Serotonin Receptor Electrophysiology in Cortical Neurons
Critical for the Response to Antidepressants

by

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Abstract

The gold standard for treating depression is a class of medicines known as selective serotonin reuptake inhibitors (SSRIs). Recent research identified a subgroup of layer V pyramidal neurons in the cerebral cortex important for the antidepressant action of SSRIs. Using transgenic mice that have these p11-expressing neurons labeled with GFP, I characterized these neurons and their neighbours under control conditions and after chronic treatment with the SSRI fluoxetine (FLX). Experiments were conducted with a particular focus on serotonin (5-HT) responses, using whole-cell recording in acute brain slices of motor and prefrontal cortex. Further investigation explored the effects of chronic FLX treatment in a mouse model of stress. I found that chronic FLX treatment has significant effects on neurons in both motor and prefrontal cortices, that p11-expressing neurons differ subtly in their response to 5-HT, and that there is evidence of an interaction between stress and FLX treatment in p11-expressing neurons.
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List of Abbreviations

5-HT: 5-Hydroxytryptamine or serotonin
5-HT₄R: 5-HT₄ receptor
aCSF: Artificial cerebrospinal fluid
AMPA: α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
ANOVA: Analysis of variance
APV: D-(-)-2-amino-5-phosphopentanoic acid, NMDA receptor antagonist
cAMP: Cyclic adenosine monophosphate
Cg1: Cingulate cortex area 1
CNQX: 6-Cyano-7-nitroquinoxaline-2,3-dione, AMPA receptor antagonist
ePSC: Excitatory postsynaptic current
FLX: Fluoxetine or Prozac
G protein: Guanine nucleotide binding protein
GABA: γ-aminobutyric acid
GFP: Green fluorescent protein
GPCR: G-protein coupled receptor
iPSC: Inhibitory postsynaptic current
KO: Knock out
M1: Motor cortex area 1 or primary motor cortex
NMDA: N-methyl-D-aspartic acid
PFC: Prefrontal cortex
PKC: Protein kinase C
TTX: Tetrodotoxin, Na⁺ channel antagonist
Chapter 1 - General Introduction

1.1 Research Summary and Aims

More than 10% of Canadians will experience major depression during their lifetimes according to the Public Health Agency of Canada, and depression is one of the leading causes of disability in terms of productive years lost to disease (Government of Canada, 2006). The gold standard treatment for depression is a class of medicines known as selective serotonin reuptake inhibitors (SSRIs), including the drug fluoxetine (FLX). When taken chronically, these medicines increase the release of the neuromodulator serotonin (5-HT) in the cerebral cortex. Healthy regulation of emotional responses depends critically on serotonergic stimulation of cortical receptors (Puig and Gulledge, 2011).

Recent research has found a subgroup of corticostriatal neurons expressing the protein p11 that are necessary for SSRI antidepressant effects (Schmidt et al., 2012). These neurons are layer V pyramidal neurons located in the motor, somatosensory, and visual cortices. In these neurons chronic SSRI exposure altered the translation of 5-HT receptors, dramatically upregulating a 5-HT receptor, 5-HT4, not typically well expressed in these neurons (Schmidt et al., 2012). These changes were specific to these p11 neurons and not seen in these same neurons after genetic deletion of p11. Yet it is not clear how chronic SSRI treatment affects the activity of these p11 neurons and their functional responses to serotonin. The first aim of this thesis was to characterize the effect of chronic FLX treatment on cortical 5-HT responses in these neurons and then to identify if this response is distinct in neurons that express p11 compared to those that do not express this protein. I hypothesized that p11 neurons would have increased excitatory activity in response to acute 5-HT after chronic FLX treatment, potentially due to increased functional expression of excitatory 5-HT receptors.

Neurons that express p11 were prominent in the primary motor cortex (M1) (Schmidt et al., 2012). This is unusual in that previous research on the cortical effects of 5-HT has focused primarily on the prefrontal cortex (PFC) because of its demonstrated role in depression (Zhong and Yan, 2011; Avesar and Gulledge, 2012; Goodfellow et al., 2012). As such the second aim of this thesis was to assess whether similar effects of chronic FLX treatment occurred in layer V neurons of a PFC region, the cingulate cortex (Cg1). As such I compared our observed chronic
FLX effects on 5-HT responses from Aim 1 with those of layer V pyramidal neurons in Cg1. I hypothesized that the expression of p11 in a large subpopulation of M1 neurons would result in different responses to 5-HT after chronic FLX than those seen previously in Cg1.

If p11 neurons are critical to the behavioural response to chronic FLX then it follows that the effects chronic FLX treatment might be altered in a mouse model of stress. Wallace et al. (2009) found that 10 weeks of social isolation produced mice with anxiety- and anhedonia-like symptoms which were reversed by chronic antidepressant treatment. As such the third aim of this thesis was to examine the effects of chronic FLX treatment on 5-HT responses in layer V pyramidal neurons from single-housed mice. I hypothesized that chronic social isolation would produce 5-HT signaling changes that would be altered by chronic FLX treatment.

The significance of this work was to further characterize a potential new target for depression treatment. Selective treatments would reduce the therapeutic delay to symptom relief currently experienced by patients taking SSRIs and potentially reduce harmful side effects such as suicidal ideation.

1.2 Literature Review

1.2.1 The serotonin system and SSRIs

Serotonergic neurons arise from the raphe nuclei located in the midbrain and project to many places in the brain, including the cortex (Lidov et al., 1980; Waterhouse et al., 1986; Araneda and Andrade, 1991). The layers of the cortex receive serotonergic innervations from the dorsal and/or median raphe nuclei, depending on the region. The motor cortex receives innervations from both the dorsal and median raphe nuclei while the PFC receives innervations from the dorsal raphe nuclei.

SSRIs act by blocking the 5-HT transporter protein and thereby inhibit reuptake of 5-HT into the “presynaptic” serotonin neuron. The term presynaptic is used loosely in this case since 5-HT is released by volume transmission, as opposed to at the more traditional synapses, in the cortex; Celada et al., 2004). Reuptake is blocked quickly, causing an initial increase in 5-HT in the raphe, which acts on autoreceptors to decrease 5-HT neuronal firing and thus 5-HT release in terminal regions. With chronic treatment over time extra-cellular 5-HT levels gradually increase.
It is this latter change in 5-HT levels which appears associated with improvements in the mood of the patient. Symptom alleviation takes weeks, with the amount of time depending on the SSRI (Marek et al., 2003; Celada et al., 2004; Cipriani et al., 2009; Papakostas et al., 2010). The time delay from starting treatment to experiencing effects of SSRIs is extremely problematic given the high incidence of suicide in depressed patients (Schloss and Williams, 1998). This suggests that there are long-term adaptive changes that occur during chronic treatment with an SSRI that lead to changes in patient mood.

Once 5-HT is released by raphe neurons (which is done in a paracrine manner; Celada et al., 2004), it binds to a group of receptors known as 5-HT receptors located on target neurons as well as on 5-HT neurons themselves (Table 1). All of these receptors (except for 5-HT3) are G-protein coupled receptors (GPCRs). 5-HT receptors consist of seven classes of receptors known as 5-HT1-7 and within these classes are multiple subclasses of receptors. Cortical pyramidal neurons express multiple 5-HT receptors including 5-HT1A, 5-HT2A, and 5-HT5A, which are the main receptors that contribute to 5-HT activity in these neurons (Pompeiano et al., 1992; Willins et al., 1997; Puig and Gulledge, 2011; Zhong and Yan, 2011; Avesar and Gulledge, 2012; Goodfellow et al., 2012).

The 5-HT1A receptor is found throughout the brain including the cortex and raphe nuclei. It couples to Gi/o proteins and its activation results in hyperpolarization through K+ channels (Araneda and Andrade, 1991; Hoyer et al., 2002; Celada et al., 2004; Benekareddy, et al., 2010; Zhong and Yan, 2011; Goodfellow et al., 2012). In the raphe nuclei 5-HT1A receptors are expressed on the soma and dendrites of cells and act to inhibit cell firing and thus the release of 5-HT (Hoyer et al., 2002). Extensive research has documented that inhibitory 5-HT1A autoreceptors located on 5-HT neurons of the dorsal raphe nucleus are desensitized by chronic SSRI treatment, thus enhancing 5-HT transmission postsynaptically in areas like the PFC (Poul et al., 2000; Celada et al., 2004; Descarries and Riad, 2012). In the cortex, by contrast, chronic FLX treatment has not been found to affect mRNA levels for 5-HT1A receptors (Poul et al., 2000) while labelling with [3H]8-OH-DPAT indicates that chronic citalopram treatment increases the density of 5-HT1A receptors (Klimek et al., 1994). As such the effect of SSRIs on 5-HT1A receptor expression in the cortex varies across drugs.
<table>
<thead>
<tr>
<th>5-HT Receptor Subtype</th>
<th>Coupling</th>
<th>Cortical Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>Gi/o</td>
<td>Cortical pyramidal neurons and interneurons</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>Gi/o</td>
<td>Cortical axon terminals</td>
</tr>
<tr>
<td>5-HT1D</td>
<td>Gi/o</td>
<td>Low levels in cortex</td>
</tr>
<tr>
<td>5-ht1E</td>
<td>Gi/o</td>
<td>Low levels in cortex</td>
</tr>
<tr>
<td>5-ht1F</td>
<td>Gi/o</td>
<td>Low levels in cortex</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>Gq/11</td>
<td>Cortical pyramidal neurons and interneurons</td>
</tr>
<tr>
<td>5-HT2B</td>
<td>Gq/11</td>
<td>None; expressed in cerebellum</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>Gq/11</td>
<td>Cortical pyramidal neurons</td>
</tr>
<tr>
<td>5-HT3</td>
<td>None</td>
<td>Cortical slow-spiking interneurons</td>
</tr>
<tr>
<td>5-HT4</td>
<td>Gs</td>
<td>Cortical pyramidal neurons</td>
</tr>
<tr>
<td>5-HT5A</td>
<td>Gi/o</td>
<td>Cortical pyramidal neurons</td>
</tr>
<tr>
<td>5-ht5B</td>
<td>?</td>
<td>None; expressed in hippocampus and medial habenulae</td>
</tr>
<tr>
<td>5-ht6</td>
<td>Gs</td>
<td>Cortical pyramidal neurons</td>
</tr>
<tr>
<td>5-HT7</td>
<td>Gs</td>
<td>Cortical pyramidal neurons (during development)</td>
</tr>
</tbody>
</table>

Table 1. 5-HT receptor subtypes, G-protein coupling, and cortical expression. (Wisden et al., 1993; Dawson et al., 2001; Hoyer et al., 2002; Puig and Gulledge, 2011; Zhong and Yan, 2011; Goodfellow et al., 2012)
The recently described 5-HT$_{5A}$ receptor is also expressed in raphe nuclei (Thomas, 2006) as well as cortical pyramidal neurons and contributes to the hyperpolarization produced by 5-HT (Goodfellow et al., 2012). This receptor also couples to G$_{i/o}$ proteins and produces hyperpolarization through inwardly rectifying K$^+$ channels (Kir3) in the rat and mouse PFC. In contrast to 5-HT$_{1A}$, 5-HT$_{5A}$ knockout (KO) mice do not display anxious behaviour (Grailhe et al., 1999) and as such its role in depression is unclear. Research in humans is inconclusive; one study found no genetic variation in 5-HT$_{5A}$ receptors in patients with affective disorders (Arias et al., 2001) while another found that at least one polymorphism in the 5-HT$_{5A}$ receptor could have a protective effect for developing affective disorders (Birkett et al., 2000). As such it has been suggested that 5-HT$_{5A}$ receptors may act as a biological safeguard to preserve hyperpolarization in cortical neurons (Goodfellow et al., 2012), although more research is needed to confirm this.

5-HT$_{2A}$ receptors are also expressed in cortical pyramidal neurons (Pompeiano et al., 1992; Willins et al., 1997; Benekareddy et al., 2010; Puig and Gullelde, 2011; Zhong and Yan, 2011; Goodfellow et al., 2012). In contrast to 5-HT$_{1A}$ and 5-HT$_{5A}$ receptors 5-HT$_{2A}$ receptors couple with G$_{q/11}$ proteins and their activation results in depolarization of cortical neurons (Araneda and Andrade, 1991; Marek et al., 2003; Benekareddy et al., 2010; Zhong and Yan, 2011; Goodfellow et al., 2012). Multiple studies have shown conflicting effects of SSRIs on 5-HT$_{2A}$ receptors (Klimek et al., 1994; Gray and Roth, 2001). Chronic citalopram treatment decreased 5-HT$_{2A}$ receptors while chronic fluoxetine treatment increased them, as measured by decreases and increases in [3H]ketanserin binding sites (Klimek et al., 1994). 5-HT$_{2A}$ antagonists have been found to augment the antidepressant effects of SSRIs (Marek et al., 2003; Marek et al., 2005), suggesting an important, yet complex, role of 5-HT$_{2A}$ receptors in affective disorders.

In summary, previous research indicates two primary (and opposing) responses to 5-HT in layer V pyramidal neurons. The first is an inhibitory response characterized by hyperpolarization which is predominantly mediated by 5-HT$_{1A}$ receptors and the second is an excitatory response characterized by depolarization predominantly mediated by 5-HT$_{2A}$ receptors (Araneda and Andrade, 1991; Marek et al., 2003; Benekareddy et al., 2010; Zhong and Yan, 2011; Goodfellow et al., 2012). Recent research found that 5-HT$_{5A}$ receptors also contribute to the inhibitory response observed in these neurons (Goodfellow et al., 2012). The balance in 5-HT activity maintained by these receptors is particularly important in layer V of the cortex. Layer V is responsible for cortical output; its primary neuron type, pyramidal neurons, projects to
multiple regions in the brain including the striatum, thalamus, nucleus accumbens, and contralateral cortex (Gabbott et al., 2005; Schmidt et al., 2012).

One previous study has examined the effect of chronic fluoxetine treatment on 5-HT-elicited activity of layer V PFC neurons (Zhong and Yan, 2011). In untreated neurons, 5-HT application elicited decreased firing rates in pyramidal neurons, an effect mediated by 5-HT$_{1A}$ receptors. Chronic FLX treatment (10 mg/kg/day for 21 days) had no effect on the intrinsic excitability of pyramidal neurons or the reductions in firing rate observed after 5-HT application. This lack of effect is consistent with chronic fluoxetine treatment having no effect on 5-HT$_{1A}$ receptor expression (Poul et al., 2000). Recently, however, a subpopulation of layer V pyramidal neurons, specifically corticostriatial projection neurons that express the protein p11, have been found to be integral to the response to SSRIs (which include FLX). Of note, p11 appears to be expressed much more strongly in M1 and sensory cortical regions than in PFC (Schmidt et al., 2012). As such FLX treatment may affect pyramidal neurons of differing regions in differing ways.

1.2.2 $\text{S}100\text{a10}/\text{p11}$ protein

One proposed target for the effects of SSRIs are a subpopulation of cortiostral projection neurons that express the adaptor protein p11 (product of the $\text{S}100\text{a10}$ gene) (Schmidt et al., 2012). The p11 protein, also known as S100a10 or annexin 2 light chain, is a small acidic protein that is insensitive to calcium. It forms symmetrical homo- and heterodimers, including a heterotetrameric complex with the well-characterized annexin A2 (Rescher and Gerke, 2008; Svenningsson et al., 2013). This complex is thought to be responsible for regulating protein trafficking and the expression of its targets in the cellular membrane (Rescher and Gerke, 2008) and as such is thought to travel within the cytoplasm from membrane to nucleus and vice versa (Oh et al., 2013). Specifically, the protein p11 has been proposed to act as a tether between proteins and annexin A2 (Rescher and Gerke, 2008).

Previous research has found that p11 interacts with a variety of ion channels, receptors, peptides, and enzymes, including sodium channels (Na$_{\text{v}}$1.8), potassium channels (TWIK-related acid-sensitive K 1), transient receptor potential channels (TRPV5 and TRPV6), acid sensing ion channels (ASIC1a), chromatin remodeling factors (SMARCA3), and serotonin receptors (5-HT$_{1B}$ and 5-HT$_{4}$) (Svenningsson et al., 2006; Rescher and Gerke, 2008; Schmidt et al., 2012; Oh et al.,
p11 is thought to bind to serotonin receptors 5-HT\textsubscript{1B} and 5-HT\textsubscript{4} and stabilize their localization and expression in the cell membrane (Svenningsson et al., 2006; Warner-Schmidt et al., 2009; Schmidt et al., 2012). Depressed patients and animal models of depression have been shown to have decreased levels of p11 in brain tissue (Svenningsson et al., 2006), an effect that is rescued by chronic SSRI treatment (Svenningsson et al., 2006; Egeland et al., 2011; Schmidt et al., 2012). Most importantly, recent research has found that knocking out cortical p11 expression abolishes the effects of chronic FLX treatment in mice, suggesting that the p11 protein is necessary for the response to SSRIs (Schmidt et al., 2012).

The Greengard and Heintz laboratories have generated S100a10 bacTRAP and S100a10 bacTRAP/p11 KO mice to characterize the expression of p11 in the cortex, which was localized to corticostriatal layer V pyramidal neurons of the motor, somatosensory, and visual cortex, with some sparse expression in cingulate areas of the PFC. Chronic FLX treatment significantly increased the expression of p11 in these areas and also altered the 5-HT receptor profile in these neurons. Pyramidal neurons are the primary neuron in layer V of the motor and PFC and typically co-express inhibitory 5-HT\textsubscript{1A} and 5-HT\textsubscript{5A} and excitatory 5-HT\textsubscript{2A} receptors (Amargos-Bosch et al., 2004; Santana et al., 2004; Puig and Gulledge, 2011; Goodfellow et al., 2012). In p11 expressing neurons however, chronic FLX treatment significantly increased expression of the excitatory 5-HT\textsubscript{4} receptor (Schmidt et al., 2012), which is not usually found in these neurons. Knocking down of the p11 expressed in these neurons also led to significant alterations in the 5-HT receptor profile, with observed decreases in 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{2C}, 5-HT\textsubscript{5A}, and 5-HT\textsubscript{7} receptor expression as well as abolishing behavioural responses to FLX (Schmidt et al., 2012).

5-HT\textsubscript{4}Rs are GPCRs that couple preferentially to the protein G\textsubscript{s}. A number of splice variants of the receptor have been previously identified, however the pharmacology of these variants is quite similar. They are expressed in the cortex in pyramidal neurons (Bockaert et al., 2004; Cochet et al., 2013). 5-HT\textsubscript{4}Rs couple positively to adenylate cyclase and modulate neurotransmitter release (including acetylcholine, dopamine, serotonin, and GABA) in the central nervous system (Hoyer et al., 2002) as well as excitability in multiple neurons types (Bockaert et al., 2004). They accomplish this by activating cyclic adenosine monophosphate (cAMP) and (PKA) pathways, which produces electrophysiological effects such as afterhyperpolarization reduction, spike accommodation reduction and depolarization increases in CA1 pyramidal neurons (Bockaert et al., 2004). Specific to the 5-HT system, 5-HT\textsubscript{4}R activation
has been found to increase 5-HT release in the hippocampus, an effect blocked by 5-HT$_4$R antagonists (GR 113808 and GR 125487) (Bockaert et al., 2004). It follows that a functional expression of 5-HT$_4$Rs in layer V pyramidal neurons of the cortex could have detectable effects on 5-HT electrophysiological responses in these neurons.

Previous research has not determined how expression of p11 affects 5-HT signaling in the cortex and how this signaling is affected by antidepressant treatment. As such the first aim of this thesis was to characterize the effects of chronic FLX treatment on 5-HT responses in neurons that express p11 and discover how they differ from neurons that do not express this protein. I hypothesized that p11 neurons would have increased excitatory activity potentially due to increased functional expression of excitatory 5-HT receptors. For this thesis I chose to focus on p11-expressing neurons of the motor cortex since I observed p11 expression to be particularly dense in this region (Figure 2. J,K).

1.2.3 The motor cortex: an unlikely target of SSRIs

Unlike the abundance of p11 neurons in layer Va of motor cortex, neurons that express p11 appear to be rare in the PFC (Schmidt et al., 2012). Yet previous research on affective disorders has focused heavily on the PFC as it is believed to play a large role in depression (Celada et al., 2004; Zhong and Yan, 2011; Avesar and Guleldege, 2012; Goodfellow et al., 2012). This is in no small part due to the reciprocal connectivity between the PFC and the raphe nuclei. The PFC receives innervations from serotonergic neurons from the dorsal raphe nucleus and pyramidal neurons from the PFC project back to the raphe, creating a feedback loop (Lidov et al., 1980; Waterhouse et al., 1986; Araneda and Andrade, 1991). The PFC also receives input from multiple brain regions and serves to process and integrate these signals. Pyramidal neurons then project these signals on to other regions including the nucleus accumbens, amygdala, hypothalamus, ventral tegmental area, substantia nigra, locus coeruleus, and other regions in the limbic system (Celada et al., 2004).

Multiple studies have investigated serotonergic signaling in the PFC and specifically in layer V. Using a mouse model of stress produced by maternal separation, Benekredddy et al., (2010) found that 5-HT-elicited signaling through 5-HT$_2$ receptors was enhanced in stressed mice. Another study investigated the effects of chronic FLX treatment on neurons from multiple
layers in the PFC, however they found that FLX had no effect on 5-HT-elicited responses in pyramidal neurons (Zhong and Yan, 2011).

In contrast very little research has focused on the motor cortex as important to affective disorders. The motor cortex receives serotonergic innervations from both the dorsal and the median raphe nuclei (Lidov et al., 1980; Waterhouse et al., 1986; Araneda and Andrade, 1991) and pyramidal neurons in layer V project to multiple brain regions including the striatum, thalamus, nucleus accumbens, and contralateral cortex (Schmidt et al., 2012). Similar to the PFC, M1 neurons also express 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{5A}$ receptors (Schmidt et al., 2012). However the recent discovery of p11-expressing neurons in the motor, somatosensory, and visual cortices indicates that layer V pyramidal neurons in the aforementioned regions are different from those in the PFC, and as such could exhibit different responses to chronic FLX treatment. To my knowledge, no one has investigated these responses in layer V pyramidal neurons from M1. As such the **second aim** of this thesis was to compare the effects of chronic FLX treatment on 5-HT responses in layer V pyramidal neurons between the M1 and cingulate cortex (Cg1), a key region from the mPFC. I hypothesized that the expression of p11 in M1 neurons would result in different responses to chronic FLX treatment than those seen previously in Cg1.

1.2.4 The motor cortex, serotonin, and depression

As mentioned above, very little research has focused on the role of the motor cortex in affective disorders and their treatment, however this area may be far more important than previously thought. The diagnosis for affective disorders includes a range of motor and psychomotor symptoms including disturbances in gross motor activity, speed of fine motor activity, psychomotor agitation, and altered speech patterns (Sobin and Sackeim, 1997; Schrijvers et al., 2008). In specific disorders, such as melancholic major depressive disorder, observable psychomotor symptoms can be the key to accurate diagnosis and treatment (Sabbe et al., 1997; Sobin and Sackeim, 1997; Parker, 2000). While these symptoms were originally believed to be due to alterations in dopaminergic neurotransmission (Schrijvers et al., 2008), evidence that motor cortex neurons are key to the response to chronic FLX treatment (Schmidt et al., 2012) suggests a role for the serotonergic system as well. A role for the serotonergic system can also be argued in that chronic FLX treatment relieves the psychomotor symptoms of affective disorders. Treatment has been found previously to relieve psychomotor agitation,
ambulation, and slow motor responses (Sabbe et al., 1997; Tollefson and Sayler, 1997; Sechter et al., 1999; Schrijvers et al., 2008).

The current gold standards for treatment of affective disorders are SSRIs, including FLX, which also suggest the role of the 5-HT system in affective disorder pathology. Some 5-HT$_{1A}$ partial agonists (ipsapirone) have failed to show any antidepressant response in human trials (Lapierre et al., 1998; Marek et al., 2003) while 5-HT$_{1A}$ antagonists have been found to accelerate the antidepressant response of SSRIs (Celada et al., 2004). These findings suggest that the desensitization of 5-HT$_{1A}$ receptors may be a long term process that is critical for the response to FLX, thus antagonizing these receptors in combination with FLX treatment may result in faster symptom alleviation. 5-HT$_2$ antagonists (M100907, risperidone, olanzapine, mirtazapine, and mianserin) have also been found to augment the antidepressant actions of SSRIs (Marek et al., 2003, 2005). This finding is supported by research in the PFC that found 5-HT$_{2A}$ receptor activity was enhanced in a mouse model of psychiatric vulnerability (without an accompanying increase in intrinsic excitability) (Benekareddy et al., 2010). In humans, postmortem analysis of frontal cortex tissue from patients with bipolar affective disorder revealed increased receptor-mediated activation of $G_q$, $G_i$, and $G_o$ proteins (Friedman and Wang, 1996), potentially indicating increased activity through activation of the 5-HT receptors (5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{5A}$ receptors) which couple to them. Together these findings suggest that 5-HT$_{1A}$ and 5-HT$_{2A}$ activity is altered as part of the pathology of affective disorders.

To my knowledge, no one has investigated responses to 5-HT and chronic FLX treatment in M1 p11 neurons from a mouse model of affective disorders. As such the third aim of this thesis was to examine the effect of chronic FLX treatment on 5-HT responses in layer V p11 pyramidal neurons in a mouse model of stress. I hypothesized that chronic stress would produce 5-HT signaling changes that would be altered by chronic FLX treatment. Wallace et al. (2009) described a model of stress induced by extended social isolation in adulthood. In this paradigm they single-housed rats for at least 10 weeks, and this isolation produced behavioural abnormalities consistent with stress (Wallace et al., 2009). In the elevated plus maze socially isolated rats spent less time in the open arms, indicating increased anxiety. Isolated rats had decreased preference for sucrose and sex, indicating anhedonia. They also displayed anxiety and agitation in the forced swim test where their locomotor activity and latency to immobility were increased. These behaviours were reversed by chronic antidepressant treatment (using
imipramine). As such I elected to use social isolation to induce a stressed phenotype in my experiments.
Chapter 2 - Methods

2.1 Experimental animals

Male and female S100a10 bacTRAP mice were a gift from the Heintz laboratory at Rockefeller University (Schmidt et al., 2012). At weaning, mice were assigned to either eight weeks of group (with 2 or 3 sibling mice of the same sex; Schmidt et al., 2012) or ten weeks of single housing (Wallace et al., 2009). In both housing conditions, transgenic mice were given *ad libitum* access to either a tap water and saccharin (1% saccharin sodium salt hydrate; Sigma Aldrich) solution or a tap water, saccharin (1%), and FLX (0.167 mg/ml; ANAWA Biomedical Services and Products) solution in a lightproof bottle for two to three weeks prior to sacrifice for recording (Schmidt et al., 2012). Solution was replaced every three to seven days. Non-treated transgenic mice were given *ad libitum* access to tap water. Group-housed mice consumed more saccharin solution than the tap water or FLX solutions (one-way ANOVA; F(2,77)=15.98, *p* < 0.001). All mice had *ad libitum* access to food. Group-housed mice received a dose of ~24 mg/kg of FLX and were 79 ± 1 days old at sacrifice. Single-housed mice received a dose of ~32 mg/kg of FLX and were 97 ± 1 days old at sacrifice. There were no apparent differences in weight across treatments, housings, or sexes.

2.2 Brain slice preparation

Coronal slices (400 µm thick) were prepared from the cerebral cortex (2.46 – 0.98 mm from bregma). Brains were rapidly cooled with 1°C oxygenated sucrose artificial cerebrospinal fluid (aCSF; 254 mM sucrose substituted for NaCl) then sliced with a DSK LinearSlicer Pro 7 (SciMedia, Irvine, CA). Slices were then transferred to oxygenated aCSF (128 mM NaCl, 10 mM D-glucose, 26 mM NaHCO3, 2 mM CaCl2, 2 mM MgSO4, 3 mM KCl, and 1.25 mM NaH2PO4, pH 7.4) at 30°C and allowed to recover for at least 1 h before recording (Goodfellow et al., 2012).
2.3 Electrophysiology

Slices were placed in a submersion chamber on the stage of an Olympus BX50WI microscope. Oxygenated aCSF flowed over slices at a rate of ~3 to 4 ml/min. Recording pipettes (2 – 4 MΩ resistance) pulled on a Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, CA) containing patch solution (120 mM potassium gluconate, 5 mM KCl, 2 mM MgCl₂, 4 mM K₂-ATP, 0.4 mM Na₂-GTP, 10 mM Na₂-phosphocreatine, and 10 mM HEPES, pH adjusted to 7.3 with KOH; Goodfellow et al., 2012) were used to patch layer V pyramidal neurons from cingulate (Cg1) and motor (M1) regions of the cerebral cortex. Pyramidal neurons were visually identified by their pyramidal shape and apical dendrites (Benekareddy et al., 2010; Zhong and Yan, 2011; Figure 2.J). GFP was visualized using an X-Cite 120Q light source (Lumen Dynamics, Mississauga, ON; Figure 2.J). Only neurons with GFP expression throughout the soma were kept for analyses (Figure 2.J). 5-HT currents and action potential activity were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). 5-HT (10 µM, 30 s) was bath applied to elicit currents and changes in firing rate. 5-HT currents were recorded at a holding potential of -75 mV and 5-HT action potential activity was recorded while injecting positive current to bring neurons to firing threshold (~4 – 5 Hz). Only neurons that returned to baseline following washout of 5-HT and neurons that fired continuously were kept for analysis. Data was acquired and low-pass filtered at 2 kHz using a Digidata 1440A data acquisition system (Molecular Devices, Sunnyvale, CA).

2.4 Pharmacology

All pharmacological agents were bath-applied to neurons. 5-HT (10 µM) was applied for 30s following a baseline period (Goodfellow et al., 2012). The 5-HT₁₅ antagonist WAY 100 635 (30 nM; Aghajanian and Marek, 1997) was applied for at least 10 min before recording 5-HT currents and action potential activity. WAY 100 635 did not wash out and was considered to be present for all recordings performed after initial bath application. Between slices the chamber was flushed with water to ensure removal of any lingering WAY 100 635. The 5-HT₂₅ anatagonist ketanserin (1 µM; Goodfellow et al., 2012) was applied for at least 10 min before recording. The 5-HT₅₅ antagonist SB-699551 (10 µM; Goodfellow et al., 2012) was bath applied
for at least 10 min before recording. The 5-HT$_{1B}$ antagonist SB-224289 (2 μM; Goodfellow et al., 2012) was bath applied for at least 10 min before recording.

The Na$^{+}$ channel antagonist tetrodotoxin (TTX, 2 μM; Goodfellow et al., 2012) was bath applied for 10 min. Before recording 5-HT currents we confirmed that action potentials had been abolished by bringing neurons to threshold with positive injected current. This block of voltage-gated Na$^{+}$ channels resulted in no action potential activity. In other experiments the AMPA receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; Zhong and Yan, 2011), NMDA receptor antagonist D-(-)-2-amino-5-phosphopentanoic acid (APV, 50 μM; Tian et al., 2011), and GABA$_{A}$ receptor antagonist bicuculline (10 μM; Zhong and Yan, 2011) were added to the bath aCSF. This altered aCSF was applied to a slice for at least 10 min prior to electrophysiological recording to allow for a complete block of synaptic activity. This receptor block resulted in the absence of EPSCs and IPSCs. In addition, FLX (10 μM; Zhong and Yan, 2011) was bath applied for at least 10 min to determine whether acute application affected 5-HT currents and action potential frequency. All pharmacological agents were obtained from Sigma-Aldrich or Tocris Bioscience and stored in stock solutions at -20°C.

2.5 Statistical Analyses

In all voltage clamp experiments peak 5-HT currents were measured using Clampfit software (Molecular Devices, Sunnyvale, CA) by subtracting the peak of the outward and/or inward current from the mean current at baseline (measured as the average current over 30s of baseline recording). Biphasic currents were divided into outward and inward components with the peak current included in analysis. Neurons with initial 5-HT currents less than 10 pA in amplitude were not included in analyses. In all current clamp experiments 5-HT action potential activity was measured using Clampfit software (Molecular Devices, Sunnyvale, CA) by subtracting the peak mean action potential frequency (measured as the average frequency over 30s following 5-HT exposure) from the mean action potential frequency at baseline (measured as the average current over 30s of baseline recording). Neurons without continuous firing were not included in analyses.

The 5-HT-elicited currents and action potential activity were examined using parametric and non-parametric statistical tests including t-tests, Chi-square tests, multivariate ANOVA,
repeated measures ANOVA, and Kruskal-Wallis tests. Between- and within-cell analyses were used to compare 5-HT currents and action potential frequency before and after application of pharmacological agents. Statistical tests and analyses were performed using SPSS 22 (IBM, Armonk, NY) and Prism 5 (GraphPad Software, La Jolla, CA) statistical software programs. Data from male and female mice was combined since no sex differences were observed (ANOVAs and unpaired t-tests, \( p > 0.05 \)). Data from mice given tap water or tap water and saccharin solution was combined into one control group since no differences between the groups were observed (unpaired t-tests, \( p > 0.05 \)). Graphs, pie charts, and tables were created using Prism 5 and Excel 2007 (Microsoft, Redmond, WA). Figures were created using Illustrator CS6 (Adobe, San Jose, CA). Example traces were obtained using Clampfit software. Tissue images were obtained using a Q-Imaging Camera (Q-Imaging, Surrey, BC) and Q Capture Pro 6.0 Software (Q-Imaging; Surrey, BC) and overlaid using Photoshop CS6 (Adobe, San Jose, CA).
Chapter 3 - Results

In order to examine how chronic FLX treatment (24 mg/kg, 2-3 weeks) altered 5-HT-elicited activity in M1 layer V pyramidal neurons, mice were treated with a tap water, saccharin (1%) and FLX (0.167 mg/ml) solution for two to three weeks. I then performed voltage- and current-clamp whole-cell in vitro electrophysiology recordings on neurons from these mice. Since roughly equal numbers of neurons were positive (eGFP+) and negative (eGFP-) for p11, I patched blind to GFP labelling and then confirmed whether it was present. First, I will present the results blind to GFP labelling (Figure 1) and then with respect to GFP labelling (Figure 2). Second I will present the results of chronic FLX treatment on neurons from Cg1 (Figure 3) and how these results compared to combined eGFP+ and eGFP- neurons from M1 (Figure 4). Last of all I will compare results from M1 neurons from group- and single-housed neurons mice (Figure 5) and how chronic FLX treatment affects neurons from single-housed mice (Figure 6).

3.1 Effects of chronic FLX treatment on 5-HT-elicited responses in M1 layer V pyramidal neurons.

Control neurons (n = 54) had a spike amplitude of 85 ± 1 mV (Figure 1.A), a resting membrane potential of -86 ± 1 mV (Figure 1.B), and an input resistance of 89 ± 5 MΩ (Figure 1.C). Compared to control neurons (n = 54), chronic FLX treatment (n = 79) significantly depolarized resting membrane potential (-83 ± 0 mV, unpaired t-test, t(131) = 5.28, p < 0.001; Figure 1.B) but did not affect spike amplitude or input resistance (unpaired t-tests, p > 0.05; Figure 1.A,C).

3.1.1 Effects of chronic FLX treatment on 5-HT-elicited currents

In neurons at rest, bath application of 5-HT (10 µM, 30 s) elicited outward (n = 48, 96%) and biphasic currents (an inward current followed by an outward current; n = 2, 4%; Figure 1.D). Chronic FLX treatment significantly increased proportions of 5-HT inward (n = 3, 4%) and biphasic currents (n = 14, 19%) and decreased proportions of 5-HT outward currents (n = 56, 77%; Chi-square(2) = 8.62, p = 0.01; Figure 1.D). In control neurons, 5-HT elicited outward currents (n = 48) with an average amplitude of 81 ± 6 pA (Figure 1.D) and duration of 104 ± 4 s (Figure 1.E). Chronic FLX treatment significantly decreased outward 5-HT current amplitude.
Figure 1. Chronic FLX treatment significantly decreases inhibitory activity through 5-HT$_{1A}$ receptors and increase excitatory activity through 5-HT$_{2A}$ receptors in M1 layer V.
**Pyramidal Neurons.** Combined data from eGFP+ and eGFP- M1 layer V pyramidal neurons from control mice and mice treated chronically with FLX (0.167 mg/ml, 2 weeks). **A,** Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps. **B,** Scatterplot showing resting membrane potential (mV) in neurons after break-in. Chronic FLX treatment significantly depolarized resting membrane potential (unpaired t-test; ***p < 0.001). **C,** Scatterplot showing input resistance (MΩ) in neurons after break-in. **D,** Scatterplot showing peak amplitude (pA) of currents elicited by bath application of 5-HT (10 µM, 30 s). Chronic FLX significantly decreased 5-HT current amplitude (unpaired t-test with Welch’s correction; ***p < 0.001). Pie charts showing proportions of neurons responding to 5-HT with outward, inward, and biphasic currents. Chronic FLX significantly increased proportions of 5-HT biphasic and inward currents (Chi-square; *p < 0.05). **E,** Scatterplot showing duration (s) of 5-HT outward currents from (D). Chronic FLX significantly increased 5-HT outward current duration (unpaired t-test with Welch’s correction; ***p < 0.001). **F,** Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). Chronic FLX significantly decreased 5-HT current amplitude (one-way ANOVA and unpaired t-test with Welch’s correction; ***p < 0.001). WAY 100 635 significantly decreased 5-HT current amplitude in neurons from control and FLX-treated mice (one-way ANOVA and unpaired t-tests with Welch’s correction; ***p < 0.001, *p < 0.05). **G,** Bar graph showing changes in 5-HT outward current amplitude (pA) after bath application of WAY 100 635 (30 nM, 10 min), Ketanserin (1 µM, 10 min), FLX (10 µM, 10 min), TTX (2 µM, 10 min), SB-224289 (2 µM, 10 min), and CNQX (20 µM)/APV (50 µM)/Bicuculline (10 µM, 10 min) in control and FLX-treated mice. WAY 100 635 significantly decreased 5-HT outward current amplitude in control and FLX-treated mice (paired t-tests; ***p < 0.001, **p < 0.01). **H,** Example traces of 5-HT-elicited decreases in spiking frequency. Chronic FLX significantly decreased the amplitude of 5-HT outward currents (unpaired t-test with Welch’s correction; ***p < 0.001). **I,** Example traces of 5-HT-elicited changes in spiking frequency. Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking frequency (Hz, bottom). Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking (unpaired t-test; ***p < 0.001). **J,** Scatterplot showing % change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (G). 5-HT significantly altered 5-HT spiking frequency in control and FLX-treated mice (paired t-tests; ***p < 0.001, **p < 0.01). Chronic FLX significantly decreased 5-HT-elicited decreases in spiking frequency (unpaired t-test with Welch’s correction; *p < 0.05). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency. **L,** Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635. Chronic FLX significantly altered 5-HT-elicited changes in spiking frequency (two-way ANOVA; *p < 0.05). **M,** Bar graph showing changes in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. WAY 100 635 significantly increased 5-HT-elicited spiking frequency in FLX-treated mice (paired t-test; *p < 0.05). Error bars represent standard error of the mean.
(44 ± 3 pA, n = 56; unpaired t-test with Welch’s correction: t(71) = 5.25, p < 0.001; Figure 1.D,F) and increased duration (132 ± 6 s; unpaired t-test with Welch’s correction: t(94) = 3.58, p < 0.001; Figure 1.E). 5-HT elicited biphasic currents (n = 2) with an average outward current of 33 ± 1 pA and inward current of -26 ± 1 pA (Figure 1.D), that had significantly longer duration (241 ± 4 s) than outward 5-HT currents (104 ± 4 s; unpaired t-test with Welch’s correction, t(6) = 23.83, p < 0.001; combined in Figure 1.E). Chronic FLX treatment significantly decreased the amplitude of the outward portion (21 ± 3 pA, n = 14; unpaired t-test with Welch’s correction: t(11) = 3.77, p = 0.003) but had no effect on the amplitude of the inward portion of 5-HT-elicited biphasic currents (unpaired t-test with Welch’s correction; p > 0.05; Figure 1.D) and decreased their duration (159 ± 17 s, n = 14; unpaired t-test with Welch’s correction: t(13) = 4.86, p < 0.001; Figure 1.E).

In control neurons, bath application of the 5-HT1A antagonist WAY 100 635 (30 nM, 10 min) significantly reduced 5-HT-elicited outward currents (before: 88 ± 12 pA; after: 27 ± 12 pA; a 65 ± 16 % reduction, n = 14; paired t-test, t(13) = 4.32, p < 0.001; Figure 1.G). After chronic FLX treatment, bath application of WAY 100 635 also significantly reduced 5-HT-elicited outward currents (before: 43 ± 6 pA; after: -5 ± 10 pA; a 97 ± 22% reduction, n = 18; paired t-test, t(17) = 3.69, p = 0.002; Figure 1.G). In preliminary experiments in control neurons, bath application of 5-HT2A antagonist ketanserin (1 µM, 10 min) appeared to reduce 5-HT-elicited inward currents and revealed 5-HT-elicited outward currents (before: -34 ± 15 pA; after: -3 ± 8 pA; an 83 ± 30% reduction, n = 3). After chronic FLX treatment, bath application of ketanserin completely blocked 5-HT-elicited inward currents and revealed 5-HT-elicited outward currents (before: -20 ± 6 pA; after: 18 ± 5 pA; a 276 ± 60% reduction, n = 8; paired t-test: t(7) = 4.70, p = 0.002). Further paired pharmacological examination of the 5-HT-elicited outward currents showed no significant effect of bath application of 5-HT2A receptor block with ketanserin, 5-HT1B receptor block with SB-224289 (2 µM, 10 min), suppression of spiking with TTX (2 µM, 10 min), or blockade of synaptic transmission with the combination of CNQX (20 µM, 10 min), APV (50 µM, 10 min), and bicuculline (paired t-tests, p > 0.05; Figure 1.G) in control neurons or neurons after chronic FLX treatment. Bath application of acute FLX (10 µM, 10 min) had no effect on 5-HT currents amplitude or duration from control neurons or neurons after chronic FLX treatment (paired t-tests, p > 0.05; Figure 1.G). Thus I concluded that 5-HT-
elicited outward currents were due to activation of 5-HT\textsubscript{1A} receptors located on the recorded M1 layer V pyramidal neurons and inward currents were due to 5-HT\textsubscript{2A} receptors.

3.1.2 Effects of chronic FLX treatment on 5-HT-elicited changes in excitability near threshold

5-HT is suggested to have membrane-dependent effects and as such I chose to examine neurons at rest and when active at threshold. To examine the effect of 5-HT on active neurons, I injected 177 ± 13 nA of current to bring neurons (n = 23) to a firing rate of 5 ± 0 Hz (Figure 1.J). Less current was required to bring neurons to firing after chronic FLX treatment (115 ± 7 nA, n = 35; unpaired t-test, \( t(36) = 4.20, p < 0.001; \) Figure 1.J), possibly because neurons were already depolarized (Figure 1.B). In order to compare neurons with similar projection targets, I recorded from layer V pyramidal neurons with continuous firing at baseline and after washout (Molnár and Cheung, 2006). Neurons with significant spike frequency accommodation or burst firing were not included in analyses.

In neurons with a consistent rate of firing at baseline (~48%), bath application of 5-HT elicited decreases (n = 11, 48%), increases (n = 8, 35%), and biphasic changes (a decrease followed by an increase; n = 4, 17%) in spiking frequency (Figure 1.K). Chronic FLX treatment had no effect on proportions of changes elicited by 5-HT (Chi-square; p > 0.05; Figure 1.K). In control neurons, 5-HT elicited a peak decrease in spiking frequency of -6 ± 1 Hz and a peak increase of 4 ± 1 Hz (Figure 1.K). Chronic FLX treatment significantly reduced 5-HT-elicited decreases in spike frequency (-3 ± 0 Hz; unpaired t-test with Welch’s correction: \( t(20) = 3.25, p = 0.004; \) Figure 1.K.L) but had no effect on 5-HT-elicited increases (unpaired t-test; p > 0.05; Figure 1.K). The increases in spiking frequency observed during 5-HT-elicited biphasic changes had substantial variability in their timing and were not included in analyses.

In preliminary experiments in control neurons, bath application of WAY 100 635 reduced the 5-HT-elicited decreases in firing frequency (before: -5 ± 1 Hz; after: -3 ± 2 Hz; a 44 ± 28% reduction, n = 4; Figure 1.M) in nearly all cells, however inhibitory activity was rarer in actively firing cells (Figure 1.K) and I did not have the power to determine significance. After chronic FLX treatment, bath application of WAY 100 635 significantly reduced 5-HT-elicited decreases in spiking frequency (before: -3 ± 0 Hz; after: -1 ± 1 Hz; a 228 ± 167% reduction, n = 6; paired
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t-test: $t(5) = 3.56, p = 0.02$; **Figure 1.M**. After chronic FLX treatment bath application of ketanserin completely blocked 5-HT-elicited increases in spiking frequency and revealed 5-HT-elicited decreases (before: $5 \pm 1$ Hz; after: $-3 \pm 1$ Hz; an $195 \pm 30\%$ reduction, $n = 6$; paired t-test: $t(5) = 5.09, p = 0.004$). Bath application of SB-224289 had no effect on 5-HT-elicited spiking frequency (paired t-test, $p > 0.05$; **Figure 1.M**).

In summary, these results demonstrate that 5-HT elicited inhibitory currents and firing activity in M1 layer V pyramidal neurons by directly activating 5-HT$_{1A}$ receptors on the neurons I recorded from. Chronic FLX directly alters 5-HT$_{1A}$ and 5-HT$_{2A}$ activity in these neurons. There is no evidence of unexplained excitatory currents or spiking in these neurons.

### 3.2 What does the expression of p11 in M1 layer V pyramidal neurons tell us about their responses to 5-HT?

In order to examine the effects of 5-HT and chronic FLX treatment on neurons that express the protein p11 I divided the neurons from **Figure 1** based on their fluorescence into eGFP- and eGFP+ neurons. Neurons from the mouse line I used have GFP tagged to the p11 promoter; as such when the neurons express p11 they also express GFP in their cytoplasm. eGFP+ neurons were found in layer Va of the motor cortex in these mice (**Figure 2.K**) and were easily identified by their fluorescent soma (**Figure 2.J**). From visual inspection approximately 50% of neurons were eGFP+ in control mice and mice after chronic FLX treatment.

As seen in **Figure 2**, the resting membrane potential of both eGFP- ($-87 \pm 1$ mV, $n = 31$) and eGFP+ ($-85 \pm 1$ mV, $n = 23$) neurons was depolarized by chronic FLX treatment (eGFP-: $-83 \pm 0$ mV, $n = 47$, eGFP+: $-83 \pm 0$ mV, $n = 32$; two-way ANOVA with posttests: $F(1,129) = 25.66, p < 0.001$; eGFP- Control vs FLX: $t = 4.82, p < 0.001$; eGFP+ Control vs FLX: $t = 2.56, p = 0.01$; **Figure 2.B**). Neuron type and chronic FLX treatment had no effect on spike amplitude or input resistance (two-way ANOVA, $p > 0.05$; **Figure 2.A,C**).
Figure 2

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O
Figure 2. Chronic FLX treatment significantly decreases 5-HT activity through inhibitory 5-HT1A and increases excitation in M1 layer V pyramidal neurons. Data from M1 layer V pyramidal neurons from control mice and mice treated chronically with FLX (0.167 mg/ml, 2 weeks) was divided into neurons expressing S100a10 (eGFP+) and neurons that do not express S100a10 (eGFP-). A, Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps. B, Scatterplot showing resting membrane potential (mV) in neurons after break-in. Chronic FLX treatment significantly depolarized resting membrane potential in eGFP- and eGFP+ neurons (two-way ANOVA; *** \( p < 0.001 \), * \( p < 0.05 \)). C, Scatterplot showing input resistance (M\( \Omega \)) in neurons after break-in. D, Scatterplot showing peak amplitude (pA) of currents elicited by bath application of 5-HT (10 \( \mu \)M, 30 s). Control eGFP- neurons had significantly larger 5-HT currents than eGFP+ neurons, a trend that continued after treatment (two-way ANOVA; ** \( p < 0.01 \)). Chronic FLX treatment significantly decreased 5-HT currents in eGFP- and eGFP+ neurons (two-way ANOVA; *** \( p < 0.001 \)). Pie charts showing proportions of neurons responding to 5-HT with outward, inward, and biphasic currents. Chronic FLX significantly increased the proportion of 5-HT biphasic and inward currents in eGFP-neurons (Chi-square; * \( p < 0.05 \)). E, Scatterplot showing duration (s) of 5-HT peak currents from (D). Chronic FLX treatment significantly increased 5-HT outward current duration in eGFP-neurons (Kruskal Wallis and unpaired \( t \)-test; *** \( p < 0.001 \)). F, Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). Chronic FLX significantly decreased 5-HT currents in eGFP-neurons (one-way ANOVA with unpaired \( t \)-tests with Welch’s correction, *** \( p < 0.001 \)). After chronic FLX, eGFP- neurons had significantly larger 5-HT currents than eGFP+ neurons (one-way ANOVA with unpaired \( t \)-tests with Welch’s correction, ** \( p < 0.01 \)). WAY 100 635 significantly decreased 5-HT current amplitude in eGFP-neurons from control mice (unpaired \( t \)-test with Welch’s correction; *** \( p < 0.001 \)). G, Bar graph showing % change in 5-HT outward current amplitude (pA) after bath application of WAY 100 635 (30 nM, 10 min) in control and FLX-treated mice. WAY 100 635 significantly decreased 5-HT outward current amplitude in control and FLX-treated mice (paired \( t \)-tests; ** \( p < 0.01 \), * \( p < 0.05 \)). H, Example traces of 5-HT outward currents after ~10 min application of CNQX, APV, and bicuculline. Control eGFP- neurons had significantly larger 5-HT outward currents than eGFP+ neurons, a trend that continued after treatment (one-way ANOVA and unpaired \( t \)-tests; ** \( p < 0.01 \), * \( p < 0.05 \)). Chronic FLX treatment significantly decreased 5-HT outward currents in eGFP- and eGFP+ neurons (Kruskal-Wallis and unpaired \( t \)-tests; *** \( p < 0.001 \), ** \( p < 0.01 \)). I, Example traces of 5-HT-elicited decreases in spiking frequency. J, Photomicrograph at high magnification of an M1 layer V pyramidal eGFP+ neuron being patched. K, Photomicrograph at low magnification of fluorescence contained in M1 layer Va. L, Scatterplots showing amount of current injected (nA; top) to reach threshold spiking frequency (Hz, bottom). Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking in eGFP- neurons (one-way ANOVA and unpaired \( t \)-test; *** \( p < 0.001 \)). M, Scatterplot showing % change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (L). 5-HT significantly altered 5-HT spiking frequency in control and FLX-treated mice (paired \( t \)-tests; *** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \)). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency. N, Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635. WAY 100 635 significantly decreased 5-HT-elicited decreases in spiking frequency in eGFP- neurons from control mice. O, Bar graph showing changes in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. Error bars represent standard error of the mean. This Figure contains data repeated from Figure 1.
3.2.1 p11 expression and the effects of chronic FLX treatment on 5-HT-elicited currents

In control eGFP- neurons, 5-HT-elicited outward currents ($n = 28$, $100\%$). Chronic FLX treatment significantly increased the proportion of 5-HT-elicited inward ($n = 2$, $4.5\%$) and biphasic currents ($n = 7$, $16\%$) and decreased the proportion of 5-HT-elicited outward currents ($n = 35$, $79.5\%$) in eGFP- neurons (Chi-square(2) = 6.55, $p = 0.04$; Figure 2.D) while having no effect on proportions in eGFP+ neurons (Chi-square, $p > 0.05$; Figure 2.D).

In control mice, eGFP- neurons had significantly larger 5-HT-elicited outward currents ($93 \pm 8$ pA, $n = 28$) than eGFP+ neurons ($64 \pm 9$ pA, $n = 20$; unpaired $t$-test with Welch’s correction: $t(42) = 2.48$, $p = 0.02$; Figure 2.D). Chronic FLX treatment significantly reduced 5-HT-elicited outward currents in eGFP- ($50 \pm 4$ pA, $n = 35$; unpaired $t$-test with Welch’s correction: $t(43) = 4.72$, $p < 0.001$; Figure 2.D) and eGFP+ neurons ($34 \pm 4$ pA, $n = 21$; unpaired $t$-test with Welch’s correction: $t(25) = 3.11$, $p = 0.005$; Figure 2.D). After chronic FLX treatment, eGFP- neurons still had significantly larger 5-HT-elicited outward currents than eGFP+ neurons (unpaired $t$-test with Welch’s correction: $t(53) = 2.71$, $p = 0.009$; Figure 2.D), and eGFP+ neurons appeared to have larger 5-HT-elicited inward currents than eGFP- neurons, although this effect only approached significance ($p = 0.052$; Figure 2.D).

Chronic FLX treatment significantly increased 5-HT-elicited outward current duration in eGFP- neurons (Control: $101 \pm 3$ s, $n = 28$; FLX: $137 \pm 9$ s, $n = 35$; unpaired $t$-test: $t(61) = 3.52$, $p < 0.001$; Figure 2.E) while having no effect on eGFP+ neurons (unpaired $t$-test, $p > 0.05$). Chronic FLX treatment had no effect on biphasic or inward current amplitude or duration (repeated measures and two-way ANOVA, $p > 0.05$; Figure 2.D,E).

Across treatments 5-HT outward currents in eGFP- and eGFP+ neurons were significantly decreased by WAY 100 635 (before: $62 \pm 7$ pA; after: $8 \pm 8$ pA; an $83 \pm 14\%$ reduction, $n = 32$; repeated measures ANOVA; $F(1,28) = 30.99$, $p < 0.001$; Figure 2.G) and responses to WAY 100 635 were similar across treatments and neurons (two-way ANOVA, $p > 0.05$; Figure 2.G). After chronic FLX treatment, bath application of ketanserin significantly reduced 5-HT-elicited inward currents in both eGFP- and eGFP+ neurons (eGFP- before: $-14 \pm 3$ pA; after: $15 \pm 7$ pA; a $281 \pm 86\%$ reduction, $n = 5$, eGFP+ before: $-31 \pm 16$ pA; after: $24 \pm 6$
pA; a 268 ± 93% reduction, n = 3; repeated measures ANOVA; F(1,6) = 30.66, p = 0.002; eGFP-: t = 3.15, p < 0.05; eGFP+: t = 4.57, p < 0.01).

3.2.2 p11 expression and the effects of chronic FLX treatment on 5-HT-elicited changes in excitability near threshold

In order to examine active neurons, I injected eGFP- neurons (n = 13) with 191 ± 14 nA of current to bring them to a firing rate of 6 ± 1 Hz and eGFP+ neurons (n = 10) with 160 ± 22 nA to bring them to a firing rate of 5 ± 1 Hz (Figure 2.L). Chronic FLX treatment significantly reduced the required injected current in eGFP- neurons (108 ± 7 nA, n = 20; one-way ANOVA; F(1,56) = 20.26, p < 0.001; unpaired t-test; t(31) = 5.78, p < 0.001; Figure 2.L) but had no effect on eGFP+ neurons or initial spiking frequency (one-way ANOVAs and posttests, p > 0.05; Figure 2.L). Chronic FLX treatment had no effect on proportions of changes or 5-HT-elicited increases or decreases in spiking frequency in eGFP- or eGFP+ neurons (Chi-square tests and two-way ANOVAs, p > 0.05; Figure 2.M).

Bath application of WAY 100 635 significantly reduced 5-HT-elicited decreases in spiking frequency (before: -4 ± 1 Hz; after: -2 ± 1 Hz; an 154 ± 101% reduction, n = 10; repeated measures ANOVA; F(1,6) = 8.80, p = 0.03) and responses to WAY 100 635 were similar across treatments and neurons (one-way ANOVAs, p > 0.05). After chronic FLX treatment, bath application of ketanserin significantly reduced 5-HT-elicited increases in spiking frequency in eGFP- neurons (before: 6 ± 2 Hz; after: -3 ± 2 Hz; an 171 ± 40% reduction, n = 4; repeated measures ANOVA; F(1,4) = 20.77, p = 0.01; eGFP-: t = 4.93, p < 0.05) and also appeared to reduced increases in eGFP+ neurons in preliminary experiments (before: 2 ± 0 Hz; after: -3 ± 0 Hz; a 243 ± 7% reduction, n = 2).

In summary, eGFP+ neurons have larger excitatory responses to 5-HT than eGFP-neurons. Chronic FLX treatment significantly increases excitation in both neuron types, but excitation may be inflated in eGFP+ neurons. Inhibitory and excitatory activity is regulated through 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in both neuron types.
3.3 Are similar changes seen in prefrontal cortex after chronic FLX treatment?

In order to compare responses to 5-HT and FLX between neurons from M1 and Cg1 I performed voltage- and current-clamp whole-cell in vitro electrophysiology recordings on neurons from Cg1 and compared that data to the M1 data described above (Figures 1, 2). Chronic FLX treatment significantly decreased spike amplitude (83 ± 1 mV, \( n = 2 \)) in Cg1 neurons (88 ± 1 mV, \( n = 21 \); two-way ANOVA: treatment: \( F(1,174) = 5.75, p = 0.02 \); interaction: \( F(1,174) = 4.30, p = 0.04 \); Cg1 Con vs FLX: \( t = 2.60, p = 0.01 \); Figure 3.A) however there were no differences between neurons from M1 and Cg1 (two-way ANOVA; \( p > 0.05 \); Figure 4.A). There were also no differences for resting membrane potential (Kruskal-Wallis and unpaired \( t \)-tests; \( p > 0.05 \); Figure 4.B). Across treatments M1 neurons had significantly smaller input resistance (Control: 89 ± 5 MΩ, \( n = 54 \); chronic FLX: 101 ± 5 MΩ, \( n = 79 \)) than Cg1 neurons (Control: 112 ± 9 MΩ, \( n = 21 \); FLX: 131 ± 13 MΩ, \( n = 24 \); Kruskal-Wallis test: Chi-square(1) = 8.75, \( p = 0.003 \); Control M1 vs Cg1: unpaired \( t \)-test: \( t(73) = 2.52, p = 0.01 \); chronic FLX M1 vs Cg1: unpaired \( t \)-test with Welch’s correction: \( t(29) = 2.21, p = 0.04 \); Figure 4.C).

### 3.3.1 Effects of chronic FLX treatment on 5-HT-elicited currents in Cg1 layer V pyramidal neurons

In Cg1 control neurons (\( n = 15 \)), 5-HT-elicited outward current (\( n = 14, 93\% \)) with a peak amplitude of 45 ± 7 pA and duration of 114 ± 11 s and a biphasic current (\( n = 1, 7\% \)) with an outward amplitude of 14 pA, an inward amplitude of -22 pA, and duration of 244 s. Chronic FLX treatment significantly increased proportions of 5-HT-elicited inward currents in Cg1 neurons (Chi-square(2) = 9.75, \( p = 0.008 \); Figure 3.D) but had no effect on 5-HT-elicited outward current amplitude or duration (Kruskal-Wallis and unpaired \( t \)-tests with Welch’s correction; \( p > 0.05 \); Figure 3.D). When all currents were combined, chronic FLX treatment significantly decreased 5-HT-elicited peak current amplitude (Control: 41 ± 8 pA, \( n = 15 \); chronic FLX: 7 ± 7 pA, \( n = 19 \); two-way ANOVA: \( F(1,153) = 30.89, p < 0.001 \); Cg1 Control vs FLX: \( t = 2.61, p = 0.01 \); Figure 3.D) but had no effect on current duration (two-way ANOVA with posttests; \( p > 0.05 \); Figure 3.E).
Figure 3. Chronic FLX treatment significantly decreases inhibitory activity through 5-HT<sub>1A</sub> receptors Cg1 layer V pyramidal neurons. Data from Cg1 layer V pyramidal neurons from control mice and mice treated chronically with FLX (0.167 mg/ml, 2 weeks). A, Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps. Chronic FLX treatment significantly decreased action potential amplitude (unpaired t-test; * p < 0.05). B, Scatterplot showing resting membrane potential (mV) in neurons after break-in. C, Scatterplot showing input resistance (MΩ) in neurons after break-in. D, Scatterplot showing peak amplitude (pA) of currents elicited by bath application of 5-HT (10 µM, 30 s). Chronic FLX significantly decreased 5-HT current amplitude (unpaired t-test; ** p < 0.01). Pie charts showing proportions
of neurons responding to 5-HT with outward, inward, and biphasic currents. Chronic FLX significantly increased proportions of 5-HT biphasic and inward currents (Chi-square; ** $p < 0.01$).  

$E$, Scatterplot showing duration (s) of 5-HT outward currents from (D).  

$F$, Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). Chronic FLX significantly decreased 5-HT current amplitude (unpaired $t$-test; ** $p < 0.01$). WAY 100 635 significantly decreased 5-HT current amplitude in neurons from control mice (unpaired $t$-tests; * $p < 0.05$).  

$G$, Bar graph showing % change in 5-HT outward current amplitude after bath application of WAY 100 635 (30 nM, 10 min) in control and FLX-treated mice. WAY 100 635 significantly decreased 5-HT outward current amplitude in control (paired $t$-test; *** $p < 0.001$).  

$H$, Scatterplots showing amount of current injected (nA; top) to reach threshold spiking frequency (Hz, bottom).  

$I$, Bar graph showing % change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (G).  

5-HT significantly altered 5-HT spiking frequency in control and FLX-treated mice (paired $t$-tests; * $p < 0.05$). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency.  

$J$, Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635.  

$K$, Bar graph showing % change in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. WAY 100 635 significantly increased 5-HT-elicited spiking frequency in control mice (paired $t$-test; * $p < 0.05$). Error bars represent standard error of the mean.
Figure 4. Chronic FLX treatment and 5-HT elicit different responses in layer V pyramidal neurons from M1 and Cg1. Data from M1 and Cg1 layer V pyramidal neurons from control mice and mice treated chronically with FLX (0.167 mg/ml, 2-3 weeks). A, Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps. Chronic FLX treatment significantly reduced spike amplitude in Cg1 neurons (two-way ANOVA; * p < 0.05). B, Scatterplot showing resting membrane potential (mV) in neurons after break-in. Chronic FLX treatment significantly depolarized resting membrane potential in M1 neurons (Kruskal-Wallis and unpaired t-tests; *** p < 0.001). C, Scatterplot showing input resistance (MΩ) in neurons after break-in. Cg1 neurons had significantly higher input resistance than M1 neurons across treatments (Kruskal-Wallis and unpaired t-tests; * p < 0.05). D, Scatterplot showing peak
amplitude (pA) of currents elicited by bath application of 5-HT (10 µM, 30 s). Cg1 neurons had significantly smaller 5-HT peak current amplitude than M1 neurons (two-way ANOVA with posttests; ** p < 0.01, * p < 0.05). Chronic FLX treatment significantly decreased 5-HT peak currents in M1 and Cg1 neurons (two-way ANOVA with posttests; *** p < 0.001, ** p < 0.01). Pie charts showing proportions of neurons responding to 5-HT with outward, inward, and biphasic currents. Chronic FLX treatment significantly increased proportions of 5-HT biphasic and inward currents in M1 and Cg1 neurons (Chi-square; ** p < 0.01, * p < 0.05). After treatment, Cg1 had significantly larger proportions of 5-HT inward currents than M1 (Chi-square; *** p < 0.001). E, Scatterplot showing duration (s) of 5-HT peak currents from (D). Chronic FLX treatment significantly increased 5-HT outward current duration in M1 neurons (two-way ANOVA with posttests; *** p < 0.001). F, Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT current amplitude in control and FLX-treated mice from M1 and control mice from Cg1 (one-way ANOVA and unpaired t-tests with Welch’s correction; *** p < 0.001; * p < 0.05). G, Bar graph showing % change in 5-HT outward current amplitude (pA) after bath application of WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT outward current amplitude in control and FLX-treated mice from M1 and control mice from Cg1 (paired t-tests; *** p < 0.001, ** p < 0.01). H, Scatterplots showing amount of current injected (nA; top) to reach threshold spiking frequency (Hz, bottom). Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking in M1 neurons (one-way ANOVA and unpaired t-test with Welch’s correction; *** p < 0.001). Control Cg1 neurons had significantly less threshold spiking than M1 neurons (two-way ANOVA; ** p < 0.01). I, Scatterplot showing % change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (H). 5-HT significantly altered 5-HT spiking frequency in control and FLX-treated mice from M1 and Cg1 (paired t-tests; *** p < 0.001, ** p < 0.01). Chronic FLX significantly reduced decreases in spiking frequency in M1 neurons (two-way ANOVA with posttests; * p < 0.05). Region M1 had significantly larger increases in spiking frequency than region Cg1 (two-way ANOVA; * p < 0.05). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency. J, Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635. K, Bar graph showing changes in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. WAY 100 635 significantly decreased changes in spiking frequency in Cg1 neurons from control mice and M1 neurons from FLX-treated mice (paired t-tests; * p < 0.05). Error bars represent standard error of the mean. This Figure contains data repeated from Figure 1.
After FLX treatment, Cg1 neurons had significantly larger proportions of 5-HT inward currents than M1 neurons (Chi-square(2) = 25.26, \( p < 0.001 \); Figure 4.D). Control CG1 neurons had significantly smaller 5-HT-elicited outward currents (45 ± 7 pA, \( n = 14 \)) than M1 neurons (81 ± 6 pA, \( n = 48 \); Kruskal-Wallis test: Chi-square(1) = 5.70, \( p = 0.02 \); unpaired \( t \)-test: \( t(60) = 2.90, p = 0.005 \)) but this difference disappeared after chronic FLX treatment (Kruskal-Wallis and unpaired \( t \)-test, \( p > 0.05 \)). There were no differences across regions for duration (one-way ANOVA; \( p > 0.05 \)). When all currents were combined, across treatments Cg1 neurons had significantly smaller 5-HT-elicited peak current amplitude (Control: 41 ± 8 pA, \( n = 15 \); chronic FLX: 7 ± 7 pA, \( n = 19 \)) than M1 neurons (Control: 79 ± 6 pA, \( n = 50 \); chronic FLX: 28 ± 5 pA, \( n = 73 \); two-way ANOVA: F(1,153) = 15.12, \( p < 0.001 \); Control Cg1 vs M1: \( t = 3.22, p = 0.002 \); chronic FLX Cg1 vs M1: \( t = 2.23, p = 0.03 \); Figure 4.D) but again there were no differences in duration (two-way ANOVA; \( p > 0.05 \); Figure 4.E).

Across treatments, bath application of WAY 100 635 significantly reduced 5-HT-elicited outward currents in Cg1 neurons (before: 58 ± 10 pA; after: 15 ± 7 pA; an 84 ± 9% reduction, \( n = 9 \); repeated measures ANOVA; F(1,37) = 25.11, \( p < 0.001 \); Figure 3.F,G). There were no differences in response to WAY 100 635 due to region or treatment (one-way ANOVAs, \( p > 0.05 \); Figure 4.F,G).

3.3.2 Effects of chronic FLX treatment on 5-HT-elicited changes in excitability near threshold in Cg1 layer V pyramidal neurons

To examine active neurons, I injected 158 ± 21 nA of current to bring neurons (\( n = 10 \)) to a firing rate of 3 ± 0 Hz (Figure 3.H). Chronic FLX treatment had no effect on the current required or initial spiking frequency (two-way and one-way ANOVA, \( p > 0.05 \); Figure 3.H). Cg1 control neurons had significantly lower initial spiking frequency than M1 control neurons (two-way ANOVA; F(1,79) = 6.06, \( p = 0.02 \), Control M1 vs Cg1: \( t = 2.53, p = 0.01 \); Figure 4.H) however this effect did not persist after chronic FLX treatment (two-way ANOVA with posttests, \( p > 0.05 \); Figure 4.H).

In neurons with a consistent rate of firing at baseline (~67% of all neurons) bath application of 5-HT elicited decreases (\( n = 4 \), 40%), increases (\( n = 5 \), 50%), and a biphasic change (a decrease followed by an increase; \( n = 1 \), 10%) in spiking frequency (Figure 3.H). 5-
HT-elicited a peak decrease in spiking frequency of $-3 \pm 1$ Hz ($n = 5$) and increase of $2 \pm 0$ Hz ($n = 5$; Figure 3.I). Chronic FLX treatment had no effect on proportions of changes or 5-HT-elicited decreases or increases (Chi-square tests and two-way ANOVAs, $p > 0.05$; Figure 3.I). Across treatments, Cg1 neurons had significantly smaller increases in spiking frequency ($2 \pm 1$ Hz, $n = 12$) than M1 neurons ($4 \pm 1$ Hz, $n = 25$; two-way ANOVA; $F(1,33) = 6.00$, $p = 0.02$; Figure 4.I).

Across treatments, bath application of WAY 100 635 significantly reduced 5-HT-elicited decreases in spiking frequency in Cg1 neurons (before: $-5 \pm 2$ Hz; after: $-3 \pm 2$ Hz; a 52 $\pm 13\%$ reduction, $n = 7$; repeated measures ANOVA; $F(1,13) = 18.26$, $p = 0.001$; Figure 3.J,K). There were no differences due to region or treatment (one-way ANOVAs, $p > 0.05$; Figure 4.J,K).

In summary M1 neurons have distinct responses to 5-HT and FLX from Cg1 neurons, potentially due to the expression of p11 protein in M1 layer V pyramidal neurons.

### 3.4 Does single-housing alter the effects of chronic FLX treatment on M1 layer V pyramidal neurons?

In order to examine the effects of 5-HT on M1 layer V pyramidal eGFP- and eGFP+ neurons I single-housed mice for 10 weeks after weaning (Wallace et al., 2009). During the last two weeks of single-housing mice were treated with a tap water and saccharin (1%) solution, a tap water, saccharin (1%) and FLX (0.167 mg/ml) solution, or tap water. I then performed voltage- and current-clamp whole-cell in vitro electrophysiology recordings on eGFP- and eGFP+ neurons from these mice. I compared this data to data from M1 layer V pyramidal neurons from group-housed mice described previously (Figures 1, 2).

In single-housed mice control eGFP- neurons ($n = 15$) had an action potential amplitude of $86 \pm 1$ mV (Figure 5.A), a resting membrane potential of $-87 \pm 1$ mV (Figure 5.B), and an input resistance of $85 \pm 10$ MΩ (Figure 5.C). Control eGFP+ neurons ($n = 9$) had an action potential amplitude of $87 \pm 2$ mV (Figure 5.A), a resting membrane potential of $-87 \pm 1$ mV (Figure 5.B), and an input resistance of $97 \pm 13$ MΩ (Figure 5.C). Housing had no effect on spike amplitude, resting membrane potential, or input resistance (three-way ANOVA, $p > 0.05$; Figure 6.A,B,C).
Figure 5. Chronic FLX treatment appears to increase excitation in active M1 layer V pyramidal neurons from single-housed mice. Data from M1 layer V pyramidal neurons from group-housed and single-housed (~10 weeks) control mice and mice treated chronically with FLX (0.167 mg/ml, 2 weeks). 

**A**, Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps in eGFP- and eGFP+ neurons from single-housed mice. After chronic FLX treatment, eGFP- neurons had significantly smaller spike amplitudes than eGFP+ neurons (three-way ANOVA; **p < 0.01**). 

**B**, Scatterplot showing resting membrane potential (mV) in eGFP- and eGFP+ neurons from single-housed mice. 

**C**, Scatterplot showing input resistance (MO) in eGFP- and eGFP+ neurons from single-housed mice.

**D**, Scatterplot showing S-H elicited current amplitude (μA) in eGFP- and eGFP+ neurons from single-housed mice. 

**E**, Scatterplot showing S-H elicited current amplitude (μA) in control and chronic FLX-treated eGFP- and eGFP+ neurons from single-housed mice.

**F**, Scatterplot showing % change in S-H elicited current amplitude in eGFP- and eGFP+ neurons from single-housed mice. 

**G**, Scatterplot showing % change in S-H elicited current amplitude in control and chronic FLX-treated eGFP- and eGFP+ neurons from single-housed mice.

**H**, Scatterplot showing injected current (μA) in eGFP- and eGFP+ neurons from single-housed mice.

**I**, Scatterplot showing % change in S-H elicited current amplitude in control and chronic FLX-treated eGFP- and eGFP+ neurons from single-housed mice.

**J**, Scatterplot showing % change in S-H elicited current amplitude in control and chronic FLX-treated eGFP- and eGFP+ neurons from single-housed mice.

**K**, Scatterplot showing % change in S-H elicited current amplitude in control and chronic FLX-treated eGFP- and eGFP+ neurons from single-housed mice.
potential (mV) in neurons after break-in in eGFP- and eGFP+ neurons from single-housed mice. Chronic FLX treatment significantly depolarized resting membrane potential in eGFP- and eGFP+ neurons (three-way ANOVA; *** $p < 0.001$, ** $p < 0.01$). C, Scatterplot showing input resistance (MΩ) in neurons after break-in in eGFP- and eGFP+ neurons from single-housed mice. Chronic FLX treatment significantly increased input resistance in eGFP- neurons (three-way ANOVA; ** $p < 0.01$). D, Scatterplot showing peak amplitude (pA) of currents elicited by bath application of 5-HT (10 µM, 30 s) in M1 neurons from single-housed control mice and mice treated chronically with FLX. Pie charts showing proportions of neurons responding to 5-HT with outward, inward, and biphasic currents. E, Scatterplot showing duration (s) of 5-HT peak currents from (D). Chronic FLX treatment significantly increased 5-HT outward current duration in eGFP+ neurons (two-way ANOVA with posttests; * $p < 0.05$). F, Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT current amplitude in control and FLX-treated mice (unpaired t-tests with Welch’s correction; ** $p < 0.01$). After WAY 100 635, eGFP- control neurons had significantly different currents than eGFP+ chronic FLX neurons (one-way ANOVA with posttests; ** $p < 0.01$). G, Bar graph showing % change in 5-HT outward current amplitude (pA) after bath application of WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT outward current amplitude in control and FLX-treated mice (paired t-tests; *** $p < 0.001$, * $p < 0.05$). H, Scatterplots showing amount of current injected (nA; top) to reach threshold spiking frequency (Hz, bottom). Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking in eGFP- neurons (two-way ANOVA with posttests; ** $p < 0.01$). After chronic FLX, eGFP+ neurons required more injected current than eGFP- neurons (two-way ANOVA with posttests; * $p < 0.05$). I, Scatterplot showing % change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (H). 5-HT significantly altered 5-HT spiking frequency in control and FLX-treated mice (paired t-tests; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency. FLX eGFP- neurons had significantly larger proportions of inward and biphasic currents than control eGFP+ neurons (Chi-square; * $p < 0.05$). J, Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635. After WAY 100 635, eGFP- control neurons had significantly different changes in spiking frequency than eGFP+ chronic FLX neurons (one-way ANOVA with posttests; * $p < 0.05$). K, Bar graph showing changes in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. Error bars represent standard error of the mean.
Figure 6. Single housing alters excitability in M1 layer V pyramidal neurons. Combined data from eGFP+ and eGFP- M1 layer V pyramidal neurons from group-housed and single-housed (~10 weeks) control mice and mice treated chronically with FLX (0.167 mg/ml, 2 weeks). A, Scatterplot showing spike amplitudes (mV) in neurons in response to depolarizing current steps in neurons from group- and single-housed mice. B, Scatterplot showing resting membrane potential (mV) in neurons after break-in in neurons from group- and single-housed mice. Chronic FLX treatment significantly depolarized resting membrane potential in neurons from group- and single-housed mice (one-way ANOVA and unpaired t-tests; *** p < 0.001). C, Scatterplot showing input resistance (MΩ) in neurons after break-in in neurons from group- and
single-housed mice. **D**, Scatterplot showing amplitude (pA) of outward currents elicited by bath application of 5-HT (10 µM, 30 s) in M1 neurons from group- and single-housed mice. Chronic FLX treatment significantly decreased 5-HT outward current amplitude in group-housed mice (two-way ANOVA; ***p < 0.001). Pie charts showing proportions of neurons responding to 5-HT with outward, inward, and biphasic currents. Control neurons from single-housed mice had significantly larger proportion of 5-HT biphasic and inward currents than group-housed mice (Chi-square; *p < 0.05). Chronic FLX treatment significantly increased proportions of 5-HT biphasic and inward currents in group-housed mice (Chi-square; *p < 0.05). **E**, Scatterplot showing duration (s) of 5-HT outward currents from (D). Chronic FLX treatment significantly increased 5-HT outward current duration in neurons from group- and single-housed mice (Kruskal-Wallis and unpaired t-tests; ***p < 0.001, *p < 0.05). After chronic FLX treatment, neurons from single-housed mice had significantly longer 5-HT outward current duration than group-housed mice (Kruskal-Wallis and unpaired t-tests; *p < 0.05). **F**, Scatterplot showing 5-HT peak currents in neurons and neurons exposed to WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT current amplitude in group- and single-housed control and chronic FLX mice (unpaired t-tests with Welch’s correction; ***p < 0.001, *p < 0.05). **G**, Bar graph showing % change in 5-HT outward current amplitude (pA) after bath application of WAY 100 635 (30 nM, 10 min). WAY 100 635 significantly decreased 5-HT outward current amplitude in control and FLX-treated mice (paired t-tests; ***p < 0.001). **H**, Scatterplots showing amount of current injected (nA; top) to reach threshold spiking frequency (Hz, bottom). Chronic FLX significantly decreased the amount of injected current required to reach threshold spiking in neurons from group-housed mice (three-way ANOVA; ***p < 0.001). **I**, Scatterplot showing percent change in spiking frequency elicited by 5-HT bath application in neurons held at threshold spiking with injected current (G). Pie charts showing proportions of neurons responding to 5-HT with decreases, increases, and biphasic changes in spiking frequency. **J**, Scatterplot showing 5-HT-elicited changes in spiking frequency in neurons and neurons exposed to WAY 100 635. **K**, Bar graph showing changes in 5-HT elicited decreases in spiking frequency after bath application of WAY 100 635. WAY 100 635 significantly reduced 5-HT-elicited decreases in spiking frequency after chronic FLX treatment (paired t-tests; **p < 0.01, *p < 0.05). Error bars represent standard error of the mean. This Figure contains data repeated from Figures 1, 4.
In single-housed mice, chronic FLX treatment significantly depolarized resting membrane potential in both eGFP- (−84 ± 1 mV, n = 18) and eGFP+ neurons (−82 ± 1 mV, n = 17; two-way ANOVA: F(1,55) = 21.30, p < 0.001; eGFP- Control vs FLX: t = 3.07, p = 0.003; eGFP+ Control vs FLX: t = 3.45, p = 0.001; Figure 5.B). Chronic FLX treatment also significantly increased input resistance in eGFP- neurons (126 ± 11 MΩ, n = 18; two-way ANOVA: Label by Treatment: F(1,55) = 4.23, p = 0.04; eGFP- Control vs FLX: t = 3.02, p = 0.004; Figure 5.C) but had no effect on eGFP+ neurons (two-way ANOVA, p > 0.05; Figure 5.C). After chronic FLX treatment eGFP- neurons had significantly smaller action potential amplitude (82 ± 1 mV, n = 18) than eGFP+ neurons (87 ± 1 mV, n = 17; two-way ANOVA: F(1,55) = 7.93, p = 0.007; FLX eGFP- vs eGFP+: t = 3.31, p = 0.002; Figure 5.A) and significantly larger input resistance (126 ± 11 MΩ, n = 18) than eGFP+ neurons (95 ± 10 MΩ, n = 17; two-way ANOVA: Label by Treatment: F(1,55) = 4.23, p = 0.04; FLX eGFP- vs eGFP+: t = 2.39, p = 0.02; Figure 5.C). Housing had no effect on spike amplitude, resting membrane potential, or input resistance (three-way ANOVA, p > 0.05; Figure 6.A,B,C).

3.4.1 Effects of chronic FLX treatment on 5-HT-elicited currents in M1 layer V pyramidal neurons from single-housed mice

In M1 eGFP- neurons from single-housed mice 5-HT elicited outward currents (n = 13, 81%) with a peak amplitude of 91 ± 8 pA and duration of 116 ± 14 s and biphasic currents (n = 3, 19%) with a peak outward amplitude of 49 ± 9 pA, inward amplitude of -45 ± 11 pA, and duration of 206 ± 18 s (combined; Figure 5.D). In M1 eGFP+ neurons from single-housed mice 5-HT elicited outward currents (n = 9, 75%) with a peak amplitude of 57 ± 11 pA and duration of 118 ± 14 s, an inward current (n = 1, 8%) with an amplitude of -18 pA and duration of 59 s, and biphasic currents (n = 2, 17%) with peak outward amplitude of 49 ± 19 pA, inward amplitude of -26 ± 5 pA, and duration of 266 ± 65 s (combined; Figure 5.D). There were no differences in neuron proportion between eGFP- and eGFP+ neurons (Chi-square test; p > 0.05; Figure 5.D).

Control eGFP- neurons had significantly larger 5-HT outward current amplitude (91 ± 8 pA, n = 13) than eGFP+ neurons (57 ± 11 pA; two-way ANOVA: Label by Treatment: F(1,42) = 5.41, p = 0.03; Control eGFP- vs eGFP+: t = 2.40, p = 0.02; Figure 5.D). Housing had no effect on 5-HT-elicited outward current amplitude (two-way ANOVA, p > 0.05; Figure 6.D). Single-
housed control eGFP- neurons had significantly longer duration (133 ± 15 s, n = 16) than group-housed neurons (101 ± 3 s, n = 28; Kruskal-Wallis Test; Chi-square (1) = 6.50, p = 0.01; unpaired t-test with Welch’s correction; t(16) = 2.12, p = 0.05; Figure 6.E).

Chronic FLX treatment significantly decreased 5-HT outward current amplitude in eGFP-neurons (59 ± 7 pA, n = 13; two-way ANOVA: Label by Treatment: F(1,42) = 5.41, p = 0.03; eGFP- Control vs FLX: t = 2.82, p = 0.007; Figure 5.D) and also increased current duration in these neurons (158 ± 14 s; one-way ANOVA: F(1,44) = 7.96, p = 0.007; unpaired t-test with Welch’s correction: t(23) = 2.21, p = 0.04; Figure 5.E). In eGFP+ neurons, FLX significantly decreased outward portions of 5-HT biphasic currents (16 ± 2 pA, n = 4; two-way ANOVA: F(1,7) = 9.91, p = 0.02; eGFP+ Control vs FLX: t = 2.64, p = 0.03; Figure 5.D). Label and treatment had no effect on 5-HT-elicited inward currents or biphasic current duration (two-way ANOVAs; p > 0.05; Figure 5.D,E). After chronic FLX treatment, single-housed eGFP+ neurons had significantly longer duration (198 ± 26 s, n = 14) than group-housed neurons (136 ± 9 s, n = 29; Kruskal-Wallis Test; Chi-square (1) = 6.50, p = 0.01; unpaired t-test with Welch’s correction; t(16) = 2.26, p = 0.04). Chronic FLX treatment and housing had no effect on current proportions (Chi-square; p > 0.05; Figure 6.D).

Bath application of WAY 100 635 significantly reduced 5-HT-elicited outward currents in eGFP- and eGFP+ neurons (before: 84 ± 7 pA; after: 17 ± 4 pA; a 77 ± 6% reduction, n = 16; repeated measures ANOVA; F(1,12) = 84.69, p < 0.001; Figure 5.G) and these changes were larger in eGFP+ neurons after chronic FLX treatment (before: 85 ± 19 pA; after: 8 ± 4 pA; an 87 ± 9% reduction, n = 3; two-way ANOVA; F(1,12) = 7.76, p = 0.02; eGFP+ Control vs FLX: t = 2.40, p = 0.03; Figure 5.G). Housing had no effect on responses to WAY 100 635 (repeated measures ANOVA, p > 0.05; Figure 6.F,G).

3.4.2 Effects of chronic FLX treatment on 5-HT-elicited changes in excitability near threshold in M1 layer V pyramidal neurons from single-housed mice

To examine active neurons from single-housed mice, I injected control eGFP- neurons (n = 8) with 151 ± 18 nA of current to bring neurons to a firing rate of 5 ± 1 Hz (Figure 5.H). In control eGFP+ neurons (n = 7) I injected 123 ± 13 nA of current to bring neurons to a firing rate of 4 ± 1 Hz (Figure 5.H). Chronic FLX treatment significantly decreased the required current in
eGFP- neurons \( (91 \pm 15 \text{ nA}, n = 8) \); two-way ANOVA; Interaction; \( F(1,30) = 5.33, p = 0.03 \), eGFP- Control vs FLX: \( t = 2.80, p = 0.009; \text{Figure 5.H} \) and after treatment eGFP+ neurons required more injected current \( (132 \pm 12 \text{ nA}, n = 11) \); two-way ANOVA; Interaction; \( F(1,30) = 5.33, p = 0.03 \); FLX eGFP- vs eGFP+: \( t = 2.06, p = 0.05; \text{Figure 5.H} \). Across treatments, neurons from single-housed mice required significantly less injected current \( (125 \pm 8 \text{ nA}, n = 34) \) than neurons from group-housed mice \( (140 \pm 8 \text{ nA}, n = 58) \); three-way ANOVA; \( F(1,84) = 4.11, p = 0.05; \text{Figure 6.H} \). Housing had no effect on initial spiking frequency (two-way ANOVA, \( p > 0.05; \text{Figure 6.H} \)).

In eGFP- neurons from single-housed mice 5-HT elicited decreases \( (n = 5, 63\%) \), increases \( (n = 2, 25\%) \), and biphasic changes \( (n = 1, 13\%) \) in action potential frequency (Figure 5.I). 5-HT elicited a peak decrease of \( 4 \pm 1 \text{ Hz} \) and a peak increase of \( 3 \pm 0 \text{ Hz} \) (Figure 5.I). In eGFP+ neurons 5-HT elicited decreases \( (n = 6, 86\%) \) and biphasic \( (n = 1, 14\%) \) changes in action potential frequency, with a peak decrease of \( 4 \pm 1 \text{ Hz} \) (Figure 5.I). Chronic FLX treatment had no effect on change proportions or 5-HT-elicited decreases in spiking frequency but did appear to increase 5-HT-elicited increases in spiking frequency, however too few increases were observed in the control groups to find significance (Chi-square tests and two-way ANOVAs, \( p > 0.05; \text{Figure 5.I} \)). Single-housed control eGFP- neurons had significantly smaller 5-HT-elicited decreases in spiking frequency \( (-4 \pm 1 \text{ Hz}, n = 6) \) than group-housed neurons \( (-6 \pm 1 \text{ Hz}, n = 11) \); three-way ANOVA; treatment by housing interaction; \( F(1,49) = 4.54, p = 0.04 \); Control eGFP- Group- vs Single-housed: \( t = 2.43, p = 0.02; \text{Figure 6.I} \). Single-housed eGFP+ neurons had significantly larger 5-HT-elicited increases in spiking frequency \( (8 \pm 2 \text{ Hz}, n = 4) \) than group-housed neurons \( (4 \pm 1 \text{ Hz}, n = 14) \); two-way ANOVA; label by housing interaction; \( F(1,31) = 4.75, p = 0.04 \); eGFP+ Group- vs Single-housed: \( t = 2.65, p = 0.01; \text{Figure 6.I} \). Single-handed control eGFP+ neurons had significantly fewer inward and biphasic and more outward 5-HT-elicited currents than group-housed neurons (Chi-square(2) = 6.68, \( p = 0.04; \text{Figure 6.I} \)).

Bath application of WAY 100 635 significantly reduced 5-HT-elicited decreases in spiking frequency in eGFP- and eGFP+ neurons (before: \(-4 \pm 1 \text{ Hz} \); after: \(-2 \pm 1 \text{ Hz} \); a \( 60 \pm 17\% \) reduction, \( n = 14 \); repeated measures ANOVA; \( F(1,10) = 25.03, p = 0.001; \text{Figure 5.K} \) and theses reductions were larger in eGFP+ neurons after chronic FLX treatment (Control: \( 16 \pm 26\% \) reduction, \( n = 4 \); chronic FLX: \( 113 \pm 6\% \) reduction, \( n = 3 \); two-way ANOVA; \( F(1,10) = 6.03, p = 0.03 \); eGFP+ Control vs FLX: \( t = 2.26, p = 0.05; \text{Figure 5.K} \)). Housing had no effect on
responses to WAY 100 635 (one-way ANOVA, $p > 0.05$; Figure 6.K). In preliminary experiments in eGFP+ neurons after chronic FLX treatment and WAY 100 635, bath application of ketanserin abolished increases and revealed decreases in spiking frequency (before: $0.4 \pm 0.1$ Hz; after: $-1.7 \pm 0.7$ Hz, $n = 3$).

In summary, 5-HT elicits inhibitory activity through 5-HT$_{1A}$ receptors in neurons from single-housed mice. Excitatory activity is notably sparse in control eGFP+ neurons, potentially due to downregulation of p11 during stress. Chronic FLX treatment significantly depolarizes resting membrane potential and increases excitatory activity in active neurons, indicating that excitatory activity might be key to the antidepressant response in these neurons. Direct differences between neurons from group- and single-housed mice are sparse. In control eGFP+ neurons from single-housed mice I observed far less excitation than in neurons from group-housed mice, potentially due to downregulated p11 expression.
Chapter 4 - Discussion

4.1 Summary of Findings

In order to characterize the effects of chronic FLX treatment on 5-HT responses in layer V pyramidal neurons I performed in vitro whole-cell electrophysiological recordings in acute cortical slices from transgenic mice. My first aim was to characterize the effects of chronic FLX treatment in neurons that express the protein p11 from M1, neurons which have been found to be important to the response to SSRIs (Schmidt et al., 2012). I hypothesized that p11 neurons would have increased excitatory activity potentially due to increased functional expression of excitatory 5-HT receptors. I found that p11-expressing control neurons responded to 5-HT with biphasic currents at rest (Figure 2.D) and significant increases in spike frequency at threshold (Figure 2.M), indicating increased excitation in these neurons. Chronic FLX treatment further excited these neurons by depolarizing their resting membrane potential (Figure 2.B) and increasing the proportion of neurons responding to 5-HT with biphasic and inward currents (Figure 2.D). Inhibitory responses were predominantly mediated by 5-HT$_{1A}$ receptors (Figure 2.F,G,N,O), and preliminary experiments suggest that excitatory responses were mediated by 5-HT$_{2A}$ receptors.

The effects of chronic FLX treatment on 5-HT responses had not been previously characterized in M1 since layer V pyramidal neurons of the PFC were thought to play a larger role in the response to SSRIs. As such my second aim was to compare the effects of chronic FLX treatment on 5-HT responses in layer V pyramidal neurons between M1 and Cg1, a key region from the PFC. I hypothesized that the expression of p11 in M1 neurons would result in different responses to chronic FLX treatment than those seen previously in Cg1. I found that Cg1 neurons had larger input resistances (Figure 4.C), smaller 5-HT-elicited currents (Figure 4.D), slower spiking frequency at threshold (Figure 4.H), and smaller 5-HT-elicited increases (Figure 4.I) than M1 neurons. Chronic FLX treatment did not depolarize the resting membrane potential (Figure 4.B) and did not reduce the required injected current to reach threshold (Figure 4.H) in Cg1 neurons as it did in M1 neurons. Inhibitory responses to 5-HT were predominantly mediated by 5-HT$_{1A}$ receptors in both M1 and Cg1 neurons (Figure 4.F,G,J,K).
To my knowledge, no one has investigated responses to 5-HT and chronic FLX treatment in M1 p11 neurons from a mouse model of affective disorders. As such my third aim was to examine the effect of chronic FLX treatment on 5-HT responses in M1 layer V p11 pyramidal neurons in a mouse model of stress. I utilized a social isolation paradigm described previously (Wallace et al., 2009) to generate stressed mice. I hypothesized that chronic stress would produce 5-HT signaling changes that would be altered by chronic FLX treatment. I found that single-housing nearly abolished excitatory responses to 5-HT in p11-expressing control neurons at threshold (Figure 5.I). Chronic FLX treatment significantly depolarized the resting membrane potential of p11-expressing neurons (Figure 5.B) and increased excitatory responses to 5-HT at threshold (Figure 5.I). In contrast to neurons from group-housed mice, chronic FLX treatment had no effect on 5-HT-elicited currents at rest (Figure 6.D). Inhibitory responses were predominantly mediated by 5-HT$_{1A}$ receptors (Figure 5.F,G,J,K).

4.2 Discussion

4.2.1 Effects of chronic FLX treatment on 5-HT responses in p11-expressing M1 layer V pyramidal neurons

4.2.1.a General Discussion

In both p11-expressing and non-p11 neurons chronic FLX treatment significantly depolarized resting membrane potential (Figure 2.B), thereby increasing excitability in both types of neurons. It is still unclear however which mechanisms are being affected by chronic FLX treatment and whether these are the same mechanisms in both neuron types. It is critical for future research to discover these mechanisms since an overall increase in intrinsic excitability on its own will significantly alter 5-HT signaling in both p11-expressing and non-p11 neurons. To determine whether p11 contributes to this effect, intrinsic properties of cells from p11-knock out (KO) mice (Svenningsson et al., 2006) could be recorded and compared to the data presented here.

In layer V pyramidal neurons from M1 (as well as from Cg1) I found that inhibitory activity was predominantly regulated by 5-HT$_{1A}$ receptors (Figure 1.F,G,L,M, 2.F,G,N,O) and excitatory activity was regulated by 5-HT$_{2A}$ receptors. This is consistent with previous research which also found that these two receptors mediate the majority of 5-HT-elicited activity in layer
V pyramidal neurons from the PFC (Araneda and Andrade, 1991; Benekareddy et al., 2010; Puig and Gulledge, 2011; Zhong and Yan, 2011; Avesar and Gulledge, 2012; Goodfellow et al., 2012).

Chronic FLX treatment significantly decreased inhibitory activity and increased excitatory activity in these neurons (Figure 1.D,E,K, 2.D,E). From my results it is unclear how 5-HT1A and 5-HT2A are altered by chronic FLX treatment, although previous research has found that FLX might increase 5-HT2A receptors in the cortex (Klimek et al., 1994) while having no effect on 5-HT1A mRNA (Poul et al., 2000). As such one possible scenario is that 5-HT1A receptors become desensitized by increased 5-HT levels after chronic FLX treatment, similar to what is observed in the raphe nuclei, while 5-HT2A receptor expression is increased, resulting in the lower inhibitory activity and increased excitatory activity I observed. Further research is needed to confirm whether this is the case.

In my experiments I observed some 5-HT-elicited hyperpolarization even in the presence of 5-HT1A antagonist WAY 100 635 (Figures 1.G,L,M, 2.F,G,N,O, 3.F,G,J,K, 4.F,G,J,K). I concluded that these currents were most likely due to 5-HT5A receptors as has been demonstrated in previous research (Goodfellow et al., 2012). 5-HT-elicited hyperpolarization could not have been due to interneuron activity since TTX, CNQX, APV, and bicuculline had no effect on 5-HT-elicited outward currents (Figure 1.G). It also could not have been due to p11-induced 5-HT1B receptor activity since it was not blocked by 5-HT1B antagonist SB-224289 (Figure 1.G) and not specific to p11 neurons (Figure 2.G). Future research could determine whether chronic FLX treatment has an effect on 5-HT1A vs 5-HT5A receptor mRNA, protein, and activity, particularly since WAY 100 635 appeared to nearly abolish 5-HT-elicited hyperpolarization in M1 neurons after chronic FLX treatment (Figures 1.G,M, 2.G,O). This future research could determine whether 5-HT5A receptors play a biological safeguard role in cortical layer V pyramidal neurons by preserving 5-HT-elicited inhibitory activity, as has been previously speculated (Goodfellow et al., 2012).

My findings that chronic fluoxetine treatment alters 5-HT responses in pyramidal neurons is inconsistent with Zhong and Yan (2011) who found that treatment produced no differences in action potential firing. However, a number of important differences exist between my experiments and theirs that could explain the differences observed. These include animal type
(rats), dosage of FLX (10 mg/kg), treatment method (intraperitoneal injection), preparation (HEPES-buffered a CSF and alternative patch solution), recording location (PFC), and neuron type (non-p11 expressing neurons).

4.2.1.b p11-expressing neurons

In p11-expressing M1 layer V pyramidal neurons (eGFP+ neurons) I also observed inhibitory and excitatory activity predominantly mediated by 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Figure 2.D,M). In comparison with control neurons that do not express p11 (eGFP- neurons), p11-expressing control neurons appeared to be responsible for the majority of excitatory activity I observed (Figure 2.D,M). This could indicate that p11 neurons express different profiles of 5-HT receptors than non-p11 neurons. In light of observations that 5-HT-elicited excitation was abolished by bath application of the 5-HT$_{2A}$ receptor antagonist ketanserin, it is possible that p11 could also affect 5-HT$_{2A}$ receptor expression. To my knowledge, however, this connection has not been previously explored. Previous research in these p11 KO found that cortical expression of mRNA for 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2C}$, 5-HT$_{5A}$, and 5-HT$_{7}$ receptors was significantly downregulated but they did not examine expression for 5-HT$_{2A}$ receptors (Schmidt et al., 2012). It is critical for future work to determine whether 5-HT$_{2A}$ receptors are also significantly affected by p11 expression and how downregulation of mRNA affects 5-HT signaling. This could be accomplished by examining the effects of chronic FLX treatment on 5-HT signaling in M1 neurons from p11 KO mice (Svenningsson et al., 2006) and by replicating Schmidt et al.’s (2012) qRT-PCR experiments for 5-HT$_{2A}$ expression.

The 5-HT-elicited excitatory activity I observed was abolished by bath application of the 5-HT$_{2A}$ antagonist ketanserin. I was surprised to find no unexplained excitatory responses to 5-HT, particularly after chronic FLX treatment, since Schmidt et al. (2012) found that chronic FLX treatment increased expression of p11 mRNA and Htr4. As such it is unclear from my results whether increased Htr4 expression results in the functional expression of 5-HT$_{4}$Rs in the membrane. It is possible however that any functional expression of 5-HT$_{4}$ receptors could result in other effects, such as the afterhyperpolarization reduction and spike accommodation reduction observed previously in CA1 pyramidal neurons (Bockaert et al., 2004). Alterations such as these could contribute to the increased excitability I observed in active p11 neurons (Figure 2.M). It is critical for future research to examine the properties of spikes and spike trains to determine
whether there is functional expression of 5-HT₄Rs. This could be accomplished by making use of the 5-HT₄ selective antagonist GR-113,808 and selective agonist BIMU8 to determine whether bath application of these drugs alters the properties mentioned above.

4.2.2 Chronic FLX treatment has different effects on M1 and Cg1 layer V pyramidal neurons

I found differences in the effects of chronic FLX treatment between M1 and Cg1 neurons. Notably, Cg1 neurons had significantly larger input resistance than M1 neurons (Figure 4.C), potentially also explaining why Cg1 neurons also had significantly slower spiking frequency (Figure 4.H) and why Cg1 neurons also had significantly smaller outward currents (Figure 4.D). This could also indicate different profiles of 5-HT receptors in these neurons; Cg1 neurons could possibly express more excitatory 5-HT₂A receptors than M1 neurons, which would reduce the amplitude of inhibitory currents produced by 5-HT₁A and 5-HT₅A receptors. Future research could determine why pyramidal neurons from two cortical regions develop different profiles of 5-HT receptor expression; I suspect that the projections from the median raphe nuclei that M1 also receives (in addition to projections from the dorsal raphe nuclei) might contribute to this difference since the region receives multiple 5-HT input.

After chronic FLX treatment Cg1 neurons also experienced increases in excitatory activity (Figure 4.D), however this did not include the depolarization of resting membrane potential experienced by M1 neurons (Figure 4.B). Also, the amplitude of inward currents in M1 neurons were significantly larger than in Cg1 neurons (Figure 4.D), potentially indicating that chronic FLX treatment results in increased excitation in M1 neurons. This could be due to a number of factors including the expression of p11 in M1 neurons, and as such the possible expression of functional 5-HT₄Rs as mentioned above. Further research is needed to determine which mechanisms facilitate the enhancement of excitation in M1 layer V pyramidal neurons after chronic FLX treatment.

As also mentioned above, my findings that chronic FLX treatment alters 5-HT responses in Cg1 layer V pyramidal neurons (Figure 3) are not consistent with previous research (Zhong and Yan, 2011) which found that chronic FLX treatment only affected 5-HT signaling in interneurons. While the potential reasons for this discrepancy are listed above, my findings
reveal that chronic FLX treatment can affect 5-HT signaling in Cg1 pyramidal neurons. In order to fully understand the effects of chronic FLX treatment on Cg1 neurons it will be critical for future research to examine the interactions between pyramidal neurons and interneurons (if any) before and after treatment. It is possible that interneurons in Cg1 might be affected whereas interneurons in M1 might not.

4.2.3 Effects of chronic FLX treatment on 5-HT signaling are altered in a mouse model of stress

One large difference I observed in stressed mice was that control p11 neurons from single-housed mice had very little excitation in active neurons (Figure 5.1). Since p11 expression has been found to be downregulated in affective disorders, my findings suggest that the excitation in active neurons from group-housed mice (Figure 2.M) might be mediated by p11. As mentioned above, it is unclear what mechanisms are behind this increase. My preliminary pharmacology work indicates that excitation after chronic FLX treatment in active neurons from single-housed mice is mediated by 5-HT2A receptors. As such there is again no evidence of a direct effect of functional 5-HT4Rs on 5-HT signaling, however these receptors might potentiate excitation by altering spike and spike train properties. The protein p11 might also have a previously undiscovered effect on 5-HT2A receptor expression as mentioned above, although an increase in 5-HT2A receptor expression after chronic FLX treatment is consistent with previous research (Klimek et al., 1994). It is most likely a combination of these effects in p11-expressing neurons, although it is critical for future research to determine whether this is the case, as mentioned above.

Chronic FLX treatment also appeared to enhance 5-HT-elicited increases in action potential frequency in non-p11 neurons (Figure 5.1), indicating that chronic FLX treatment increased excitability in both neuron types. This suggests that stress alters excitatory signaling in all M1 layer V pyramidal neurons. As mentioned above chronic FLX treatment has been found to increase 5-HT2A receptor expression (Klimek et al., 1994) and as such an added potentiation from the effects of p11 protein could account for the differences between p11-expressing and non-p11 neurons. Again however it is not clear whether different mechanisms regulate excitability changes in p11 and non-p11 neurons; future research is needed to determine which mechanisms mediate these changes in excitability. Future research could examine the effect of
single-housing on p11 KO mice to determine how p11 influences 5-HT and FLX responses in neurons from stressed mice.

4.3 Summary and conclusions

In summary, chronic FLX treatment has different effects on p11-expressing M1 layer V pyramidal neurons than non-p11 neurons. Effects on M1 layer V pyramidal neurons differ from those observed on Cg1 neurons. In all regions, 5-HT elicits inhibition predominantly mediated by 5-HT$_{1A}$ receptors and excitation mediated by 5-HT$_{2A}$ receptors. Stress alters M1 neurons’ response to 5-HT, especially in p11-expressing neurons, where little excitation is observed, however excitation is increased by chronic FLX treatment. Across all experiments chronic FLX treatment increased excitation in layer V pyramidal neurons. My results suggest that 5-HT receptor activity is altered by both chronic FLX treatment and chronic stress, although how it is altered is a subject for future research. The most critical future research is to examine the mechanisms behind the decreases in inhibition and increases in excitation observed in these neurons. Important steps include determining which 5-HT receptors p11 interacts with, whether 5-HT$_4$Rs play a role in these alterations, and how the typical 5-HT receptor profile (5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{5A}$) is altered by chronic FLX treatment. Utilizing p11-KO mice to complete these future experiments is vital to fully understanding the role of p11.

In conclusion, 5-HT signaling in the cortex is immensely complex and not fully characterized. As such, the effects of chronic stress on the 5-HT system are not well understood, but seem to be integral to understanding many diseases, including affective disorders. This is particularly since SSRIs (including FLX) have been so successful in treating these disorders in the past. These treatments are far from perfect however and the pressure to develop faster-acting treatments with specific targets and to further develop our understanding of affective disorders remains high. Research describing the p11 neurons integral to the SSRI response is a step in the right direction, however much work still remains before we understand why this protein is so important.
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