute paroxetine treatment may have been attenuated by depleted presynaptic 5-HT levels. Future experiments should therefore administer paroxetine immediately following the baseline period to confirm the lack of effect following discontinuation.

Overall, these data suggest that 5-HT terminals were more sensitive to depolarising stimuli following paroxetine discontinuation. Nonetheless, caution is needed when interpreting these findings. For example, changes in extracellular 5-HT do not solely arise from changes in terminal 5-HT release, and could also reflect increased neuronal firing rates, changes in firing pattern, or reduced 5-HT uptake. Moreover, local potassium infusion depolarises all neurons around the microdialysis probe, not just 5-HT terminals. This means that the activation of other neurons, for example GABA interneurons (Pei et al., 1989), may have influenced extracellular 5-HT concentrations. Future experiments could use optogenetics or chemogenetics to specifically activate 5-HT neurons to evoke 5-HT release. Furthermore, the spatial and temporal specificity of microdialysis is low. Fluorescent 5-HT sensors could therefore be used to monitor the dynamic changes in

extracellular 5-HT during discontinuation. Finally, experiments should be repeated in awake, behaving mice, to confirm that there were no artefacts generated by anaesthesia.

4.5 Two days of paroxetine discontinuation reduced 5-HT$_{1A}$ autoreceptor expression

The results presented thus far suggest that paroxetine discontinuation was associated with increased 5-HT synthesis and terminal sensitivity, likely via feedback mechanisms involving the 5-HT autoreceptors. To further explore this hypothesis, the expression of *5-HT$_{1A}$* and *5-HT$_{1B}$* receptor mRNA was quantified in the raphe nuclei, hippocampus, and PFC following paroxetine discontinuation. In initial experiments, neither two nor five days of paroxetine discontinuation was associated with changes in 5-HT-related gene expression. Yet, when the experiment was repeated and tissue samples taken earlier on the second day of discontinuation, *5-HT$_{1A}$* receptor expression was reduced in the raphe nuclei and PFC compared to continued paroxetine treatment.

These findings support the hypothesis that reduced 5-HT$_{1A}$ autoreceptor activation increased 5-HT synthesis and neuronal excitability following paroxetine discontinuation, as well as evidence of increased 5-HT neuronal activity that was reported in the previous chapter. Nonetheless, there have been no other studies on the effects of SSRI discontinuation on 5-HT$_{1A}$ receptor expression with which to compare these findings. Thus, further work is needed to confirm this effect and to determine if changes in mRNA translate to protein expression. Moreover, these findings cannot attest to the functionality of 5-HT$_{1A}$ autoreceptors, such as the extent of their desensitisation, following paroxetine discontinuation.

In contrast, there was no change in *5-HT$_{1B}$* receptor mRNA after paroxetine cessation. This is supported by previous studies that suggested that *5-HT$_{1B}$* mRNA in the hippocampus and DRN did not differ from saline controls one week after fluoxetine discontinuation (Neumaier et al., 1996; Piñeyro & Blier, 1999; Anthony et al., 2000). This could suggest that the feedback mechanisms involved in the effects of paroxetine discontinuation were predominantly mediated by 5-HT$_{1A}$ autoreceptors.

Interestingly, paroxetine discontinuation was not associated with changes in 5-HTT expression. Nonetheless, this finding may have been confounded by the lack of effect of repeated paroxetine treatment on 5-HTT. Although the literature is inconsistent on the effects of SSRI treatment on 5-HTT expression (for example, Spurlock et al., 1994, and Johnson et al., 2009), *in situ* hybridisation and radioligand binding studies in rats have suggested that chronic SSRI administration reduces 5-HTT expression compared to vehicle controls (Lesch et al., 1993; Piñeyro et al., 1994; Neumaier et al., 1996; Benmansour et al., 1999; Benmansour et al., 2002). Consequently, it has been reported that reduced 5-HTT expression recovered to saline control levels within a week of fluoxetine and sertraline discontinuation (Neumaier et al., 1996; Benmansour et al., 1999). While this agrees with the current finding that *5-HTT* mRNA did not differ from saline controls at any point following paroxetine discontinuation, this may simply be because repeated paroxetine itself did not alter 5-HTT expression.

Similarly, the lack of effect of paroxetine treatment and its discontinuation on *Tph2* mRNA was surprising given the evidence of altered 5-HT synthesis. It is therefore likely that these effects were not mediated by changes in TPH2 expression but instead by its activity, which cannot be determined from mRNA data.

The expression of other key components of the 5-HT system, including postsynaptic 5-HT receptors, were also investigated. The lack of effects of paroxetine discontinuation on *5-HT$_{2A}$* and *5-HT$_{4}$* mRNA expression could suggest that most of the changes to 5-HT neurochemistry were presynaptic, rather than related to the postsynaptic detection of 5-HT. Nonetheless, further work is needed to explore the effect of SSRI discontinuation on other receptors. These experiments would also benefit from increased spatial specificity, for example by analysing the DRN and MRN separately, or subregions of the hippocampus and PFC, for example using *in situ* hybridisation.

4.6 The dopaminergic system was unaffected by paroxetine discontinuation

Unlike the changes to the 5-HT system following paroxetine discontinuation, neither paroxetine nor citalopram discontinuation altered regional tissue DA or DOPAC levels. The concentration of DOPAC in the microdialysate also did not differ from that of saline controls following paroxetine discontinuation. These findings agree with another study that found no evidence of changes to DA synthesis in the hippocampus following fluoxetine discontinuation in rats (Esteban et al., 1999; Karanges et al., 2011). Although there is some evidence that fluoxetine discontinuation was associated with increased brain DOPAC concentrations (Gardier et al., 1994), the current experiments suggest that paroxetine and citalopram discontinuation were associated with altered 5-HT but not DA neurochemistry. Nonetheless, the effects of SSRI discontinuation on other neurotransmitter systems, such as noradrenaline and acetylcholine, have not yet been explored.

4.7 Conclusions

The current experiments provide evidence of a rebound increase in 5-HT synthesis following paroxetine discontinuation. Although basal hippocampal 5-HT levels fell within two days of paroxetine discontinuation, 5-HT terminals in the VH appeared more sensitive to depolarising stimuli. Increased sensitivity to stimulation and reduced 5-HT_{1A} autoreceptor expression in the raphe nuclei supports immunohistochemical evidence of increased 5-HT neuron activation (Chapter 4).

To explain these experimental observations, it is hypothesised that reduced extracellular 5-HT following paroxetine discontinuation may reduce 5-HT_{1A} autoreceptor activation. Combined with their reduced expression, this would disinhibit 5-HT neurons, increase 5-HT synthesis, and make the neurons more sensitive to depolarising stimuli. This notion is consistent with the oppositional model of drug tolerance as an explanation for withdrawal symptoms (Chapter 1 section 7). During SSRI treatment, compensatory homeostatic mechanisms work against the chronic suppression of 5-HT neurons to normalise 5-HT transmission. With this inhibition relieved following discontinuation, the system may become unrestrained, driving increased 5-HT synthesis and 5-HT release.

Furthermore, these findings raise the question as to whether altered 5-HT transmission could be responsible for the increased anxiety-like behaviour two days after paroxetine discontinuation. They present two potential ways in which changes to the 5-HT system may mediate these effects: firstly, the initial fall in extracellular 5-HT may trigger the heightened anxiety-like behaviours (as previously speculated in reviews); or secondly, the rebound increase in 5-HT transmission may be the causal mediator. Experiments in the next chapter explored the role of changes to 5-HT neurochemistry in the behavioural effects of SSRI discontinuation.

Chapter 6
Examination of the causal link between the neurobiological and behavioural effects of paroxetine discontinuation

1 Introduction

Based on data obtained in Chapters 4 and 5, it is hypothesised that SSRI discontinuation in mice is associated with a rebound increase in 5-HT transmission. Following SSRI cessation, a sudden fall in extracellular 5-HT likely reduces 5-HT_{1A} autoreceptor activation, disinhibiting 5-HT neurons and thus increasing 5-HT transmission. Given evidence that heightened 5-HT transmission can increase anxiety-like behaviour in rodents (e.g., Ohmura et al., 2014; Teissier et al., 2015; Ohmura et al., 2020), a rebound activation of the 5-HT system may have contributed to the putative anxiogenic effects of paroxetine discontinuation observed in Chapter 3. To investigate the role of 5-HT in these effects, experiments in this chapter investigated whether pharmacologically manipulating the 5-HT system could precipitate the behavioural effects and neuronal activation patterns evident two days after paroxetine discontinuation. These experiments aimed to establish whether the initial fall in extracellular 5-HT or the subsequent rebound increase in transmission was the causal trigger for these effects.

1.1 Precipitating CNS drug discontinuation

The lack of previous research on the mechanisms of SSRI discontinuation symptoms means insights must be taken from other CNS drug withdrawal states, for instance those associated with opiate withdrawal. Chronic opioid use is associated with

the constant activation of μ-opioid receptors, and acute opiate withdrawal produces a robust and debilitating discontinuation syndrome (Farrell, 1994). Naloxone is used clinically to reverse opiate overdoses by competitively antagonising the μ-opioid receptor (Pathan & Williams, 2012). Naloxone-precipitated withdrawal has also been used as an experimental model to explore the mechanisms underlying the opiate withdrawal syndrome. For example, naloxone was used to elucidate the role of noradrenergic rebound in the behavioural effects of opiate withdrawal in rodents (Taylor et al., 1988; Kamei & Ohsawa, 1997).

A similar approach could theoretically be applied to SSRI discontinuation to investigate the causal role of changes to 5-HT transmission in its behavioural effects in mice. Unlike naloxone-precipitated opiate withdrawal, however, there are no drugs that can competitively and selectively block SSRIs binding to the 5-HTT. Although it was previously thought that tianeptine could potentiate 5-HT reuptake via the 5-HTT (Mocaër et al., 1988), there is growing evidence that tianeptine acts indirectly to reduce 5-HT transmission rather than increasing 5-HT recovery (Gassaway et al., 2014). Manipulation of the 5-HT system using 5-HT_{1A} receptor pharmacology was therefore chosen as a first attempt to precipitate the behavioural effects and neuronal activation patterns of SSRI discontinuation. This pharmacological approach could test whether reducing extracellular 5-HT with a 5-HT_{1A} receptor agonist or increasing 5-HT transmission with a 5-HT_{1A} receptor antagonist could induce paroxetine discontinuation.

1.2 Neurochemical and behavioural effects of 5-HT_{1A} receptor agonists

The 5-HT_{1A} receptor agonist 8-OH-DPAT was chosen to test the causal involvement of the fall in extracellular 5-HT in the behavioural effects of paroxetine

discontinuation. It is well known that the activation of 5-HT_{1A} autoreceptors reduces 5-HT transmission. For instance, when administered systemically to rodents, 5-HT_{1A} receptor agonists reduced the firing rate of 5-HT neurons in the DRN and MRN of anaesthetised rats (Hajós et al., 1995; Blier et al., 1998). 8-OH-DPAT also reduced c-FOS expression in 5-HT neurons in the raphe nuclei of stressed rats compared to vehicle-treated controls (Rioja et al., 2006), demonstrating its ability to reduce 5-HT neuron activity. *In vivo* microdialysis experiments confirmed that 8-OH-DPAT also reduced extracellular 5-HT in the rodent raphe nuclei (Bosker et al., 1994; Adell & Artigas, 1998; Adell & Artigas, 1999) and forebrain (Sprouse & Aghajanian, 1987; Sharp et al., 1989b; Sharp et al., 1989c; Sharp & Hjorth, 1990; Hjorth & Sharp, 1991). Moreover, these effects were recapitulated by the local infusion of 8-OH-DPAT into the DRN or MRN (Hjorth et al., 1982; Vandermaelen & Aghajanian, 1983; Sharp et al., 1989a; Kreiss & Lucki, 1994), indicating that this was an autoreceptor-mediated effect.

Notably, 5-HT_{1A} autoreceptors have been reported to lose sensitivity with persistent activation (reviewed in Raymond et al., 1999, and Descarries & Riad, 2012), although the extent of this desensitisation is debated (see Chapter 5 section 1.1). For instance, recent data suggests that this is more likely to represent a reduction in sensitivity rather than a complete loss of 5-HT_{1A}-dependent signalling (Guiard et al., 2012). Accordingly, 8-OH-DPAT was able to reduce extracellular 5-HT even in rats repeatedly treated with citalopram (Cremers et al., 2000b).

Ideally, a drug to precipitate the behavioural effects of SSRI discontinuation should not alter anxiety-like behaviour itself; instead, its actions on the 5-HT system of SSRI-treated mice should cause its behavioural effects. Although 5-HT_{1A} receptor agonists are typically thought to have anxiolytic properties (for example, Noguerón-

Merino et al., 2015, Jastrzębska-Więsek et al., 2018, Cai et al., 2019), reports on the effects of 8-OH-DPAT vary (such as Moser et al., 1990, Critchley et al., 1992, Handley & McBlane, 1993, and McCreary et al., 1996). Nonetheless, the behavioural effects of 8-OH-DPAT are dose dependent, and low doses (0.1 mg/kg or lower) have been shown to produce minimal effects on behaviour, including on entries to the open arms of the EPM and on LMA in rats (Critchley & Handley, 1987; Hillegaart et al., 1989; Blanchard et al., 1992). Crucially, however, these low doses still reduced extracellular 5-HT in forebrain regions (Casanovas et al., 1997). It was therefore hypothesised that, in the current experiments, low doses of 8-OH-DPAT would reduce 5-HT neuron activation and hippocampal extracellular 5-HT without altering behaviour. Thus, any behavioural effects in paroxetine-treated mice would reflect the precipitation of the anxiogenic effects of paroxetine discontinuation and not the inherent effects of 8-OH-DPAT.

1.3 Neurochemical and behavioural effects of 5-HT_{1A} antagonists

To test the alternative hypothesis that increased 5-HT transmission directly causes the behavioural effects of paroxetine discontinuation, the 5-HT_{1A} receptor antagonist WAY-100635 was used to increase 5-HT neuron activity. Theoretically, 5-HT_{1A} receptor antagonists disinhibit 5-HT neuron activity and increase 5-HT transmission. Nonetheless, experimental evidence suggests that WAY-100635 produces few effects on the 5-HT system and is considered a ‘silent’ antagonist, despite its high affinity for the 5-HT_{1A} receptor (Forster et al., 1995; Fletcher et al., 1996). For instance, the perfusion of WAY-100635 onto rat hippocampal slices did not significantly alter neuronal firing rates, although it could block the decrease in neuronal firing caused by 5-HT perfusion (Corradetti et al., 1996; Fletcher et al., 1996). WAY-100635 also had no effect on the

firing of 5-HT neurons in the guinea pig DRN *in vivo* (Mundey et al., 1994), or on extracellular 5-HT in anaesthetised rats (Gartside et al., 1995).

Yet, there is some evidence that acute WAY-100635 administration increased the firing rate of 25% of 5-HT neurons in rat midbrain slices (Fletcher et al., 1996). Moreover, there is consistent evidence from microdialysis studies that 5-HT_{1A} receptor antagonists augment the effects of SSRIs on extracellular 5-HT (Gartside et al., 1995; Invernizzi et al., 1996; Dawson & Nguyen, 2000; Hervás et al., 2000). This is likely because 5-HT_{1A} receptor antagonists block the 5-HT_{1A} autoreceptor-mediated inhibitory effects of SSRI administration, thus enhancing 5-HT transmission.

Importantly, evidence suggests that 5-HT_{1A} receptor antagonists have few effects on rodent behaviour. For example, systemic WAY-100635 administration did not alter the time rats spent in the light zone of the LDB (Fletcher et al., 1996) or EPM performance in mice (Noguerón-Merino et al., 2015). Thus, acute WAY-100635 administration appears to disinhibit 5-HT neurons without affecting anxiety-like behaviour, suggesting it could be used to investigate whether increasing 5-HT neuron activation could precipitate paroxetine discontinuation.

1.4 Hypothesis and aims

This chapter aimed to establish if pharmacologically manipulating the 5-HT system could precipitate the behavioural effects of SSRI discontinuation in mice. It was hypothesised that if a reduction in extracellular 5-HT is the causal trigger, then reducing 5-HT neuronal activation and thus extracellular 5-HT with 8-OH-DPAT would induce anxiety-like behaviour in mice receiving continued paroxetine treatment. On the other

hand, if increased 5-HT neuron activation is ultimately causal, disinhibiting 5-HT neurons with WAY-100635 would increase anxiety-like behaviour.

Firstly, pilot experiments aimed to determine the effects of 8-OH-DPAT and WAY-100635 alone on anxiety-like behaviour on the EPM and on LMA. c-FOS immunohistochemistry was also used to confirm that the 5-HT_{1A} ligands altered 5-HT neuronal activation as hypothesised. *In vivo* microdialysis experiments were also conducted to assess the effects of 8-OH-DPAT and WAY-100635 on hippocampal extracellular 5-HT under the current conditions. Finally, mice receiving continued paroxetine treatment were given an acute injection of 8-OH-DPAT or WAY-100635 before testing on the EPM to determine if these ligands could induce the behavioural effects of paroxetine discontinuation. c-FOS expression was further quantified in the DRN, MRN and VH to determine if the 5-HT_{1A} ligands could also precipitate the neuronal activation patterns seen two days after paroxetine cessation.

2 Experimental Methods

2.1 Animals

C57BL/6J male mice (Charles River, 7 weeks old) were group housed (4-6 per cage) with their littermates in open-top cages or IVCs for at least one week before the start of treatment.

2.2 Drugs and reagents

Paroxetine, 8-OH-DPAT, and WAY-100635 were dissolved in saline and administered *s.c*. Drug doses were chosen based on previous studies (Fletcher et al., 1996; Hajós et al., 2007).

2.3 Experimental design

Three types of experiment were conducted to investigate the ability of 8-OH-DPAT and WAY-100635 to precipitate the behavioural effects of SSRI discontinuation in mice: i) pilot experiments to determine the effects of 8-OH-DPAT and WAY-100635 alone on anxiety-like behaviour, homecage LMA, and c-FOS expression in untreated mice; ii) precipitation experiments to test the ability of 8-OH-DPAT or WAY-100635 to induce the behavioural effects of SSRI discontinuation in mice receiving continued paroxetine treatment; iii) *in vivo* microdialysis experiments to confirm the effects of 8-OH-DPAT and WAY-100635 on hippocampal extracellular 5-HT. Individual experiments are summarised in *Table 6.1*. A summary of the results of each experiment is presented at the end in section 3.8 (*Table 6.10*).

Table 6.1 Experimental design. In order of appearance in Results section. HCA, homecage assessment; IHC, immunohistochemistry. *Ex vivo* analysis in brackets. † Also included in Chapter 4 section 3.2.

Experiment	Group size	Pre-treatment (days 1-13)	Experiment on day 14
0.1 mg/kg 8-OH-DPAT EPM pilot experiment	N=5-8	Untreated	EPM, HCA (IHC)
0.1 mg/kg 8-OH-DPAT precipitation experiment †	N=12	12-13 days once-daily 10 mg/kg paroxetine or saline	EPM (IHC)
8-OH-DPAT microdialysis experiment	N=4-5	Untreated	Microdialysis
Continued paroxetine 8-OH-DPAT microdialysis experiment	N=3-5	13 days once-daily 10 mg/kg paroxetine	Microdialysis
0.5 mg/kg 8-OH-DPAT EPM pilot experiment	N=6-8	Untreated	EPM, HCA (IHC)
WAY-100635 EPM pilot experiment	N=6	Untreated	EPM, HCA (IHC)
WAY-100635 microdialysis experiment	N=5	Untreated	Microdialysis
Continued paroxetine WAY-100635 microdialysis experiment	N=3	13 days once-daily 10 mg/kg paroxetine	Microdialysis
0.5 mg/kg 8-OH-DPAT and WAY-100635 precipitation experiment	N=12	13 days once-daily 10 mg/kg paroxetine or saline	EPM (IHC)

Pilot experiments. Mice were assigned to treatment groups by stratified randomisation. Mice were habituated to a holding room adjacent to the testing room for three days before behavioural testing and for 1 h on the day of testing. On the morning of the experiment, mice were weighed and injected with saline, or the drug being tested: 0.1 mg/kg 8-OH-DPAT, 0.5 mg/kg 8-OH-DPAT or 1.0 mg/kg WAY-100635. Sixty minutes after the injection, mice were tested on the EPM to measure anxiety-like behaviour, or their LMA recorded in a clean homecage, for 5 min. All behavioural testing was conducted by an experimenter blind to treatment groups. Mice were transcardially perfused with PBS and 4% PFA 90 min after the start of behavioural testing and the brains

were processed for c-FOS immunoreactivity in the DRN, MRN and VH (*Fig. 6.1*).

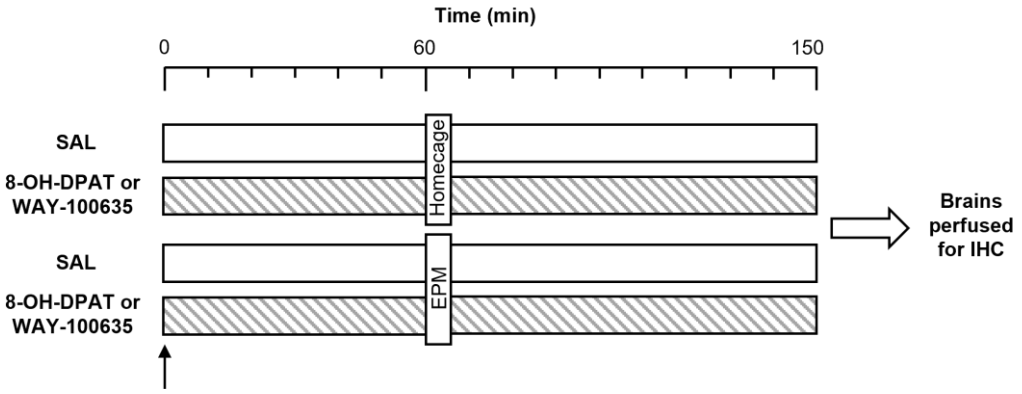


Figure 6.1 Experimental design of pilot experiments. *S.c.* injection (arrow) of either saline, 0.1 mg/kg, or 0.5 mg/kg 8-OH-DPAT, or 1.0 mg/kg WAY-100635. Separate mice for EPM and HCA.

Precipitation experiments. Mice were allocated to treatment groups by stratified randomisation. Mice received 12-13 days of once-daily 10 mg/kg paroxetine treatment or saline. On the morning of the experiment (day 14), mice were injected with saline or the drug being tested. Sixty minutes after the injection, mice were tested on the EPM for 5 min. Mice were perfused 90 min after the start of the EPM and the brains were processed for c-FOS immunohistochemistry.

In the first experiment, there were four groups: i) saline group (SAL-SAL): days 1-13 saline + day 14 saline; ii) continuation group (CON-SAL): days 1-13 paroxetine + day 14 saline; iii) precipitation group (CON-DPAT): days 1-13 paroxetine + day 14 0.1 mg/kg 8-OH-DPAT; iv) discontinuation group (DIS-SAL): days 1-12 paroxetine + day 13 saline + day 14 saline (*Fig. 6.2A*). c-FOS expression from the SAL-SAL, CON-SAL and DIS-SAL groups was also presented in Chapter 4 section 3.2 but is shown here for comparison to the CON-DPAT group (all slices processed at the same time).

In the second experiment, there were also four groups: i) saline group (SAL-SAL): days 1-13 saline + day 14 saline; ii) continuation group (CON-SAL): days 1-13 paroxetine + day 14 saline; iii) 0.5 mg/kg 8-OH-DPAT precipitation group

(CON-DPAT): days 1-13 paroxetine + day 14 0.5 mg/kg 8-OH-DPAT; iv) 1.0 mg/kg WAY-100635 precipitation group (CON-WAY): days 1-13 paroxetine + day 14 1.0 mg/kg WAY-100635 (*Fig. 6.2B*).

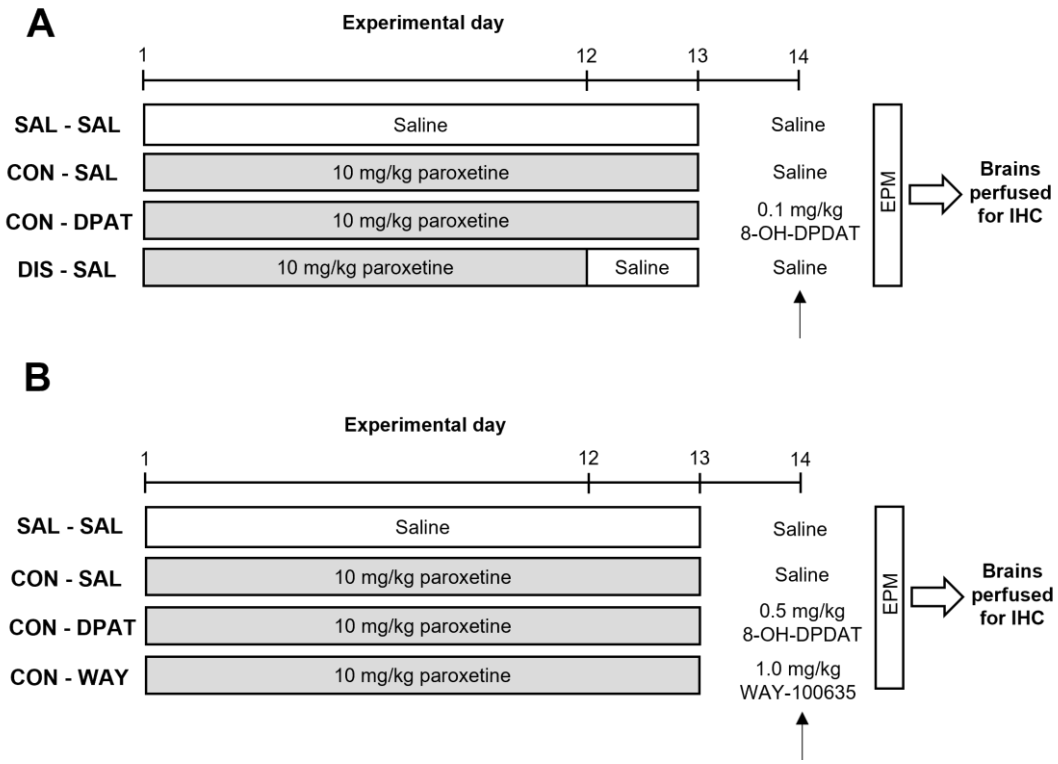


Figure 6.2 Experimental design of precipitation experiments. First precipitation experiment with 0.1 mg/kg 8-OH-DPAT (A) and second with 0.5 mg/kg 8-OH-DPAT and WAY-100635. *S.c.* injection (arrow) 60 min before testing on the EPM.

In vivo microdialysis experiments. Untreated mice were used to investigate the effects of 8-OH-DPAT and WAY-100635 on hippocampal extracellular 5-HT levels. Subsequent experiments tested the effects of these drugs on mice pre-treated with 13 days of once-daily paroxetine injections. Mice were anaesthetised and a microdialysis probe implanted in the VH [AP: -3.0 mm, ML: ±2.9 mm, DV: -4.0 mm] using stereotaxic surgery. Probes were continuously perfused at 2.0 µl/min with ACSF. The ACSF used for untreated mice included 1 µM citalopram to increase baseline extracellular 5-HT to make changes following 8-OH-DPAT and WAY-100635 easier to detect (Sharp &

Hjorth, 1990). Microdialysate samples were collected every 20 min into Eppendorf tubes containing 10 µl 0.09 M PCA and analysed with HPLC-ED (as in Chapter 2 section 8).

After a 60 min baseline period, mice received an injection of saline or the drug being tested and the microdialysate was sampled for a further 120 min (*Fig. 6.3, top*). In the WAY-100635 experiment, untreated mice received an injection of WAY-100635 then 60 min later 0.5 mg/kg 8-OH-DPAT. Samples were taken for a further 120 min (*Fig. 6.3, bottom*). At the end of the experiments, brains were perfused to identify the probe tracts (as in Chapter 2 section 8.3).

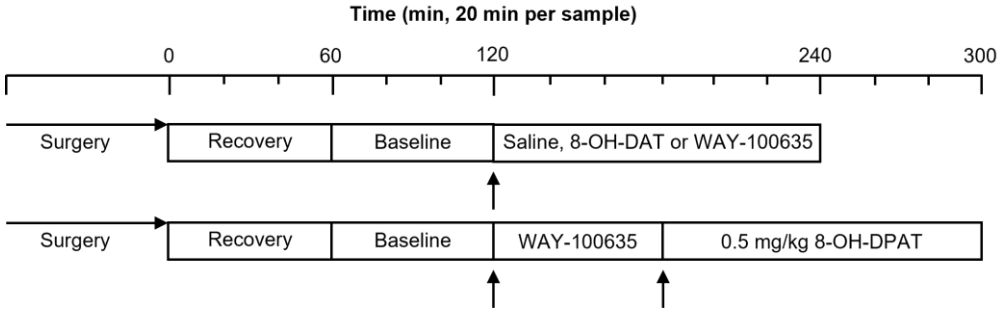


Figure 6.3 Experimental design of *in vivo* microdialysis experiments. *S.c.* (arrow) of saline, 0.1 mg/kg, or 0.5 mg/kg 8-OH-DPAT, or WAY-100635. Top, 8-OH-DPAT microdialysis experiments and continued paroxetine WAY-100635 microdialysis experiment; bottom, WAY-100635 microdialysis experiment.

2.4 Immunohistochemistry and c-FOS quantification

c-FOS expression was analysed as in Chapter 2 section 6. Coronal brain slices containing the DRN, MRN and VH were immunohistochemically stained for c-FOS and NeuN. DRN and MRN slices were also stained for TPH2. Images were taken using epifluorescence microscopy (20x magnification for the DRN and MRN, two images at 10x magnification for the VH) and c-FOS immunoreactivity was quantified using Image J. In the dorsal DRN, and the MRN, the number of c-FOS+ neurons, c-FOS+ TPH2+ neurons, c-FOS+ TPH2− neurons, TPH2+ neurons, and the total number of NeuN+ neurons were counted. In the DG of the VH, the total number of c-FOS+ neurons

were counted. Images were analysed from three slices per region per mouse, then data were averaged per mouse. Slice staining, imaging, and counting were conducted by an experimenter blind to treatment group. c-FOS immunoreactivity in the DH, dl-PAG and PLC was also quantified but there were no effects of treatment in any experiment, so the data are not shown.

2.5 Statistical analysis

Pilot experiments. For the behavioural experiments, if data were determined to be normally distributed with D'Agostino-Pearson's test for normality, then Student's t-tests were used. If the data were non-parametric, a Mann-Whitney U test was used. Two-way ANOVA with post-hoc Tukey's test was used to assess the effects of test, treatment, and test*treatment interactions on c-FOS expression.

Precipitation experiments. If data were normally distributed, then one-way ANOVA with post-hoc Fisher's LSD was used. If data were non-parametric, then Kruskal Wallis with post-hoc Uncorrected Dunn's test was used.

In vivo microdialysis experiments. Data were expressed as a change in 5-HT compared to the mean of the three baseline samples for each animal (Δ5-HT). As the sample sizes were small (n=3-5), Kruskal Wallis with Uncorrected Dunn's test and Mann Whitney U tests were used to assess the effects of 8-OH-DPAT and WAY-100635 60 min after the injection. Two-way ANOVA with post-hoc Tukey's test was also used to compare the response to 8-OH-DPAT between untreated and paroxetine pre-treated mice.

All analyses were conducted using GraphPad Prism (v8). Data are presented as mean ± SEM or median ± IQR values. P values of $p<0.05$ were considered statistically significant. All analyses were performed by an experimenter blind to treatment groups.

3 Results

3.1 Effect of 0.1 mg/kg 8-OH-DPAT on anxiety-like behaviour and c-FOS expression

To investigate the effect of 0.1 mg/kg 8-OH-DPAT alone on anxiety-like behaviour, mice were tested on the EPM 60 min after a *s.c.* injection of 8-OH-DPAT. There were no significant effects of 8-OH-DPAT on EPM performance compared to saline controls (*Table 6.2*). There was also no effect on 8-OH-DPAT on the distance travelled in a clean homecage (*Table 6.2*). Thus, 0.1 mg/kg 8-OH-DPAT did not alter anxiety-like behaviour or LMA.

Table 6.2 Effect of 0.1 mg/kg 8-OH-DPAT on the EPM. 5 min testing on the EPM or in a homecage. Each value is a mean ± SEM, analysed using Student's t-test. N=8/group EPM, n=5/group HCA.

	Group (mean ± SEM)		
Parameter	**SAL**	**8-OH-DPAT**	**Student's t-test**
EPM			
Time in open arms (s)	38.1 ± 6.8	42.7 ± 10.9	t(14)=0.3615, p=0.7231
Entries to open arms	9.1 ± 1.3	8.1 ± 1.4	t(14)=0.5143, p=0.6151
Latency to enter open arms (s)	20.6 ± 6.4	35.9 ± 7.3	t(14)=1.578, p=0.1370
Head dips	18.6 ± 4.1	23.5 ± 3.8	t(14)=0.8711, p=0.3984
Distance travelled (m)	9.7 ± 0.6	9.3 ± 0.7	t(14)=0.4210, p=0.6801
HCA			
Distance travelled (m)	6.7 ± 0.3	6.7 ± 0.4	t(8)=0.0017, p=0.9987

c-FOS immunoreactivity was then quantified to investigate the effect of 0.1 mg/kg 8-OH-DPAT on neuronal activation. In the DRN, 8-OH-DPAT reduced the percentage of c-FOS+ TPH2+ neurons compared to saline controls following EPM, but not homecage, exposure (effect of test: F(1,22)=0.4122, p=0.5275; effect of treatment: F(1,22)=2.765, p=0.1105; test*treatment interaction: F(1,22)=10.76, p=0.0034; post-hoc EPM SAL vs 8-OH-DPAT p=0.0006; *Fig. 6.4A*). There was a trend towards an effect of treatment on the percentage of c-FOS+ TPH2+ neurons in the MRN, but no significant

effect of test or a test*treatment interaction (test: F(1,22)=1.621, p=0.2175; treatment: F(1,22)=3.727, p=0.0665; interaction: F(1,22)=2.353, p=0.1393; *Fig. 6.4B*). There were no effects of EPM exposure, 8-OH-DPAT, or test*treatment interactions on the total percentage of c-FOS+ neurons, the percentage of c-FOS+ TPH2− neurons, or the percentage of neurons that were TPH2+ in either the DRN or MRN (*Table 6.3*).

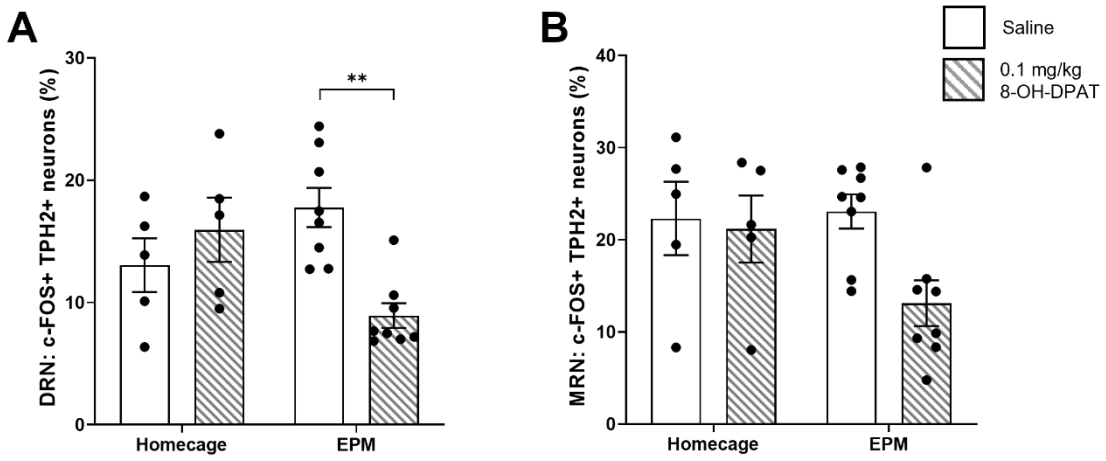


Figure 6.4 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS expression in 5-HT neurons. Bars represent the mean ± SEM values for the percentage of c-FOS+ TPH2+ neurons in the DRN (A) and MRN (B) following homecage and EPM exposure. Homecage (n=5/group), EPM (n=8/group). Individual values are indicated by dots. Two-way ANOVA with post-hoc Tukey's test, within-test significant differences ** p<0.01.

Table 6.3 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS immunoreactivity in the DRN and MRN. DPAT, 8-OH-DPAT. Effects of treatment, test, and test*treatment interactions. Each value is a mean ± SEM, analysed using two-way ANOVA with Tukey's test. N=5/group HCA, n=8/group EPM.

	Group (mean ± SEM)						
	HCA		EPM		Two-way ANOVA		
Parameter	**SAL**	**DPAT**	**SAL**	**DPAT**	**Treatment**	**Test**	**Interaction**
DRN							
c-FOS+ (%)	16.3 ± 1.5	17.8 ± 1.5	15.9 ± 1.2	12.1 ± 1.9	F(1,22)=3.285 p=0.0836	F(1,22)=0.5144 p=0.4808	F(1,22)=2.432 p=0.1332
c-FOS+ TPH2− (%)	21.7 ± 4.2	19.5 ± 1.8	23.8 ± 2.6	18.1 ± 2.9	F(1,22)=0.0061 p=0.9383	F(1,22)=0.8853 p=0.3570	F(1,22)=1.542 p=0.2274
TPH2+ (%)	47.2 ± 2.4	45.3 ± 1.3	38.7 ± 4.3	42.6 ± 3.1	F(1,22)=0.7233 p=0.4042	F(1,22)=2.449 p=0.1319	F(1,22)=0.6446 p=0.4307
MRN							
c-FOS+ (%)	18.6 ± 0.6	17.7 ± 1.3	16.2 ± 2.7	13.7 ± 1.9	F(1,22)=0.5691 p=0.4586	F(1,22)=2.128 p=0.1587	F(1,22)=0.1485 p=0.7037
c-FOS+ TPH2− (%)	17.9 ± 1.5	16.7 ± 2.3	15.4 ± 3.2	14.0 ± 2.5	F(1,22)=0.1145 p=0.7382	F(1,22)=2.389 p=0.1365	F(1,22)<0.0001 p=0.9979
TPH2+ (%)	20.8 ± 1.7	20.3 ± 1.6	16.7 ± 3.4	23.0 ± 2.9	F(1,22)=0.7233 p=0.4042	F(1,22)=0.0411 p=0.8412	F(1,22)=0.9629 p=0.3371

In the VH, EPM exposure increased the number of c-FOS+ neurons compared to homecage exposure, but there was no effect of 8-OH-DPAT administration compared to saline controls (test: F(1,22)=4.914, p=0.0373; treatment: F(1,22)=0.3233, p=0.5754; interaction: F(1,22)=0.2395, p=0.6294; *Fig. 6.5*).

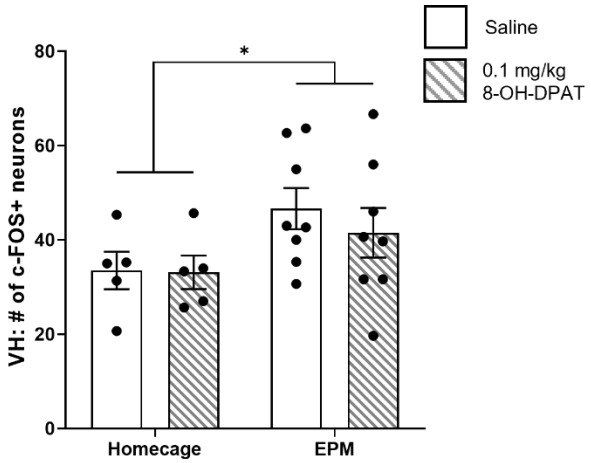


Figure 6.5 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS expression in VH. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the VH. Homecage (n=5/group), EPM (n=8/group). Individual values are indicated by dots. Two-way ANOVA with Tukey's test, between test significant difference, * $p<0.05$.

In summary, 0.1 mg/kg 8-OH-DPAT did not alter anxiety-like behaviour on the EPM but reduced the percentage of c-FOS+ TPH2+ neurons in the DRN (and tended to in the MRN) after EPM exposure. Thus, this dose of 8-OH-DPAT was sufficiently low that it did not produce behavioural effects, but it could reduce 5-HT neuron activation.

3.2 Effect of 0.1 mg/kg 8-OH-DPAT on anxiety-like behaviour and c-FOS expression in mice treated with paroxetine

To assess whether 0.1 mg/kg 8-OH-DPAT could precipitate the behavioural effects of paroxetine discontinuation, mice receiving continued paroxetine treatment were administered 0.1 mg/kg 8-OH-DPAT before testing on the EPM (*Fig. 6.6*).

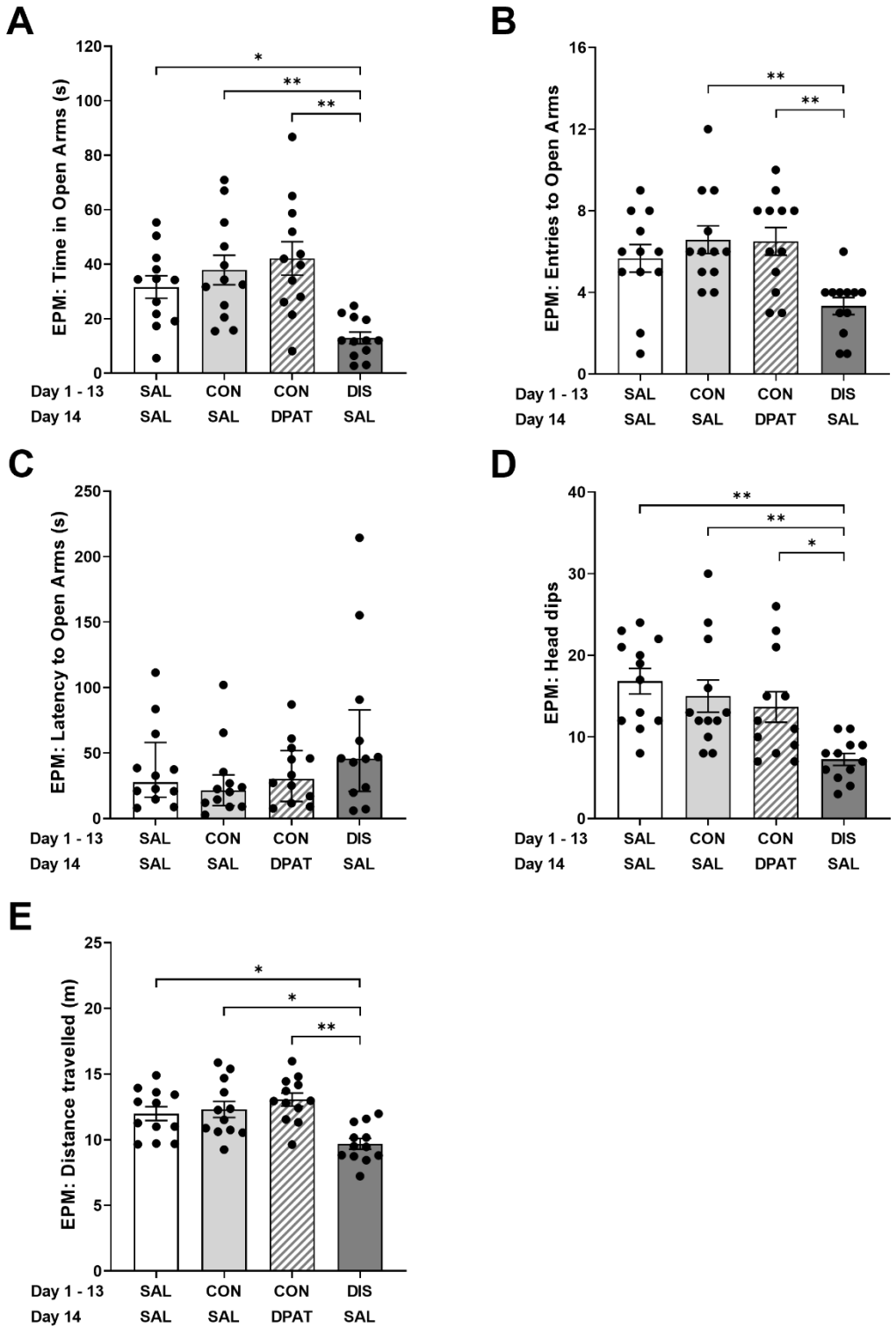


Figure 6.6 Effect of 0.1 mg/kg 8-OH-DPAT on the EPM in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM (A-B, D-E) or median ± IQR (C) values for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL-SAL (n=12), CON-SAL

(n=12), CON-DPAT (n=12), DIS-SAL (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

There were no effects of 0.1 mg/kg 8-OH-DPAT administered to mice receiving continued paroxetine treatment on anxiety-like behaviour on the EPM compared to paroxetine-treated mice that received a saline injection before the EPM or saline controls. Unlike previous experiments, continued paroxetine also did not increase anxiety-like compared to saline controls. On the other hand, paroxetine discontinuation increased anxiety-like behaviour on the EPM compared to continued paroxetine, saline controls, and mice that received 8-OH-DPAT (time: $F(3,44)=7.466$, $p=0.0004$, post-hoc SAL-SAL vs DIS-SAL $p=0.0365$, CON-SAL vs DIS-SAL $p=0.0029$, CON-DPAT vs DIS-SAL $p=0.0004$; *Fig. 6.6A*; entries: $F(3,44)=5.912$, $p=0.0018$, post-hoc CON-SAL vs DIS-SAL $p=0.0034$, CON-DPAT vs DIS-SAL $p=0.0044$; *Fig. 6.6B*; latency: $H(3)=2.990$, $p=0.3932$; *Fig. 6.6C*; head dips: $F(3,44)=6.685$, $p=0.0008$, post-hoc SAL-SAL vs DIS-SAL $p=0.0007$, CON-SAL vs DIS-SAL $p=0.0076$, CON-DPAT vs DIS-SAL $p=0.0352$; *Fig. 6.6D;* distance: $F(3,44)=7.59$, $p=0.0003$, post-hoc SAL-SAL vs DIS-SAL $p=0.0120$, CON-SAL vs DIS-SAL $p=0.0043$, CON-DPAT vs DIS-SAL $p=0.0002$; *Fig. 6.6E*). Thus, 0.1 mg/kg 8-OH-DPAT did not precipitate the behavioural effects of paroxetine discontinuation.

c-FOS immunoreactivity was then quantified to assess if 8-OH-DPAT had precipitated the neuronal activation patterns characteristic of paroxetine discontinuation. In the DRN, neither continued paroxetine treatment nor 8-OH-DPAT administered to mice receiving continued paroxetine treatment altered the percentage of c-FOS+ TPH2+ neurons compared to continued paroxetine or saline controls. On the other hand, paroxetine discontinuation increased the percentage of c-FOS+ TPH2+ neurons

compared to the other groups ($F(3,21)=11.74$, $p<0.0001$, post-hoc SAL-SAL vs DIS-SAL $p=0.0036$, CON-SAL vs DIS-SAL $p<0.0001$, CON-DPAT vs DIS-SAL $p<0.0001$; *Fig. 6.7A*). There was no effect of treatment on the percentage of c-FOS+ TPH2+ neurons in the MRN ($F(3,20)=1.925$, $p=0.1589$; *Fig. 6.7B*).

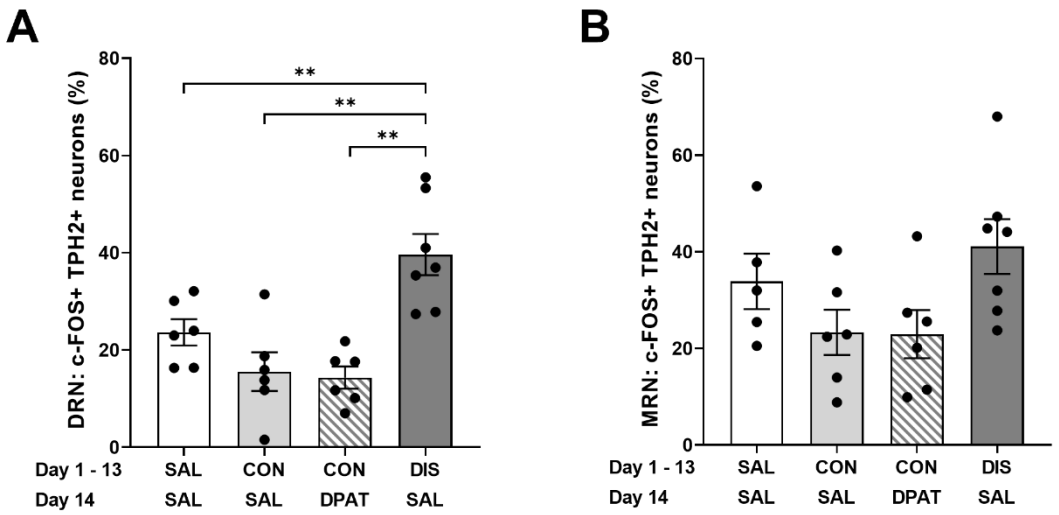


Figure 6.7 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS expression in 5-HT neurons in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM values for the percentage of c-FOS+ TPH2+ neurons in the DRN (A) and MRN (B). SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), DIS-SAL (n=7). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, ** $p<0.01$.

As previously reported, paroxetine discontinuation increased the total percentage of c-FOS+ neurons in the DRN compared to controls (*Table 6.4*; post-hoc CON-SAL vs DIS-SAL $p=0.0003$; CON-DPAT vs DIS-SAL $p=0.0012$). In the MRN, continued paroxetine treatment reduced the total percentage of c-FOS+ neurons compared to saline controls, and 8-OH-DPAT did not have any further effects on c-FOS immunoreactivity; thus, the percentage of c-FOS+ neurons was lower than in saline controls. On the other hand, paroxetine discontinuation increased the percentage of c-FOS+ neurons back to saline control levels (*Table 6.4*; post-hoc SAL-SAL vs CON-SAL $p=0.0153$, SAL-SAL vs CON-DPAT $p=0.0239$, CON-SAL vs DIS-SAL $p=0.0042$, CON-DPAT vs DIS-SAL

p=0.0070). There were no effects on the percentage of c-FOS+ TPH2− neurons or the percentage of neurons that were TPH2+ in the DRN or MRN (*Table 6.4*).

Table 6.4 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS immunoreactivity in the DRN and MRN. Each value is a mean ± SEM, analysed using one-way ANOVA with Fisher's LSD. Bold values indicate significant values. * p<0.05 SAL-SAL vs CON-SAL, # p<0.05 SAL-SAL vs CON-DPAT, † p<0.05, †† p<0.01, CON-SAL vs DIS-SAL, ‡ p<0.05 CON-DPAT vs DIS-SAL. SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), DIS-SAL (n=7).

	Group (mean ± SEM)				
Parameter	**SAL-SAL**	**CON-SAL**	**CON-DPAT**	**DIS-SAL**	**ANOVA**
DRN					
c-FOS+ (%)	27.1 ± 1.6	17.3 ± 3.5	19.9 ± 2.7	35.6 ± 3.5 † ‡	**F(3,21)=7.847 p=0.0011**
c-FOS+ TPH2− (%)	33.4 ± 4.2	19.1 ± 2.9	24.3 ± 4.4	37.4 ± 8.3	F(3,21)=2.196 p=0.1186
TPH2+ (%)	46.7 ± 5.4	47.8 ± 4.8	39.9 ± 2.5	51.5 ± 4.3	F(3,21)=1.120 p=0.3306
MRN					
c-FOS+ (%)	22.0 ± 1.6	14.4 ± 1.5 *	15.0 ± 2.0 #	22.9 ± 2.2 †† ‡	**F(3,20)=5.535 p=0.0062**
c-FOS+ TPH2− (%)	18.3 ± 2.9	11.6 ± 1.9	13.2 ± 2.1	18.6 ± 2.1	F(3,20)=2.612 p=0.0796
TPH2+ (%)	23.2 ± 1.1	22.3 ± 3.3	19.6 ± 2.4	22.3 ± 2.3	F(3,20)=0.3783 p=0.7697

In the VH, neither continued paroxetine treatment nor 8-OH-DPAT administered to mice receiving continued paroxetine treatment altered the number of c-FOS+ neurons. On the other hand, paroxetine discontinuation increased the number of c-FOS+ TPH2+ neurons compared to paroxetine-treated mice that received 8-OH-DPAT before the EPM and saline controls (F(3,21)=3.987, p=0.0215, post-hoc SAL-SAL vs DIS-SAL p=0.0030, CON-DPAT vs DIS-SAL p=0.0314; *Fig. 6.8*).

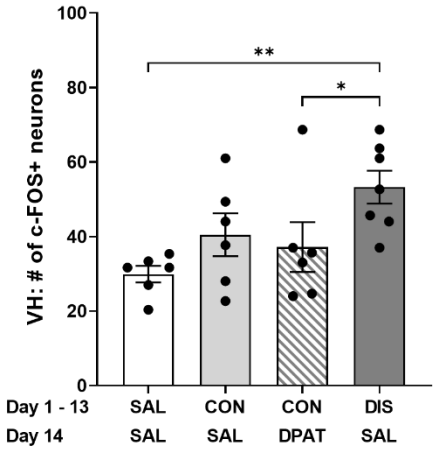


Figure 6.8 Effect of 0.1 mg/kg 8-OH-DPAT on c-FOS expression in the VH in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in VH. SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), DIS-SAL (n=7). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

In summary, 0.1 mg/kg 8-OH-DPAT administration to mice receiving continued paroxetine treatment did not precipitate the behavioural effects or neuronal activation patterns typical of two days of paroxetine discontinuation. In contrast, these experiments replicated the anxiogenic effects of paroxetine discontinuation detected in Chapter 3. Moreover, 8-OH-DPAT could no longer reduce the percentage of c-FOS+ TPH2+ neurons in the DRN compared to saline controls in mice repeatedly treated with paroxetine (as it did in the pilot experiment), suggesting that the 5-HT_{1A} autoreceptors were desensitised by repeated paroxetine treatment.

3.3 Effect of 8-OH-DPAT on hippocampal extracellular 5-HT

To investigate whether 8-OH-DPAT could reduce extracellular 5-HT as well as somatic activation, *in vivo* microdialysis was performed in the VH of anesthetised mice. Preliminary data showed that an acute *s.c.* injection of 0.1 or 0.5 mg/kg 8-OH-DPAT reduced the concentration of 5-HT in the microdialysate after 60 min compared a saline

injection in untreated mice (effect of injection: H(2)=6.012, p=0.0398, post-hoc SAL vs 0.1 DPAT p=0.0364, SAL vs 0.5 DPAT p=0.0258; *Fig. 6.9A-B*).

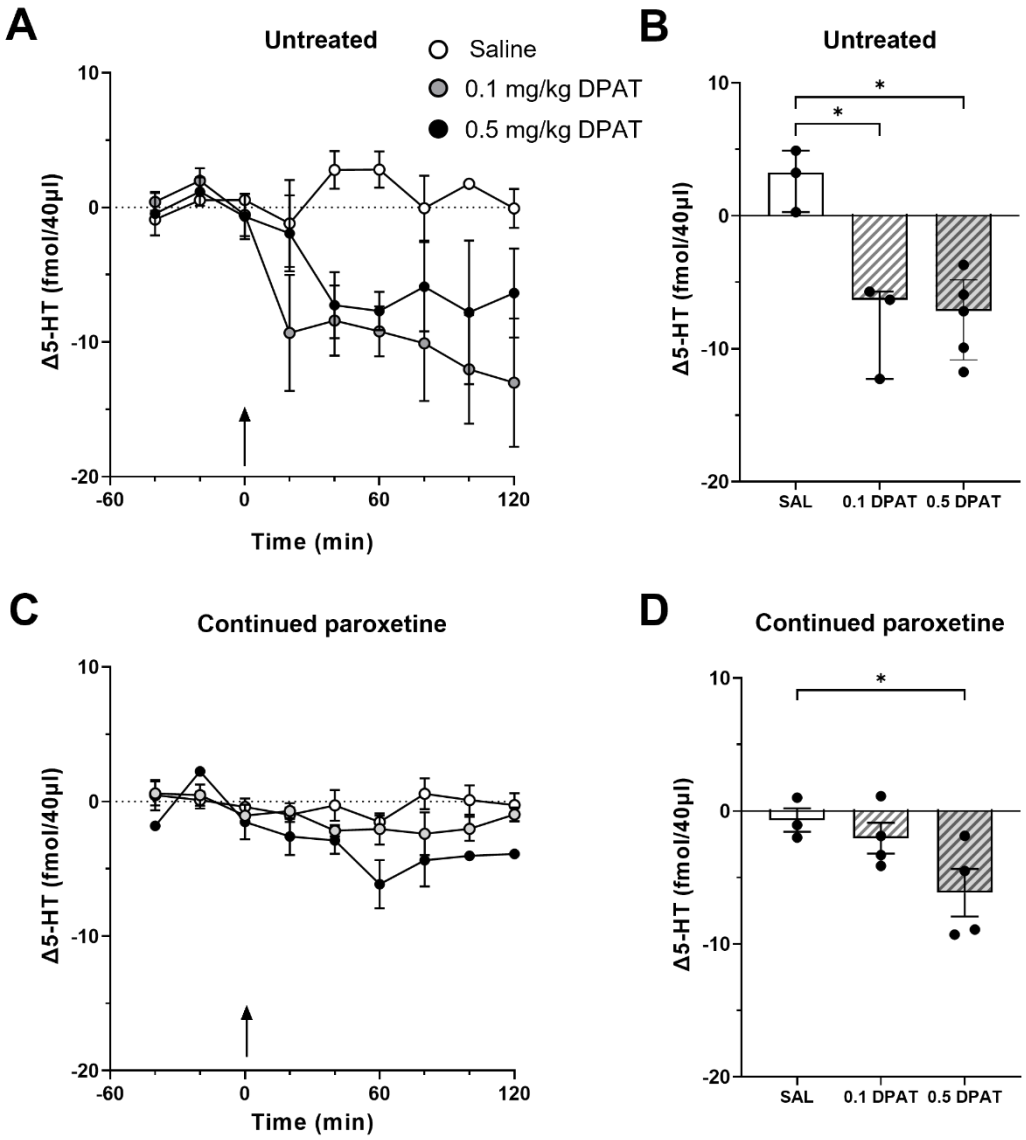


Figure 6.9 Effect of 8-OH-DPAT on hippocampal extracellular 5-HT. Points represent mean ± SEM of change in extracellular 5-HT (Δ5-HT, fmol/40µl) following a *s.c.* injection (arrow) of saline or 8-OH-DPAT (A, C). Bars represent median ± IQR of t=60 values, individual values are indicated by dots (B, D). Untreated: saline (n=3), 0.1 mg/kg 8-OH-DPAT (0.1 DPAT, n=4 one outlier excluded from t=60), 0.5 mg/kg 8-OH-DPAT (0.5 DPAT, n=5) (A-B). Continued paroxetine treatment: saline (n=3); 0.1 mg/kg 8-OH-DPAT (n=4); 0.5 mg/kg 8-OH-DPAT (n=4) (C-D). Difference in extracellular 5-HT at t=60 analysed using Kruskal Wallis with Uncorrected Dunn's test, * p<0.05, ** p<0.01.

In mice continuously treated with paroxetine, however, there was a trend towards an overall effect of the treatment, and post-hoc tests showed that 0.5 but not 0.1 mg/kg 8-OH-DPAT reduced the concentration of 5-HT in the microdialysate 60 min after the injection ($H(2)=3.982$, $p=0.0631$, post-hoc SAL vs 0.5 DPAT $p=0.0303$; *Fig. 6.9C-D*). Adding pre-treatment with paroxetine as a factor in a two-way ANOVA found a significant interaction with the dose of 8-OH-DPAT and paroxetine treatment. Post-hoc tests showed that there were no differences in the response to a saline injection or 0.5 mg/kg 8-OH-DPAT between the untreated and paroxetine-treated mice, but there was a significantly smaller reduction in extracellular 5-HT following 0.1 mg/kg 8-OH-DPAT in the paroxetine-treated mice compared to the untreated controls (effect of treatment: $F(2,16)=13.81$, $p=0.0003$; effect of pre-treatment: $F(1,16)=1.154$, $p=0.2987$; interaction: $F(2,16)=4.235$, $p=0.0334$, post-hoc 0.1 DPAT untreated vs continued paroxetine $p=0.0465$).

Thus, these preliminary data suggest that both doses of 8-OH-DPAT could reduce hippocampal extracellular 5-HT in untreated mice. In mice treated with paroxetine, however, 0.5 but not 0.1 mg/kg 8-OH-DPAT reduced extracellular 5-HT. This suggests that the 5-HT_{1A} autoreceptors had lost sensitivity with repeated paroxetine treatment and that 0.1 mg/kg 8-OH-DPAT was too low a dose to overcome this desensitisation. Subsequent experiments therefore tested if 0.5 mg/kg 8-OH-DPAT could precipitate the behavioural effects of paroxetine discontinuation (section 3.7). WAY-100635 was also tested to determine if an increase in 5-HT neuronal activation would instead precipitate discontinuation (section 3.7).

3.4 Effect of 0.5 mg/kg 8-OH-DPAT on anxiety-like behaviour and c-FOS expression

To investigate the behavioural effects of 0.5 mg/kg 8-OH-DPAT alone, mice were tracked on the EPM or in a homecage 60 min after 8-OH-DPAT administration. 8-OH-DPAT reduced the number of head dips, produced a trend towards a reduction in the number of entries to the open arms and produced a numerical decrease in the time spent in the open arms compared to saline controls, suggesting it had a slight anxiogenic effect (*Table 6.5*). There were no effects of treatment the latency to enter the open arms, or the distance travelled on the EPM (*Table 6.5*). There was no effect of 8-OH-DPAT on the distance travelled in a clean homecage, meaning it did not alter LMA (*Table 6.5*).

Table 6.5 Effect of 0.5 mg/kg 8-OH-DPAT on the EPM. 5 min testing on the EPM or in a homecage. Each value is a mean ± SEM or median and IQR [25th percentile, 75th percentile], analysed using Student's t-test or Mann Whitney U. Bold values indicate significant effects of treatment, N=8/group EPM, n=6/group HCA.

	Group (mean ± SEM, median [25th, 75th])		
Parameter	**SAL**	**8-OH-DPAT**	**Student's t-test**
EPM			
Time in open arms (s)	54.0 ± 7.4	36.4 ± 9.3	t(14)=1.481, p=0.1609
Entries to open arms	9.5 ± 1.0	6.6 ± 1.1	t(14)=1.918, p=0.0758
Latency to enter open arms (s)	10.5 [8.3,15.9]	17.8 [8.7,62.7]	U=22, p=0.3283
Head dips	30.0 ± 3.0	21.3 ± 2.1	**t(14)=2.379, p=0.0321**
Distance travelled (m)	13.4 ± 0.6	12.1 ± 0.7	t(14)=1.456, p=0.1674
HCA			
Distance travelled (m)	7.6 ± 0.2	7.0 ± 0.6	t(10)=0.8883, p=0.3953

c-FOS immunoreactivity was then quantified to determine the effect of 0.5 mg/kg 8-OH-DPAT on neuronal activation. In the DRN, 8-OH-DPAT reduced the percentage of c-FOS+ TPH2+ neurons compared to saline controls after EPM, but not homecage, exposure (test: F(1,24)=1.394, p=0.2492; treatment: F(1,24)=1.213, p=0.2817; interaction: F(1,24)=6.400, p=0.0184; post-hoc EPM SAL vs 8-OH-DPAT p=0.0485;

Fig. 6.10A). There was also a trend towards a reduction in the percentage of c-FOS+ TPH2+ neurons in the MRN following 8-OH-DPAT administration compared to saline controls (test: F(1,24)=0.1632, p=0.6898; treatment: F(1,24)=4.242, p=0.0505; interaction: F(1,24)=0.6196, p=0.4389; *Fig. 6.10B*). There were no effects of EPM exposure, 8-OH-DPAT, or test*treatment interactions on the total percentage of c-FOS+ neurons, the percentage of c-FOS+ TPH2− neurons, or the percentage of neurons that were TPH2+ in either the DRN or MRN (*Table 6.6*).

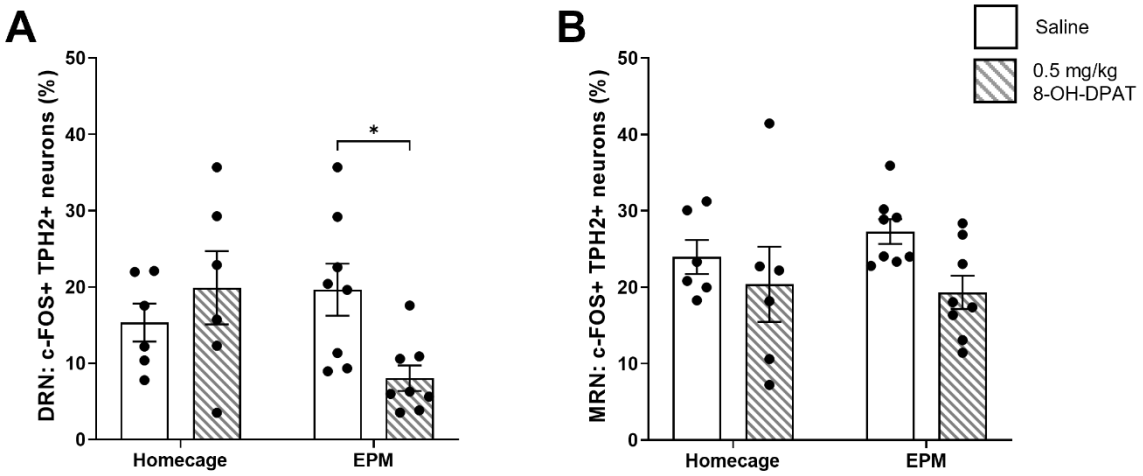


Figure 6.10 Effect of 0.5 mg/kg 8-OH-DPAT on c-FOS expression in 5-HT neurons. Bars represent the mean ± SEM values for the percentage of c-FOS+ TPH2+ neurons in the DRN (A) and MRN (B) following homecage and EPM exposure. Homecage (n=6/group), EPM (n=8/group). Individual values are indicated by dots. Two-way ANOVA with post-hoc Tukey's test, within-test significant differences shown, * p<0.05.

Table 6.6 Effect of 0.5 mg/kg 8-OH-DPAT on c-FOS immunoreactivity in the DRN and MRN. Effects of treatment, test, and test*treatment interactions. Each value is a mean ± SEM, analysed using two-way ANOVA with Tukey's test. N=6/group HCA, n=8/group EPM.

	Group (mean ± SEM)						
	HCA		EPM		Two-way ANOVA		
Parameter	**SAL**	**DPAT**	**SAL**	**DPAT**	**Treatment**	**Test**	**Interaction**
DRN							
c-FOS+ (%)	29.5 ± 3.1	30.8 ± 2.8	30.0 ± 4.6	34.8 ± 8.2	F(1,24)=0.292 p=0.5938	F(1,24)=0.152 p=0.7000	F(1,24)=0.090 p=0.7665
c-FOS+ TPH2− (%)	22.1 ± 2.9	25.4 ± 2.7	24.8 ± 3.9	19.8 ± 2.6	F(1,24)=0.071 p=0.7911	F(1,24)=0.1927 p=0.6646	F(1,24)=1.661 p=0.2098
TPH2+ (%)	52.2 ± 2.9	46.4 ± 4.2	45.8 ± 3.4	54.9 ± 4.3	F(1,24)=0.178 p=0.6761	F(1,24)=0.076 p=0.7849	F(1,24)=3.664 p=0.0676
MRN							
c-FOS+ (%)	16.2 ± 2.0	14.1 ± 1.2	14.9 ± 2.7	16.4 ± 1.9	F(1,24)=0.019 p=0.8916	F(1,24)=0.0560 p=0.8087	F(1,24)=0.706 p=0.4089
c-FOS+ TPH2− (%)	17.7 ± 1.4	15.3 ± 1.5	17.9 ± 2.0	16.6 ± 1.6	F(1,24)=1.061 p=0.3132	F(1,24)=0.176 p=0.6783	F(1,24)=0.110 p=0.7428
TPH2+ (%)	26.8 ± 1.5	19.7 ± 3.0	26.9 ± 3.5	26.7 ± 2.3	F(1,24)=1.695 p=0.2053	F(1,24)=1.590 p=0.2194	F(1,24)=1.483 p=0.2351

In the VH, there were also no effects of EPM exposure or 8-OH-DPAT administration on c-FOS immunoreactivity (test: F(1,24)=3.186, p=0.0878; treatment: F(1,24)=0.1.413, p=0.2462; interaction: F(1,24)=1.462, p=0.2385; *Fig. 6.11*).

In summary, 0.5 mg/kg 8-OH-DPAT had a small anxiogenic effect on the EPM, but it also reduced the percentage of c-FOS+ TPH2+ neurons in the DRN following EPM exposure, replicating the effect of 0.1 mg/kg 8-OH-DPAT.

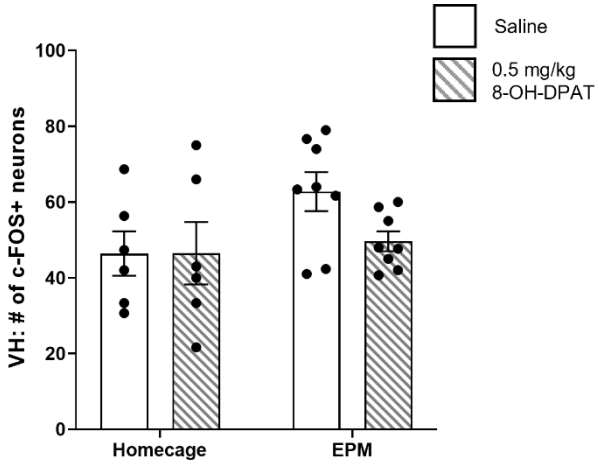


Figure 6.11 Effect of 0.5 mg/kg 8-OH-DPAT on c-FOS expression VH. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the VH. Homecage (n=6/group), EPM (n=8/group). Individual values are indicated by dots. Two-way ANOVA with post-hoc Tukey's test.

3.5 Effect of WAY-100635 on anxiety-like behaviour and c-FOS expression

In a pilot experiment, there were no effects of WAY-100635 on EPM performance or distance travelled in a homecage compared to saline controls (*Table 6.7*).

Table 6.7 Effect of WAY-100635 on the EPM. 5 min testing on the EPM or in a homecage. Each value is a mean ± SEM or median and IQR [25th percentile, 75th percentile], analysed using Student's t-test or Mann Whitney U. N=6/group EPM, n=6/group HCA.

	Group (mean ± SEM, median [25th, 75th])		Student's t-test/ Mann Whitney
Parameter	**SAL**	**WAY-100635**	
EPM			
Time in open arms (s)	44.7 ± 8.5	43.4 ± 8.7	t(10)=0.1058, p=0.9179
Entries to open arms	7.6 ± 1.1	6.5 ± 1.3	t(10)=0.3885, p=0.7058
Latency to enter open arms (s)	22.4 [7.5,46.1]	31.6 [3.9,54.5]	U=18, p>0.9999
Head dips	13.5 ± 1.2	12.2 ± 2.1	t(10)=0.5438, p=0.5985
Distance travelled (m)	11.4 ± 0.9	10.1 ± 0.3	t(10)=1.387, p=0.1956
HCA			
Distance travelled (m)	10.5 ± 0.6	10.6 ± 0.6	t(10)=0.0774, p=0.9398

WAY-100635 increased the percentage of c-FOS+ TPH2+ neurons compared to saline controls in both the DRN and MRN, irrespective of test exposure (DRN, test: $F(1,20)=0.0974$, $p=0.7582$; treatment: $F(1,20)=27.12$, $p<0.0001$; interaction: $F(1,20)=0.8051$, $p=0.3802$; *Fig. 6.12A*; MRN, test: $F(1,20)=0.2880$, $p=0.5974$; treatment: $F(1,20)=17.86$, $p=0.0004$; interaction: $F(1,20)=0.9340$, $p=0.3454$; *Fig. 6.12B*). WAY-100635 also increased the total percentage of c-FOS+ neurons compared to saline controls in the DRN, and there was a trend towards such an effect in the MRN (*Table 6.8*). There were no effects of EPM exposure, WAY-100635, or test*treatment interactions on the percentage of c-FOS+ TPH2− neurons, or the percentage of neurons that were TPH2+, in either the DRN or MRN (*Table 6.8*).

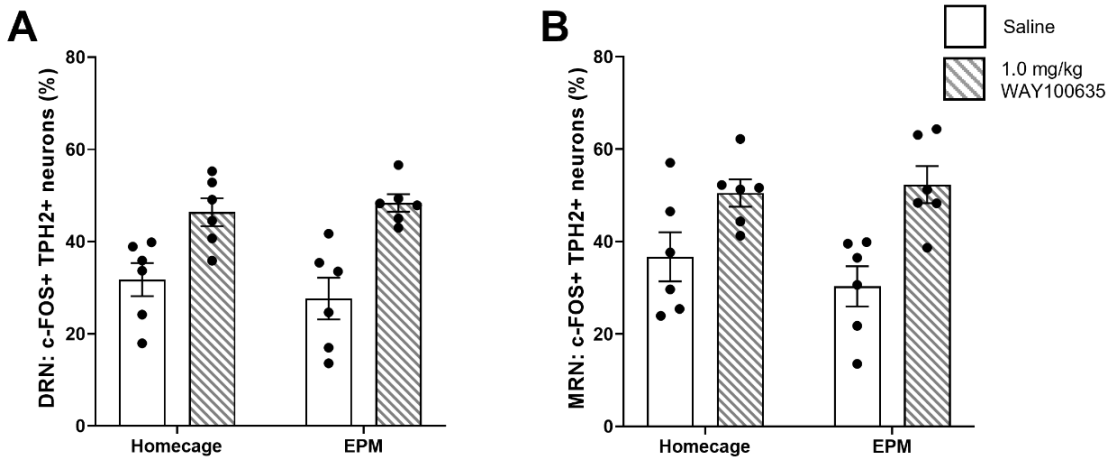


Figure 6.12 Effect of WAY-100635 on c-FOS expression in 5-HT neurons. Bars represent the mean ± SEM values for the percentage of c-FOS+ TPH2+ neurons in the DRN (A) and MRN (B) following homecage and EPM exposure. Homecage (n=6/group), EPM (n=6/group). Individual values are indicated by dots. Two-way ANOVA with post-hoc Tukey's test.

Table 6.8 Effect of WAY-100635 on c-FOS immunoreactivity in the DRN and MRN. WAY, WAY-100635. Effects of treatment, test, and test*treatment interactions. Each value is a mean ± SEM, analysed using two-way ANOVA with Tukey's test. Bold values indicate significant effects, N=6/group HCA, n=6/group EPM.

	Group (mean ± SEM)						
	HCA		EPM		Two-way ANOVA		
Parameter	**SAL**	**WAY**	**SAL**	**WAY**	**Treatment**	**Test**	**Interaction**
DRN							
c-FOS+ (%)	36.5 ± 2.2	44.8 ± 3.3	35.4 ± 3.4	45.2 ± 1.9	**F(1,20)=10.61 p=0.0039**	F(1,20)=0.0124 p=0.9125	F(1,20)=0.0755 p=0.7864
c-FOS+ TPH2− (%)	41.5 ± 4.2	43.5 ± 5.8	42.6 ± 2.9	43.0 ± 2.2	F(1,20)=0.0840 p=0.7750	F(1,20)=0.0050 p=0.9423	F(1,20)=0.0385 p=0.8465
TPH2+ (%)	49.7 ± 1.6	56.7 ± 1.3	47.5 ± 1.5	47.0 ± 3.1	F(1,20)=2.649 p=0.1193	F(1,20)=8.767 p=0.0770	F(1,20)=3.364 p=0.0816
MRN							
c-FOS+ (%)	26.7 ± 3.0	30.1 ± 4.8	25.0 ± 1.8	35.8 ± 3.5	F(1,20)=4.222 p=0.0532	F(1,20)=0.3292 p=0.5725	F(1,20)=1.125 p=0.3014
c-FOS+ TPH2− (%)	24.5 ± 2.6	24.5 ± 5.0	24.2 ± 2.3	31.3 ± 4.1	F(1,20)=0.9428 p=0.3432	F(1,20)=0.8198 p=0.3760	F(1,20)=0.9559 p=0.3399
TPH2+ (%)	22.0 ± 1.5	23.3 ± 2.4	24.4 ± 1.9	22.3 ± 2.5	F(1,20)=0.0398 p=0.8439	F(1,20)=0.1173 p=0.7356	F(1,20)=0.6529 p=0.4286

In the VH, WAY-100635 increased the number of c-FOS+ neurons compared to saline-treated mice, again irrespective of behavioural condition (test: F(1,20)=0.0128, p=0.9110; treatment: F(1,20)=18.93, p=0.0003; interaction: F(1,20)=0.0197, p=0.8899; *Fig. 6.13*).

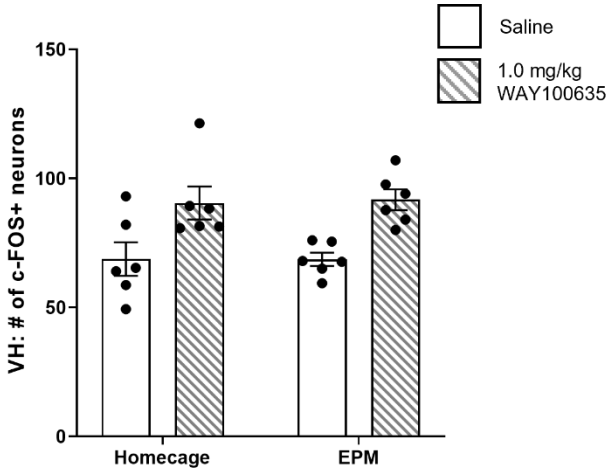


Figure 6.13 Effect of WAY-100635 on c-FOS expression in the VH. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the VH. Homecage: n=6/group, EPM: n=6/group. Individual values are indicated by dots. Two-way ANOVA with post-hoc Tukey's test.

In summary, WAY-100635 had no effect on anxiety-like behaviour in control animals but increased the percentage of c-FOS+ TPH2+ neurons, and the number of c-FOS+ neurons in the VH. This suggests it increased neuronal activation by antagonising inhibitory 5-HT_{1A} receptors.

3.6 Effect of WAY-100635 on extracellular 5-HT

To investigate whether 1.0 mg/kg WAY-100635 could increase extracellular 5-HT as well as somatic activation, *in vivo* microdialysis was performed in the VH of anesthetised mice. As WAY-100635 does not typically alter extracellular 5-HT (Gartside et al., 1995), mice received a *s.c.* injection of WAY-100635 60 min before 0.5 mg/kg 8-OH-DPAT, which was previously shown to reduce extracellular 5-HT (*Fig. 6.9*). In these preliminary experiments, WAY-100635 did not alter hippocampal extracellular 5-HT, but it blocked the effect of 0.5 mg/kg 8-OH-DPAT (effect of time: H(2)=2.660, p=0.2839; *Fig. 6.14A-B*). WAY-100635 administration to mice receiving continued paroxetine treatment also had no effect on the concentration of 5-HT in the microdialysate

60 min later (effect of time: H(1)=1.00, p=0.2000; *Fig. 6.14C-D*).

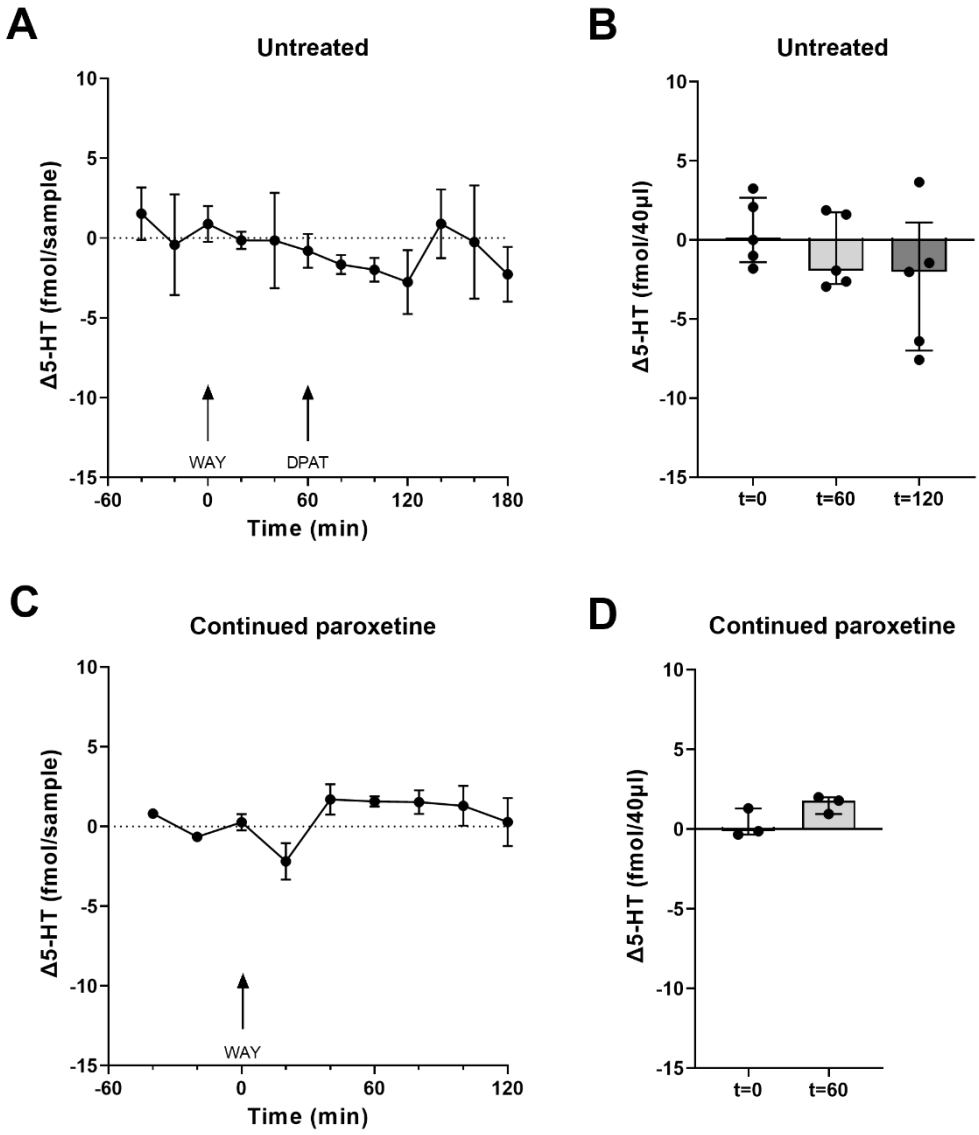


Figure 6.14 Effect of WAY-100635 on hippocampal extracellular 5-HT. Points represent mean ± SEM of change in extracellular 5-HT (Δ5-HT, fmol/40µl) (A, C). Bars represent median ± IQR of t=60 and t=120 values, individual values are indicated by dots (B, D). Untreated (n=5): 1.0 mg/kg WAY-100635 injection (*s.c.*) at first arrow (t=0), 0.5 mg/kg 8-OH-DPAT injection (*s.c.*) at second arrow (t=60) (A). Continued paroxetine treatment: 1.0 mg/kg WAY-100635 injection (*s.c.*) at arrow (t=0) (n=3) (B). Difference in extracellular 5-HT between t=0, t=60 and t=120 (effect of time) analysed using Kruskal Wallis with Uncorrected Dunn's test (B) or Mann Whitney U test (D).

Thus, 1.0 mg/kg WAY-100635 could antagonise the 5-HT_{1A} autoreceptor, although this did not alter hippocampal extracellular 5-HT in untreated or paroxetine-treated mice,

at least under baseline conditions. Yet, these data should be interpreted with caution, as the experiment did not include a saline control group and was underpowered. Nevertheless, as c-FOS immunohistochemical data showed that WAY-100635 could disinhibit 5-HT neurons in the raphe nuclei, WAY-100635 was still used to test if increasing 5-HT neuron activation could precipitate paroxetine discontinuation.

3.7 Effect of 0.5 mg/kg 8-OH-DPAT and WAY 100635 on anxiety-like behaviour and c-FOS expression in mice treated with paroxetine

To assess whether 0.5 mg/kg 8-OH-DPAT or 1.0 mg/kg WAY-100635 could precipitate the behavioural effects of paroxetine discontinuation, mice receiving paroxetine treatment were given the ligands before testing on the EPM. 8-OH-DPAT administration to paroxetine-treated mice did not alter EPM performance compared to paroxetine-treated mice that received a saline injection before the EPM, suggesting it did not precipitate the behavioural effects of discontinuation (*Fig. 6.15*). Nonetheless, 8-OH-DPAT reduced the time spent in the open arms, the number of open arm entries and the number of head dips compared to saline controls, suggesting it had an anxiogenic effect (time: $F(3,44)=7.080$, $p=0.0006$, post-hoc SAL-SAL vs CON-DPAT $p=0.0194$; *Fig. 6.15A*; entries: $F(3,44)=3.163$, $p=0.0338$, post-hoc SAL-SAL vs CON-DPAT $p=0.0063$; *Fig. 6.15B*; head dips: $F(3,44)=5.897$, $p=0.0018$, post-hoc SAL-SAL vs CON-DPAT $p=0.0376$; *Fig. 6.15D*). On the other hand, WAY-100635 given to paroxetine-treated mice increased the time mice spent in the open arms compared to both continued paroxetine and saline controls, and increased the number of head dips compared to continued paroxetine, suggesting it had an anxiolytic effect (time: post-hoc

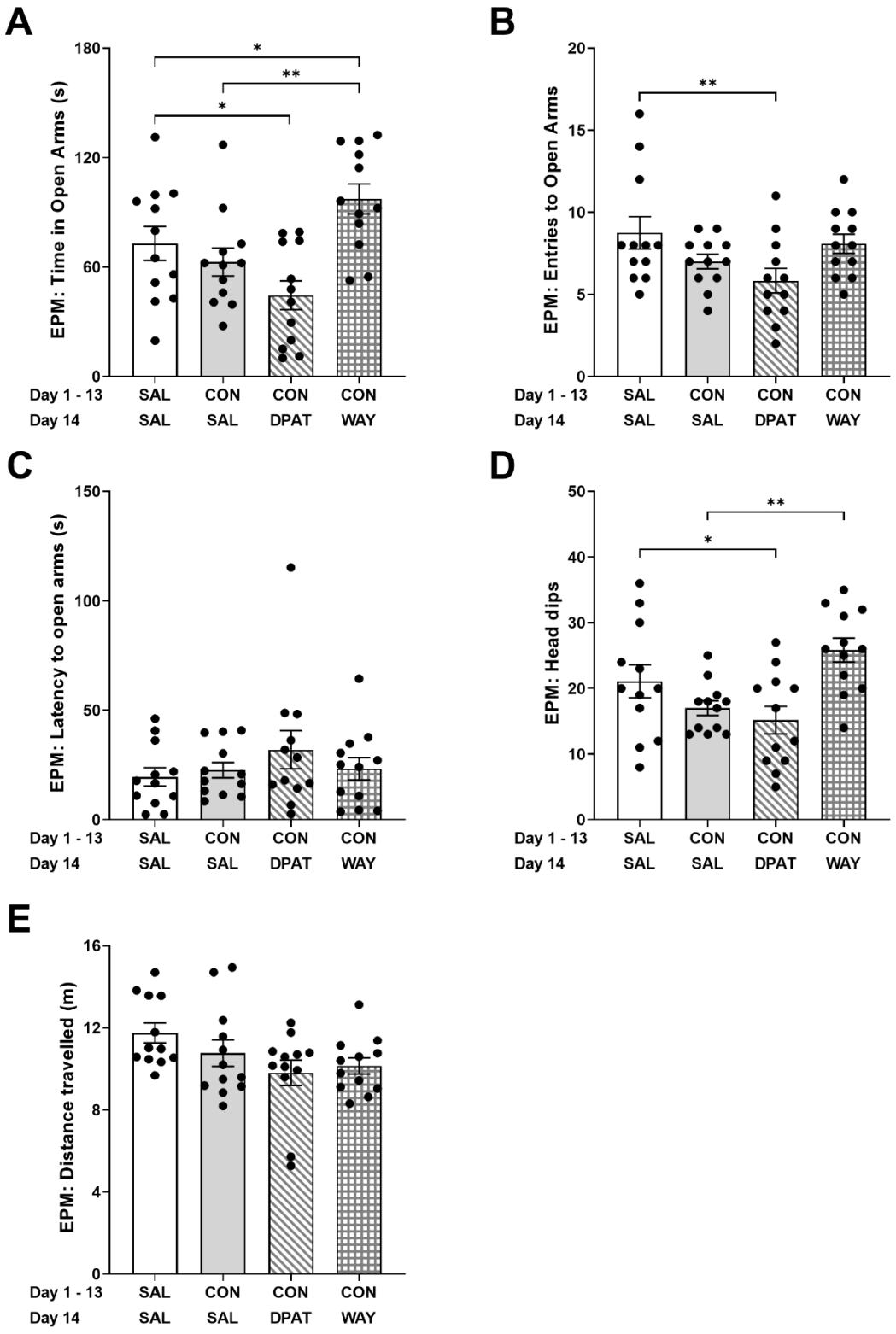


Figure 6.15 Effect of 0.5 mg/kg 8-OH-DPAT and WAY-100635 on the EPM in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM values for time spent in the open arms (A), number of entries to the open arms (B), latency to enter the open arms (C), number of head dips (D) and distance travelled on the EPM (E). 5 min testing on the EPM. SAL-SAL (n=12), CON-SAL (n=12),

CON-DPAT (n=12), CON-WAY (n=12). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * p<0.05, ** p<0.01.

SAL-SAL vs CON-WAY p=0.0430, CON-SAL vs CON-WAY p=0.0050; *Fig. 6.15A*; head dips: post-hoc CON-SAL vs CON-WAY p=0.0025; *Fig. 6.15D*).

WAY-100635 did not alter the number of entries to the open arms, and neither 8-OH-DPAT nor WAY-100635 altered the latency to enter the open arms or the distance travelled on the EPM compared to controls (latency: $F(3,44)=0.8565$, $p=0.4707$; *Fig. 6.15C*; distance: $F(3,44)=2.439$, $p=0.1770$; *Fig. 6.15E*). Thus, neither 5-HT_{1A} ligand precipitated the behavioural effects of paroxetine discontinuation. In this experiment, continued paroxetine treatment also did not alter anxiety-like behaviour compared to saline controls.

Moreover, neither continued paroxetine treatment nor 8-OH-DPAT administered to paroxetine-treated mice altered the percentage of c-FOS+ TPH2+ neurons compared to saline controls in the DRN (*Fig. 6.16A*) or MRN (*Fig. 6.16B*). 8-OH-DPAT also did not alter the total percentage of c-FOS+ neurons in the DRN and MRN (*Table 6.9*). 8-OH-DPAT reduced the percentage of c-FOS+ TPH2− neurons compared to saline controls in the MRN (*Table 6.9*; post-hoc SAL-SAL vs CON-DPAT p=0.0215), but not in the DRN (*Table 6.9*).

By comparison, WAY-100635 administration to paroxetine-treated mice increased the percentage of c-FOS+ TPH2+ neurons compared to paroxetine-treated mice that received saline before the EPM and saline controls in the DRN ($F(3,20)=11.92$, $p=0.0001$, post-hoc SAL-SAL vs CON-WAY p=0.0015, CON-SAL vs CON-WAY p<0.0001; *Fig. 6.16A*) but not in the MRN ($F(3,20)=0.4272$, $p=0.9681$; *Fig. 6.16B*). WAY-100635 administration to paroxetine-treated mice also increased the total percentage of c-FOS+

neurons compared to continued paroxetine treatment in the DRN and MRN (*Table 6.9*; post-hoc DRN: CON-SAL vs CON-WAY p=0.0077; MRN: CON-SAL vs CON-WAY p=0.0255). WAY-100635 increased the percentage of c-FOS+ TPH2− neurons compared to continued paroxetine treatment in the MRN (*Table 6.9*; post-hoc SAL-SAL vs CON-WAY p=0.0293), but not in the DRN (*Table 6.9*). There was no effect of treatment on the percentage of neurons that were TPH2+ (*Table 6.9*).

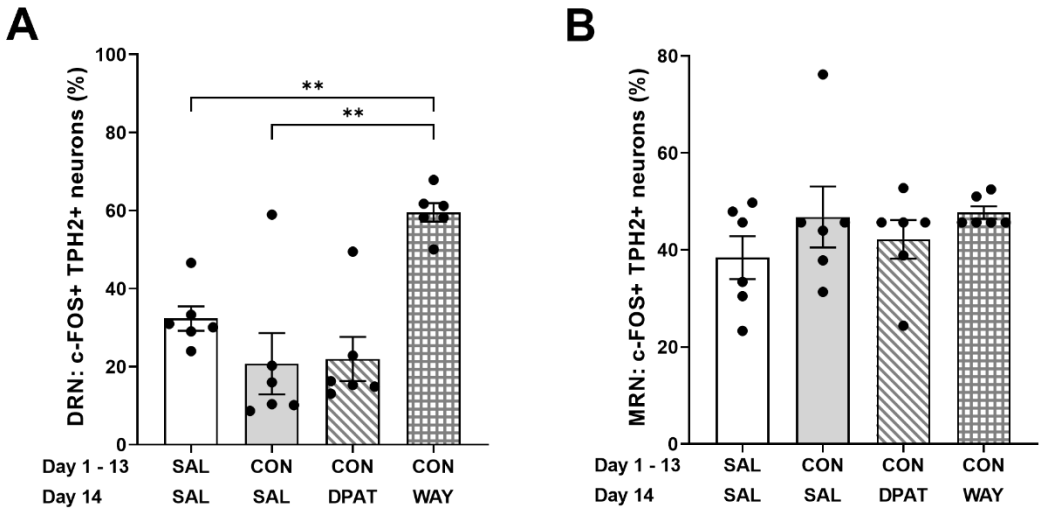


Figure 6.16 Effect of 0.5 mg/kg 8-OH-DPAT and WAY-100635 on c-FOS expression in the DRN and MRN in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM values for the percentage of c-FOS+ TPH2+ neurons in the DRN (A) and percentage of c-FOS+ TPH2+ neurons in the MRN (B). SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), CON-WAY (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$, ** $p<0.01$.

Table 6.9 Effect of 0.5 mg/kg 8-OH-DPAT and WAY-100635 on c-FOS immunoreactivity in the DRN and MRN. Each value is a mean ± SEM, analysed using one-way ANOVA with Fisher's LSD. Bold values indicate significant values, # p<0.05 SAL-SAL vs CON-DPAT, † p<0.05, †† p<0.01 CON-SAL vs CON-WAY. SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), CON-WAY (n=6).

	Group (mean ± SEM)				
Parameter	**SAL-SAL**	**CON-SAL**	**CON-DPAT**	**CON-WAY**	**ANOVA**
DRN					
c-FOS+ (%)	19.2 ± 1.9	15.7 ± 3.6	15.9 ± 2.2	25.8 ± 0.9 ††	**F(3,20)=3.977 p=0.0225**
c-FOS+ TPH2− (%)	14.8 ± 2.0	13.8 ± 2.3	13.5 ± 2.3	15.1 ± 0.8	F(3,20)=0.2070 p=0.8903
TPH2+ (%)	27.2 ± 1.7	24.9 ± 2.5	24.0 ± 1.5	24.2 ± 1.8	F(3,20)=0.5873 p=0.6304
MRN					
c-FOS+ (%)	10.2 ± 0.9	9.7 ± 1.2	7.6 ± 0.7	13.0 ± 0.9 †	**F(3,20)=5.418 p=0.0068**
c-FOS+ TPH2− (%)	7.5 ± 0.8	6.5 ± 0.6	5.3 ± 0.5 #	8.6 ± 0.6 †	**F(3,20)=5.033 p=0.0093**
TPH2+ (%)	8.6 ± 0.4	9.7 ± 0.5	7.5 ± 0.3	8.8 ± 1.1	F(3,20)=1.784 p=0.1825

In the VH, 8-OH-DPAT administered to paroxetine-treated mice did not significantly alter the number of c-FOS+ neurons, whereas WAY-100635 increased the number of c-FOS+ neurons compared to paroxetine-treated mice that received saline before the EPM and saline controls (F(3,20)=6.229, p=0.0037, post-hoc SAL-SAL vs CON-WAY p=0.0171, CON-SAL vs CON-WAY p=0.0012; *Fig. 6.17*).

Thus, 0.5 mg/kg 8-OH-DPAT did not precipitate the behavioural effects nor c-FOS expression patterns characteristic of paroxetine discontinuation in mice still receiving paroxetine treatment. WAY-100635 administration to paroxetine-treated mice increased c-FOS immunoreactivity in the DRN and VH, like the effects of two days of paroxetine discontinuation. Nonetheless, this was associated with an anxiolytic effect, meaning it also did not precipitate the putative anxiogenic effect of discontinuation.

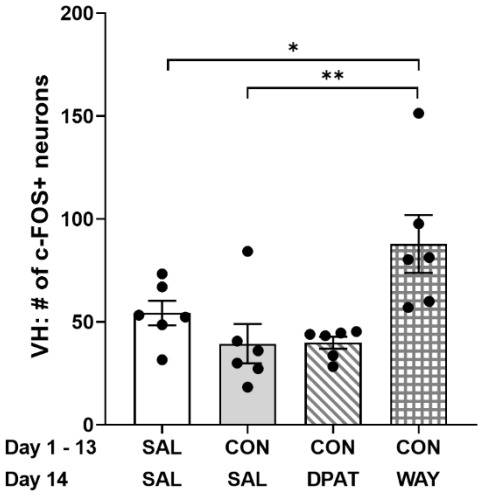


Figure 6.17 Effect of 0.5 mg/kg 8-OH-DPAT and WAY-100635 on c-FOS expression in the VH in mice receiving continued paroxetine treatment. Bars represent the mean ± SEM values for the number of c-FOS+ neurons in the VH. SAL-SAL (n=6), CON-SAL (n=6), CON-DPAT (n=6), CON-WAY (n=6). Individual values are indicated by dots. One-way ANOVA with post-hoc Fisher's LSD, * $p<0.05$ * $p<0.01$.

3.8 Summary of experimental results

Table 6.10 Summary of effects of 8-OH-DPAT and WAY-100635 on behaviour, hippocampal extracellular 5-HT, and c-FOS immunoreactivity. ↑ increased; ↓ decreased; - no effect; $[5\text{-HT}]_{ex}$, concentration of extracellular 5-HT. * Preliminary findings.

Effect	0.1 mg/kg 8-OH-DPAT	0.5 mg/kg 8-OH-DPAT	1.0 mg/kg WAY-100635
Untreated control mice			
EPM performance	-	Anxiogenic	-
Homecage LMA	-	-	-
5-HT neuron c-FOS immunoreactivity	↓	↓	↑
VH c-Fos expression	-	-	↑
$[5\text{-HT}]_{ex}$ in VH*	↓	↓	-
Continuously paroxetine treated mice			
EPM performance	-	Anxiogenic	Anxiolytic
5-HT neuron c-FOS immunoreactivity	-	-	↑
VH c-Fos expression	-	-	↑
$[5\text{-HT}]_{ex}$ in VH*	-	↓	-

4 Discussion

Experiments in this chapter investigated whether manipulating 5-HT transmission using 5-HT_{1A} receptor ligands could precipitate both the behavioural effects and the regional patterns of c-FOS immunoreactivity evident two days after paroxetine discontinuation. It was hypothesised that if a reduction in extracellular 5-HT caused the discontinuation effects, then the 5-HT_{1A} receptor agonist 8-OH-DPAT would precipitate such effects in mice continuously treated with paroxetine. Alternatively, if an increase in 5-HT transmission was causal, then the 5-HT_{1A} receptor antagonist WAY-100635 would precipitate discontinuation. Nonetheless, neither 8-OH-DPAT nor WAY-100635 administration precipitated the anxiety-like effects or the neuronal activation patterns observed two days after paroxetine discontinuation.

4.1 8-OH-DPAT did not precipitate paroxetine discontinuation

In the first precipitation experiment, the lower dose of 8-OH-DPAT (0.1 mg/kg) did not induce the behavioural effects of paroxetine discontinuation. Data from the pilot experiments suggested that 8-OH-DPAT reduced the percentage of c-FOS+ TPH2+ neurons in the DRN and MRN, meaning it reduced 5-HT neuron activation. Moreover, *in vivo* microdialysis experiments confirmed that 0.1 mg/kg 8-OH-DPAT administration reduced hippocampal extracellular 5-HT in control mice. This finding is consistent with previous microdialysis studies in rats (Sharp et al., 1989a; Sharp et al., 1989b; Sharp & Hjorth, 1990; Hjorth & Sharp, 1991).

In the current study, however, 0.1 mg/kg 8-OH-DPAT did not alter c-FOS expression in the DRN, or extracellular 5-HT in the VH, in mice receiving continued paroxetine treatment. Similar studies have also reported that chronic SSRI treatment

attenuated the ability of 0.1 mg/kg 8-OH-DPAT to reduce extracellular 5-HT in rats (Rutter et al., 1994; Cremers et al., 2000a; Dawson & Nguyen, 2000; Ceglia et al., 2004). Moreover, chronic paroxetine treatment abolished the effects of 8-OH-DPAT on c-FOS expression in several brain regions in rats (de Jong et al., 2005). Together, these results suggest that 0.1 mg/kg 8-OH-DPAT may not have been a sufficiently high dose to precipitate paroxetine discontinuation.

To account for this possibility, the dose of 8-OH-DPAT was increased to 0.5 mg/kg. In untreated mice, 0.5 mg/kg 8-OH-DPAT reduced c-FOS expression in TPH2+ neurons in the DRN and reduced extracellular 5-HT in the VH. Critically, though, it also reduced extracellular 5-HT in paroxetine-treated mice, suggesting that this higher dose was sufficient to overcome the loss of 5-HT_{1A} autoreceptor sensitivity.

Nonetheless, 0.5 mg/kg 8-OH-DPAT also did not precipitate the behavioural effects of paroxetine discontinuation. In mice receiving continued paroxetine treatment, 0.5 mg/kg 8-OH-DPAT reduced the time mice spent in the open arms, the number of open arm entries and head dips, suggesting an anxiogenic effect. However, this was only significant relative to saline controls and not paroxetine pre-treated mice that received a saline injection before the EPM. This mirrored the results of the pilot study, which suggested that 0.5 mg/kg 8-OH-DPAT alone had a mild anxiogenic effect. Thus, the reduction in open arm exploration with 8-OH-DPAT administration likely arose from the drug itself rather than its effects on 5-HT transmission in mice pre-treated with paroxetine. Moreover, in these same mice receiving continuous paroxetine treatment, 0.5 mg/kg did not significantly reduce c-FOS expression in TPH2+ neurons.

It is interesting that these experiments found evidence of an anxiogenic effect of 0.5 mg/kg 8-OH-DPAT given its well-reported anxiolytic effects in rodents (Engel et al.,

1984; File et al., 1996; Noguerón-Merino et al., 2015; Jastrzębska-Więsek et al., 2018; Cai et al., 2019). Nonetheless, other researchers have reported anxiogenic effects of 8-OH-DPAT (Moser et al., 1990; Critchley et al., 1992; Handley & McBlane, 1993; McCreary et al., 1996). The balance of 5-HT_{1A} receptor activation could account for this variation. In SSRI-treated animals, 8-OH-DPAT will have activated the 5-HT_{1A} heteroreceptors to a greater extent than the desensitised autoreceptors[6]. Local infusion of 8-OH-DPAT into the hippocampus, amygdala or mPFC, thus selectively activating the 5-HT_{1A} heteroreceptors, increased anxiety-like behaviour on a range of unconditioned anxiety tests (Hodges et al., 1987; De Almeida et al., 1998; Cervo et al., 2000; Cheeta et al., 2000; Dos Santos et al., 2008; Solati et al., 2011). Heteroreceptor activation may therefore have driven the anxiogenic effect of 0.5 mg/kg 8-OH-DPAT seen here.

Overall, 8-OH-DPAT administration to mice receiving continued paroxetine treatment did not precipitate either the anxiogenic behaviours or the patterns of c-FOS expression characteristic of SSRI discontinuation. These results could be taken to suggest that a reduction in extracellular 5-HT itself does not trigger the effects observed in mice following paroxetine cessation.

4.2 WAY-100635 also did not precipitate paroxetine discontinuation

Experiments using the 5-HT_{1A} receptor antagonist WAY-100635 were then conducted to test the hypothesis that increasing 5-HT neuron activity would precipitate paroxetine discontinuation. In pilot experiments, WAY-100635 did not affect EPM performance, but c-FOS immunohistochemical evidence suggested that it activated 5-HT

[6] 5-HT_{1A} heteroreceptor sensitivity appears to be minimally affected by repeated SSRI treatment (Piñeyro & Blier, 1999; Le Poul et al., 2000; Hensler, 2002).

neurons in the DRN and MRN. Elevated c-FOS expression in 5-HT neurons was also observed when WAY-100635 was administered to paroxetine-treated mice. These findings are consistent with electrophysiological recordings showing that WAY-100635 activated 5-HT neurons in rat DRN slices (Fletcher et al., 1996). This activation was still evident following repeated SSRI treatment – for example, WAY-100635 increased *in vivo* 5-HT neuron firing in mice repeatedly treated with escitalopram (Guiard et al., 2012).

Despite these promising results, WAY-100635 did not induce the putative anxiogenic effects of paroxetine discontinuation on the EPM; in fact, it increased the time mice spent in the open arms and the number of head dips, suggesting it had an anxiolytic effect. Other studies have also observed anxiolytic effects of WAY-100635 in rodents. For example, high doses of WAY-100635 increased the number of times rats reared in the light compartment of the LDB, interpreted as a reduction in anxiety-like behaviour (Fletcher et al., 1996). It is likely that these anxiolytic effects are mediated by the blockade of 5-HT_{1A} heteroreceptors. For instance, directly infusing the 5-HT_{1A} receptor antagonist NAN-190 into the hippocampus or mPFC produced a robust increase in the time rats and mice spent in the open arms of the EPM compared to vehicle-treated controls (Nunes-de-Souza et al., 2002; Solati et al., 2011). Thus, during repeated paroxetine treatment when the sensitivity of the 5-HT_{1A} autoreceptors was low, the action of WAY-100635 at the 5-HT_{1A} heteroreceptors may have produced anxiolytic effects.

Although WAY-100635 did not precipitate the behavioural effects of paroxetine discontinuation, it produced patterns of c-FOS immunoreactivity resembling those seen two days after paroxetine discontinuation in mice receiving continued paroxetine treatment. Specifically, WAY-100635 increased the proportion of c-FOS+ TPH2+ neurons in the DRN and increased the number of c-FOS+ neurons in the VH of

paroxetine-treated mice. This is not evidence that WAY-100635 precipitated the neuronal activation patterns of paroxetine discontinuation, as WAY-100635 alone produce the same patterns of c-FOS immunoreactivity. However, the fact that 5-HT_{1A} receptor antagonism mimicked the patterns of neuronal activation observed two days after paroxetine discontinuation provides further evidence that SSRI cessation may be associated with reduced 5-HT_{1A} autoreceptor signalling. Nevertheless, reproducing this pattern of neuronal activation did not generate the anxiogenic effects of paroxetine discontinuation. This could be taken as evidence that the activation of these neural circuits may be a consequence, but not a cause, of the behavioural effects of paroxetine cessation.

Notably, WAY-100635 administration to paroxetine-treated mice did not increase basal hippocampal extracellular 5-HT in the microdialysis experiments. This finding was surprising as several studies have demonstrated that WAY-100635 can potentiate the SSRI-induced increase in extracellular 5-HT in rats (Gartside et al., 1995; Invernizzi et al., 1996; Dawson & Nguyen, 2000; Hervás et al., 2000; Hervás et al., 2001), even during chronic SSRI treatment (Cryan et al., 1999; Dawson et al., 2002). Nonetheless, these preliminary experiments were underpowered and lacked saline control animals, meaning caution should be taken when interpreting these findings. The microdialysis experiments should therefore be repeated to increase the power of the experiment. Moreover, potassium-evoked 5-HT release could be studied following WAY-100635 administration to differentiate between basal levels of extracellular 5-HT and its stimulated release, a dissociation that was evident following paroxetine discontinuation.

Overall, WAY-1001635 did not precipitate the behavioural effects of SSRI discontinuation in mice receiving continued paroxetine treatment. This would suggest

that increased 5-HT neuron activation does not cause the heightened anxiety-like behaviour evident two days after paroxetine discontinuation.

4.3 Complex changes in 5-HT transmission may occur during paroxetine discontinuation

In the current study, neither 8-OH-DPAT nor WAY-100635 precipitated the behavioural effects of discontinuation in mice still receiving paroxetine. At face value, these findings suggest that neither an increase nor decrease in 5-HT transmission causes the anxiogenic effect of paroxetine cessation in mice. Nonetheless, these results do not preclude the possibility that altered 5-HT signalling produces the discontinuation effects, but the current experiments may have failed to reproduce the temporal dynamic changes that occur in the 5-HT system. As proposed in Chapter 5, the serotonergic response to SSRI discontinuation appears to involve two, bidirectional phases of 5-HT signalling, which simple manipulations with 5-HT_{1A} receptor ligands could not recapitulate.

For instance, 8-OH-DPAT reduced extracellular 5-HT, but its constant agonism of the 5-HT_{1A} autoreceptors would have prevented them from detecting the fall in 5-HT. Consequently, the neurons were not disinhibited, and excitability was not enhanced, potentially explaining why there was no increase in anxiety-like behaviour. Conversely, WAY-100635 increased 5-HT neuron activation, which may have increased 5-HT release. Yet, extracellular 5-HT would still have been elevated due to the blockade of the 5-HTT by paroxetine. An initial decrease in extracellular 5-HT may therefore be needed for neuronal disinhibition to produce a great enough change in 5-HT transmission to drive anxiety-like behaviour.

Thus, rather than implying that neither increased nor decreased 5-HT transmission is responsible for the discontinuation effects, these experiments could be interpreted as evidence that both phases are needed. Consequently, neither increasing nor decreasing 5-HT in isolation was able to precipitate the effects of paroxetine cessation. Nonetheless, this explanation is currently theoretical, meaning further experiments are needed to investigate this theory.

4.4 Limitations of the experimental approach

Another explanation for why neither 8-OH-DPAT nor WAY-100635 was able to precipitate paroxetine discontinuation is that the systemic administration of 8-OH-DPAT and WAY-100635 will have altered both auto- and heteroreceptor activation. This may have obscured the behavioural effects of specifically altering autoreceptor activity. To circumvent this problem, the local infusion of 8-OH-DPAT and WAY-100635 into the raphe nuclei could be used to specifically target the autoreceptors. Opto- or chemogenetics could also be used to selectively manipulate 5-HT neuron activity to investigate the role of 5-HT transmission in the effects of paroxetine discontinuation.

Moreover, such manipulations of the 5-HT system may have precipitated other effects of paroxetine discontinuation, such as sleep disturbances, which were not monitored here. The current experiments were therefore limited as they used EPM performance as the only readout of discontinuation effects. Further experiments should therefore investigate whether SSRI cessation in mice produces other correlates of the human discontinuation syndrome. These could then be used to further investigate the causal role of 5-HT in the behavioural effects of discontinuation in mice.

Another limitation was the short time (60 min) between administering 8-OH-DPAT or WAY-100635 and behavioural testing. Pilot EPM and microdialysis experiments showed that 60 min was sufficient for 8-OH-DPAT and WAY-100635 to alter extracellular 5-HT or c-FOS expression. Yet, more sustained changes to 5-HT transmission may be needed to generate the behavioural effects of paroxetine discontinuation. For instance, feedback mechanisms may need longer to be activated. A greater and longer-lasting reduction in extracellular 5-HT might be produced by tryptophan depletion, which has previously been suggested to induce discontinuation symptoms in patients taking SSRIs (Delgado, 2006). However, tryptophan depletion is a difficult and slow procedure in rodents and does not necessarily translate to changes in extracellular or tissue 5-HT (Biskup et al., 2012; Sánchez et al., 2015). Nonetheless, it could be trialled and optimised for future precipitation experiments.

4.5 The lack of anxiogenic effect of continued paroxetine treatment

Finally, in the current precipitation experiments, repeated paroxetine treatment did not increase anxiety-like behaviour on the EPM. This contrasted with the robust anxiogenic effects of 12 days of paroxetine treatment reported in Chapter 3. This variation mirrors the high heterogeneity between studies in the meta-analysis of the effects of repeated SSRI treatment in mice (Appendix 2). One explanation for the disparity between these experiments is the difference in the absolute levels of anxiety in the saline controls relative to mice receiving continued paroxetine treatment. For instance, saline control mice spent on average 50 s in the open arms of the EPM in Chapter 3 (section 3.2, *Fig. 3.5*); but saline-treated mice only spent 30 s in the open arms in this chapter (section 3.2, *Fig. 6.6*). By comparison, paroxetine-treated mice spent 30-40 s in the open arms in both

experiments. The increased anxiety-like behaviour in the saline controls in this chapter likely arose from the additional saline injection 60 min before EPM testing. Thus, the lack of effect of repeated paroxetine treatment here may reflect an increase in anxiety in the control mice rather than variable effects of continued paroxetine treatment.

4.6 Conclusions

Overall, neither 8-OH-DPAT nor WAY-100635 precipitated the behavioural effects of SSRI discontinuation in mice receiving continued paroxetine treatment. These results could suggest that reduced extracellular 5-HT and increased neuronal activation are not causal in the behavioural effects of paroxetine discontinuation. Alternatively, complex changes in 5-HT transmission may be needed to produce SSRI discontinuation effects, which pharmacological manipulations of the 5-HT_{1A} receptors cannot reproduce. Moreover, the approach to precipitating SSRI discontinuation may not have been optimal for testing these hypotheses. Thus, based on data in the previous chapters, a rebound increase in 5-HT transmission appears to be a consequence of stopping SSRI treatment, but it is not yet possible to say whether this causes the elevated anxiety-like behaviour. Further work is therefore needed using different experimental approaches to determine if changes to 5-HT transmission are causal in the behavioural effects of paroxetine discontinuation in mice.

Chapter 7
Effect of paroxetine discontinuation on sleep and circadian rhythms

1 Introduction

In the previous chapters, increased anxiety-like behaviour was used as a readout of the behavioural effects of paroxetine discontinuation in mice. Immunohistochemical and neurochemical experiments suggested that this was associated with a rebound increase in 5-HT transmission. However, pharmacologically manipulating the 5-HT system did not precipitate the anxiogenic effects of paroxetine discontinuation. Yet, altered 5-HT transmission may be responsible for other, as yet unstudied effects of SSRI cessation. For instance, insomnia and sleep disturbances are common in patients undergoing SSRI discontinuation, and 5-HT is known to regulate sleep and circadian rhythms. Here, passive activity monitoring was used to track mouse behaviour and to identify sleep disturbances in the two weeks following paroxetine discontinuation. These experiments aimed to establish whether other behaviours could be used to study the role of 5-HT transmission in the discontinuation syndrome in future experiments.

1.1 Sleep disturbances following SSRI discontinuation

Sleep disturbances are common symptoms of SSRI discontinuation in patients and include initial and middle insomnia, interrupted sleep, and vivid dreams (Coupland et al., 1996; Haddad, 1997; Zajecka et al., 1998; Haddad, 2001; Tonks, 2002; Jha et al., 2018). The first evidence that SSRI discontinuation was linked to sleep disturbances came from case reports of insomnia following fluoxetine and paroxetine cessation (Cooper, 1988;

Stoukides & Stoukides, 1991; Barr et al., 1994; Keuthen et al., 1994; Dominguez & Goodnick, 1995; Frost & Lal, 1995; Yasui-Furukori & Kaneko, 2011). Clinical trials have since demonstrated that insomnia and sleeping difficulties occur in a significant proportion of patients stopping SSRI medications (Coupland et al., 1996; van Geffen et al., 2005; Fava et al., 2007). This includes following discontinuation from paroxetine (Baldwin et al., 2006b), escitalopram (Hindmarch et al., 2000; Judge et al., 2002; Baldwin et al., 2006b), and sertraline (Benazzi, 2002). Other symptoms linked to sleep disturbances include daytime tiredness, lethargy, and somnolence, the feeling of sleepiness and the desire to go to sleep (Mallya et al., 1993; Barr et al., 1994; Haddad et al., 1998; Zajecka et al., 1998). In a double-blind clinical trial, 4% of patients experienced daytime somnolence in the first two weeks following fluoxetine cessation, whereas there were no reported cases in continued treatment controls (Black et al., 1993).

Vivid dreaming, abnormal dreams and nightmares can also occur following SSRI discontinuation (Louie et al., 1994; Dominguez & Goodnick, 1995; Fava & Grandi, 1995; Coupland et al., 1996; Zajecka et al., 1998). Patients reported vivid dreaming in double-blind, placebo-controlled trials of fluoxetine, paroxetine and sertraline cessation (Michelson et al., 2000; Judge et al., 2002; Sir et al., 2005), and in a smaller study of venlafaxine and escitalopram discontinuation (Montgomery et al., 2004b).

Sleep disturbances following SSRI discontinuation have also been studied using cortical EEG, focusing on its effects on REM sleep. REM sleep is characterised by high frequency, low voltage theta EEG rhythms, resembling those of the awake brain although the eyes remain closed. It is also the stage of sleep in which dreams often occur (although they can also occur in other stages) (Hobson, 2009; Lowry et al., 2010). REM sleep differs from the slow wave activity (delta waves) that occurs during non-REM (NREM) sleep

(Borbély & Achermann, 1992; Vyazovskiy et al., 2017) and the high frequency, low amplitude beta EEG rhythms characteristic of wakefulness (Lowry et al., 2010; Vyazovskiy et al., 2017).

EEG studies have revealed that patients often experience a rebound increase in REM sleep following SSRI discontinuation. This effect was first observed in 40 patients with MDD discontinuing paroxetine treatment (Staner et al., 1995). While patients were taking paroxetine, REM sleep was suppressed compared to pre-treatment levels (discussed further in section 1.4). Within just two days of discontinuation, however, the time spent in REM sleep increased above pre-medication levels (Staner et al., 1995). REM rebound was also detected four to six days after fluoxetine discontinuation (Trivedi et al., 1999; Feige et al., 2002), and in the six days following citalopram cessation (van Bemmel et al., 1993). It has therefore been speculated that an increase in abnormal and vivid dreaming following SSRI discontinuation may be linked to REM rebound.

1.2 The neural basis of sleep-wake control

Sleep is a ubiquitous phenomenon, and its regulation comprises of two main mechanisms: sleep homeostasis, and circadian rhythms (Borbély & Tobler, 2011). Sleep homeostasis is the process in which sleep pressure, or the propensity to fall asleep, increases during wakefulness and dissipates during sleep (Tobler et al., 1983; Trachsel et al., 1992; Borbély et al., 2016). The proposed role of this homeostatic promotion of sleep is to off-set wakefulness with recovery periods. This is thought to restore metabolic balance, network function and levels of synaptic strength to avoid saturation and high levels of metabolic demand (Vyazovskiy et al., 2017). Increased sleep pressure therefore

arises from prolonged periods of wakefulness, as well as from increased local metabolic or cognitive demands (Dijk et al., 1987; Dijk et al., 1990; Borbély et al., 2016).

Sleep is also synchronised to environmental cues such as daylight, known as entrainment, by endogenous circadian rhythms. Circadian rhythms ensure that we sleep at appropriate times of day – this manifests in humans as both wake promotion later in the day to counteract the rising homeostatic drive to sleep and sleep promotion during the rest phase at night (Mistlberger, 2005). The opposite is true in nocturnal rats and mice (Borbély et al., 2016). Homeostatic and circadian processes therefore interact to control when we are awake and when we need to sleep (Borbély & Achermann, 1992; Deboer et al., 2003; Deboer et al., 2007).

Several brain regions have been identified as key components of the sleep-wake circuitry. Wake-promoting systems originate in the midbrain and ascend to subcortical structures via two pathways. The dorsal pathway innervates the hypothalamus, thalamus and cortex, and has been implicated in the control of consciousness (Braun et al., 1997; Scammell et al., 2017). The ventral pathway, on the other hand, directly innervates the basal forebrain (BF), which projects to the cortex and other arousal-promoting brain regions. This pathway controls the behavioural state of wakefulness (Scammell et al., 2017). Its activity is greatest during wakefulness (Jones, 2017) and is negatively correlated with NREM sleep (Saper & Fuller, 2017).

Nonetheless, sleep does not simply arise from low activity in wake-promoting brain regions. It is also associated with the activation of distinct sleep-promoting subcortical regions such as the ventrolateral preoptic nucleus (VLPO) and the median preoptic nucleus in the hypothalamus (Donlea, 2017; Saper & Fuller, 2017). These regions send GABAergic projections to arousal systems to inhibit their activity and hence promote

sleep (Alam et al., 2014; Kroeger et al., 2017). The firing rates of these neurons are greatest during sleep deprivation and reduce during recovery sleep (Alam et al., 2014). REM sleep is specifically mediated by several small subcortical nuclei, including the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT), and medial pontine reticular formation (mPRF) (Monti & Jantos, 2005; Scammell et al., 2017).

Sleep-wake activity is further regulated by the suprachiasmatic nucleus (SCN) in the hypothalamus, which is often referred to as the mammalian 'master clock' (Hattar et al., 2002; Peirson et al., 2018). The SCN receives projections from melanopsin-containing retinal ganglion cells, allowing the SCN to synchronise sleep to external daylight cues. The SCN also synchronises clocks in peripheral tissues – hundreds of genes exhibit circadian expression patterns that are entrained to environmental timing cues, known as *zeitgebers* (ZT) (Dibner et al., 2010; Scammell et al., 2017).

1.3 5-HT and sleep

The 5-HT system also regulates sleep-wake activity. It was previously believed that monoaminergic transmission, including 5-HT signalling, controlled wakefulness and behavioural arousal (Saper & Fuller, 2017). It is now understood, however, that 5-HT modulates behavioural arousal by promoting wakefulness and inhibiting REM sleep (Trulson & Jacobs, 1979). The context in which changes in 5-HT signalling occur, for example the time of day, the region of the brain and the extent of 5-HT neuron activation, also determines its effect on sleep.

5-HT is thought to regulate sleep via its extensive innervation of wake- and sleep-promoting brain regions, such as the frontal cortex, BF and preoptic nuclei (Jones, 2003; Morin & Allen, 2006). Electrophysiological evidence demonstrates that 5-HT neural

activity varies between sleep states. For instance, recordings from juxtacellular-labelled 5-HT neurons in the DRN of non-anaesthetised rats showed that firing progressively fell and lost its regularity during the transition from wakefulness to NREM sleep (McGinty & Harper, 1976; Trulson & Jacobs, 1979). Furthermore, 5-HT tone was virtually absent during REM sleep (Trulson & Jacobs, 1979).

5-HT is also thought to promote wakefulness by hyperpolarising GABAergic neurons in the VLPO, disinhibiting arousal systems and promoting wakefulness (Diering et al., 2017). Moreover, evidence suggests that 5-HT suppresses REM sleep by inhibiting the LDT/PPT and other REM-promoting regions (Monti, 2011). For example, direct infusion of 8-OH-DPAT into the DRN reduced 5-HT release and increased REM sleep (Portas et al., 1996). Moreover, mice lacking inhibitory 5-HT_{1A} receptors exhibited more REM sleep than wildtype littermates (Boutrel et al., 2002). It is therefore thought that reduced 5-HT release promotes REM sleep by reducing 5-HT_{1A} receptor signalling and disinhibiting REM-promoting brain regions.

5-HT neurons also innervate the SCN (Aghajanian et al., 1969), where the rhythmical variations in 5-HT release are thought to regulate circadian rhythms (Block & Zucker, 1976). Oscillations in extracellular 5-HT were first reported in microdialysis experiments in the SCN of freely behaving hamsters, with 5-HT output peaking at the transition from the light to the dark phase (i.e. as activity increased) (Dudley et al., 1998). 5-HT can also cause phase shifts, meaning sleep-wake transitions occur earlier or later than would be predicted from environmental light cues, as evident in *in vitro* brain slices[7]

[7] Before brain slicing, rats were housed under a constant 12:12 light/dark cycle. Electrophysiological recordings show that even after brain slicing, the SCN retained its circadian firing patterns, which can be manipulated by 5-HT application or 5-HT-promoting drugs in this same manner as *in vivo* (Sprouse et al. 2004, 2006).

(Sprouse et al., 2004; Sprouse et al., 2006) and *in vivo* (Tominaga et al., 1992; Edgar et al., 1993; Glass et al., 1995). These effects are thought to be mediated by 5-HT_{1A}, 5-HT_{5A} and 5-HT_7 receptor activation (Prosser et al., 1990; Sprouse et al., 2004).

In summary, 5-HT is thought to promote wakefulness and supress sleep by activating arousal circuitry and modulating SCN activity. 5-HT also potently suppresses REM sleep, an effect that is in part mediated by 5-HT_{1A} receptor signalling.

1.4 Effects of SSRI treatment on sleep

SSRIs have been shown to disrupt sleep and promote wakefulness (Wichniak et al., 2017). Insomnia is especially common at the start of SSRI treatment (van Bemmel et al., 1993; Wilson & Argyropoulos, 2005; Hickie et al., 2013). For instance, approximately 17% of patients experienced emergent insomnia in the first few weeks of SSRI treatment, compared to only 9% of placebo controls (Thompson, 2002). Insomnia then tends to improve with prolonged SSRI treatment.

SSRIs also potently suppress REM sleep (Trivedi et al., 1999; Palagini et al., 2013; Wichniak et al., 2017), as expected given the effects of 5-HT described above. In human EEG studies, SSRI administration reduced REM sleep by as much as 85% compared to vehicle treatment (Saletu et al., 1991; Staner et al., 1995; McCarthy et al., 2016). Unlike insomnia, REM suppression appears sustained throughout SSRI treatment – for example, four weeks of paroxetine treatment was also associated with increased latency to enter REM sleep and reduced the total time spent in REM in healthy subjects (Staner et al., 1995; Schlösser et al., 1998) as well as in depressed patients (Argyropoulos et al., 2009).

REM suppression is also evident in preclinical studies of SSRIs (Kleinlogel & Burki, 1987). For instance, acute paroxetine treatment reduced the number of REM

episodes by 65-85% compared to vehicle treatment in rats (Gervasoni et al., 2002; Kantor et al., 2017). A similar reduction in REM sleep was evident after acute citalopram, and chronic citalopram or paroxetine treatment in rats and mice (Neckelmann et al., 1996; Monaca et al., 2003; Kantor et al., 2017). The direct infusion of fluoxetine into the LDT also reduced REM sleep in rats (Monti & Jantos, 2005).

The REM suppressant effects of SSRIs likely arise from elevated extracellular 5-HT. Exemplifying this, the ability of citalopram to supress REM sleep was absent in 5-HT_{1A} KO mice (Monaca et al., 2003; Adrien, 2004), demonstrating that 5-HT signalling was critical in mediating this effect. Increased extracellular 5-HT during SSRI treatment can also disrupt the control of circadian rhythms by the SCN, for example causing phase advancement (Prosser et al., 1990; Sprouse et al., 2006; Nomura et al., 2008).

Overall, there is substantial evidence to suggest that SSRIs promote wakefulness and inhibit sleep by increasing extracellular 5-HT. This often presents as insomnia, which is particularly common at the beginning of treatment. SSRIs also suppress REM sleep in both rodents and humans, an effect that persists even with chronic SSRI treatment.

1.5 Effect of SSRI discontinuation on sleep in rodents

The effects of SSRI cessation on sleep in rodents has not been extensively investigated. In the only preclinical study to investigate sleep architecture immediately following SSRI discontinuation, Gervasoni et al. (2002) used EEG to show that three weeks of paroxetine treatment suppressed REM sleep in rats, yet this had reversed within 24 h of discontinuation. Notably, in this rodent study there was no evidence of a rebound increase in REM sleep above vehicle control levels, despite its consistent detection in patients (Gervasoni et al., 2002). Similarly, repeated paroxetine treatment reduced REM

sleep compared to vehicle-treated controls in a mouse model of Huntington's disease. When EEG was repeated two weeks after paroxetine discontinuation, however, the time spent in REM sleep did not differ from vehicle controls (Kantor et al., 2017).

These preclinical studies suggest that paroxetine-induced changes to sleep architecture rapidly reverse following SSRI cessation, and that there are no changes two weeks after paroxetine discontinuation. Nonetheless, there is currently no data available on whether sleep disruption may emerge and then spontaneously resolve in the gap between when these studies were conducted. Experiments in the previous chapters found evidence of changes in the 5-HT system for at least five days following paroxetine cessation. This may therefore be a critical window in which to detect other correlates of the human discontinuation syndrome. Thus, there was a need for an experiment to screen sleep in mice during the two weeks following paroxetine discontinuation to detect transient changes in sleep-wake activity.

1.6 Passive infrared (PIR) monitoring to track activity and sleep in mice

While EEG is considered the gold standard for determining sleep state in rodents, researchers have also developed sensitive but non-invasive techniques to monitor sleep-wake activity and circadian rhythms. The Continuous Open Mouse Phenotyping of Activity and Sleep Status (COMPASS) system was developed in Oxford as an alternative to EEG. This approach uses PIR monitoring to measure circadian rhythms in mice. Mice are single housed in light tight chambers (LTCs) and their activity is recorded using infrared cameras positioned above the cages. The cameras simply detect movement within the cage, which is expressed as the percentage of time mice spent active in a 10 s epoch.

Sleep can then be estimated from these activity measurements. EEG and infrared beam break studies have shown that the minimum sleep bout duration is approximately 40 s in C57BL/6 mice (Pack et al., 2007). Periods of at least 40 s without movement can therefore be identified as 'behaviourally-defined' sleep. When this criterion was applied to activity data, it was 92% accurate compared to EEG scoring of sleep states (Pack et al., 2007). Behaviourally-defined sleep, as calculated by the COMPASS system, has also been validated with EEG (Brown et al., 2016), including in a mouse model of sleep disruption (Krone et al., 2021).

The COMPASS system can therefore be used to simultaneously track homecage activity and to study disrupted sleep in mice (Brown et al., 2019; Tam et al., 2021). Activity data can also be used to calculate the interday stability (IS), a measure of the variation in the activity of an individual in 24 h periods (Brown et al., 2019). Intraday variation (IV) can also be used to quantify the stability of a mouse's sleep and wake periods, based on the number of sleep-wake transitions it undergoes in a 24 h period. The percentage of time mice spend asleep, the latency to fall asleep (the time between the start of the light phase and the first sleep episode), and the number of sleep bouts in each day, light or dark phase can also be calculated (Wichniak et al., 2017; Ang et al., 2021). Notably, though, PIR monitoring cannot differentiate between REM and NREM sleep.

PIR monitoring has several advantages over other techniques to measure sleep. Its non-invasive nature means there is no need for surgical EEG electrode placement. This eliminates the inflammation resulting from neurosurgery and device implantation, which can produce changes in local neurotransmitter concentrations and behaviour (Balzekas et al., 2016; Albrecht et al., 2018). It can also be studied over an extended period of time to investigate longitudinal changes in sleep.

Other non-invasive techniques have also been applied to study sleep in rodents, but the COMPASS system is again advantageous. For example, infrared beam break methods, as used for measuring spontaneous LMA in Chapter 3, can also be used. Nonetheless, this approach requires specialised cages that are unsuitable for long-term housing (Brown et al., 2016). The position of the sensors on or near the cage floor also makes them susceptible to obstruction by bedding. This issue is mitigated by placing infrared cameras above the cage with the COMPASS system. Automated video scoring has also been used to assess sleep, but this requires sophisticated software to analyse behaviours (Fisher et al., 2012). On the other hand, the PIR system generates data that can be analysed with simple computational methods. This also makes the technique cheaper than other approaches and more applicable to exploratory experiments.

1.7 Hypothesis and aims

The aim of this chapter was to use PIR monitoring of activity to detect evidence of insomnia and other sleep disturbances in the two weeks following discontinuation from repeated paroxetine treatment (although REM sleep could not specifically be assessed). Continued paroxetine treatment was hypothesised to reduce sleep, due to the wake-promoting effects of elevated extracellular 5-HT. Based on evidence that paroxetine discontinuation is associated with a rapid reversal of the effects of its repeated treatment in mice, it was hypothesised that any effects of paroxetine treatment may reverse to baseline levels following discontinuation. Nonetheless, given that SSRI discontinuation is associated with insomnia and sleep disturbances in patients, it was also predicted that paroxetine discontinuation would reduce time asleep, increase latency to fall asleep and increase sleep fragmentation during the discontinuation period.

2 Experimental Methods

2.1 Animals

C57BL/6J male mice (Charles River, 7 weeks old) were single housed in large opaque open-top cages lined with sawdust. Cages contained a small amount of sizzle nest bedding and mice had *ad libitum* access to food and water. Mice were housed in LTCs on a 12:12 light-dark cycle (light ON 05:00, lights OFF 17:00) for seven days before the start of the experiment to habituate them to single housing.

2.2 Drugs and reagents

Paroxetine was dissolved in saline and administered *s.c*.

2.3 Experimental design

Mice were allocated to one of two experimental groups by stratified randomisation: i) saline group: saline injections throughout; ii) paroxetine group: paroxetine injections during dosing period + saline during discontinuation period (*Fig. 7.1*). Mice were handled for three days to habituate them to daily treatment. Mice were weighed daily throughout the experiment. All mice received once-daily 10 ml/kg *s.c.* saline injections for four days to establish a baseline weight and activity levels (baseline period). Mice then received 12 days of either once-daily *s.c.* injections of saline or 10 mg/kg paroxetine (dosing period). In an initial experiment, all mice received saline injections for seven days to monitor the effects of paroxetine discontinuation. This period was extended to 13 days in a second experiment (discontinuation period; *Fig. 7.1*). Injections were given between 16:00 and 17:00 each day to minimise disruption to

circadian rhythms, as activity naturally increases in the hour before the dark phase (Peirson et al., 2018). After 17:00 on the final day of the experiment, mice were killed by cervical dislocation and the brains were immediately frozen in dry ice-cold isopentane and stored at -80^{o}C.

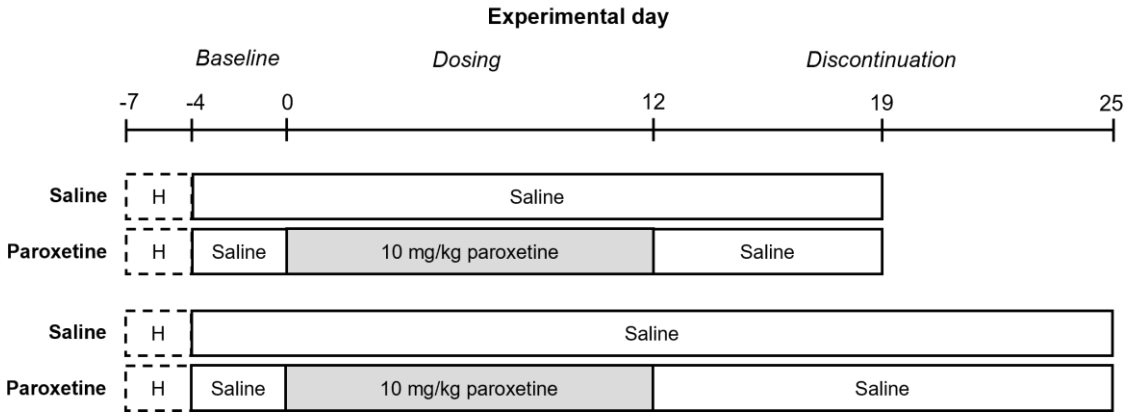


Figure 7.1 Experimental design. First experiment (top) followed discontinuation for seven days. Second experiment (bottom) following discontinuation for 13 days. H, handling.

2.4 Activity and circadian rhythm analysis

The activity of the mice was continually recorded using the PIR monitoring system as described in detail in Chapter 2 section 10 (Brown et al., 2016). Briefly, infrared cameras were positioned above the cages and data were collected every 100 ms and binned into 10 s epochs. Representative actograms of the baseline, dosing, and discontinuation periods were produced from the raw data using freely available scripts.

Data were binned into 1 h epochs using MATLAB R2021b to calculate the mean activity. Hourly activity data were plotted over 24 h and averaged over the whole day, dark phase, or light phase for each mouse. Behaviourally-defined sleep was defined as ≥40 s of immobility (Pack et al., 2007; Fisher et al., 2012; Brown et al., 2016). Sleep was calculated from the activity data as the rolling sum over the last four 10 s bins and

expressed as a binary sequence of awake (0) or asleep (1). Sleep data were binned into 1 h epochs to calculate the percentage of time asleep per hour, then averaged over the whole day, dark phase, or light phase per mouse. The number of sleep bouts per day, and in each dark and light phase, were counted. The duration of each sleep bout was calculated and durations grouped into bins of <1 min, 1-10 min, 10-60 min or >60 min (Ang et al., 2021). Sleep bout duration data were expressed as a percentage of the total number of sleep bouts that day, dark phase, or light phase. The median sleep bout durations were also calculated for each day, and in each dark and light phase (data were skewed towards short bout durations, hence medians and not means were used). The latency to the first sleep bout after the lights turned on was calculated for each mouse (assigned value of 0 s if the mouse was already asleep when the light was turned on). The interday stability (IS) and intraday variability (IV) of activity were also calculated for each mouse.

2.5 Statistical analysis

Data were combined from the two experiments (n=12/group). Treatment day 0 represents the mean value for each mouse in the baseline period. Mean activity, percentage of time asleep, number of sleep bouts, sleep bout durations and body weight were analysed using a mixed-effects model with Geisser-Greenhouse correction and Bonferroni's test for multiple comparisons (between-subject effects: saline vs paroxetine group; within-subject effects: day 0 vs days 1-25, and day 12 vs days 13-25). Repeated measures ANOVA could not be used as there was no data for mice in the first experiment for discontinuation days 8 to 13. Data from day 25 were not included in analyses of the light phase as mice were culled before 17:00 that day.

Representative treatment days (day 0, SAL; day 12, CON; day 14, DIS 2; day 17, DIS 5) were chosen to relate findings to experiments in Chapter 3. Sleep latencies and median sleep bout durations on these representative treatment days, as well as interday stability, intraday variability, and weight changes were assessed for normality with the D'Agostino Pearson's test. Interday stability and intraday variability data were parametric and thus were analysed with Student's t-tests. Weight change was calculated as the difference in weight between day 0 to day 12, day 12 to 14, and day 12 to 17, and analysed with Student's t-tests. Sleep latencies were non-parametrically distributed. Mixed-effect models can still robustly detect differences in distributions even with large skews or biases (Schielzeth et al., 2020). Nonetheless, when the distributions violate the underlying assumptions of statistical tests, results must be interpreted with caution. Thus, individual Mann Whitney U tests were used to confirm the results of the mixed-effects model on each representative treatment day. There was little to no variation in the median sleep bout durations, hence data were not analysed statistically. All analyses were conducted using GraphPad Prism (v8). P values of $p<0.05$ were considered statistically significant. All analyses were performed by an experimenter blind to treatment groups.

3 Results

3.1 Effects of paroxetine continuation and discontinuation on hourly activity

To investigate the effect of repeated paroxetine treatment and its discontinuation on daily activity, infrared cameras monitored activity every 10 s for the duration of the experiment. Representative actographs show that, in a saline-treated mouse, activity was similar each day across the experiment (*Fig. 7.2A*). In the paroxetine group, however, paroxetine administration during the dosing phase attenuated activity at the beginning of the dark phase, but elevated activity in the last few hours of the dark phase compared to the baseline period (*Fig. 7.2B*). In general, activity was low in the light phase, except for a spike in activity at ZT 11.5, corresponding to the daily injections.

Mean hourly activity was then averaged across animals in the same treatment group to visualise how activity changed during the light/dark cycle. In all mice, activity was low in the light phase (*Fig 7.3*). In saline-treated mice, activity sharply increased in the first 2 h of the dark phase (ZT 12-13), and then gradually reduced through the dark period, reaching its lowest point 9 h into the dark period (ZT 21; *Fig. 7.3A*). There was a second, smaller peak in activity in the 2 h before the light phase (ZT 23-24). Overall, there were no significant changes in activity patterns over time in the saline-treated mice (effect of treatment day: $F(1,22)=0.0696$, $p=0.5595$; day*ZT interaction: $F(23,506)=1.111$, $p=0.2546$; *Fig. 7.3A*).

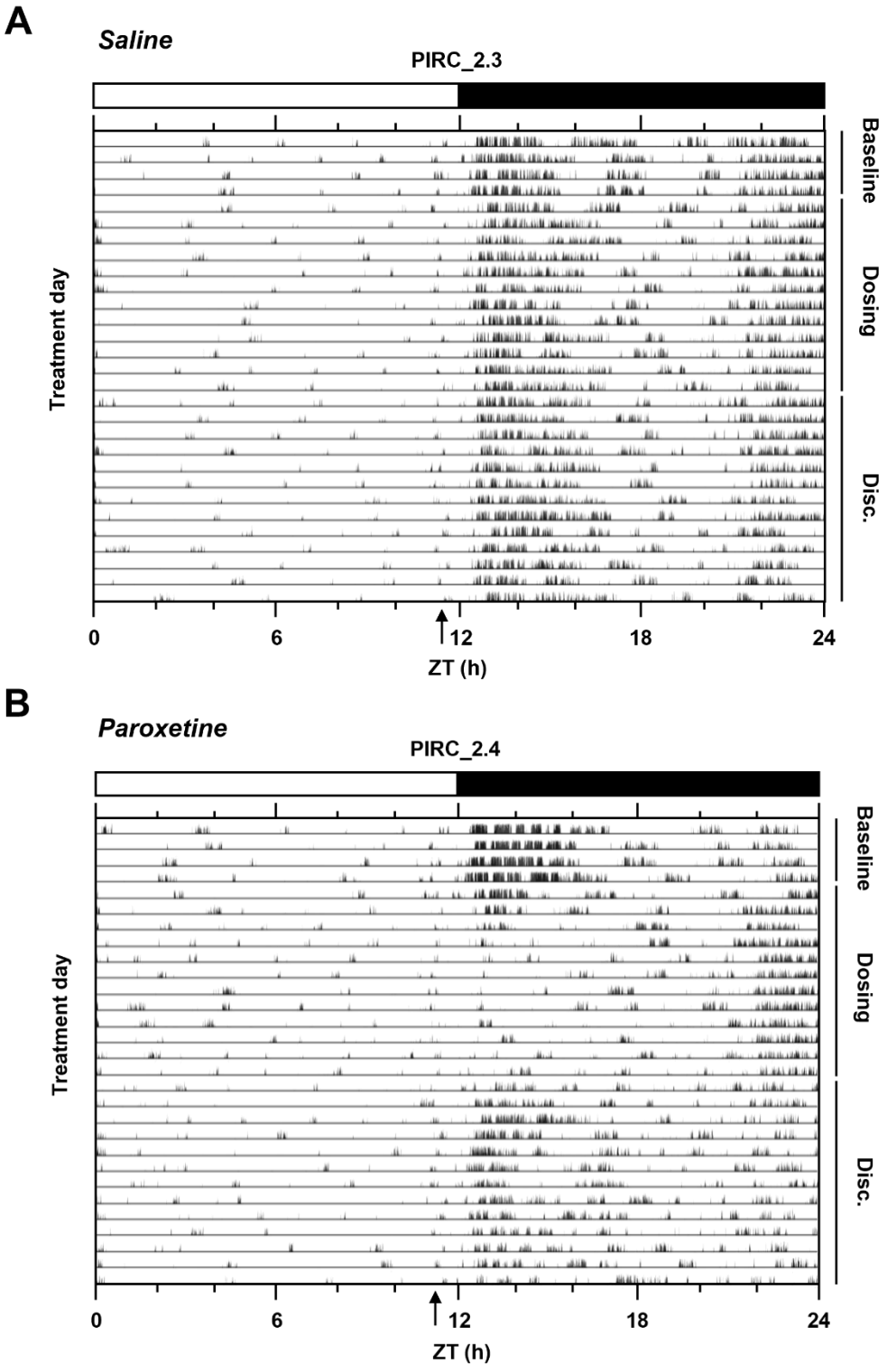


Figure 7.2 Representative actographs. Activity of a mouse receiving saline injections (saline group) (A) and another receiving paroxetine treatment (paroxetine group) (B). Each row represents 24 h of activity (zeitgeist time, ZT). Each spike represents activity (arbitrary units). Horizontal white bar represents light phase (05:00 to 17:00), black bar represents dark phase (17:00 to 05:00). Arrow represents time of daily injections.

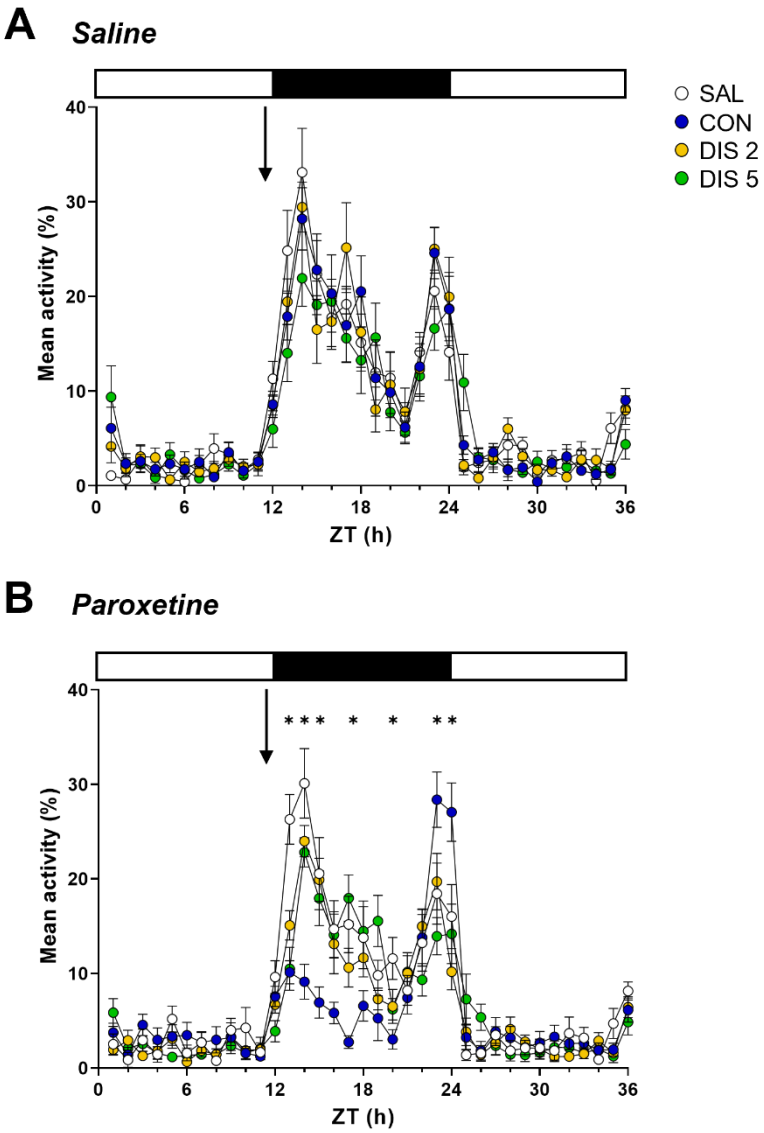


Figure 7.3 Effects of continued paroxetine and discontinuation on mean activity per hour. Points represent the mean ± SEM values for hourly activity (ZT) for the baseline period (treatment day 0, SAL), end of continued paroxetine treatment (day 12, CON) discontinuation day two (day 14, DIS 2) and discontinuation day five (day 17, DIS 5) in the saline (A) and paroxetine group (B). White bar represents light phase (05:00 to 17:00), black bar represents dark phase (17:00 to 05:00). Arrow represents time of daily injections. Saline group (n=12), paroxetine group (n=12). Repeated measures ANOVA with post-hoc Bonferroni's test (only the first 24 h included in analysis), * $p<0.05$ between treatment days.

Continued paroxetine treatment suppressed activity in the first 8 h of the dark phase compared to the baseline period, but activity was significantly elevated in the final 2 h of the dark phase compared to the baseline period (effect of day: $F(1,22)=3.334$, $p=0.0278$;

day*ZT interaction: F(23,506)=5.263, p<0.0001; post-hoc SAL vs CON: ZT 13 p=0.0003, ZT 14 p=0.0006, ZT 15 p=0.0305, ZT 17 p=0.0092, ZT 20 p=0.0188, ZT 23 p=0.01862, ZT 24 p=0.0454; *Fig. 7.3B*). The effects of continued paroxetine treatment reversed following discontinuation, although activity was still lower in the first hour of the dark phase (ZT 13) two and five days after paroxetine discontinuation than during the baseline period (SAL vs DIS 2 p=0.0106, SAL vs DIS 5 p=0.0010; *Fig. 7.3B*). Consequently, activity was significantly greater during paroxetine discontinuation in ZT 14, 15 and 17 than it was at the end of paroxetine treatment, but activity was significantly lower during discontinuation in ZT 23 and 24 than at the end of paroxetine treatment (ZT 14: CON vs DIS 2 p<0.0001, CON vs DIS 5 p<0.0001; ZT 15: CON vs DIS 2 p=0.0010, CON vs DIS 5 p=0.0247; ZT 17: CON vs DIS 2 p=0.0174, CON vs DIS 5 p=0.0003; ZT 23: CON vs DIS 5 p=0.0036; ZT 24: CON vs DIS 2 p=0.0011, CON vs DIS 5 p=0.0475; *Fig. 7.3B*).

In summary, continued paroxetine treatment suppressed activity in the first 8 h of the dark period but increased activity above that of the baseline period in the final 2 h of the dark period. The inhibitory effects of continued paroxetine rapidly reversed following discontinuation. There were no effects on activity during the light phase.

3.2 Effects of paroxetine continuation and discontinuation on daily activity

Mean daily activity, and mean activity in the light or dark phase, were then analysed. The saline-treated and paroxetine-treated groups were compared on each treatment day (between-subject comparisons), and activity was compared to the baseline period (day 0) and the end of continued paroxetine treatment (day 12) within each treatment group (within-subject comparisons).

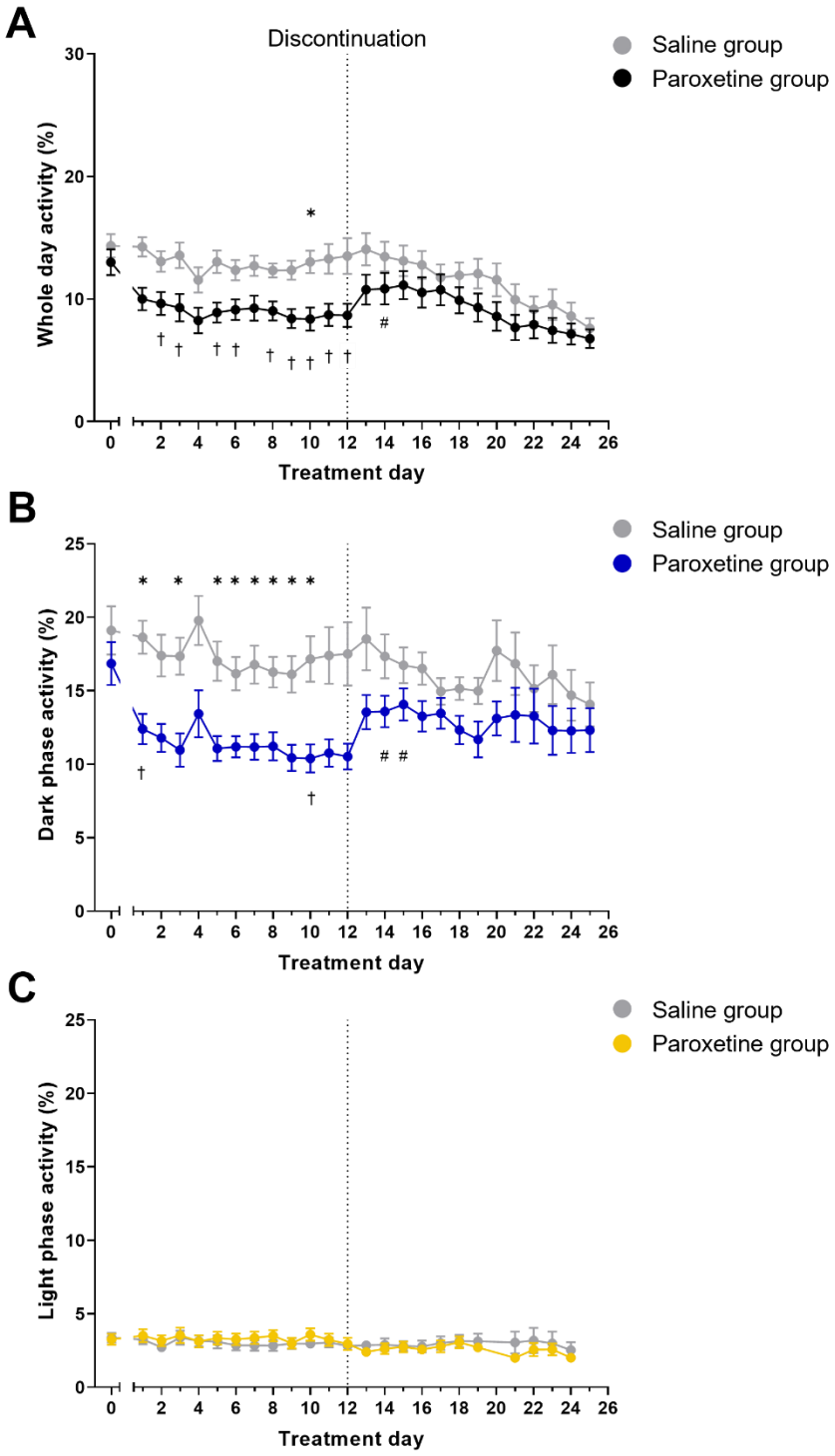


Figure 7.4 Effects of continued paroxetine and discontinuation on daily activity. Points represent the mean ± SEM values for activity per day (A) or during the dark phase (B) and light phase (C). Dotted vertical line represents paroxetine discontinuation. Saline group (n=12), paroxetine group (n=12). Data from day 20 removed from light phase analysis due to outliers. Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test, * $p<0.05$ between-subject comparisons, † $p<0.05$ within-subject comparison treatment day vs day 0, # $p<0.05$ treatment day vs day 12.

Continued paroxetine treatment reduced daily activity, which reversed back to control levels following discontinuation. A mixed-effect model found that overall daily activity was lower in the paroxetine group than in the saline group (effect of treatment: $F(1,22)=4.993$, $p=0.0359$). There was a significant effect of day and a treatment*day interaction (effect of day: $F(25,550)=2.782$, $p<0.0001$; interaction: $F(25,550)=6.242$, $p=0.0013$; *Fig. 7.4A*). Between-subject pairwise comparisons showed that activity was significantly lower in paroxetine-treated mice than in saline-treated mice on day 10 (post-hoc $p=0.0427$). Within-subject pairwise comparisons found no significant changes in activity over time in the saline group. In the paroxetine group, however, within-subject comparisons found that activity was lower during paroxetine treatment than it was during the baseline period, but this reversed to baseline levels within two days of discontinuation (post-hoc day 0 vs day 2 $p=0.0020$, vs day 3 $p=0.0382$, vs day 5 $p=0.0199$, vs day 6 $p=0.0439$, vs day 8 $p=0.0331$, vs day 9 $p=0.0154$, vs day 10 $p=0.0041$, vs day 11 $p=0.0051$, vs day 12 $p=0.0012$; day 14 vs day 12 $p=0.0079$; *Fig. 7.4A*).

As seen in the hourly activity graph, continued paroxetine treatment reduced activity during the dark phase, which reversed to control levels following discontinuation. Overall, activity was lower in the paroxetine group than in the saline group (treatment: $F(1,22)=8.144$, $p=0.0092$). There was a significant effect of day and a treatment*day interaction (day: $F(25,550)=5.012$, $p=0.0031$; interaction: $F(25,550)=3.240$, $p<0.0001$; *Fig. 7.4B*). Between-subject pairwise comparisons showed that activity was significantly lower in mice receiving paroxetine treatment than it was in saline-treated mice (post-hoc day 1 $p=0.0122$, day 3 $p=0.0289$, day 5 $p=0.0357$, day 6 $p=0.0398$, day 7 $p=0.0497$, day 8 $p=0.0428$, day 9 $p=0.0346$, day 10 $p=0.0395$). Within-subject pairwise comparisons found no significant changes in dark phase activity in the saline group. In the paroxetine

group, however, within-subject comparisons found that activity was lower during paroxetine treatment than it was during the baseline period, but this reversed to baseline levels within two days of discontinuation (post-hoc day 0 vs day 1 p=0.0402, vs day 10 p=0.0351; day 12 vs day 14 p=0.0142, vs day 15 p=0.0036; *Fig. 7.4B*).

Neither continued paroxetine treatment nor discontinuation altered activity in the light phase. There was no effect of treatment or a treatment*day interaction, although there was a significant effect of day (treatment: $F(1,22)<0.0001$, p=0.9886; day: $F(23,506)=3.155$, p=0.0179; interaction $F(23,506)=1.524$, p=0.5770; *Fig. 7.4C*).

In summary, continuous paroxetine treatment reduced daily activity, which was particularly evident in the dark phase. This effect rapidly reversed following paroxetine discontinuation. There were no effects of paroxetine treatment in the light phase.

3.3 Effects of paroxetine continuation and discontinuation on time spent asleep

Given the effects of paroxetine continuation and discontinuation were different in the dark and light phases, data are henceforward presented separately but not as an average of the whole day. Continued paroxetine treatment increased behaviourally-defined sleep in the dark phase, which reversed back to control levels following discontinuation. Overall, the percentage of time spent asleep was greater in the paroxetine group than in the saline group (treatment: $F(1,22)=8.419$, p=0.0083). There was also a significant effect of day and a treatment*day interaction (day: $F(25,550)=6.067$, p=0.0003; interaction $F(25,550)=5.913$, $p<0.0001$; *Fig. 7.5A*).

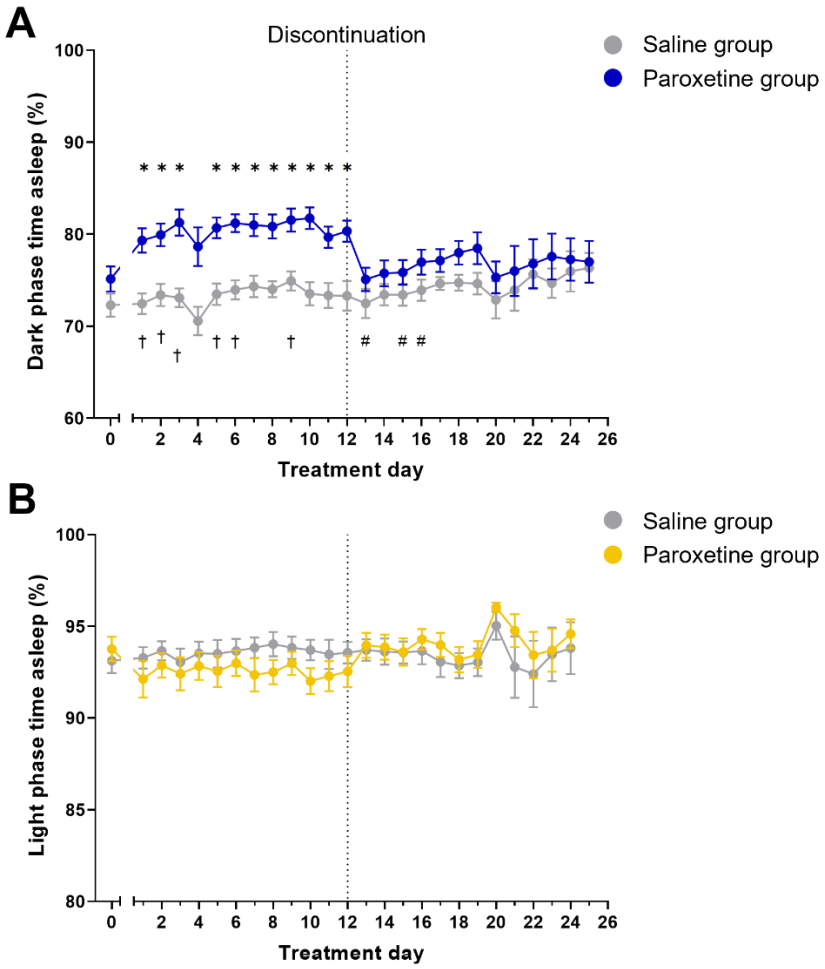


Figure 7.5 Effects of continued paroxetine and discontinuation on the percentage of time asleep. Points represent the mean ± SEM values for the percentage of time spent asleep in the dark phase (A) or light phase (B). Dotted vertical line represents paroxetine discontinuation. Saline group (n=12), paroxetine group (n=12). Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test, * $p<0.05$ between-subject comparisons, † $p<0.05$ within-subject comparison vs day 0, # $p<0.05$ within-subject comparison vs day 12.

Between-subject pairwise comparisons showed that the percentage of time asleep was significantly greater in mice receiving paroxetine treatment than it was in saline-treated mice (post-hoc day 1 p=0.0184, day 2 p=0.0265, day 3 p=0.0039, day 5 p=0.0053, day 6 p=0.0011, day 7 p=0.0180, day 8 p=0.0093, day 9 p=0.0144, day 10 p=0.0024, day 11 p=0.0472, day 12 p=0.0426). Within-subject pairwise comparisons found no significant changes in sleep during the dark phase in the saline group. In the paroxetine group,

however, within-subject comparisons found that the percentage of time spent asleep was significantly greater during paroxetine treatment than it was during the baseline period, but this reversed to baseline levels within one day of discontinuation (post-hoc day 0 vs day 1 p=0.0470, vs day 2 p=0.0198, vs day 3 p=0.0194, vs day 5 p=0.0308, vs day 6 p=0.0111, vs day 9 p=0.0324; day 12 vs day 13 p=0.0082, vs day 15 p=0.0289, vs day 16 p=0.0100; *Fig. 7.5A*).

Neither continued paroxetine treatment nor discontinuation altered the time spent asleep in the light phase. There was no effect of treatment on the percentage of time spent asleep per hour (treatment: F(1,22)=0.0428, p=0.8379). Although there was a significant effect of day and a significant treatment*day interaction (day: F(24,528)=3.496, p=0.0115; interaction F(24,528)=2.518, p=0.0001; *Fig. 7.5B*), there were no significant post-hoc differences between paroxetine-treated and saline-treated mice.

In summary, continuous paroxetine treatment increased the percentage of time mice spent asleep in the dark phase compared to saline controls. This effect reversed following paroxetine discontinuation. There were no effects of paroxetine treatment on sleep during the light phase.

3.4 Effects of paroxetine continuation and discontinuation on sleep latency

Most mice were asleep at the start of the light phase, so the sleep latency was calculated as 0 min, meaning that the data were skewed and non-parametrically distributed. To exemplify the lack of difference between the treatment groups, sleep latencies on representative treatment days were analysed using a mixed-effects model. To account for the non-parametric distribution of the data, separate Mann Whitney U tests were performed for each representative treatment day to compare between the

paroxetine-treated and saline-treated mice. Neither continued paroxetine treatment nor discontinuation altered sleep latency. There were no significant effects of treatment or day, or a treatment*day interaction (treatment: F(1,22)=0.1451, p=0.7070; day: F(3,66)=0.9104, p=0.4093; interaction: F(3,66)=2.156, p=0.1015; *Table 7.1*).

Table 7.1 Effects of continued paroxetine and discontinuation on sleep latency. Each value is a median and IQR [25th percentile, 75th percentile] of sleep latency in min, analysed using a mixed-effects model with Bonferroni's test and separate Mann Whitney U tests. Saline group (n=12), paroxetine group (n=12).

	Group (median [25th,75th])			
Treatment group	**SAL**	**CON**	**DIS 2**	**DIS 5**
Saline	0.04 [0.0,0.1]	0.00 [0.0,0.1]	0.00 [0.0,0.0]	0.00 [0.0,0.0]
Paroxetine	0.00 [0.0,0.1]	0.00 [0.0,0.0]	0.00 [0.0,0.0]	0.00 [0.0,0.3]
Mann Whitney comparisons	U=54.50, p=0.2945	U=59.50, p=0.3416	U=65.50, p=0.7391	U=57.00, p=0.2520

3.5 Effects of paroxetine continuation and discontinuation on the number of sleep bouts

Continued paroxetine treatment reduced the number of sleep bouts in the dark phase, which reversed back to control levels following discontinuation. Overall, the number of sleep bouts was lower in the paroxetine group than in the saline group (treatment: F(1,22)=5.373, p=0.0301). There was a significant effect of day and a treatment*day interaction (day: F(25,550)=5.887, p<0.0001; interaction: F(25,550)=4.084, p<0.0001; *Fig. 7.6A*). Between-subject pairwise comparisons showed that mice receiving paroxetine treatment had significantly fewer sleep bouts compared to saline-treated mice (post-hoc day 3 p=0.0333, day 4 p=0.0251, day 5 p=0.0349, day 6 p=0.0171, day 8 p=0.0280, day 9 p=0.0397, day 10 p=0.0086). Within-subject pairwise comparisons found no significant changes over time in the dark phase in the saline group. In the paroxetine group, however, within-subject comparisons found that the number of

sleep bouts was significantly lower on the sixth day of paroxetine treatment compared to the baseline period (post-hoc day 0 vs day 6 p=0.0435). The number of sleep bouts was greater one day after paroxetine discontinuation than at the end of repeated paroxetine treatment but this was not significantly different from the baseline period (day 12 vs day 13 p=0.0140; *Fig. 7.6A*).

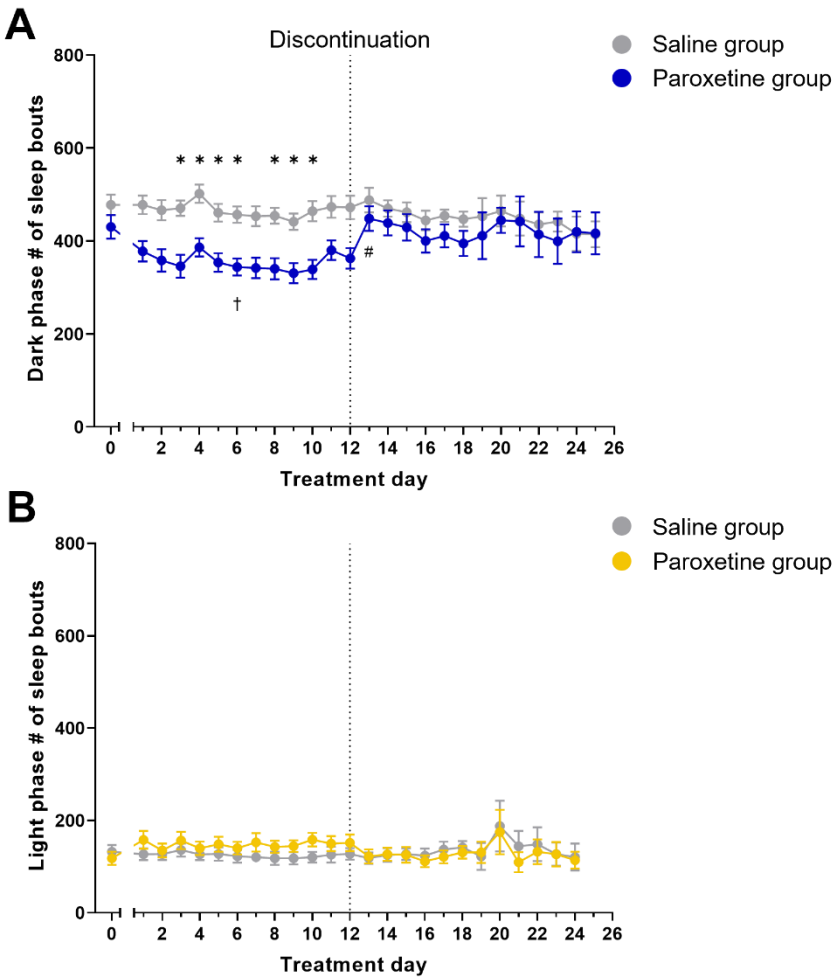


Figure 7.6 Effects of continued paroxetine and discontinuation on the number of sleep bouts per day. Points represent the mean ± SEM values for the number of sleep bouts in the dark phase (A) and light phase (B). Dotted vertical line represents paroxetine discontinuation. Saline group (n=12), paroxetine group (n=12). Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test, * $p<0.05$ between-subject comparisons, † $p<0.05$ within-subject comparisons vs day 0, # $p<0.05$ within-subject comparisons vs day 12.

Neither continued paroxetine nor discontinuation altered the number of sleep bouts in the light phase. There was no effect of treatment on the number of sleep bouts ($F(1,22)=0.1342$, $p=0.7176$). Although there was a significant effect of day and a treatment*day interaction (day: $F(24,528)=3.031$, $p=0.0254$; interaction: $F(24,528)=1.750$, $p=0.0145$; *Fig. 7.6B*), there were no significant differences between paroxetine-treated and saline-treated mice.

In summary, continued paroxetine treatment reduced the number of sleep bouts during the dark phase compared to saline-treated controls. This effect reversed following paroxetine discontinuation.

3.6 Effects of paroxetine continuation and discontinuation on sleep bout duration in the dark phase

Overall, paroxetine discontinuation was associated with a smaller proportion of short sleep bouts (<1 min duration) in the dark phase compared to continued paroxetine treatment and saline controls. There was a trend towards an effect of treatment, in that the overall proportion of <1 min sleep bouts was lower in the paroxetine group than in the saline group ($F(1,22)=3.484$, $p=0.0754$). There was a significant effect of day and a treatment*day interaction (day: $F(25,550)=4.731$, $p=0.0001$; interaction: $F(25,550)=1.466$, $p=0.0495$; *Fig. 7.7A*). Between-subject pairwise comparisons found that the percentage of <1 min sleep bouts was significantly lower in mice during the seven days following paroxetine discontinuation than it was in saline-treated mice (post-hoc day 14 $p=0.0083$, day 15 $p=0.0153$, day 16 $p=0.0140$, day 17 $p=0.0320$, day 19 $p=0.0498$). The percentage of <1 min sleep bouts was also significantly lower on the first day of paroxetine treatment compared to saline controls (day 1 $p=0.0095$). Within-subject

pairwise comparisons found no significant changes in the percentage of <1 min sleep bouts over time in the saline group. In the paroxetine group, however, within-subject comparisons found that the percentage of <1 min sleep bouts was significantly lower on discontinuation day two and five than during the baseline period (day 0 vs day 14 p=0.0402, vs day 17 p=0.0329; *Fig. 7.7A*).

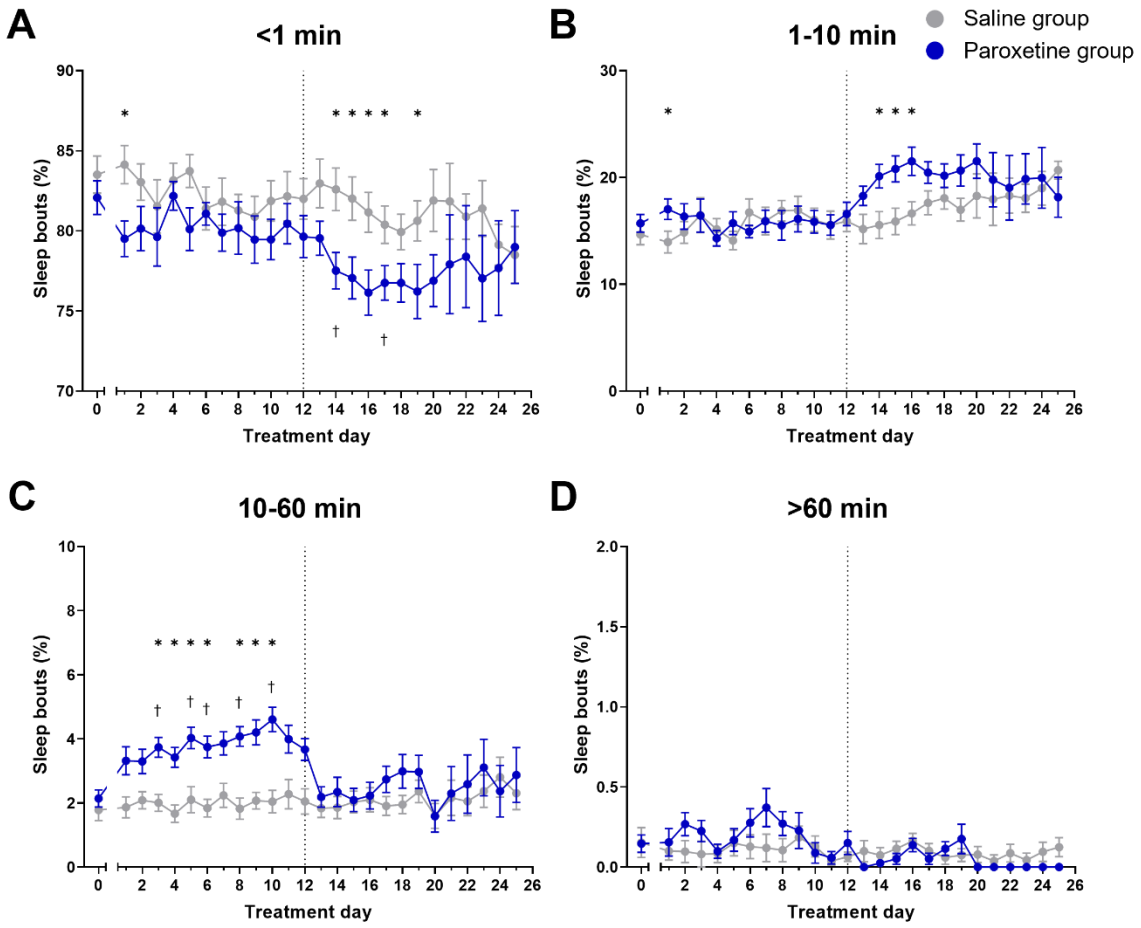


Figure 7.7 Effects of continued paroxetine and discontinuation on sleep bout duration in the dark phase. Points represent the mean ± SEM of sleep bouts of <1 min (A), 1-10 min (B), 10-60 min (C) and >60 min duration (D). Dotted vertical line represents paroxetine discontinuation. Saline group (n=12), paroxetine group (n=12). Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test, * p<0.05 between-subject comparisons, † p<0.05 within-subject comparisons vs day 0.

By comparison, the percentage of 1-10 min sleep bouts was significantly greater in the four days following paroxetine discontinuation than in saline-treated mice. There was

no overall effect of treatment ($F(1,22)=1.727$, $p=0.2024$), but there was a significant effect of day and a treatment*day interaction (day: $F(25,550)=5.833$, $p<0.0001$; interaction: $F(25,550)=2.785$, $p<0.0001$; *Fig. 7.7B*). Between-subject pairwise comparisons found that the percentage of 1-10 min sleep bouts was greater in mice two and four days after paroxetine discontinuation than it was in saline-treated controls (post-hoc day 14 $p=0.0125$, day 15 $p=0.0098$, day 16 $p=0.0095$). The percentage of 1-10 min sleep bouts was also significantly greater on the first day of paroxetine treatment compared to saline controls (day 1 $p=0.0399$). Within-subject pairwise comparisons found no significant changes in the percentage of 1-10 min bouts over time in either the saline group or the paroxetine group (*Fig. 7.7B*).

Continued paroxetine treatment increased the percentage of 10-60 min sleep bouts compared to saline controls, but this reversed following paroxetine discontinuation. There were significant effects of treatment and day, and a treatment*day interaction (treatment: $F(1,22)=6.730$, $p=0.0166$; day: $F(25,550)=5.184$, $p=0.0010$; interaction: $F(25,550)=4.563$, $p<0.0001$; *Fig. 7.7C*). Between-subject pairwise comparisons found that the percentage of 10-60 min sleep bouts was significantly greater in mice receiving paroxetine treatment compared to saline-treated mice, but there were no significant differences after discontinuation (post-hoc day 3 $p=0.0084$, day 4 $p=0.0088$, day 5 $p=0.0401$, day 6 $p=0.0070$, day 8 $p=0.0014$, day 9 $p=0.0054$, day 10 $p=0.0016$). Within-subject pairwise comparisons found no significant changes over time in the saline group. In the paroxetine group, however, within-subject comparisons found that the percentage of 10-60 min sleep bouts was significantly higher during continued paroxetine treatment than during the baseline period, but again this reversed following

discontinuation (post-hoc day 0 vs day 3 p=0.0272, vs day 5 p=0.0348, vs day 6 p=0.0099, vs day 8 p=0.0069, vs day 10 p=0.0180; *Fig. 7.7C*).

There were no significant effects of treatment or time, or a treatment*time interaction on the percentage of >60 min duration sleep bouts (treatment: F(1,22)=0.1036, p=0.7506; time: F(25,550)=2.430, p=0.3090; interaction: F(25,550)=1.742, p=0.1521; *Fig. 7.7D*). The median sleep bout duration did not vary between treatment groups (0.5 [0.0, 0.5] min in each group on the representative treatment days), hence data were not statistically analysed.

In summary, the proportion of <1 min sleep bouts was reduced but the proportion of 1-10 min sleep increased in the seven days following paroxetine discontinuation compared to saline-treated controls. Although the median sleep bout duration was not altered, these data suggest that paroxetine discontinuation lengthened sleep bouts in the dark phase. Repeated paroxetine treatment increased the percentage of long sleep bouts (10-60 min) compared to saline controls

3.7 Effects of paroxetine continuation and discontinuation on sleep bout duration in the light phase

There were no significant effects of treatment, time, or treatment*time interactions on any sleep bout duration during the light phase (<1 min, treatment: F(1,22)=2.124, p=0.1591; time: F(24,528)=0.9302, p=0.5023; interaction: F(24,528)=1.162, p=0.2720; *Fig. 7.8A*; 1-10 min, treatment: F(1,22)=2.667, p=0.1167; time: F(24,528)=0.7722, p=0.6392; interaction: F(24,528)=1.098, p=0.3416; *Fig. 7.8B*; 10-60 min, treatment: F(1,22)=0.0014, p=0.9708; time: F(24,528)=1.538, p=0.1314; interaction: F(24,528)=0.8730, p=0.6403; *Fig. 7.8C*; >60 min, treatment: F(1,22)=0.0007, p=0.9362;

time: F(24,528)=1.254, p=0.2923; interaction: F(24,528)=1.497, p=0.0624; *Fig. 7.8D*). There was little variation in the median durations of sleep bouts, hence data were not statistically analysed (*Table 7.2*).

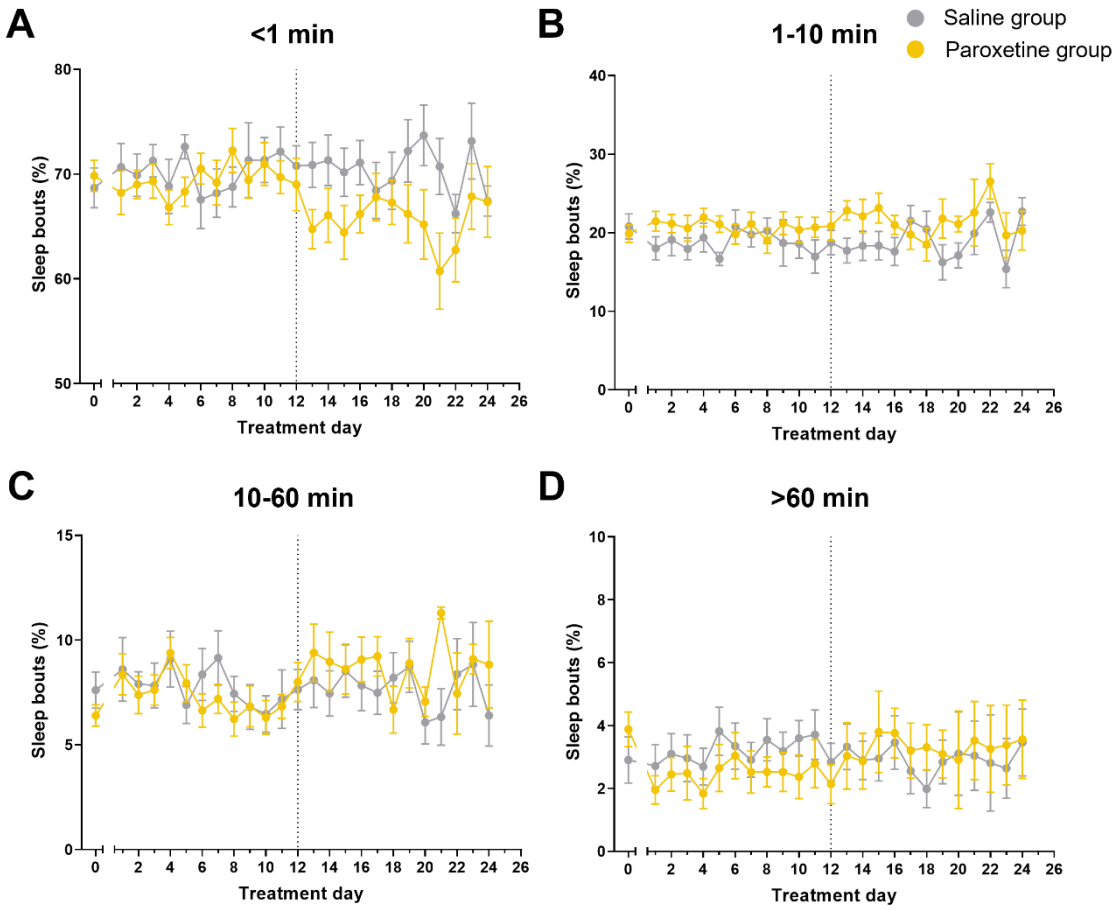


Figure 7.8 Effects of continued paroxetine and discontinuation on sleep bout duration in the light phase. Points represent the mean ± SEM of sleep bouts of <1 min (A), 1-10 min (B), 11-60 min (C) and >60 min duration (D). Dotted vertical line represents paroxetine discontinuation. Saline group (n=12), paroxetine group (n=12). Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test.

Table 7.2 Effects of continued paroxetine and discontinuation on median sleep bout duration in the light phase. Each value is a median and IQR [25th percentile, 75th percentile] in min. Saline group (n=12), paroxetine group (n=12).

	Group (median [25th,75th])			
Treatment group	**SAL**	**CON**	**DIS 2**	**DIS 5**
Saline	0.6 [0.5,0.7]	0.6 [0.5,0.7]	0.60 [0.5,0.7]	0.7 [0.5,0.7]
Paroxetine	0.6 [0.5,0.6]	0.5 [0.5,0.7]	0.50 [0.5,0.7]	0.7 [0.5,0.7]

3.8 Effects of paroxetine continuation and discontinuation on the stability of circadian rhythms

Across the experiment, the interday stability (the variation within one day compared to the rest of the experiment) was significantly lower in the paroxetine group than in the saline group ($t(22)=4.358$, $p=0.0003$; *Fig. 7.9A*). Nonetheless, there were no differences in the interday stability between paroxetine- and saline-treated mice when the baseline, dosing period (treatment days 1-12) or discontinuation periods (treatment days 13-25) were considered separately (baseline: $t(22)=0.7883$, $p=0.4384$; dosing: $t(22)=0.8558$, $p=0.4013$; discontinuation: $t(22)=1.115$, $p=0.2769$; *Fig. 7.9B*). The intraday variability (how the sleep-wake transitions varied within one day) did not differ between the saline and paroxetine groups ($t(22)=0.1730$, $p=0.8640$; *Fig. 7.9C*), or when the baseline, dosing and discontinuations periods were considered separately (baseline: $t(22)=0.3680$, $p=0.7164$; dosing: $t(22)=1.918$, $p=0.0682$; discontinuation; $t(22)=1.399$, $p=0.1751$; *Fig. 7.9D*).

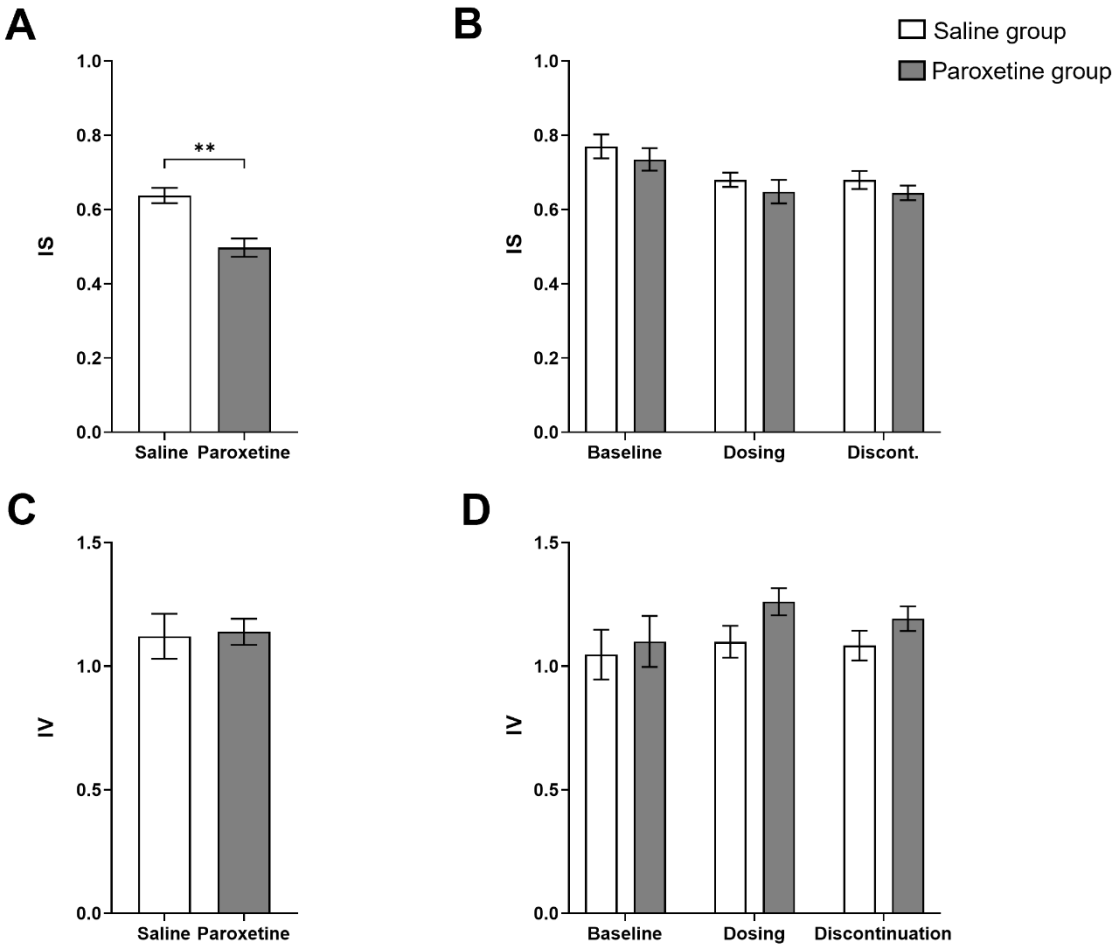


Figure 7.9 Effects of continued paroxetine and discontinuation on the stability of circadian rhythms. Bars represent mean ± SEM values of the interday stability (IS) (A) and IS by treatment period (B), and the intraday variability (IV) (C) and IV by treatment period (D). Saline group (n=12), paroxetine group (n=12). Student's t test, ** p<0.01.

3.9 Effects of paroxetine continuation and discontinuation on weight

Overall, mice receiving continued paroxetine treatment gained more weight than saline controls, whereas mice undergoing paroxetine discontinuation gained less weight than controls. All mice gained weight during the experiment (time: $F(25,550)=32.05$, $p<0.0001$). There was no effect of treatment but there was a significant treatment*day interaction (treatment: $F(1,22)=1.110$, $p=0.3036$; interaction: $F(25,550)=5.057$, $p<0.0001$; *Fig. 7.10A*). Between-subject pairwise comparisons found that mice receiving

continued paroxetine weighed significantly more than saline-treated mice at the end of paroxetine treatment (post-hoc day 10 p=0.0027, day 11 p=0.0142, day12 p=0.0367, day 14 p=0.0229; *Fig. 7.10A*).

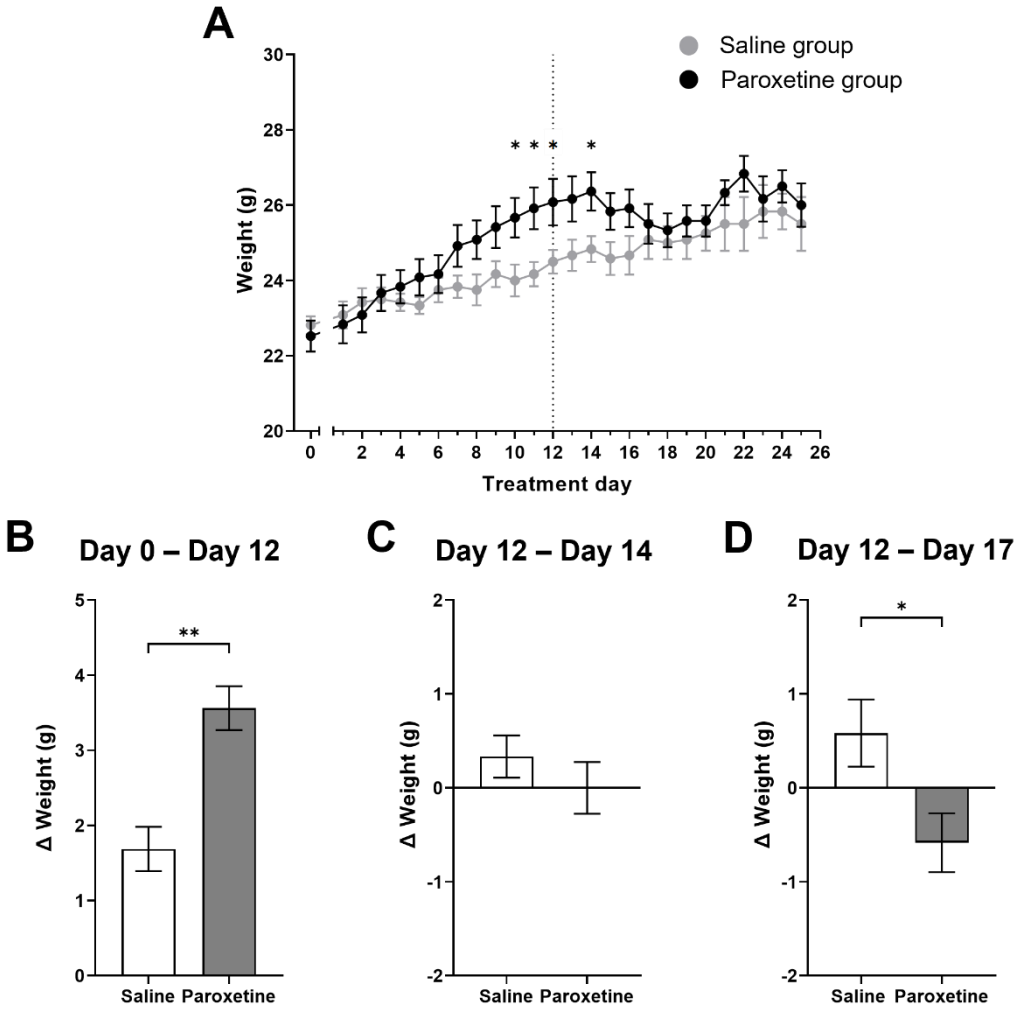


Figure 7.10 Effects of continued paroxetine and discontinuation on body weight. Points represent the mean ± SEM values for body weight each day (A). Dotted line represents paroxetine discontinuation. Bars represent the mean ± SEM values for weight change during paroxetine treatment (B), two days of discontinuation compared to the end of paroxetine treatment (C) and five days discontinuation compared to the end of paroxetine treatment (D). Saline group (n=12), paroxetine group (n=12). Mixed-effects model with Geisser-Greenhouse correction with post-hoc Bonferroni's test (A) or Student's t test (B-D), * p<0.05 ** p<0.01 between-subject differences.

Subsequent analysis showed that mice receiving paroxetine injections gained more weight than saline controls (t(22)=4.513, p=0.0002 treatment day 0 vs day 12; *Fig.*

7.10B). By comparison, two days after paroxetine discontinuation, there was no difference in weight gain between the paroxetine and saline groups (day 12 vs 14: $t(22)=0.9381$, $p=0.3584$; *Fig. 7.10C*). Mice five days after paroxetine discontinuation had gained significantly less weight than saline controls (day 12 vs 17: $t(22)=2.454$, $p=0.0225$; *Fig. 7.10D*).

4 Discussion

Insomnia and sleep disturbances are common symptoms of SSRI discontinuation in patients. The current chapter investigated whether passive, non-invasive monitoring of activity and sleep could be used to detect similar disruption in mice undergoing paroxetine discontinuation.

Continuous paroxetine treatment reduced activity in the dark phase compared to saline-treated controls, an effect that was particularly apparent in the first 8 h of the dark phase. Paroxetine administration was also associated with an increase in the time mice spent asleep (as defined by activity measurements) during the dark phase compared to controls, and specifically an increase in the proportion of sleep bouts of 10-60 min duration.

The effects of continuous paroxetine treatment reversed within 24 h of discontinuation. Importantly, during discontinuation there was evidence of a reduction in the number of <1 min sleep bouts and an increase in the number of 1-10 min sleep episodes, suggesting a lengthening of sleep bouts. This effect was evident for seven days after paroxetine cessation, providing the first evidence in this thesis of a behavioural effect of paroxetine discontinuation that persists beyond the second day of discontinuation. Notably, paroxetine discontinuation did not affect behaviour in the light phase. A summary of the results is presented in *Table 7.3*.

Table 7.3 Summary of the effects of continued paroxetine treatment and discontinuation on daily activity and sleep. ↑ increase, ↓ decrease, - no change, relative to saline controls or within-subject saline baseline period.

	Treatment		
Parameter	**Continued paroxetine**	**Discontinuation day 2**	**Discontinuation day 5**
Mean activity			
Dark phase	↓	-	-
Light phase	-	-	-
Percentage of time spent asleep			
Dark phase	↑	-	-
Light phase	-	-	-
Sleep latency	-	-	-
Number of sleep bouts			
Dark phase	↓	-	-
Light phase	-	-	-
Sleep bout duration			
Dark phase	↑ 10-60 min bouts	↓ <1 min bouts ↑ 1-10 min bouts	↓ <1 min bouts ↑ 1-10 min bouts
Light phase	-	-	-
Median sleep bout duration			
Dark phase	-	-	-
Light phase	-	-	-
Body weight	Increased weight gain	-	Reduced weight gain

4.1 Saline-treated mice followed typical activity and sleep patterns

In the current experiments, the activity of saline-treated mice was consistent throughout the experiment. Their activity peaked in the first 2 h of the dark phase, fell during the middle of the dark phase, and increased again in the final 2 h before the light phase. This regular pattern of sleep-wake activity is typical of wildtype C57BL/6J mice (Pernold et al., 2021). For instance, the peak in activity early in the dark phase is a common feature of C57BL/6 behaviour and likely reflects increased food consumption after sleep (Hossain et al., 2004; Peirson et al., 2018). The second activity peak in the final few hours of the dark phase is also characteristic, and reflects that mice are typically most active during light/dark transitions (Peirson et al., 2018).

By comparison, activity was low and sleep was high in the light phase. Light is the strongest environmental factor to which mice entrain their circadian rhythms (Peirson et al., 2018) and it is a much greater influence on sleep-wake behaviours than it is in humans. The reduction in activity during the light phase in control mice confirms that they did not have any underlying sleep disruption, which typically presents as increased activity in the light (Brown et al., 2019). The activity patterns were also stable throughout the experiment, with interday stability and intraday variability values mirroring those typical of C57BL/6 mice (Brown et al., 2019). This suggests that receiving daily saline injections did not disrupt the activity patterns of the mice and further validates the use of PIR monitoring for assessing circadian behaviours in this model.

4.2 Continued paroxetine treatment reduced activity and increased sleep in the dark phase

Although not the focus of the current study, continued paroxetine treatment produced marked changes in activity and sleep. Specifically, paroxetine reduced activity during the dark phase, an effect that was already apparent on the first day of its administration. The amount of time spent asleep also increased during the dark phase, with the number of sleep bouts decreasing but the proportion of longer sleep bouts increasing. The effects of paroxetine treatment on activity were generally very stable throughout the dosing period. Consequently, although the interday stability was lower in the paroxetine-treated mice (likely reflecting the changes from the baseline period), there was no difference in interday stability or intraday variability in any specific treatment period. This suggests that the activity patterns were equally stable in both groups, albeit at different absolute levels.

The activity-suppressant effects of paroxetine agree with previous reports. In a mouse model of Huntington's disease, chronic paroxetine treatment was associated with reduced activity in the dark phase compared to vehicle-treated controls, without changes to light phase behaviour (Kantor et al., 2017; Ouk et al., 2018). Reduced spontaneous LMA was also detected in mice treated for 28 days with paroxetine compared to saline controls (Chapter 3). Moreover, the most robust effects of paroxetine were seen in the first 8 h after its administration, mirroring the effect it produced in a previous study of circadian activity in rats (Gervasoni et al., 2002). The timing of this effect may reflect the short half-life of paroxetine (Kreilgaard et al., 2008), with the greatest effects occurring when its plasma, and ultimately brain, concentrations were at their highest.

It is noteworthy that paroxetine-treated mice exhibited increased activity in the final 2 h of the dark phase. Although the cause of this effect cannot be ascertained from the current data, it could reflect compensatory activity following suppression earlier in the dark phase. As C57BL/6 mice typically spend the first few hours of the dark phase eating (Hossain et al., 2004), paroxetine treatment may have initially reduced food consumption, hence mice needed to eat more later in the dark phase. Alternatively, this altered activity pattern could be evidence of phase shifting and circadian disruption arising from increased 5-HT signalling in the SCN (Wichniak et al., 2017). Further experiments characterising the behaviour of these mice throughout the dark phase, for example video tracking to simultaneously assess eating, or measuring food intake with other in-cage methods, are needed to determine the exact behavioural changes that occurred.

Mice receiving continuous paroxetine treatment also showed evidence of increased sleep compared to saline controls. The COMPASS system uses 40 s of immobility to estimate 'behaviourally-defined sleep' (Pack et al., 2007; Fisher et al., 2012; Brown et

al., 2016), hence reduced activity was interpreted as increased sleep. There was also a reduction in the number of sleep episodes during the dark phase, but sleep was enriched with longer sleep bouts of 10 to 60 min in duration, explaining the overall increase in the percentage of time spent asleep.

Other studies also suggest that SSRIs can increase sleep in rodents, and specifically slow wave sleep (Wichniak et al., 2017). For instance, the same dose of paroxetine as used here increased the total time mice spent in NREM sleep in the dark phase but had no effect in the light. Another study also reported that paroxetine reduced the number of NREM sleep episodes compared to vehicle-treated mice (Kantor et al., 2017). These findings could imply mice receiving paroxetine treatment had increased sleep pressure, which presents as increased sleep when the opportunity arises (Campbell & Tobler, 1984). Alternatively, it could indicate weaker circadian rhythms, as the mice were less able to stay awake despite light cues (i.e., darkness).

It is also possible that paroxetine has a sedative effect. Other classes of antidepressants, such as TCAs, have well known sedative effects due to their anticholinergic and antihistaminergic properties (Steriade, 2004; Wichniak et al., 2017). Paroxetine also has a relatively high affinity for muscarinic acetylcholine receptors (Wilson & Argyropoulos, 2005; Sanchez et al., 2014), potentially contributing to its propensity to suppress activity and promote sleep.

4.3 Discontinuation reversed the effects of paroxetine treatment within 24 h

Common symptoms of SSRI discontinuation in humans include insomnia and sleep disturbances, suggesting SSRI discontinuation could be associated with reduced sleep and increased sleep fragmentation in mice. This effect would be consistent with the findings

in Chapters 4 and 5 that paroxetine discontinuation increased 5-HT transmission. On the other hand, the rapid fall in basal hippocampal extracellular 5-HT following paroxetine discontinuation (Chapter 5) would hypothetically increase sleep. These conflicting hypotheses made the effects of paroxetine discontinuation on sleep-wake behaviours difficult to predict.

In the current experiments, paroxetine discontinuation was associated with a rapid reversal of the effects of continuous paroxetine treatment. Reduced daily activity and evidence of increased sleep returned to baseline levels within just 24 h of the first missed dose of paroxetine, where they remained for the rest of the two week discontinuation period. Previous studies support these findings. Following three weeks of repeated treatment in rats, the REM-suppressant effects of paroxetine reversed within 24 h of its washout (Gervasoni et al., 2002). NREM also did not differ from saline controls two weeks after discontinuation of paroxetine treatment in mice (Kantor et al., 2017). Although different sleep states could not be determined here, these findings support the notion that sleep rapidly reverts to control levels following treatment cessation.

There were also no effects of paroxetine discontinuation on activity or sleep during the light phase. Increased light phase activity is generally a hallmark of circadian disruption (Brown et al., 2019), so the lack of effect here provides further evidence that paroxetine discontinuation was not associated with broad circadian disruption.

4.4 Paroxetine discontinuation increased sleep bout duration in the dark phase

While there was little evidence to suggest that paroxetine discontinuation caused significant disruption to the sleep-wake cycle, there was subtle evidence that sleep architecture may have changed immediately after paroxetine discontinuation. In the seven

days after the end of paroxetine treatment, the proportion of sleep bouts lasting less than 1 min was reduced, and the proportion of 1-10 min sleep bouts increased, compared to the baseline period. These changes suggest a lengthening of sleep episodes during the dark phase, in that when mice slept, they slept for longer.

There are several interpretations of this finding. Firstly, it could suggest weakened SCN activity. The role of the SCN is to prevent sleep at inappropriate times, meaning mice are kept awake during the dark phase despite their increasing homeostatic drive to sleep (Peirson et al., 2018). The enrichment of dark phase sleep with longer sleep bouts could imply a weakened clock mechanism, hence mice would have been more susceptible to homeostatic drive and thus have slept for longer in each episode.

Alternatively, these data could be related to a rebound increase in REM sleep, which is reported to occur following SSRI cessation. For rodents, the probability of entering REM sleep grows as the length of the sleep bout increases (Kantor et al., 2017). Moreover, mice with REM deficits showed evidence of reduced sleep overall, suggesting changes in REM can alter overall sleep structure (Banks et al., 2020). By this logic, increased REM sleep could present as increased sleep bout durations. There is also evidence that when REM suppression is lifted, for instance after chronic SSRI treatment, more REM sleep occurs to compensate for the previous deficit (Vogel et al., 1975). Thus, longer sleep bouts could suggest mice were more likely to be in REM sleep than controls.

Nevertheless, this effect is purely speculative, as the PIR system is not able to differentiate REM from NREM sleep. Other studies have also not detected REM rebound in rodents following paroxetine washout, at least at similar time points to those studied here (Gervasoni et al., 2002). EEG studies are therefore needed to determine if a rebound increase in REM sleep can be detected in the week following paroxetine discontinuation.

4.5 Persistent effects of paroxetine discontinuation

Previously, increased anxiety-like behaviour was evident two days after paroxetine discontinuation, but this effect had dissipated by day five (Chapter 3). The shift in the distribution of sleep episodes up to seven days after paroxetine discontinuation therefore provides the first evidence in this thesis that SSRI cessation produces behavioural effects in mice beyond the second day after treatment cessation.

Moreover, the lack of weight gain five days after paroxetine discontinuation, an effect that was also seen in Chapter 3 (section 3.3), suggests there may be hitherto unappreciated physiological changes in mice following paroxetine discontinuation. These results could indicate increased stress, as weight loss and low appetite can be indicative of poor wellbeing in mice (Talbot et al., 2019). Alternatively, a lack of weight gain could indicate GI disruption, another common symptom of SSRI discontinuation syndrome. Weight loss could also relate to metabolic changes following paroxetine discontinuation, as there is extensive evidence that 5-HT is involved in the central and peripheral control of metabolism (Yabut et al., 2019).

Thus, there may be ongoing changes to appetite, metabolism, or general wellbeing in the week following paroxetine discontinuation that measures of anxiety-like behaviour were not able to detect. These enduring effects of paroxetine discontinuation more closely mirror the prolonged symptoms often experienced by patients, adding to the face validity of the mouse model. These findings warrant further experiments to characterise the other behavioural and somatic changes associated with SSRI discontinuation in mice.

4.6 Limitations of PIR activity monitoring

Passive, non-invasive monitoring is a valuable continuous screen of mouse activity and sleep in the weeks following paroxetine discontinuation. It was advantageous over the test batteries used in Chapter 3, in that the same behaviour could be measured repeatedly over time. Consequently, more data could be collected from fewer mice. It was also a simple, non-invasive approach to monitor sleep indices in mice, avoiding the issues that can arise during chronic EEG recordings (discussed in section 1.6).

Nevertheless, PIR monitoring cannot differentiate between sleep states. The inability to measure REM sleep with this method meant that key questions regarding REM rebound during SSRI discontinuation could not be addressed. Moreover, although PIR estimations of sleep based on 40 s of inactivity are very similar to EEG-defined sleep (Brown et al., 2016; Krone et al., 2021), they tend to overestimate sleep due to the lack of differentiation between quiet wakefulness and sleep (Pack et al., 2007). As ever, EEG recordings are needed to explore sleep-wake architecture to validate what is an interesting but ultimately preliminary result.

The light/dark cycle that mice were housed under also somewhat limits our ability to detect sleep effects. Light is the main cue to entrain sleep to the environment in rodents (Peirson et al., 2018), and it has been shown to supress activity via the SCN and outputs to the sympathetic nervous system. Thus, light can force sleep in rodents regardless of sleep pressure (Mrosovsky & Hattar, 2003). Consequently, turning the lights on at the same time each day could have obscured underlying circadian dysregulation that would have been evident in free running (24 h darkness) conditions. An interesting future experiment would be to investigate paroxetine discontinuation under constant darkness conditions to avoid the sleep-promoting effects of light.

Furthermore, mice had to be single housed in the current experiment, which may have produced different behavioural consequences to group housing in the previous chapters. The PIR system provides data on total cage movement; thus, it is not possible to track the activity of individual mice if they are group housed. Nonetheless, group housing may have affected activity due to thermoregulatory effects of multiple animals living together, or the different social dynamics (e.g., Hennessy et al., 2009; Lidster et al., 2019; Karamihalev et al., 2020). It is therefore difficult to determine if the behaviour of these animals was similar to the group housed mice in previous experiments.

4.7 Role of 5-HT in sleep during paroxetine discontinuation

It is interesting to consider how changes to 5-HT signalling may have modulated sleep following paroxetine discontinuation. Given extensive evidence that 5-HT promotes wakefulness (discussed in section 1.3), the reduction in sleep following paroxetine discontinuation (back to saline control levels) is consistent with the evidence of increased 5-HT transmission in Chapters 4 and 5.

On the other hand, the lengthening of sleep episodes in the week following SSRI cessation would suggest reduced 5-HT transmission. Low extracellular 5-HT would also promote REM sleep and potentially contribute to REM rebound in patients. Moreover, the 5-HT_{1A} receptor is a key mediator of the effects of 5-HT on sleep and reduced 5-HT_{1A} receptor function is associated with increased REM sleep (Monaca et al., 2003). The reduction in *5-HT_{1A}* receptor mRNA two days after paroxetine discontinuation, as reported in Chapter 5, could contribute to the increase in REM sleep that patients reportedly experience following discontinuation, and potentially the effects on sleep episode duration seen here.

Nonetheless, there is no clear association between the behavioural effects of SSRI cessation on sleep-wake activity and unidirectional changes to 5-HT transmission. Further work is therefore needed to investigate how changes in 5-HT signalling following paroxetine discontinuation may be modulating sleep and circadian activity in mice.

4.8 Conclusions

Experiments in this chapter found evidence that paroxetine discontinuation was associated with a rapid reversal of the effects of continuous paroxetine treatment on homecage activity and sleep. Nonetheless, there was also evidence of a lengthening of sleep episodes during the dark phase for seven days following paroxetine cessation, providing the first evidence of a persistent behavioural effect of discontinuation in this model. These findings present another way in which the behavioural effects of paroxetine discontinuation can be monitored. Future experiments could investigate how these alterations in sleep architecture may relate to changes in 5-HT transmission.

Chapter 8
General Discussion

1 Summary of main findings

Cessation of SSRI treatment can produce a debilitating discontinuation syndrome, including symptoms such as anxiety and sleep disturbances. Prior to this thesis, however, there had been very few preclinical studies of the condition. Increased anxiety-like behaviour had been demonstrated in the week following citalopram discontinuation in rats, and there was some evidence of changes to 5-HT neurochemistry (Caccia et al., 1993; Trouvin et al., 1993; Bosker et al., 2010; Guiard et al., 2012). Nonetheless, there had not been a systemic preclinical investigation of the effects of SSRI discontinuation, and it was not known if other symptoms of the syndrome could be detected in mice. Finally, there had been no investigations of the potentially causal link between altered 5-HT transmission and the behavioural effects of SSRI discontinuation.

This thesis aimed to establish a mouse model of SSRI discontinuation syndrome, which could be used to investigate changes in the 5-HT system. Experiments focused on paroxetine due to its propensity to cause discontinuation symptoms (Price et al., 1996; Gastaldon et al., 2022). Experiments in Chapter 3 found evidence that two days of paroxetine discontinuation increased anxiety-like behaviour and behavioural inhibition in male mice on the EPM compared to continued paroxetine and saline controls. This effect was short-lasting, having dissipated by discontinuation day five, and required at least 12 days of paroxetine treatment before its emergence. There was also a small increase in anxiety following citalopram and venlafaxine discontinuation.

c-FOS immunohistochemistry experiments in Chapter 4 found that paroxetine discontinuation was associated with the persistent and specific activation of 5-HT neurons in the raphe nuclei, including a rebound increase in DRN 5-HT neuron activation. In Chapter 5, analysis of regional brain tissue samples provided evidence that reduced 5-HT synthesis and metabolism during continued paroxetine recovered quickly after discontinuation and rebounded above saline controls on discontinuation day five. *In vivo* microdialysis experiments showed that elevated basal hippocampal extracellular 5-HT fell to control levels within two days of paroxetine discontinuation. Yet, 5-HT terminals in the VH appeared more sensitive to potassium-evoked depolarisation at two and five days after discontinuation. Gene expression analysis also found evidence of a reduction in *5-HT$_{1A}$* receptor mRNA on the second day after paroxetine cessation. It was therefore hypothesised that altered 5-HT transmission may cause the heightened anxiety-like behaviour evident following discontinuation. Nonetheless, neither reducing 5-HT neuronal activation (and thus extracellular 5-HT) nor increasing neuronal activation with 5-HT$_{1A}$ receptor ligands precipitated the behavioural effects of discontinuation in mice receiving paroxetine treatment (Chapter 6).

Finally, the effect of paroxetine discontinuation on sleep-wake behaviour was explored in Chapter 7. These experiments found evidence of changes in activity patterns that could suggest altered sleep architecture for seven days after paroxetine cessation. These findings provided the first evidence in this thesis that behavioural effects persisted past the second day of discontinuation, and that this mouse model could produce other behavioural correlates of the clinical discontinuation syndrome.

In summary, two days after paroxetine discontinuation mice showed evidence of an increase in anxiety-like behaviour. This was temporally associated with a reduction in

extracellular 5-HT in the VH, suggesting that 5-HT fell following paroxetine cessation. Nonetheless, there was also evidence of a rebound activation of the 5-HT system, in that there was an increase in 5-HT neuron activation in the DRN, a recovery of regional brain tissue levels of 5-HT and 5-HIAA, and a rebound increase in the sensitivity of 5-HT terminals to potassium-evoked depolarisation above control levels.

2 By discontinuation day five, however, the anxiogenic effect of paroxetine discontinuation had dissipated. While basal extracellular 5-HT remained at saline-control levels, there was still evidence of a rebound activation of the DRN and increased 5-HT terminal sensitivity, as well as an emergent rebound in 5-HT synthesis and metabolism. Thus, the short-lived behavioural effects of paroxetine discontinuation were dissociated from the ongoing changes to the 5-HT system. Role of 5-HT in SSRI discontinuation symptoms

2.1 Effect of SSRI discontinuation on the 5-HT system

The current experiments found evidence that paroxetine discontinuation is associated with a rebound increase in 5-HT transmission following a period of prolonged 5-HT neuron suppression. Microdialysis experiments showed that continued paroxetine treatment increased basal hippocampal extracellular 5-HT, which likely activated 5-HT_{1A}

autoreceptors and suppressed 5-HT neuron excitability (*Fig 8.1A*). Within two days of paroxetine discontinuation, however, extracellular 5-HT fell to control levels, likely due to increased 5-HTT availability and 5-HT reuptake, reducing 5-HT_{1A} autoreceptor activation. Combined with evidence of reduced *5-HT$_{1A}$* receptor mRNA in the raphe nuclei, it is hypothesised that this reduction in autoreceptor activation would have disinhibited 5-HT neurons (*Fig. 8.1B*), increasing 5-HT release.

This hypothesised mechanism suggests that complex temporal changes occurred in the 5-HT system in the days following discontinuation, whereby an initial reduction in extracellular 5-HT led to a rebound increase in 5-HT neuron excitability. This idea partially supports the hypothesis put forward in several reviews that a drop in extracellular 5-HT may be the trigger for the discontinuation syndrome (Blier & Tremblay, 2006; Warner et al., 2006; Renoir, 2013; Harvey & Slabbert, 2014). It would also be consistent with suggestions that tryptophan depletion induces discontinuation reactions in patients taking SSRIs (Delgado, 2006). Yet, this theory also agrees with the extensive preclinical literature suggesting that acute increases in anxiety-like behaviour are associated with increased 5-HT transmission in rodents (for example, Ohmura et al., 2014, Ohmura et al., 2020, and Teissier et al., 2015).

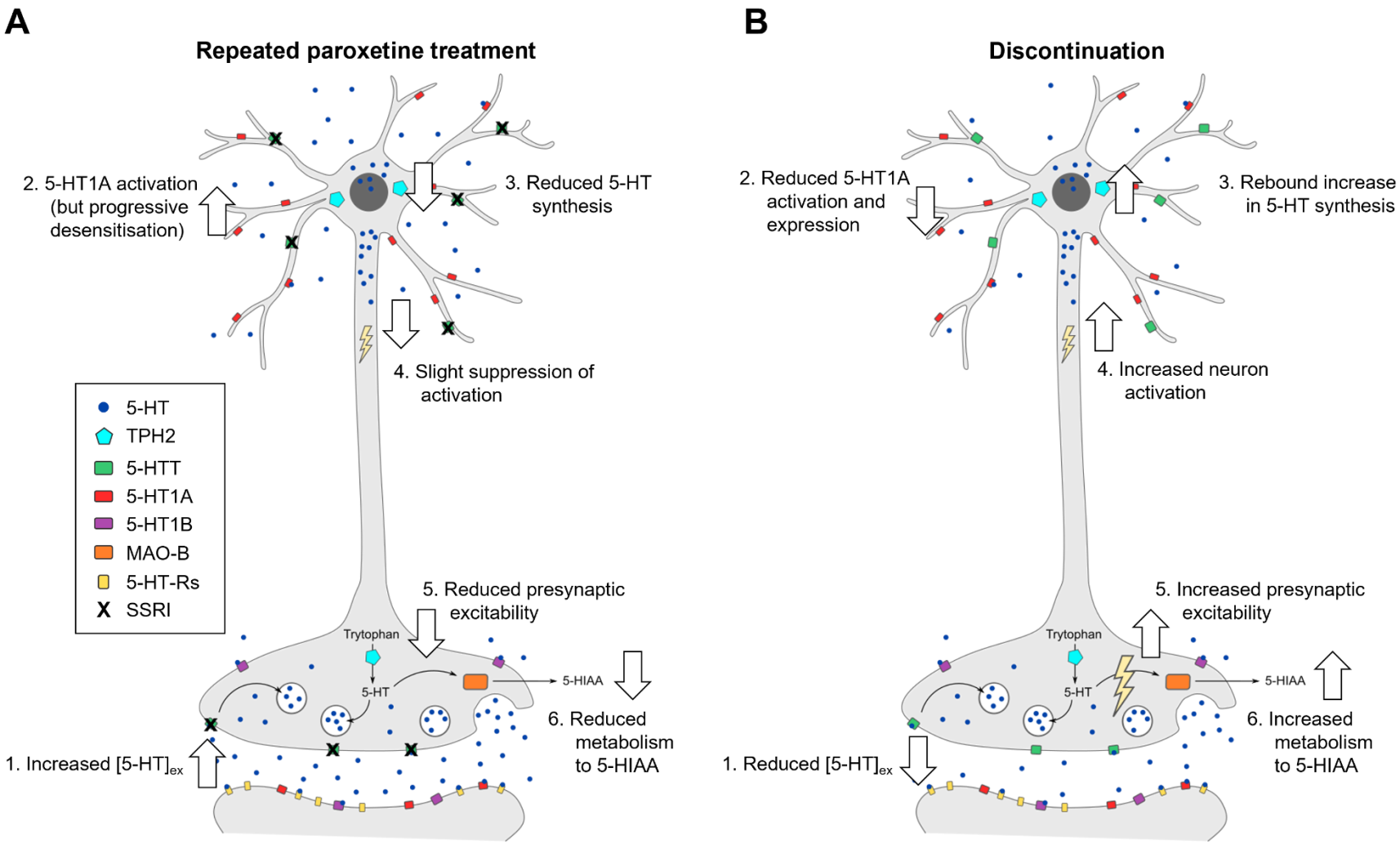


Figure 8.1 Proposed mechanism underlying the rebound increase in 5-HT transmission associated with paroxetine discontinuation. 5-HT neuron during repeated paroxetine treatment (A) and following discontinuation (B). $[5\text{-HT}]_{ex}$, extracellular 5-HT. Adapted from Harvey & Slabbert (2014) and extended.

This theory also suggests that basal and evoked 5-HT transmission may change in opposite directions following discontinuation. For example, at rest 5-HT transmission may return to baseline levels, hence basal hippocampal 5-HT did not differ from that of saline controls. Yet, 5-HT neurons appeared more sensitive to potassium infusions, suggesting that 5-HT release could be evoked by smaller stimuli. Thus, when the 5-HT system was stimulated, 5-HT neuron disinhibition became apparent as a greater response to that stimulus.

Nonetheless, from the current data it is not possible to conclude whether such changes in 5-HT transmission directly mediated the behavioural effects of SSRI discontinuation. For instance, the temporal dissociation between the behavioural and neurochemical effects of paroxetine discontinuation suggest that increased 5-HT transmission was not causal in the elevated anxiety-like behaviour, as it persisted even after behaviour recovered to control levels.. Moreover, pharmacologically manipulating the 5-HT system did not precipitate paroxetine discontinuation. The recovery of sleep to control levels but the lengthening of sleep bouts in the dark phase during paroxetine discontinuation can also not solely explained by the changes in 5-HT transmission outlined above. Yet, the possibility that a rebound increase in 5-HT transmission could have produced the behavioural effects should not be excluded. For instance, the experimental approach used to precipitate paroxetine discontinuation may not have been optimal (as discussed in Chapter 6 section 4.4). Moreover, increased 5-HT transmission may have increased anxiety on discontinuation day two, but there may have been other compensatory influences on behaviour on discontinuation day five (discussed in Chapter 4 section 4.2). These might reflect changes in the sensitivity or functionality of postsynaptic 5-HT receptors, inputs from other brain regions or changes in the balance of

activity in different subregions of the DRN or MRN (Ren et al., 2018; Ohmura et al., 2020). Thus, 5-HT neuron activity may have remained high, but the effect on anxiety-like behaviour was no longer evident.

Furthermore, increased 5-HT neuron activity could contribute to other aspects of the discontinuation syndrome, such as the somatic symptoms, which have not yet been investigated in this model. For instance, neuronal hyperexcitability has been linked to the 'electric-shock like' sensations (Coupland et al., 1996), and increased 5-HT_3 receptor activation can induce nausea and vomiting (Navari, 2015). Vertigo and dizziness have also been linked to altered 5-HT transmission in the vestibular system (Wilson et al., 2005; Soto et al., 2013). Although these symptoms would be more difficult to study in rodents, they present interesting opportunities to explore the role of 5-HT transmission in the broader discontinuation syndrome.

Thus, while the current experiments suggest that a rebound increase in 5-HT transmission was a consequence of SSRI cessation, they could not demonstrate that altered 5-HT transmission caused the behavioural effects. Nonetheless, an alternative approach to precipitating SSRI discontinuation may be able to prove this causal link. These data also cannot preclude the possibility that altered 5-HT transmission is responsible for other discontinuation symptoms.

2.2 Evidence implicating the 5-HT_{1A} autoreceptors

According to the current hypothesis, reduced 5-HT_{1A} autoreceptor function may have contributed to the rebound increase in 5-HT transmission following paroxetine discontinuation. There is also evidence that altered 5-HT_{1A} autoreceptor activation may be linked to the discontinuation syndrome in humans. For example, the C(-1019)G

polymorphism in the 5-HT_{1A} receptor gene was associated with an increased risk of experiencing symptoms after paroxetine discontinuation (Huang et al., 2004; Murata et al., 2010). Moreover, reducing 5-HT_{1A} receptor activation with WAY-100635 increased REM sleep in rats (Boutrel et al., 2002; Monaca et al., 2003), resembling REM rebound during SSRI discontinuation. Together, these studies further implicate the 5-HT_{1A} receptor as being involved in the mechanism underlying the discontinuation symptoms.

Nonetheless, it is difficult to imply the causal role of 5-HT_{1A} receptor signalling as the rate at which the autoreceptors resensitise following SSRI cessation is uncertain. Notably, other studies suggest that the 5-HT_{1A} autoreceptors remained desensitised for one to three days after SSRI discontinuation in rats (Invernizzi et al., 1994; Bosker et al., 1995). Further work is therefore needed to determine how the function of the 5-HT_{1A} autoreceptors changes over time following paroxetine cessation.

Notably, while the 5-HT_{1A} autoreceptors have been the focus of these experiments, the 5-HT_{1B} autoreceptors may also be involved. The rapid drop in extracellular 5-HT upon discontinuation would also have reduced the activation of 5-HT_{1B} autoreceptors and disinhibited the 5-HT terminals. This would have increased the excitability of presynaptic 5-HT terminals, potentially explaining the increase in depolarisation-evoked 5-HT release. Given 5-HT_{1A} receptor agonists and antagonists were unable to precipitate paroxetine discontinuation, future experiments could investigate if changes in 5-HT_{1B} autoreceptor activation, and thus terminal 5-HT release, contribute to the behavioural effects of paroxetine discontinuation.

2.3 Evidence implicating the DRN and VH

c-FOS immunohistochemical studies demonstrated that the DRN and to some extent the MRN were persistently activated following paroxetine discontinuation, and several experiments implicated a role for the VH. *Ex vivo* and *in vivo* neurochemical analysis found evidence of an upregulation of 5-HT synthesis and release in the hippocampus. c-FOS immunohistochemistry also showed that two days of paroxetine discontinuation was associated with elevated VH activation, and yet this effect was not present five days after cessation. These results could suggest that the activation of 5-HT projections to the VH are involved in generating the anxiety-like effects of paroxetine discontinuation, as VH activity shows a clearer relationship with the behavioural effects of discontinuation than 5-HT neuron activation. Increased VH activity during elevated anxiety also agrees with the findings of other studies (Ohmura et al., 2020) and the theorised role of 5-HT in promoting anxiety in rodents via the recruitment of the VH (McNaughton & Gray, 2000; Bannerman et al., 2003; Bannerman et al., 2004).

Nonetheless, the analysis of brain regions following paroxetine discontinuation was far from exhaustive, meaning similar effects may have occurred in other emotion-related brain structures. These include other subregions of the amygdala, cortical regions such as the ACC, and other components of the limbic system. It is also difficult to speculate which, if any, 5-HT-containing projections are responsible for the behavioural effects of paroxetine discontinuation. This is due to the complex, bidirectional circuits between the raphe nuclei, hippocampal subregions, and other components of the anxiety-promoting circuitry (Jacobs & Azmitia, 1992; Felix-Ortiz et al., 2013; Parfitt et al., 2017).

Furthermore, it is unlikely that the same neural circuits are responsible for the diverse behavioural effects of SSRI cessation. As previously discussed, sleep and anxiety are controlled by distinct brain regions. Yet, regional brain tissue analysis demonstrated

that paroxetine discontinuation produced broad, non-region-specific changes to 5-HT synthesis and metabolism. Thus, SSRI discontinuation likely affects the 5-HT system indiscriminately. Nevertheless, specific symptoms may be driven by different raphe nuclei, subregions of the DRN, or 5-HT-containing projections. For instance, the raphe nuclei may activate the VH to mediate anxiety-like behaviours, whereas projections to the BF may regulate sleep-wake behaviours. Future experiments could use optogenetics or chemogenetics to activate specific 5-HT pathways to investigate their causal role in the behavioural effects of SSRI discontinuation in mice. Additionally, fMRI in mice (such as in Salvan et al., 2022) could be used to identify novel brain circuits involved in the discontinuation symptoms in a less targeted, more exploratory manner.

2.4 Potential role of other neurotransmitters

Given that SSRIs target the 5-HTT, these experiments focused on the 5-HT system. Nonetheless, other neurotransmitters may also be involved in the generation of SSRI discontinuation symptoms. For example, increased noradrenergic transmission has been linked to heightened anxiety-like behaviour in rodents (reviewed in Morris et al., 2020), and NA has been implicated in the human discontinuation syndrome (Blier & Tremblay, 2006). 5-HT also interacts with the noradrenergic system, creating a complex relationship between 5-HT, NA, and behaviour (Lucki & O'Leary, 2004). Moreover, paroxetine inhibits NET to a greater extent than other SSRIs (Delgado, 2006; Renoir, 2013) and SNRIs also produce discontinuation symptoms (Fava et al., 2015; Gastaldon et al., 2022), further alluding to a role for noradrenergic signalling in the symptoms.

Enhanced cholinergic transmission has also been linked to antidepressant discontinuation symptoms (Dilsaver & Greden, 1984; Blier & Tremblay, 2006; Renoir,

2013). Cholinergic signalling in the VH has also been shown to promote anxiety-like behaviours in mice (Mei et al., 2020). Given paroxetine also antagonises M1 acetylcholine receptors (Fujishiro et al., 2002; Sanchez et al., 2014), cholinergic rebound may also contribute to the behavioural effects seen here. Future *ex vivo* and *in vivo* neurochemical experiments should therefore measure tissue and extracellular concentrations of NA and acetylcholine to determine if other neuromodulators are disrupted following SSRI cessation.

Glutamate has also been implicated in the discontinuation syndrome (Harvey et al., 2002; Harvey et al., 2003). Discontinuation from chronic imipramine treatment increased the density of N-methyl-D-aspartate (NMDA) receptor binding sites in the mouse cortex compared to on-drug controls, suggesting increased glutamate signalling may be involved in the discontinuation symptoms (Skolnick et al., 1996). As yet, the possibility that SSRI discontinuation alters glutamatergic transmission has not been explored experimentally. Nevertheless, increased glutamatergic transmission in the VH has been shown to promote anxiety-like behaviour in rodents (Häckl & Carobrez, 2007). Moreover, a subpopulation of 5-HT neurons co-release glutamate (Sengupta et al., 2017), and some of these neurons project from the DRN to the VH (Fortin-Houde et al., 2022). Thus, 5-HT neuron activation following paroxetine discontinuation may also alter fast glutamatergic co-release in downstream brain regions. The behavioural effects of disrupting the balance of 5-HT and glutamate transmission should therefore be explored following paroxetine discontinuation, for example in transgenic mice lacking 5-HT-glutamte co-transmission (Mansouri-Guilani et al., 2019).

Finally, the behavioural effects of paroxetine discontinuation in mice could reflect altered stress responses by the hypothalamic-pituitary-adrenal (HPA) axis (Harvey et al.,

2003). Numerous studies have shown that 5-HT activates the HPA axis (such as Young et al., 1994, Harmer et al., 2003b, Heisler et al., 2007, and Goel et al., 2014). Moreover, corticosterone has been shown to increase 5-HT transmission in the VH; and vice versa, the VH modulates HPA activation (Jacobson & Sapolsky, 1991; Kjelstrup et al., 2002; Barr & Forster, 2011). This suggests a complex relationship between 5-HT and glucocorticoid signalling in the VH that could be affected by abrupt SSRI cessation. This could be explored further by measuring plasma corticosterone levels in mice during SSRI discontinuation. The activation of key components of the HPA axis, such as the paraventricular nucleus of the hypothalamus, could also be studied using c-FOS immunohistochemistry.

Thus, altered noradrenergic, cholinergic, and glutamatergic transmission, as well as changes in the neuroendocrine system, could be involved in the symptoms of SSRI cessation. These systems may also interact with 5-HT transmission to complicate the relationship between the neurochemical and behavioural effects of SSRI cessation.

3 Evaluation of the model

3.1 Similarities between the mouse model and the human condition

The principal aim of this thesis was to optimise a mouse model of SSRI discontinuation syndrome. Experiments in Chapter 3 found that 12 days of once-daily paroxetine administration produced a robust increase in anxiety-like behaviour. Like the clinical syndrome, the behavioural effects of paroxetine discontinuation were greater than those of citalopram or venlafaxine (Price et al., 1996; Fava et al., 2015; Gastaldon et al., 2022). Furthermore, increased anxiety-like behaviour was evident just two days after

paroxetine cessation, mirroring when patients first report symptoms (Michelson et al., 2000). Assessment of the circadian rhythms of mice undergoing paroxetine discontinuation detected changes in sleep structure for seven days following treatment cessation (Chapter 7), which resembles the period of sleep disturbances often experienced by patients (although see caveats in section 3.2). These findings could be taken to suggest that the current model has good face and translational validity for the human discontinuation syndrome.

3.2 Limitations of the model

Conversely, this model has several caveats. Firstly, mice were only treated for 12 days before discontinuation. This duration was based on a previous study of repeated paroxetine treatment in mice (Elizalde et al., 2008). Moreover, 12 days of treatment produced similar anxiogenic effects following discontinuation to 28 days of treatment,, yet they were not present after just 7 days of treatment. These findings suggest that the behavioural and neurochemical effects observed were not just those of acute paroxetine treatment. Nonetheless, patients often take antidepressants for months and even years before discontinuation. Consequently, there may be additional adaptive changes to 5-HT transmission or neuroplasticity in patients that this model could not recapitulate, which may have limited the subsequent discontinuation effects

While the SSRI doses used here are typical for preclinical experiments (Elizalde et al., 2008; Karlsson et al., 2011; Venzala et al., 2012), they are much higher than the equivalent doses used in humans (Taylor et al., 2021). Although this accounts for the faster metabolism of SSRIs in rodents, it may have contributed to the anxiogenic effects of paroxetine (Sánchez & Meier, 1997). Moreover, the bolus *s.c.* administration of the

drugs may have limited the plasma steady state of paroxetine and thus the neuroadaptive changes it could produce. Future work could investigate whether similar discontinuation effects could be produced by lower doses of paroxetine but administered continually, for example via the drinking water or osmotic minipumps, to avoid its confounding anxiogenic effect. This may also better model the effects of prolonged SSRI treatment and their discontinuation in patients.

Another feature of the model was that increased anxiety-like behaviour was evident for just two days following paroxetine discontinuation and changes in sleep structure had recovered to baseline levels within a week. In contrast, there are reports of symptoms lasting for many months in some patients (Davies & Read, 2019). This incongruency suggests that mice may only experience acute withdrawal effects and not those of post-discontinuation syndromes. This limits the face validity of this model as it could not recapitulate one of the key issues regarding SSRI discontinuation syndrome – that some patients can experience severe symptoms for long periods after treatment cessation. Thus, while this paradigm may be able to detect immediate behavioural effects of SSRI discontinuation, its long-term effects on behaviour cannot be studied.

Finally, these experiments used wild type mice rather than high anxiety models or mice with depressive traits. Given that SSRIs are prescribed for a range of mental health and physiological illnesses, it would have been premature to investigate SSRI cessation in the context of just one of these conditions. The use of wild type mice also differentiated the effects of discontinuation from relapse, as there was no previous anxious or depressive state to return to. Nonetheless, it is possible that some discontinuation effects may depend on the changes SSRIs make to disrupted neurotransmitter systems or deficits in neuroplasticity present in a mood disorder. For instance, one effect of SSRI treatment is

to counteract the sleep disruption characteristic of depression (Abad & Guilleminault, 2005; Kaplan & Harvey, 2009; Wulff et al., 2010). Paroxetine treatment and its subsequent discontinuation may therefore have produced greater or qualitatively different effects on sleep if treatment had been given to 'depressed' or 'anxious' mice. It would therefore be interesting to investigate the effects of SSRI discontinuation in mouse models of mood disorders, for example mice exposed to a chronic stress paradigm or early life maternal separation (Surget et al., 2008; David et al., 2009; Venzala et al., 2012). This may elucidate additional behavioural or neurochemical effects of SSRI cessation. It would also be pertinent to confirm the generalisability of the current findings to diseased states.[8]

3.3 Limitations of animal models of mood disorders

There are also broader issues with animal models of psychiatric disorders. One of the biggest problems facing preclinical models is that they aim to mimic subjective and self-reported human experiences. This is a particular challenge for modelling mood disorders; in the case of anxiety, the human feelings of worry, unease and tension are equated to behaviours such as approach-avoidance conflict in rodents (Griebel & Holmes, 2013; Slattery & Cryan, 2014; Sartori & Singewald, 2019). A great deal of anthropomorphising is needed to relate the performance of a mouse on a test such as the EPM to the human condition. Moreover, performance in these paradigms can be confounded by other behavioural effects of treatment, such as LMA suppression, or potentially by somatic effects of drug withdrawal such as dizziness and GI disturbances.

[8] On the other hand, it could be argued that the anxiogenic effects of repeated paroxetine treatment alone meant that discontinuation experiments were performed in a mouse model of anxiety. This could be taken to suggest that the behavioural effects seen here are likely to generalise to other mouse models of anxiety.

Heterogeneity in the cause, experience or vulnerability to psychiatric disorders is also difficult to study in inbred, genetically identical mice.

This raises questions about the relevance of behavioural changes in mice to human disorders. In Chapter 7, for example, the increase in sleep bout duration in the dark phase could be equated to humans sleeping more in the daytime. While this resembles daytime somnolence, which is a symptom of the clinical discontinuation syndrome, the structure of mouse sleep is far removed from human circadian activity. Thus, it is difficult to equate mice sleeping for slightly longer in several hundred sleep bouts per day to human sleep.

It is therefore clear that a mouse model cannot replicate a human disorder, especially one as complex and heterogeneous as SSRI discontinuation syndrome. A change in how we view preclinical models is therefore needed. Instead of expecting them to reproduce subjective aspects of a condition, objective outputs such as EPM performance or sleep architecture should be seen as biomarkers of an experimental manipulation. These outputs can be used to test theories about the mechanism of action of a drug or the symptoms of a disease state, which can then be translated to humans. In the case of SSRI discontinuation, this could mean testing whether new drugs produce discontinuation reactions, or trialling tapering strategies that might prevent or minimise symptoms.

Overall, the current mouse model produces some behavioural effects of SSRI cessation that resemble common symptoms in patients, although it is far from an exact replica of the human discontinuation syndrome. Nonetheless, it is still a useful system in which to investigate the neurochemical effects of SSRI cessation that could later be translated into clinical studies.

4 Sex and gender factors in SSRI discontinuation

Until recently, the clinical literature was unclear as to whether sex and gender factors affect an individual's risk of SSRI discontinuation syndrome. Moreover, there had not been any preclinical studies of SSRI cessation using female animals, so it was not known if sex effects would be evident in mice. Interestingly, a report from the World Health Organisation in November 2022 found that men are not only more likely to experience discontinuation symptoms, but the symptoms are also more severe (Gastaldon et al., 2022). The current experiments also found, at least with this dosing regimen, that male mice exhibited behavioural effects of discontinuation whereas female mice did not (Chapter 3 section 3.1).

Thus, there appears to be a biological basis for the sex differences observed in the rates of withdrawal reactions in men and women. This could involve variation in the 5-HT system, for example differential expression of the 5-HTT between male and female mice (Hodes et al., 2010). Alternatively, different rates of SSRI metabolism (Renoir et al., 2011) may have made female mice less susceptible to experiencing discontinuation effects (discussed in detail in Chapter 3 section 4.6). Varying the dose, frequency and duration of paroxetine treatment could therefore be trialled to determine if modifying the dosing protocol would generate discontinuation effects in female mice.

Another explanation for the lack of effects in female mice is that the stage of oestrous the mice were in could have influenced their behaviour and thus masked any effects of paroxetine discontinuation (for example, Yohn et al. (2020a) reported different effects of repeated fluoxetine treatment on unconditioned tests of anxiety during different oestrous stages). Moreover, oestrogen has been shown to modulate the activity and plasticity of key components of the anxiety-promoting brain circuitry, including the

hippocampus (Mendoza-Garcés et al., 2011). This potentially produces different effects on anxiety-like behaviours, or the lack thereof, in female mice. Further work is needed to explore how sexual variation in the 5-HT system and its regulation by sex hormones interact with SSRI discontinuation to alter behaviour in rodents.

Furthermore, while it is beyond the scope of this thesis to discuss them in detail, there could also be societal factors that contribute to the gender differences in the rates of SSRI discontinuation syndrome. For example, men taking antidepressant medications were reported to consult primary care doctors 8% less than women (Wang et al., 2013), hence they may be less likely to seek advice on how best to discontinue or taper their SSRI. Although gender cannot account for the sex differences in the discontinuation effects in mice, it may contribute to the variable incidence of SSRI discontinuation syndrome in patients and should also be considered when identifying risk factors.

5 Similarities to other withdrawal states

As noted throughout this thesis, SSRI discontinuation syndrome resembles other drug withdrawal states, including opioid and amphetamine withdrawal. Shared symptoms include nausea, dizziness, and headaches, as well as affective symptoms including anxiety and agitation. It is also becoming apparent that the neurochemical effects associated with psychotropic drug discontinuation are similar. Discontinuation reactions from opiates and older classes of antidepressants are associated with rebound increases in neuronal activity. These include noradrenergic rebound following morphine withdrawal (reviewed in Gold & Pottash, 1981, and Kosten & George, 2002) and cholinergic rebound after TCA discontinuation (Dilsaver & Greden, 1984; Renoir, 2013). Together with the evidence of a rebound in 5-HT function during SSRI discontinuation, these neurobiological effects of

drug cessation support the oppositional model of tolerance as an explanation for withdrawal reactions (Chouinard & Chouinard, 2015; Fava, 2020).

Nonetheless, the variation in symptoms between different withdrawal states likely reflects the different neurotransmitter systems and neural circuits the drugs target. In the case of SSRIs, the current data suggest that the rebound increase occurs in the 5-HT system, hence anxiety and sleep disturbances are common following SSRI cessation. By comparison, increased dopaminergic, noradrenergic, and cholinergic signalling have been observed following TCA withdrawal, leading to symptoms such as cardiac abnormalities, movement disorders and cognitive deficits (Dilsaver et al., 1987; Colzato et al., 2021). Future work could explore whether changes in cellular physiology, for example hyperexcitability or autoreceptor activation, may provide a unifying mechanism that explains the effects of CNS drug cessation.

6 Clinical implications

The current data have several implications for the study of SSRI discontinuation syndrome in humans. Firstly, the model could be used to trial strategies to mitigate discontinuation symptoms, for example tapering. The current clinical evidence on the benefit of tapering is inconsistent (Dominguez & Goodnick, 1995; Rosenbaum et al., 1998; Tint et al., 2008; Fava et al., 2015; Jha et al., 2018). This model could therefore be used to establish if strategies such as hyperbolic tapering or switching to an SSRI with a longer half-life could lessen discontinuation reactions (Gabriel & Sharma, 2017; Ruhe et al., 2019). The influence of genetic risk factors on the likelihood of experiencing SSRI discontinuation symptoms, such as variation in 5-HT_{1A} receptor expression or CYP2D6

genotype (hence the rate of SSRI metabolism; Sindrup et al., 1992; Özdemir et al., 1999), could also be explored using transgenic mice.

The current data can also inform future clinical trials. For example, objective measurements of common discontinuation symptoms could be performed repeatedly in the weeks following SSRI cessation to better understand the time course of symptoms. PET imaging could also be performed to investigate if the changes in extracellular 5-HT seen in mice translate to humans. Furthermore, fMRI could be used to identify other brain regions whose activity is modulated by SSRI cessation.

Future experiments could also lead to a model in which SSRI discontinuation can be precipitated, for example using tryptophan depletion, firstly in mice and then in humans. This would allow for more controlled discontinuation studies and remove the need for patients to discontinue SSRI therapy. This could make clinical trials more accessible to individuals who are not yet in remission or ready to stop their treatment. This approach could also be used to elucidate the effects of drug non-compliance.

Finally, these findings raise awareness of the behavioural and neurochemical effects of SSRI discontinuation within neuropharmacological research. Just two days after paroxetine washout, mouse behaviour and brain neurochemistry were vastly different to both controls and animals maintained on-drug. Researchers should therefore consider the time between the end of treatment and when animals are tested to avoid inadvertently measuring effects of discontinuation rather than the drug itself. This could generate more reproducible results and ultimately lead to the better translation of therapies into humans.

7 Conclusions

In this thesis, SSRI discontinuation produced a transient increase in anxiety-like behaviour, and some evidence of changes to sleep structure, in mice. These effects were evident after discontinuation from several SSRIs but most notably paroxetine, mimicking its propensity to cause discontinuation symptoms in patients. Neurochemical and c-FOS immunohistochemical experiments showed that paroxetine discontinuation was associated with a fall in extracellular 5-HT but a rebound increase in 5-HT neuron excitability. The current data suggest these changes to the 5-HT system may be a consequence of paroxetine cessation, but it is not yet possible to conclude whether they caused the behavioural effects.

Overall, these experiments provide the first evidence that SSRI discontinuation can be modelled in mice and offer new insights into the effects of paroxetine cessation on the 5-HT system. Further work is needed to establish if changes to 5-HT transmission are causal in the behavioural effects seen here, as well as to investigate other behavioural and neurochemical correlates of the clinical discontinuation syndrome.

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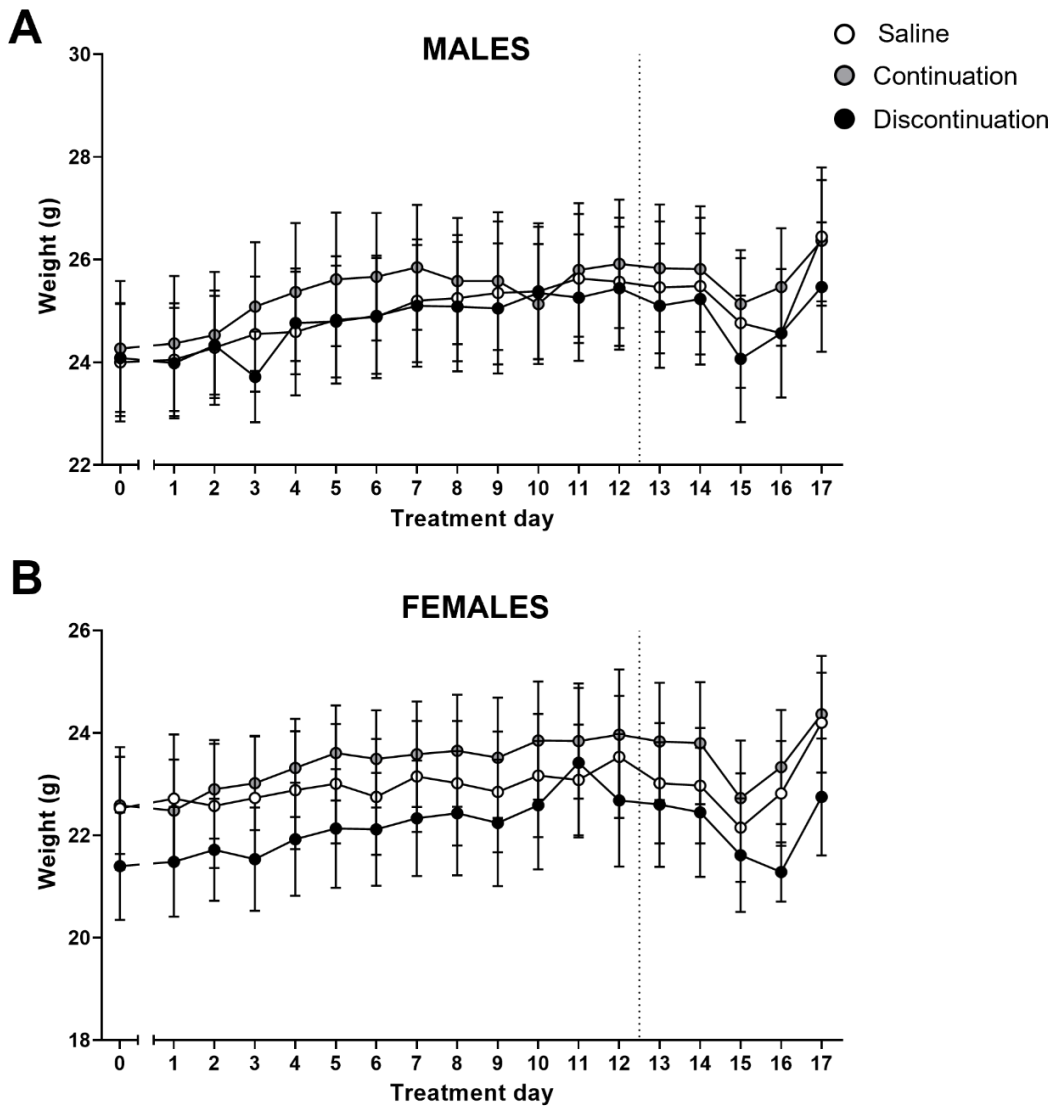
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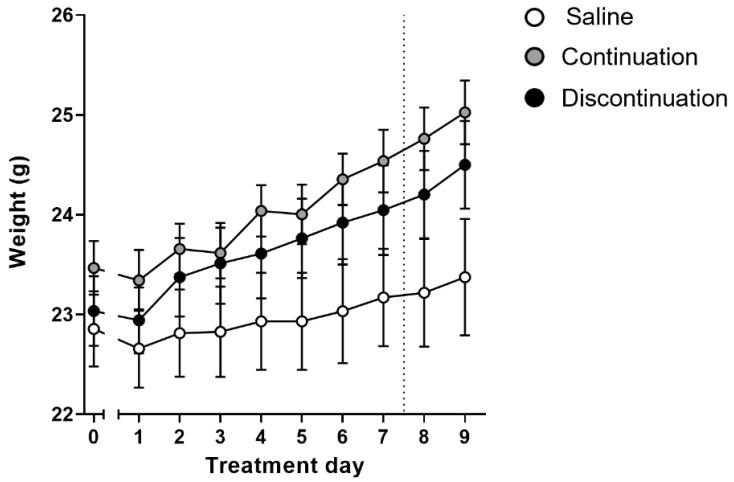
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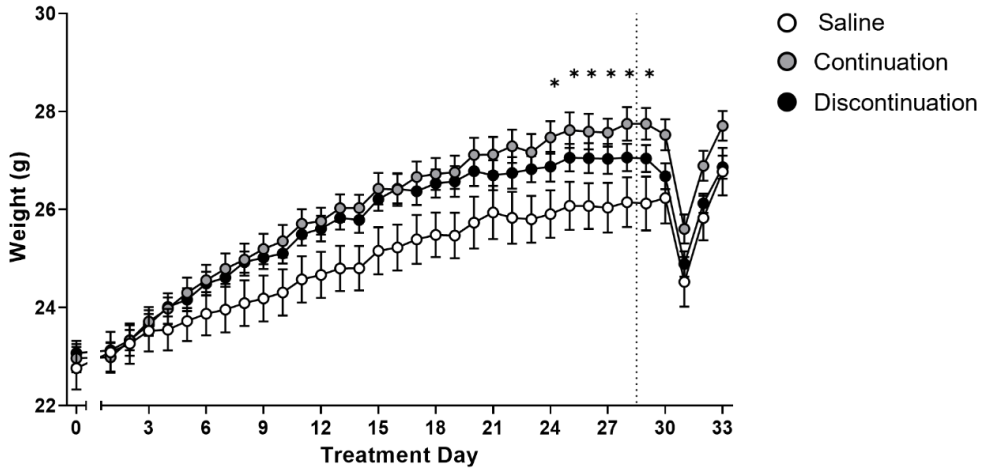
Appendix 1
Effect of SSRI discontinuation on mouse weight



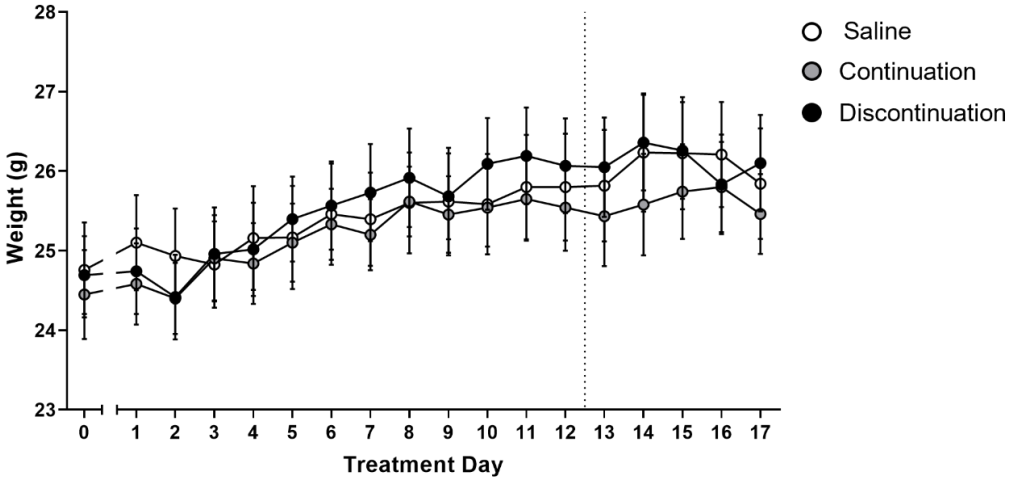
Suppl. Figure 1.1 Effect of discontinuation from 12 days of once-daily paroxetine treatment on the weight of male and female mice. Points represent the mean ± SEM values for weight each day. Saline (males n=6, females n=6), Continuation (males n=6, females n=5-6), Discontinuation (males n=6, females n=6). Dotted line represents discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Males: effect of treatment $F(2,15)=0.610$, $p=0.883$, effect of time $F(17,255)=1.182$, $p=0.308$, treatment*time interaction $F(34,255)=0.037$, $p>0.999$. Females: effect of treatment $F(2,14)=0.518$, $p=0.943$, effect of time $F(17,238)=6.023$, $p=0.003$, treatment*time interaction $F(34,238)=0.048$, $p>0.999$.



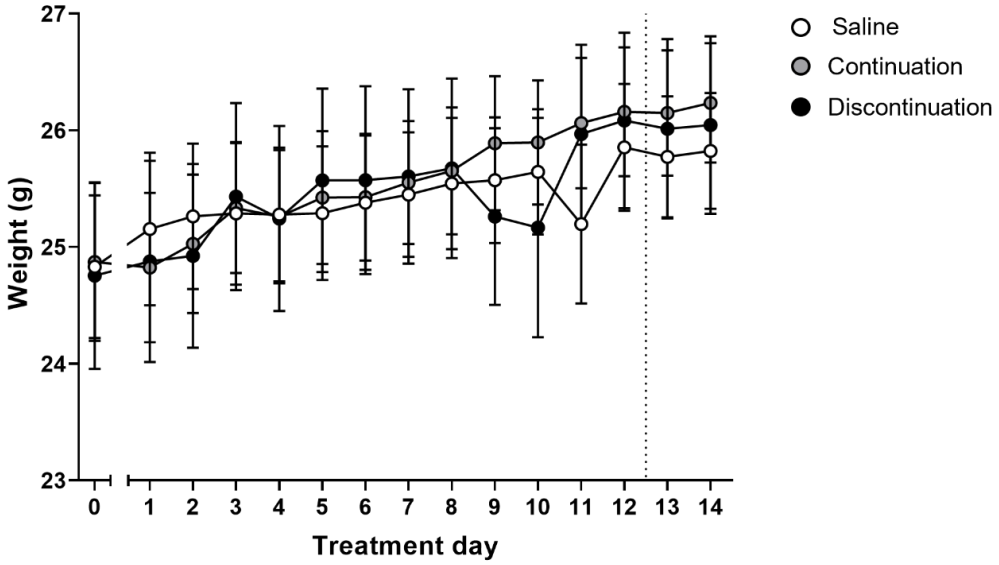
Suppl. Figure 1.2 Effect of discontinuation from seven days of once-daily paroxetine treatment on weight. Points represent the mean ± SEM values for weight each day. Saline (n=12), Continuation (n=12), Discontinuation (n=12). Dotted line represents discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Effect of treatment $F(2,33)=1.978$, $p=0.154$, effect of time $F(9,297)=14.649$, treatment*time interaction $F(18,297)=1.787$, $p=0.026$.



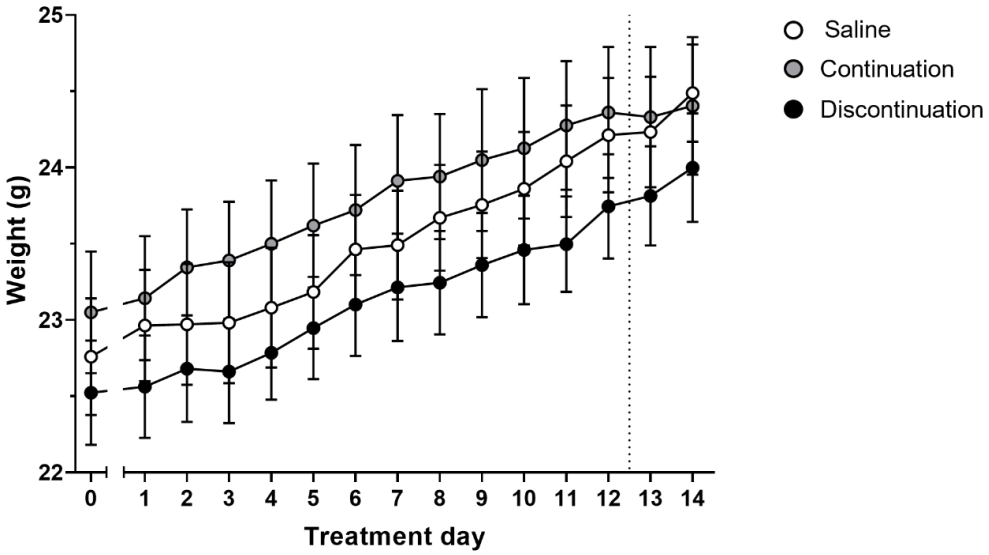
Suppl. Figure 1.3 Effect of discontinuation from 28 days of once-daily paroxetine treatment on weight. Points represent the mean ± SEM values for weight each day. Saline (n=12), Continuation (n=12), Discontinuation (n=12). Dotted line represents discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Effect of treatment $F(2,33)=2.567$, $p=0.092$, effect of time $F(33,1089)=274.5$, $p<0.001$, treatment*time interaction $F(66,1089)=3.831$, $p<0.001$. * $p<0.05$ between groups at specific timepoint.



Suppl. Figure 1.4 Effect of discontinuation from 12 days of twice-daily paroxetine treatment on weight. Points represent the mean ± SEM values for weight each day. Saline (n=12), Continuation (n=12), Discontinuation (n=12). Dotted line represents discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Effect of treatment $F(2,33)=0.112$, $p=0.895$, effect of time $F(17,561)=22.08$, $p<0.0001$, treatment*time interaction $F(34,561)=0.894$, $p=0.643$.



Suppl. Figure 1.5 Effect of discontinuation from 12 days of twice-daily citalopram treatment on weight. Points represent the mean ± SEM values for weight each day. Saline (n=18), Continuation (n=18), Discontinuation (n=18). Dotted line represents discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Effect of treatment $F(2,51)=0.017$, $p=0.983$, effect of time $F(14,714)=10.31$, $p<0.001$, treatment*time interaction $F(28,714)=1.034$, $p=0.418$.



Suppl. Figure 1.6 Effect of discontinuation from 12 days of twice-daily venlafaxine treatment on weight. Points represent the mean ± SEM values for weight each day. Saline (n=12), Continuation (n=12), Discontinuation (n=12). Dotted line represents paroxetine discontinuation. Repeated measures ANOVA with post-hoc Bonferroni's test. Effect of treatment $F(2,33)=0.750$, $p=0.480$, effect of time $F(14,462)=105.287$, $p<0.001$, treatment*time interaction $F(28,462)=0.938$, $p=0.559$.

Appendix 2
Effect of repeated SSRI treatment on anxiety-like behaviour on the EPM in mice: a meta-analysis

The following sections detail the results of the meta-analysis of the effects of repeated SSRI treatment on behaviour on the EPM. Other anxiety tests (AOF, LDB, NSF) and tests for depressive-like behaviours (FST, TST) were analysed but data are not shown.

1 Aims

This meta-analysis aimed to review the literature on the effects of repeated SSRI treatment on anxiety-like behaviours in mice. Factors that affect anxiety-like behaviour in mice, including biological (e.g., strain, sex, age) and environmental (e.g., number of mice per cage) variables, were also investigated.

2 Experimental Methods

2.1 Search strategy

A comprehensive literature search was conducted using Google Scholar (https://scholar.google.com/), PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Web of Science (https://www.webofscience.com/wos/woscc/basic-search). Searches were conducted using the key words ‘chronic’ or ‘repeated’, ‘anxiety’, ‘mice’ and ‘SSRI’, with additional individual searches with SSRI non-proprietary names (fluoxetine, paroxetine,

citalopram, escitalopram, sertraline, and fluvoxamine) as well as individual searches of the behavioural tests used ('elevated plus maze' or 'EPM').

2.2 Inclusion and exclusion criteria

Titles and abstracts were reviewed to determine their eligibility using the following inclusion criteria: behavioural tests were performed in healthy, wild-type mice over the age of 8 weeks at the point of testing; behaviour had to be assessed on the EPM after at least two days of treatment with any SSRI via any route of administration, within 24 h of the last dose. All studies were published in English before May 2020. Full-text articles were assessed, and data extracted. Papers were subsequently excluded for not containing the necessary data to conduct the meta-analysis (group means, either in tabular or graphical form, variation, or group sizes). Bibliographies were also searched to identify other eligible papers. The methodology followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

2.3 Data extraction

The following data were extracted from the included studies: i) biological variables: sex, mouse strain, age of mice at the start of dosing (when given in months there was assumed to be four weeks in every month); ii) experimental variables: housing conditions (number of mice housed per cage), SSRI, dose (mg/kg), duration of treatment (days), route of administration, time between last dose and testing (washout, h); iii) outcomes: the time spent in the open arms (raw or percentage, but not adjusted for distance travelled in the experiment), mean, SEM or standard deviation, sample size per experiment group (n). Data were extracted from tabular results or estimated from graphs.

2.4 Meta-analysis

The meta-analysis was conducted using a random effects model with continuous variables, calculated using the effect size of each experimental comparison (odds ratio, difference between the control and experimental group), weighted by the group size of each experiment. Revman Review Manager Version 5.3. (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) was used to calculate individual effect sizes and the overall statistical significance. Data is presented as effect size ± 95% confidence interval using GraphPad Prism (v8). Heterogeneity was calculated from the Chi-squared statistic (Q) and degrees of freedom (df), as $I^2 = ((Q – df) / Q)*100$. If I^2 was greater than 50%, the data were considered heterogeneous.

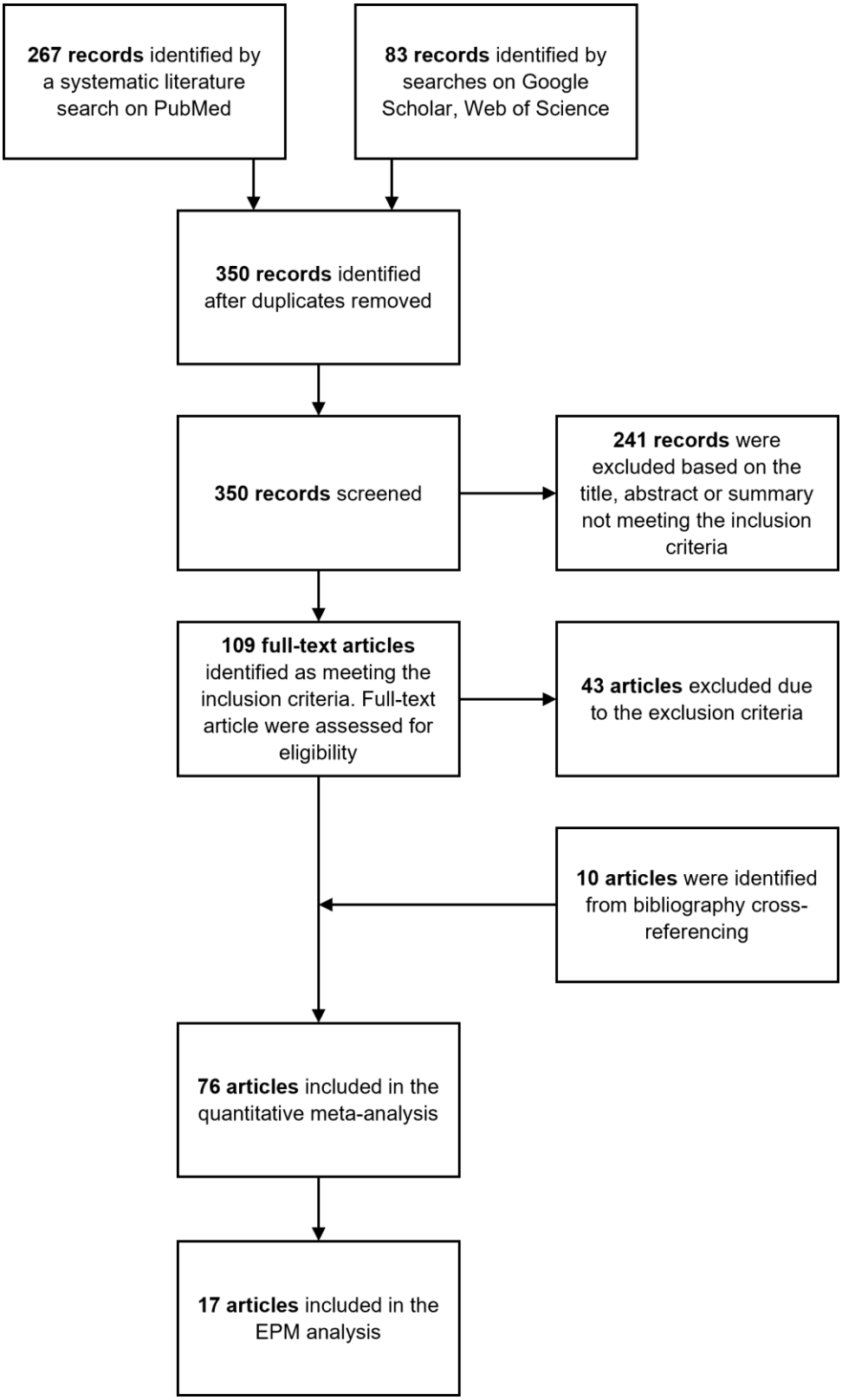
The relationships between the age at the start of treatment, number of mice per cage, SSRI dose, duration of treatment, and washout period were determined using Spearman's rank correlation. Where a range was given for these variables, the lower bound was used for the correlation analysis. Papers that did not give specific values for any variable (e.g., 'adult' for age or 'group-housed' for number of mice per cage) were not included in the correlations, although this is reported in the table for each test for reference. Effect sizes were also grouped by mouse strain, SSRI, route of administration and sex and analysed using Kruskal Wallis with Dunn's post-hoc test to assess the effect of treatment in these subgroups. Effect sizes were also grouped by housing condition (single or group housed) and analysed using the Mann Whitney U test. All correlations and subsequent analyses were conducted and presented using GraphPad Prism. $P<0.05$ is considered statically significant throughout.

3 Results

3.1 Study characteristics

A total of 109 studies matched the inclusion criteria. 43 papers were subsequently excluded because they did not include the relevant data to perform the meta-analysis. 76 papers were identified as meeting all the inclusion criteria and included at least one of the following behavioural tests: EPM, OF, LD, NSF, NIH, TST, FST, marble burying (*Suppl. Fig. 2.1*). The included studies were performed between 1995 and 2020.

Seventeen papers were identified that used the EPM to assess the effect of repeated SSRI treatment on anxiety-like behaviours (*Suppl. Table 2*). 11 papers (64.7%) exclusively used male mice, and only two papers (11.8%) used female mice. Four papers (23.5%) used mixed sex groups, and one paper compared male and female mice (McEuen et al., 2009). Eleven strains were used, with the most common being substrains of C57BL/6 mice (11 papers, 64.7%) and Swiss mice (four papers, 23.5%). Only one paper compared two different strains (Oh et al., 2009). Twelve papers (70.6%) stated the age of the mice used; the other six papers (35.5%) simply stated they used ‘adult’ mice. Of the papers that stated age, the mice were between 8-12 weeks old at the start of the experiment, with the most common age being eight weeks (six papers, 35.5%).

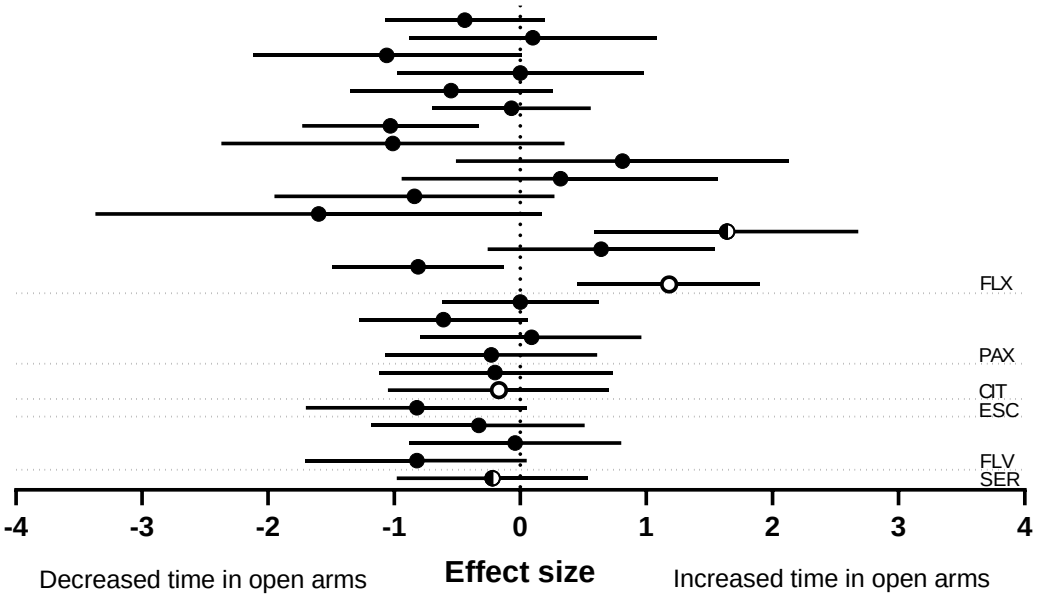


Suppl. Figure 2.1 PRISMA flow-chart. Details of studies identified in the systematic literature search and those included in meta-analysis.

Nine papers (52.9%) group-housed mice, eight used single-housed mice (47.1%) and one did not state how mice were housed. Six SSRIs were used: 11 papers (64.7%) used fluoxetine, four papers (23.5%) used paroxetine, and there was one paper for each of the other SSRIs. Two papers compared different SSRIs (Goeldner et al., 2005; Turcotte-Cardin et al., 2019). The dose of SSRI used varied between 2 mg/kg/day and 30 mg/kg/day, with the mode dose being 10 mg/kg/day (nine papers, 52.9%). Four papers (23.5%) compared different doses of the same SSRI. The duration of SSRI treatment ranged from 14 to 30 days, with the mode duration being 28 days (six papers, 35.5%). Four routes of administration were used. Eight papers (47.1%) used *i.p.* injections, seven used drinking water administration (41.2%), one used oral gavage (Thoeringer et al., 2010) and one used both drinking water and oral gavage (Yohn et al., 2020). Nine papers (52.9%) stated the time between the last dose of SSRI and the behavioural testing. Four papers (23.5%) were assumed to have no washout time between dosing and testing due to the continuous nature of the route of administration. There were four papers (23.5%) in which the washout period was not stated, although all were tested within 24 h of the last dose. All included studies are summarised in *Suppl. Table 2*.

3.2 Elevated plus maze (EPM)

Overall, repeated SSRI treatment did not significantly alter the time mice spent in the open arms of the EPM compared to vehicle-treated controls (overall effect: -0.20 [-0.44, 0.04]; effect of treatment: Z=1.62, p=0.11; 630 mice in total; *Suppl. Fig. 2.2*). There was a substantial variation between the studies (I^2=52%).



Suppl. Figure 2.2 EPM meta-analysis. Dots and lines represent the mean and 95% confidence interval of the effect size of each study. Black, all male mice; white, all female mice; half black, mixed sex. Fluoxetine, FLX; Paroxetine, PAX; Citalopram, CIT; Escitalopram, ESC; Fluvoxamine, FLV; Sertraline, SERT.

There were no significant correlations between the age at the beginning of treatment (r=-0.1860, p=0.4600), the number of mice per cage (r=-0.0500, p=0.8252), the dose of SSRI used (r=0.0286, p=0.8874), the duration of treatment (r=0.0384, p=0.8491) or the washout time (r=-0.2812, p=0.2168) on the effect sizes observed.

Kruskal Wallis tests revealed no significant effects of mouse strain (H(5)=2.130, p=0.8309), SSRI (H(5)=1.305, p=0.9344), route of administration (H(3)=3.571, p=0.3116) or sex (H(2)=2.392, p=0.3025) on the effect size of chronic SSRI treatment on the EPM. There was also no difference in the effect sizes between mice that were single versus grouped housed (U=76, p=0.5681).

Suppl. Table 2 Details of the elevated plus maze (EPM) meta-analysis and other experimental variables. Not specified, NS; intraperitoneal injections, i.p.

SSRI	Mouse strain	Sex	Age at start (weeks)	Animals per cage	Dose (mg/kg/day)	Treatment duration (days)	Route of administration	Washout time (h)	Effect [95% CI]	Reference
Fluoxetine	Swiss	Males	Adult	20	10	27	i.p.	0.3	-0.44 [-1.07, 0.19]	Goeldner et al. (2005)
	C57BL/6J	Males	8	1	10	14	Drinking water	NS	0.10 [-0.88, 1.08]	Kobayashi et al. (2008)
	C57BL/6	Males	12	1	12	28	Drinking water	NS	0.00 [-0.98, 0.98]	Oh et al. (2009)
	Swiss Webster	Males	8	1	18	28	Drinking water	NS	-1.06 [-2.12, 0.01]	Oh et al. (2009)
	C57BL/6J	Males	9	1	14	28	Drinking water	1.5	-0.55 [-1.35, 0.26]	Kobayashi et al. (2011)
	C57BL/6J	Males	9	1	22	28	Drinking water	1.5	-0.07 [-0.70, 0.56]	Kobayashi et al. (2011)
	C57BL/6	Males	8	NS	10	30	i.p.	21-24	-1.03 [-1.73,-0.33]	Venzala et al. (2012)
	Swiss	Males	Adult	5	10	15	i.p.	0.5	-1.01 [-2.37, 0.35]	Sorregotti et al. (2013)
	Swiss	Males	Adult	5	20	15	i.p.	0.5	0.81 [-0.51, 2.13]	Sorregotti et al. (2013)
	Swiss	Males	Adult	5	30	15	i.p.	0.5	0.32 [-0.94, 1.57]	Sorregotti et al. (2013)
	C57BL/6J	Males	9	2	20	14	i.p.	1	-0.84 [-1.95, 0.27]	Baek et al. (2015
	C57BL/6J	Males	9	2	20	14	i.p.	1	-1.60 [-3.37, 0.17]	Baek et al. (2015)
	C57BL/6N	Mixed	11	1	18	22	Drinking water	0	1.64 [0.59, 2.68]	Vahid-Ansari et al. (2017)
	Black Swiss	Males	Adult	1	10	19	i.p.	NS	0.64 [-0.26, 1.54]	Simard et al. (2018)
	C57BL/6N	Mixed	10	1	18	15	Drinking water	0	-0.81 [-1.49,-0.13]	Turcotte-Cardin et al. (2019)
	C57BL/6J	Females	8	Up to 5	18	At least 28	Drinking water + oral gavage	Up to 24	1.18 [0.45, 1.90]	Yohn et al. (2020)
Paroxetine	Swiss	Males	Adult	20	5	27	i.p.	0.3	0.00 [-0.62, 0.62]	Goeldner et al. (2005)
	C57BL/6J	Males	8-10	1	10	18	i.p.	21-24	-0.61 [-1.28, 0.06]	Elizalde et al. (2008)
	C57BL/6JolaHsd	Males	9-12	1	10	19	Oral gavage	1	0.09 [-0.79, 0.96]	Thoeringer et al. (2010)
	C57BL/6NTac	Mixed	Adult	4-5	18	At least 28	Drinking water	0	-0.23 [-1.07, 0.61]	Guilloux et al. (2011b)
Citalopram	C57BL6/129Sv-crossed	Males	9	Group	10	At least 28	Drinking water	0	-0.20 [-1.12, 0.73]	McEuen et al. (2009)
	C57BL6/129Sv-crossed	Females	9	Group	10	At least 28	Drinking water	0	-0.17 [-1.05, 0.70]	McEuen et al. (2009)
Escitalopram	C57BL/6N	Mixed	10	1	30	15	Drinking water	0	-0.82 [-1.70, 0.05]	Turcotte-Cardin et al. (2019)
Fluvoxamine	CD1	Males	Adult	10-11	2	21	i.p.	0.5	-0.33 [-1.18, 0.51]	Rodgers et al. (1997)
	CD1	Males	Adult	10-11	4	21	i.p.	0.5	-0.04 [-0.88, 0.80]	Rodgers et al. (1997)
	CD1	Males	Adult	10-11	8	21	i.p.	0.5	-0.82 [-1.70, 0.05]	Rodgers et al. (1997)
Sertraline	FVB/N	Mixed	8	Group	20	28	i.p.	NS	-0.22 [-0.98, 0.54]	Hult Lundh et al. (2013)

Appendix 3
Additional neurochemical analyses

Suppl. Table 3.1 Effect of two days discontinuation from 12-days once-daily paroxetine on tissue DA and DOAPC levels. DA and DOPAC per mg of wet tissue (nmol/mg). Repeated measures ANOVA with Bonferroni's test. N=8/group. DA: effect of treatment $F(2,21)=0.844$, $p=0.433$, effect of region $F(3,63)=74.49$, $p<0.001$, treatment*region interaction $F(6,63)=0.263$, $p=0.953$. DOPAC: effect of treatment $F(2,21)=0.364$, $p=0.696$, effect of region $F(3,63)=51.21$, $p<0.001$, treatment*region interaction $F(6,63)=0.256$, $p=0.956$.

	Group (mean ± SEM)		
Brain region	**SAL**	**CON**	**DIS**
DA			
Midbrain	0.111 ± 0.016	0.135 ± 0.014	0.134 ± 0.025
Hippocampus	0.132 ± 0.021	0.162 ± 0.032	0.148 ± 0.029
Striatum	3.025 ± 0.529	3.448 ± 0.415	3.514 ± 0.610
Frontal cortex	0.657 ± 0.114	0.921 ± 0.140	1.219 ± 0.493
DOPAC			
Midbrain	0.030 ± 0.002	0.026 ± 0.003	0.031 ± 0.006
Hippocampus	0.020 ± 0.004	0.020 ± 0.007	0.021 ± 0.002
Striatum	0.289 ± 0.067	0.330 ± 0.053	0.309 ± 0.051
Frontal cortex	0.120 ± 0.022	0.135 ± 0.011	0.174 ± 0.047

Suppl. Table 3.2 Effect of five days discontinuation from 12 days once-daily paroxetine on tissue DA and DOPAC levels. DA and DOPAC per mg of wet tissue (nmol/mg). Repeated measures ANOVA with Bonferroni's test. N=9/group. DA: effect of treatment $F(2,24)=2.325$, $p=0.109$, effect of region $F(3,72)=15.00$, $p<0.001$, treatment*region interaction $F(6,72)=0.539$, $p=0.708$. DOPAC: effect of treatment $F(2,24)=2.483$, $p=0.092$, effect of region $F(6,72)=71.07$, $p<0.001$, treatment*region interaction $F(6,72)=0.827$, $p=0.554$.

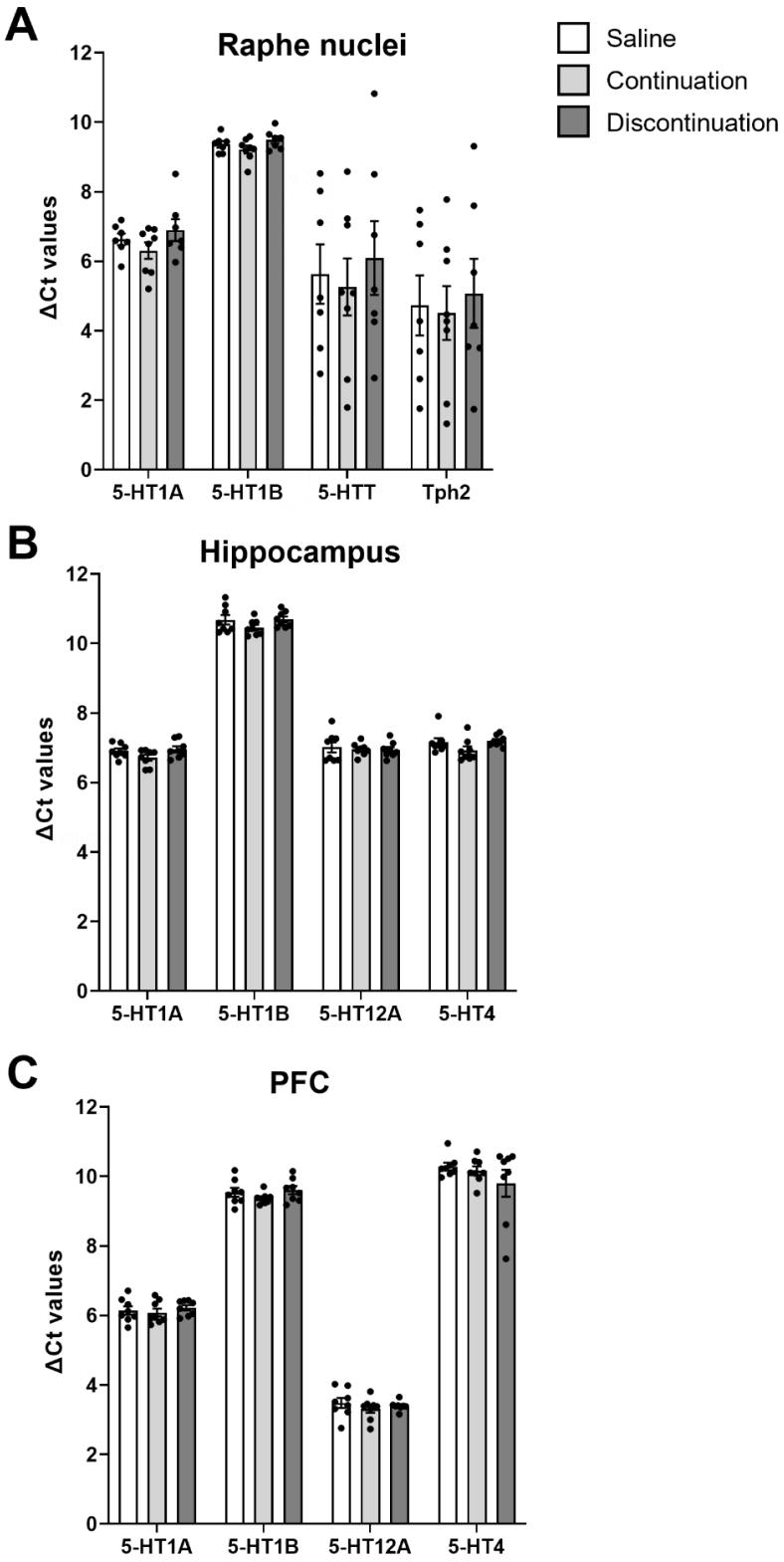
	Group (mean ± SEM)		
Brain region	**SAL**	**CON**	**DIS**
DA			
Midbrain	0.048 ± 0.010	0.063 ± 0.15	0.067 ± 0.013
Hippocampus	0.074 ± 0.025	0.084 ± 0.025	0.163 ± 0.047
Striatum	0.975 ± 0.271	1.935 ± 0.399	2.190 ± 0.498
Frontal cortex	0.419 ± 0.115	0.423 ± 0.142	0.613 ± 0.128
DOPAC			
Midbrain	0.025 ± 0.004	0.028 ± 0.004	0.056 ± 0.034
Hippocampus	0.013 ± 0.004	0.020 ± 0.003	0.033 ± 0.008
Striatum	0.291 ± 0.063	0.342 ± 0.058	0.391 ± 0.044
Frontal cortex	0.073 ± 0.017	0.086 ± 0.023	0.128 ± 0.025

Suppl. Table 3.3 Effect of five days discontinuation from 12 days twice-daily paroxetine on tissue DA and DOPAC levels. DA and DOPAC per mg of wet tissue (nmol/mg). Repeated measures ANOVA with Bonferroni's test. N=6/group. DA: effect of treatment $F(2,15)=0.411$, $p=0.665$, effect of region $F(3,45)=50.59$, $p<0.001$, treatment*region interaction $F(6,45)=0.523$, $p=0.789$. DOPAC: effect of treatment $F(2,15)=0.003$, $p=0.997$, effect of region $F(3,45)=24.75$, $p<0.001$, treatment*region interaction $F(6,45)=0.309$, $p=0.930$.

	Group (mean ± SEM)		
Brain region	**SAL**	**CON**	**DIS**
DA			
Midbrain	0.134 ± 0.030	0.415 ± 0.276	0.167 ± 0.055
Hippocampus	0.246 ± 0.058	0.108 ± 0.024	0.137 ± 0.035
Striatum	4.054 ± 0.912	5.494 ± 1.058	4.715 ± 1.014
Frontal cortex	1.289 ± 0.257	1.319 ± 0.364	1.592 ± 0.288
DOPAC			
Midbrain	0.055 ± 0.008	0.182 ± 0.132	0.067 ± 0.015
Hippocampus	0.043 ± 0.016	0.025 ± 0.006	0.042 ± 0.009
Striatum	0.366 ± 0.088	0.427 ± 0.096	0.436 ± 0.100
Frontal cortex	0.309 ± 0.117	0.279 ± 0.057	0.222 ± 0.026

Suppl. Table 3.4 Effect of five days discontinuation from 12 days twice-daily citalopram on tissue DOPAC levels. DOPAC per mg of wet tissue (nmol/mg). Repeated measures ANOVA with Bonferroni's test. N=8/group. Effect of treatment $F(2,21)=0.324$, $p=0.724$, effect of region, $F(3,63)=121.8$, $p<0.001$, treatment*region interaction $F(6,63)=0.289$, $p=0.941$.

	Group (mean ± SEM)		
Brain region	**SAL**	**CON**	**DIS**
Midbrain	0.028 ± 0.004	0.029 ± 0.002	0.032 ± 0.007
Hippocampus	0.022 ± 0.002	0.028 ± 0.004	0.026 ± 0.004
Striatum	0.370 ± 0.044	0.350 ± 0.058	0.328 ± 0.032
Frontal cortex	0.079 ± 0.017	0.099 ± 0.013	0.070 ± 0.013



Suppl. Fig. 3.1 Relative abundance of each GOI in qPCR experiments. Relative expression the GOI in the raphe nuclei (RN) (A), hippocampus (B) and prefrontal cortex (PFC) (C). ΔCt value represent the Ct values (number of cycles to reach a given threshold during PCR) of the housekeeping gene β-actin subtracted from the Ct of the GOI, where lower ΔCt values indicate greater amounts of GOI mRNA in the sample. N=5-8/treatment per region.