The role of the 5-HT1A autoreceptor in response to antidepressant treatment

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Abstract

Selective Serotonin Reuptake Inhibitors (SSRIs) are the first-line treatment for major depression, but require several weeks to elicit a clinical effect. One mechanism that may underlie this delay in SSRI action implicates the gradual desensitization of 5-HT1A autoreceptors, leading to enhanced firing and increasing serotonin (5-HT) at the synapse. I hypothesized that in absence of 5-HT1A autoreceptors, fluoxetine (FLX) would improve behavior faster and more effectively. To specifically knock out 5-HT1A receptors in 5-HT neurons, we crossed TPH2-CRE^{ERT2} and flx5HT1A-YFP mice to generate the flx1A mice, a tamoxifen-inducible conditional knockout. Tamoxifen-induced recombination in adult flx1A-/- mice induced YFP expression and reduced 5-HT1A receptor levels by over 90%, specifically in TPH-positive cells of the raphe. To test the response to sub-chronic SSRI treatment, the mice were treated for 9 days with SSRIs FLX or escitalopram and examined for anxiety and depression-like behavior using multiple tests, including the novelty suppressed feeding test (NSF) that responds to chronic but not acute SSRI treatment. Subchronic FLX treatment had no effect in flx1A +/+ control mice, but resulted in an unexpected anxiogenic effect in flx1A -/- littermates, in both the NSF and elevated plus maze tests. Subchronic treatment with escitalopram also increased anxiety-like behavior in the NSF in flx1A-/-, but not wild-type mice. To determine whether FLX treatment differentially affected brain activity in these mice, the number of FosB-stained cells was determined as an index of chronic activation. In flx1A -/- vs. +/+ mice, the number of FosB-positive cells was reduced in several brain regions linked to anxiety and depression including hippocampus and entorhinal cortex, and FLX

treatment reduced activation in the flx1A +/+ compared to -/- mice these areas and in 5-HT neurons of the median raphe nucleus. These results suggest that in the absence of 5-HT1A autoreceptors, SSRIs have a pro-anxiety effect that may involve reduced inactivation anxiety-related brain regions.

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List of Abbreviations

^{125}I-MPPI 4-(20-methopxyphenyl)-1-[20- (n-200-pyridinyl)-p-[125I]iodobenzamido]ethylpiperazine

5-HT Serotonin; 5-hydroxytryptamine

5-HIAA 5-hydroxyindoleacetic acid

5-HTP 5-Hydroxytryptophan

5-HT1A Serotonin 1A receptor

AADC Aromatic amino acid decarboxylase

ANOVA Analysis of variance

BLA Basolateral amygdala

BP Base pair

cAMP cyclic adenosine monophosphate

CSF Cerebrospinal fluid

DA Dopamine

DR Dorsal raphe

EC Entorhinal cortex

EPM Elevated plus maze

ER Estrogen receptor

ESC Escitalopram

FLX Fluoxetine

FST Forced swim test

LHB Lateral habenula

Het Heterozygous

HPC Hippocampus

KD knockdown

KO Knockout

LD Light-dark box

LS Lateral septum

MR Median raphe

MS Medial septum

Nacc Nucleus accumbens

NDS Normal donkey serum

NSF Novelty Suppressed Feeding

OF Open field test

PBS Phosphate Buffered Saline

PCR Polymerase chain reaction

PFA Paraformaldehyde

PFC Prefrontal cortex

SERT Serotonin transporter

SNRI Selective Noradrenalin reuptake inhibitor

SSRI Selective serotonin reuptake inhibitor

TAM Tamoxifen

TCA Tricyclic antidepressant

TPH Tryptophan hydroxylase

TS Tail suspension

VTA Ventral tegmental area

WT Wild type

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Introduction

Major Depressive Disorder

Depression and anxiety are heterogeneous and multifactorial illnesses that negatively affect mood and emotions (El Yacoubi, Bouali et al. 2003, aan het Rot, Mathew et al. 2009, Albert, Vahid-Ansari et al. 2014). Major depressive disorder (MDD) is a severe mental illness characterized by low mood, anhedonia, alterations of circadian rhythm and appetite, low self-esteem and feeling of hopelessness. This debilitating illness causes significant impairments with everyday tasks, social interactions and judgment.

In Canada, MDD has a lifetime prevalence of 9.9% (Patten, Williams et al. 2015) and 11.3% of Canadians will experience a major depressive episode during their lifetime and an increasing trend has been noted in the last decade. Also, prevalence in women is 1.5 to 3 fold higher than in men (Albert, Vahid-Ansari et al. 2014). MDD is considered the third contributor to the worldwide disease burden and it is estimated that it will be the major contributor by 2030 (Branchi, Santarelli et al. 2013).

Diagnosis

MDD can be diagnosed using different approaches including nosological/categorical, syndromal or dimensional/functional. The nosological approach is a rigid and strict system. It categorizes each psychiatric disorder as a discrete entity with specific symptomatology, course, outcome and pathophysiology. The inherent problem with this approach is that patients need to meet all the criteria to qualify for a particular diagnosis,

leading to under diagnosis. MDD is heterogeneous, thus, the symptoms vary depending on the person, the environment and the context.

The syndromal approach emphasizes the syndrome, thus, symptomatology is the only criterion used. The inherent problem with this approach is that syndromes may appear incomplete to fit one mental illness or mixed with other syndromes.

The dimensional/functional approach dissects the abnormal mental state into compartments and assesses the severity of each of them. This method provides the clinician a detailed, precise and scientific depiction of abnormal mental states and helps direct targets for treatment.

These diagnostic strategies are now combined to create a multi-tier diagnosing. It characterizes the nosological cluster the mental illness corresponds to and depicts a precise syndrome analysis to categorize them. However, several mental illnesses share the same physical, cognitive and emotional symptoms. This can lead to misdiagnosis, thus, mistreatment. Furthermore, patients that do not fit in a category can be overlooked because their symptoms are not severe enough. It is important and crucial that every patient with abnormal mental states is properly diagnosed and treated to avoid further complications like suicide.

Serotonin System and Depression

The first scientific hypothesis to explain MDD is the monoamine hypothesis where low synaptic monoamines (in particular 5-HT and noradrenaline) were thought to trigger MDD (Schildkraut and Kety 1967). This reduction in monoamines was thought to be due to lower synthesis, reuptake, metabolization or storage processes (Hasselmann 2014). An

alternate possibility is that 5-HT levels are normal but ineffective because of receptor malfunction (Hindmarch 2001).

Serotonin (5-HT) is a monoamine neurotransmitter derived from the essential amino acid tryptophan. In the brain raphe nuclei, this amino acid is converted into 5-hydroxytryptophan by the rate-limiting enzyme Tryptophan Hydroxylase 2 (TPH2), which is then converted to 5-HT by aromatic amino acid decarboxylase (AADC) (Cote, Thevenot et al. 2003). In the body, 5-HT is widespread and is synthesized by TPH1 in the gastrointestinal system, and is accumulated in the blood in platelets that express the 5-HT transporter (Amireault, Hatia et al. 2011). Thus, its function differs depending on the region it is expressed. 5-HT neurons send projections throughout the brain including to regions linked to anxiety and depression (e.g. prefrontal cortex, nucleus accumbens, basal ganglia, ventral hippocampus) (Albert and Lemonde 2004). The major implications of these neurons are in the cognitive and integrative function and also in mediating mood and emotions. Thus, dysregulation of this system can lead to mood disorders, including anxiety and depression, and ultimately to suicide (Mann 1999).

Based on the monoamine hypothesis, researchers started thinking that vulnerability to depression was due to a decrease in serotonergic activity (Mann 1999). A few years later, the 5-HT system was studied in depressed patients to further understand the neurochemical changes. Initially, the level of 5-HIAA, the major 5-HT metabolite and an indicator of 5-HT release and turnover, was shown to be reduced in the cerebrospinal fluid (CSF) of depressed patients (Dencker, Malm et al. 1966, Mendels, Frazer et al.

1972). Also, there was a negative correlation between 5-HIAA levels in CSF of depressed patients and the severity of suicidal behavior, but not severity of depression (Mann, Malone et al. 1996).

Another useful approach to study the role of 5-HT in depression in humans is using depletion to reduce brain 5-HT. This could be achieved by either blocking the tryptophan hydroxylase (TPH) using para-chlorophenylalanine (PCPA) or via diet by acute tryptophan depletion, which drastically reduces tryptophan and 5-HT levels (Shopsin, Friedman et al. 1976, Delgado, Price et al. 1994). These studies showed that acute tryptophan depletion leads to relapse in recovered depressed patients and this effect can be reversed by the addition of tryptophan.

Advances in technology led to the development of Positron Emission Tomography (PET), a brain imaging technique that uses specific probes to determine metabolic brain activity or label receptors in the brain of depressed living patients. Using this technique, there is evidence of a reduction of 5-HT precursor l-5-HTP transported to the brain in patients with MDD in comparison to healthy controls (Agren, Reibring et al. 1991). Also, there is a reduction in binding potential of 5-HT transporter (SERT) in depressed patients and a more pronounced reduction in depressed patient who never received antidepressant medication (Parsey, Hastings et al. 2006). Interestingly, this research group also showed an increase in 5-HT1A binding potential in MDD patient and among subjects with remitted MDD (Miller, Brennan et al. 2009). Furthermore, other studies using post-mortem brain analysis of suicide victims showed an increase in 5-HT1A autoreceptors

(Albert and Lemonde 2004). These findings suggest a relationship between MDD and suicide patients and regulation of 5-HT1A autoreceptors expression.

In summary, MDD is associated with an altered monoamine system, in particular 5-HT system, which results in decrease 5-HT and 5-HIAA levels. MDD is also associated with increased levels of 5-HT1A receptors. These results provide evidence that depression is linked to a reduction in serotonergic activity.

Treatment for Depression

The main target of drugs most commonly used to treat depression is the 5-HT system. The first antidepressants consisted of three classes: tricyclic antidepressants (TCA), monoamine oxidase A inhibitors (MAOIs) and the selective serotonin reuptake inhibitors (SSRIs) (Hasselmann 2014). TCAs act by blocking both SERT and noradrenaline transporters. Since TCAs act on two different monoamine systems, several side effects have been noted including dizziness, drowsiness and sexual dysfunction. For these reasons, they are rarely prescribed. MAOIs act by inhibiting the enzyme that converts monoamines to their respective metabolites, the increasing all monoamines. Patients under this medication need to be careful with their diet since a diet rich in tryptophan could trigger a hypertensive episode or 5-HT syndrome. Even though MAOIs have similar side effects as TCAs, they are still prescribed for atypical depression, treatment-resistant depression and bipolar depression (Thase 2012). SSRIs directly target the 5-HT system by blocking specifically SERT and remain the most commonly prescribed antidepressants due to their specificity and tolerability. However, it has been reported

that SSRI treatment in adolescents might trigger suicidal behavior (Gibbons, Brown et al. 2007, Barbui, Esposito et al. 2009).

These antidepressant treatments all have a delay of several weeks before an appreciable antidepressant effect is seen. Furthermore only 50% of depressed patients respond to the treatment, and even fewer (30%) show remission (Rush, Warden et al. 2009).

Other strategies can be used to treat severe depression. Electroconvulsive therapy (ECT) has been shown to be the most effective treatment for depression (Lisanby 2007). It consists in the application of electricity to the scalp to induce seizure activity to enhance brain activity.

Once the patient has failed to improve following multiple medications, psychotherapy and ECT, the depression is considered treatment-resistant. There is no proven treatment for this type of depression but promising results have been noted with deep brain stimulation (DBS) which consists in implanting electrodes in specific brain regions and applying electrical impulses (Mayberg, Lozano et al. 2005). Studies in animal models suggest a key role for 5-HT in the antidepressant actions of DBS (Hamani and Nobrega 2012). Lastly, low dose of ketamine was proven to treat acutely suicidal patient (Zigman and Blier 2013, Murrough, Soleimani et al. 2015).

Serotonin System

5-HT action is mediated by the 5-HT receptors. There are seven families of 5-HT receptors (5-HT1 to 5-HT7), with subtypes within a family identified by letter (e.g., 5-HT1A-F, 5-HT2A-C). With the exception of the 5-HT3 receptor, which is a ligand-gated

ion channel, 5-HT receptors are all G-protein coupled receptors. The 5-HT4, 6 and 7 are coupled to G_s subunit, which activates adenylyl cyclase and increases the production of cAMP leading to neuronal activation. The 5-HT1 and 5-HT5 are classified as inhibitory receptors coupled to G_i/G_o proteins. Upon 5-HT binding to the receptor, $G_{I/o}$ alpha subunit inhibits adenylyl cyclase, which decreases the production of cAMP. This molecule is a key component of several intracellular cascades including PKA-CREB pathways. In addition, release of $G_{beta/gamma}$ subunits changes the conductance of voltage-gated potassium and calcium channels leading to hyperpolarization of the cell (Gross, Zhuang et al. 2002) and reduction in neuronal firing. The 5-HT1A autoreceptor (5-HT1AR) is considered the strongest negative regulator of 5-HT firing, thus it has a strong influence on the activity of the 5-HT system (Blier and El Mansari 2013).

The 5-HT1A receptor

Auto- and hetero-receptor

The 5-HT1AR is encoded by a single intronless gene and has no spliced variant in the coding sequence (Albert, Zhou et al. 1990). The 5-HT1AR is expressed presynaptically (as an autoreceptor) and postsynaptically (as a heteroreceptor). The presynaptic 5-HT1A autoreceptors are found on the cell body and dendrites of 5-HT neurons located in the dorsal and median raphe (Verge, Daval et al. 1985). These receptors regulate 5-HT release by mediating a negative feedback inhibition via their activation and are considered to be important regulators of the 5-HT system (Albert 2012).

The 5-HT1A heteroreceptors are expressed on non-serotonergic neurons like pyramidal cortical neurons and interneurons and mediate the action of serotonin on target neurons. They are abundantly expressed within the limbic system including amygdala, septum, hippocampus and prefrontal cortex (Albert and Lemonde 2004) where they regulate the action of serotonin on fear, anxiety, stress and cognitive function (Albert, Vahid-Ansari et al. 2014). Also, the 5-HT1A heteroreceptors indirectly regulate 5-HT release by inhibition of pyramidal cortical neurons projecting to the raphe nuclei (Celada, Puig et al. 2004).

Transcriptional Regulation of Htr1a

The promoter of the 5-HT1AR gene (Htr1a) contains several binding sites for transcription factors (Figure 1). Five-prime repressor element under dual repression (Freud) is one of them that represses 5-HT1A gene expression in raphe 5-HT neurons but also in non-serotonergic neurons (Ou, Lemonde et al. 2003). Deformed epidermal autoregulatory factor 1 (Deaf1) is another regulator that binds to a site located upstream of the promoter. In the raphe nuclei, Deaf1 represses Htr1a but, in non-serotonergic cells, it enhances the promoter activity (Czesak, Le Francois et al. 2012). In the Deaf1 binding site, there is a C(-1019)G polymorphism in the 26 bp palindrome. Analysis of this polymorphism showed that Deaf1 and Hes5 could bind to the C allele but not the G allele (Lemonde, Turecki et al. 2003, Albert, Le Francois et al. 2011). The G/G genotype leads to an increased level of 5-HT1AR in the raphe and decrease post-synaptic 5-HT1AR. A study performed on depressed patient showed that the ones with the G/G genotype have increase 5-HT1AR binding potential (Parsey, Oquendo et al. 2006). Thus, alteration of

the transcription factors impacts the levels of the 5-HT1A receptor which can influence mood and emotions.

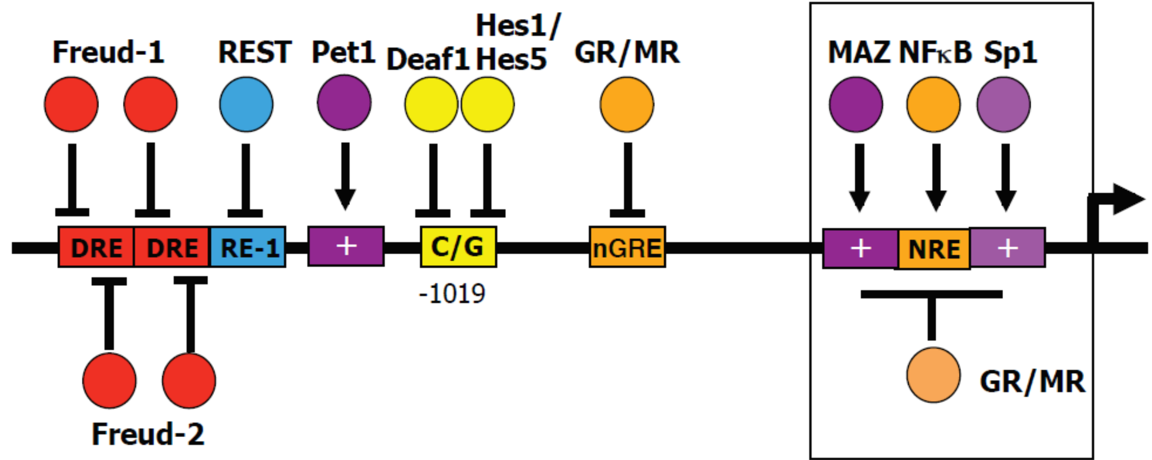

Figure 1:Promoter elements of the human 5-HT1A receptor gene (Htr1A)
From (Albert, Le Francois et al. 2011). Identified activator (arrow) and repressor (bar) are shown. Freud-1, Freud-2, REST, Deaf1 and Hes1/5 repress the expression of the 5-HT1A gene. There is a glucocorticoid response element (nGRE) that mediated the repression by GR/MR. Pet-1 is a strong enhancer specifically in 5-HT neurons.

Implication in SSRI action

The 5-HT1AR is at the center of the most widely accepted theory to explain the 3-4 weeks' delay in SSRI action (Pineyro and Blier 1999, Albert 2012). The latter involves the desensitization of the receptor after chronic treatment. When the drug is administered to the patient, there is a transient increase of 5-HT at the synapse due to the inhibition of SERT. However, because of projections to the cell body, the local release of 5-HT in the raphe nuclei activates the 5-HT1A autoreceptors. This activation leads to a reduction in neuronal firing to rebalance the enhancement of the synaptic 5-HT levels induced by the SSRI treatment. Thus, the short-term effect of SSRI on 5-HT neurotransmission is thought to be attenuated by signaling of the 5-HT1A autoreceptors. However, after chronic SSRI treatment, there is a reduction in the activity and number of 5-HT1A autoreceptors via desensitization. The resulting disinhibition of 5-HT neurons enhances their firing rate, leading to an increase in 5-HT release. These delayed changes are thought to underlie the beneficial effects of SSRI treatment.

Studying Mental illness in rodents

Anxiety and Depression

The approach to study mental illness in rodents is different than in humans and mainly relies on analysis of stereotype behaviors. Anxiety and depression are transformed into measurable and quantifiable properties to be assessed in rodents (Table 1). Rodent models are advantageous to study mechanisms of mental illness because they can be

genetically modified to examine the role of specific genes, or they can be treated with different drugs to model drug action or dissect the roles of specific drug targets.

Symptom	How might symptom be modelled in mice?
Avoidance of places from which escape could be difficult (agoraphobia)	Increased avoidance of exposed, well-lit areas
Sudden onset of intense fearfulness, often with respiratory distress and fear of 'going crazy' (panic attack)	Increased flight from a predator
Anxiety provoked by social situations, leading to avoidance behaviour (social phobia)	Low social interaction with unfamiliar conspecific
Anxiety provoked by a specific feared object, leading to avoidance behaviour (specific phobia)	Conditioned taste avoidance
Re-experiencing a traumatic event, leading to increased arousal and avoidance of stimuli associated with the event (post-traumatic stress disorder)	Increased freezing response to fear-conditioned cue or context
Anxiety-provoking obsessions and anxiety-reducing compulsions (obsessive–compulsive disorder)	Increased marble burying and excessive grooming
Difficulty concentrating or mind going blank (generalized anxiety disorder)	Impaired sustained attention
Sleep disturbance/insomnia	Abnormal sleep architecture (measured using electroencephalogy)
Autonomic hyperarousal (tachycardia, blushing, sweating and frequent urination)	Radiotelemetric measurement of heart rate dynamics during anxiety-provocation, such as increased stress-induced hyperthermia
Flashbacks of traumatic events	Impairment in extinction of fear memory
Cognitive bias towards ambiguous or weak threat cues	Increased fear conditioning to partial threat cue
Heightened startle response, particularly in threatening contexts	Increased acoustic startle response and fear-potentiated startle response
Separation anxiety	Increased ultrasonic vocalizations in pups separated from their mother
Feelings of losing control or going crazy during a panic attack	Cannot be modelled

*Symptoms used in the *Diagnostic and Statistical Manual-IV* diagnosis of anxiety disorders.

Table 1: Symptoms of anxiety disorders in humans (left) and how they are modeled in mice (right). From (Cryan and Holmes 2005)

There are several assays that have been developed and are considered valid to study anxiety in mice (Belzung and Griebel 2001). It is important to note that mice have a natural aversion for bright light, open areas and heights. They are also naturally curious animals that explore any new environment they are placed in. These assays are using this conflict to assess the anxiety-like behavior. Typically, the light-dark box (LD), elevated plus maze (EPM) and the open field tests (OF) are used to assess an acute anxiety-like behavior (Albert, Vahid-Ansari et al. 2014). The more time spent in bright lid or open area, the less the anxiety-like phenotype (Lister 1987, Borsini, Podhorna et al. 2002, Cryan and Holmes 2005). The novelty-suppressed feeding test is also used to assess the anxiety levels and importantly it has been shown to respond to chronic but not acute antidepressant treatment (Santarelli, Saxe et al. 2003). Therefore, this paradigm can be used to assess the anxiolytic effect of certain drugs.

The tests typically used to assess the depression-like phenotype are based on behavioral despair in an inescapable stress. The forced swim test (FST) and tail suspension test (TS) are two inescapable paradigm based on the immobility of the animal as a measure of despair. In the FST, the mouse in placed in a cylinder of water. After a short period of habituation, the mouse will start floating instead of swimming. Typically, climbing and time spent immobile are measured. Similarly, in the TS, the mouse is suspended by its tail and its movements (struggling period or immobility) are recorded (Steru, Chermat et al. 1985, Ripoll, David et al. 2003, Cryan, Mombereau et al. 2005). The FST and TS respond to acute antidepressant treatment. Therefore, they are often used

to assess antidepressant-like effect of different drugs (Petit-Demouliere, Chenu et al. 2005, Castagne, Moser et al. 2010).

5-HT1A Transgenic Models

Over the years, the design of transgenic and knockout mice has evolved with our knowledge and the advance in technologies. Therefore, there are several genetic models altering 5-HT1AR expression in mice that have been used to study the role of 5-HT1AR in anxiety and depression (Table 2).

Genetic model	Target gene expression			Adult behavior	References
	Embryo (%)	Early PN (%)	Adult (%)		
5-HT1A					
5-HT1A$^{-/-}$	–	–	–	Anxiety, antidepressed	Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998
5-HT1A auto-iS (Pet1-tTX)	+	+	−30	Antidepressed, SSRI+	Richardson-Jones et al., 2010
5-HT1A auto-iS (Pet1-tTX)	+	−40	+	Anxiety, social−	Donaldson et al., 2014
5-HT1A auto-iS (Pet1-tTX)	−80	−80	−80	Anxiety	Richardson-Jones et al., 2011
5-HT1A hetero-iS (CamKII-tTX)	−95	−95	−95	Depression	Richardson-Jones et al., 2011
5-HT1A auto-siRNA	+	+	−80	Anti-depressed, SSRI+	Bortolozzi et al., 2012; Ferres-Coy et al., 2013
5-HT1A hetero-rescue (CaMKII-tTA iTg)	–	+	–	Anti-anxiety	Gross et al., 2002
5-HT1A-heteroOE (5-HT1A-tg)	+	++	+	Anti-anxiety, Anti-depressed	Kusserow et al., 2004
5-HT1A-autoOE (Tph2-tg)	+300	+300	+300	Aggression, anxiety	Audero et al., 2013; Piszczek et al., 2013
5-HT1A-auto rescue (Tph2-tg)	+300	+300	+300	Anxiety, anti-depressed	Audero et al., 2013; Piszczek et al., 2013
5-HT					
TPH2$^{-/-}$	–	–	–	Anti-Anxiety, depression, aggression	Mosienko et al., 2012
TPH2-R439H KI	−80	−80	−80	Anxiety, depression	Jacobsen et al., 2012b; Sachs et al., 2013
PET1$^{-/-}$	−80	−80	−80	Aggression, anxiety	Hendricks et al., 2003
Pet1-adult-iKO	+	+	−80	Anxiety	Liu et al., 2010
En1/Pet1-TetTX IT	–*	–*	–*	Anti-Anxiety, cognitive+	Kim et al., 2009
5-HTT					
5-HTT-/-	–	–	–	Anxiety, depression anti-aggression	Holmes et al., 2002, 2003; Lira et al., 2003; Kalueff et al., 2006
5-HTT-FLX	+	–	+	Anxiety	Ansorge et al., 2004
5-HTT-G56A KI	++*	++*	++*	Social−, SSRI+	Veenstra-Vanderweele et al., 2012
5-HTT-tg	+100	+100	+100	Anti-anxiety	Jennings et al., 2006

Table 2: Genetic models involving the 5-HT system.
From (Albert, Vahid-Ansari et al. 2014). The promoter used is indicated in parentheses. The effect on the indicating genetic model on target gene expression (-, none; +, normal; ++, increased; +X, increase by X%) at indicated developmental time [embryo, early postnatal (PN) or adult] associated with its adult behavior phenotype or response to SSRIs.

The initial genetic model of the 5-HT1AR was a constitutive and global 5-HT1AR knockout. The vector used was designed to replace a portion of protein-coding region with a neomycin resistance cassette. This replacement caused a loss of function due to a truncated 5-HT1AR at the third intracellular loop (Heisler, Chu et al. 1998, Parks, Robinson et al. 1998, Ramboz, Oosting et al. 1998). The knockout mice displayed a consistent increase in anxiety-like behavior in several assays, including OF and EPM. However, a global knockout does not address the effect of losing autoreceptors versus heteroreceptors. Thus, the enhanced anxiety-like behavior cannot be associated with the loss of one of the 5-HT1AR populations.

A complementary study used a transgenic model where the 5-HT1AR were overexpressed in the CNS (Kusserow, Davies et al. 2004). They found a significant decrease in anxiety-like behavior of both male and female mice confirming the importance of the 5-HT1AR in mediating anxiety-like behavior. Once again, these studies were limited due to their lack of specificity and embryonic alteration of the 5-HT system that was made. Embryonically, 5-HT is thought to regulate the maturation of target brain regions. Thus, early life dysregulation of the system may lead to permanent developmental alterations in brain function and behavior (Whitaker-Azmitia 2001).

To tease out the importance of each 5-HT1AR population, researchers started using tissue-specific promoters to drive the expression of the 5-HT1AR or of other proteins like Cre recombinase. The idea is that the 5-HT1AR or Cre enzyme will only be expressed in a certain cell type or tissue (e.g. Pet-1 or TPH2 for 5-HT neurons and CAMKIIα for glutamatergic pyramidal in the forebrain (Albert, Vahid-Ansari et al. 2014)) resulting in a

conditional knockout/knockin model. To prevent the embryonic alteration of 5-HT system, temporal control of gene modification is now possible using a fusion protein consisting of Cre and a mutated estrogen receptor ER^T or ER^{T2} that will only bind tamoxifen to allow the excision of the flanked sequence (Dhaliwal and Lagace 2011).

To address whether the loss of the 5-HT1A heteroreceptors is responsible for the anxiety behavior previously seen in the global knockout, Gross and colleagues (Gross, Zhuang et al. 2002) used a tissue-specific and conditional 5-HT1A receptor rescue mouse model. The 5-HT1AR gene was modified by the insertion of a STOP cassette at the 5' untranslated region of the gene to block its transcription. A tetO binding site upstream of the protein-coding sequence was inserted to permit the gene transcription in presence of the bacterial transcription factor tTa. Furthermore, the CaMKIIα promoter was used to drive the expression of the tTa in the forebrain glutamatergic pyramidal neurons. On the 5-HT1AR -/- background, the rescued mice had the same level of anxiety as the global 5-HT1A knockout mice when the receptor was rescued in adulthood. However, when the forebrain 5-HT1A receptors were rescued in early postnatal development, they observed a rescued anxiety-like phenotype. These results suggest that a normal anxiety level in adults is likely due to proper signaling through the 5-HT1A heteroreceptors in forebrain during the early postnatal period.

As previously mentioned, the 5-HT1A autoreceptor is considered to be one of the main regulators of 5-HT neuronal activity and has been implicated in mood disorders. For these reasons, this receptor has been studied extensively to understand its role in the 5-HT system. One study used a partial knockdown (30-40%) of the 5-HT1A autoreceptor

induced during adulthood. This transgenic mouse had slightly increased activity of 5-HT neurons, but no change in anxiety-like behavior but increased resilience to stress (Richardson-Jones, Craige et al. 2010, Donaldson, Piel et al. 2014).Interestingly, when the 5-HT1A autoreceptor was reduced (by 80%) from after birth, these mice displayed increased anxiety; transient knockdown by 40% in early life (between p14-p30) resulted in a mild anxiety phenotype in the EPM but not the NSF test (Donaldson, Piel et al. 2014). Thus the extent and developmental timing of knockdown of 5-HT1A autoreceptors appears to dictate the severity of the anxiety phenotype.

The next interrogation was based on the central role of the 5-HT1A autoreceptor in regulating the 5-HT system. Researchers wanted to know whether the rescue of the 5-HT1A autoreceptor was sufficient to restore a normal anxiety in a global 5-HT1A knockout (Piszczek, Schlax et al. 2013). To specifically restore the autoreceptors, the TPH2 promoter was used to target the 5-HT neurons in the 5-HT1AR knockout background. Rescue mice had recovery of normal 5-HT1A-mediated inhibition of 5-HT neuron firing and a normal response during the 8-OH-DPAT induced hypothermia. Surprisingly, their anxiety behavior was indistinguishable from the global 5-HT1A knockout mice. These results suggest that in absence of the heteroreceptors, the autoreceptors are unable to restore a normal anxiety level. Thus, the autoreceptors are necessary but not sufficient to modulate the anxiety.

Antidepressant studies

As previously mentioned, desensitization of 5-HT1A autoreceptors might account for the delay in SSRI action. Therefore, researchers tested antidepressant action in transgenic 5-HT1AR mouse models to further explore this theory.

The first study was performed using global 5-HT1A KO mice that were treated with either the SSRI Fluoxetine (FLX), or TCAs imipramine or desipramine (Santarelli, Saxe et al. 2003). The NSF was used as a measure specific for response to chronic but not acute antidepressant treatment. Interestingly, the KO mice were insensitive to chronic FLX treatment, but responded to both imipramine and desipramine (TCAs), which target noradrenaline in addition to 5-HT. Furthermore, administration of the 5-HT1A-selective agonist 8-OH-DPAT caused a significant decrease in latency to feed in the WT but not in the KO mice. Taken together, these results provide evidence that SSRI action is mediated through the 5-HT1AR. However, this study did not distinguish between the two (auto- vs. heteroreceptor) receptor populations since the authors used a global 5-HT1A KO.

In order to reduce the expression of 5-HT1A autoreceptor, another group has infused siRNA in the dorsal raphe nucleus (DRN) to silence the 5-HT1A autoreceptors during adulthood, leading to 80% knockdown (KD) (Bortolozzi, Castane et al. 2012). They found a reduction in time spent immobile during the FST and TS for the KD mice, but no change in time spent in the open arm in the EPM test. These results show that reducing the number of autoreceptors acutely during adulthood has an antidepressant-like effect and does not affect the anxiety levels.

As discussed above, Richardson Jones and colleagues induced a partial knockdown (30%) of the 5-HT1A autoreceptor to create a mouse line with a lower level of receptors called 1A-low (Richardson-Jones, Craige et al. 2010). They showed that during adulthood, the KD results in an increase in chronic stress resilience. Furthermore, the 1A-low mice treated with FLX for 8 days had a significant decrease in latency to feed during the NSF while the WT mice showed no response. These results are suggesting an acceleration of the SSRI action when the 5-HT1A autoreceptors are partially reduced.

In agreement with these findings, another group used the same 1A-low mouse line to see the effect of another newer SSRI-5-HT1A partial agonist, Vilazodone (Garcia-Garcia, Navarro-Sobrino et al. 2016). They used FLX to compare the effect of the new SSRI to determine if they were giving similar results. After 8 days of treatment with Vilazodone, they saw a robust reduction in latency to feed during the NSF. Both of these studies show strong evidence that the delay in SSRI action is mediated through the partial removal of the 5-HT1A autoreceptors.

Hypothesis and Objectives

Based on previous findings presented above, I hypothesize that the beneficial action of SSRIs will be greater and faster in the absence of the 5-HT1A autoreceptors during adulthood.

To test my hypothesis, I used an inducible conditional 5-HT1A KO mouse model to knockout the autoreceptors during adulthood. The mice were sub-chronically treated with Fluoxetine or Escitalopram in the drinking water and assessed through a battery of behavior tests: NSF, LD, EPM, TS and FST.

The brain tissues were used to perform immunofluorescence to confirm the KO and to determine differences in neuronal activation patterns between the WT and KO but also untreated and treated.

Methods

General animal procedures

The University of Ottawa Animal Care Committee approved all animal studies and experimental procedures in accordance with guidelines established by the Canadian Council of Animal Care. Mice were kept under a 12-hour light/dark cycle with the light onset at 7:00 AM with food and water *ad libitum*. Cages were changed every other week or as needed. Also, equal numbers of male/female were used and pooled since there were no gender differences in all the behavior tests.

TPH2-Cre^{ERT2}-flx5HT1A mouse

The TPH2-Cre^{ERT2} mice (mixed background) were obtained from Jackson Labs (United States) and the flx-5HT1A mice (C57BL/6 background) were obtained from Dr. Rene Hen (Columbia University, New York City, NY)(Samuels, Anacker et al. 2015). TPH2-Cre^{ERT2} mice, containing the Cre^{ERT2} construct fused to the TPH2 promoter, were bred to flx-5HT1A mice, containing the 5-HT1A receptor gene flanked by loxP sites (floxed) with YFP integrated downstream, in the University of Ottawa Animal Facility to create an inducible and conditional 5-HT1A knockout (here referenced as flx1A mice). To maintain the mouse line, heterozygous mice for the flx-5-HT1A gene and hemizygous for the TPH2-Cre^{ERT2} gene were mated to obtain WT and KO littermates. The Cre^{ERT2} construct consists of the Cre enzyme fused with a mutated estrogen receptor (ERT2) that binds tamoxifen, but not estrogen. Once the tamoxifen is added, it binds to the ER ligand binding domain freeing the Cre enzyme allowing it to translocate into the nucleus to

excise the floxed gene. Also, since the construct is driven by the TPH2 promoter, Cre expression is specifically targeted to the serotonin-producing cells in the brain, located in the raphe nuclei. Furthermore, the floxed 5-HT1A gene contains a STOP codon in front of a YFP sequence inserted 3' to the 5-HT1A gene. The excision of the 5-HT1A gene allows the expression of the YFP, which is used as reporter of recombination (Figure 2).

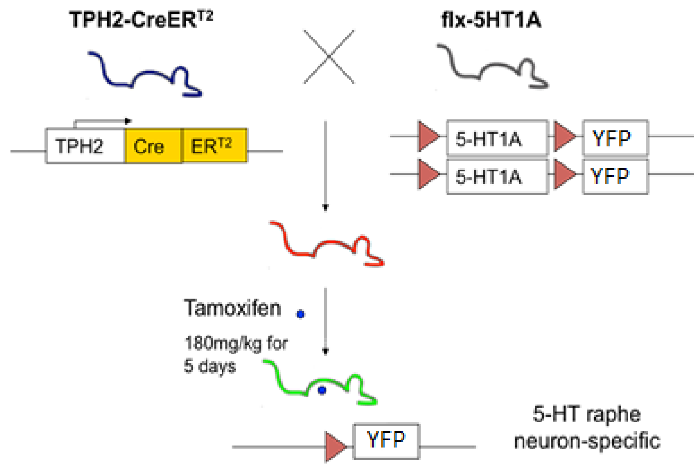

Figure 2: Inducible conditional knockout of the 5-HT1A receptor
Adapted from Christine Luckhart, M.Sc. thesis, 2014). TPH2-CreERT2 and flx-5HT1A mice were bred to generate the flx-1A mouse model in which the 5-HT1A gene is knocked out and YFP is expressed in TPH-positive serotonin neurons.

Genotyping

DNA was extracted from mouse (3 week-old) ear samples using the REDExtract-N-AMP^{TM} Tissue PCR kit (Sigma). The extracted DNA was amplified by two different sets of PCR primers to amplify the 5-HT1A and Cre genes. The primers for the flx-5HT1A gene are: flx1A: 1A-5 5'-GGG CGT CCT CTT CAC GTA G-3' and 1A-7 5'-CAG GGA CGT TGT GGT GTT GT-3'. The PCR cycle is: 94°C for 2 min; 94°C for 30 sec, 68°C for 30 sec/-0.5°C cycle, 68°C for 20 sec, 15 cycles; 94°C for 30 sec, 68°C for 30 sec, 68°C for 20 sec, 20 cycles; 68°C for 5 min. This PCR generates 254bp (WT) and 292bp (KO) bands. The primers for the TPH2-Cre^{ERT2} are: 5'-GCT GAG AAA GAA AAT TAC ATC G-3', 5'-TGG CTT GCA GGT ACA GGA GG-3', 5'-CAA ATG TTG CTT GTC TGG TG-3' and 5'-GCT AGT CGA GTG CAC AGT TT-3'. The PCR cycle is: 94°C for 1 min; 94°C for 15 sec, 57°C for 20 sec, 72°C for 10 sec, 35 cycles; 72°C for 2 min. This PCR results in 200bp WT and 300bp transgenic bands. Each mouse was genotype before weaning and after sacrificed.

Tamoxifen injection

At seven and half weeks postnatal, tamoxifen (180 mg/kg final concentration in sunflower oil; Sigma) was injected intraperitoneally. The injected side was alternated for three consecutive days. Mice were closely monitored during the treatment. After the treatment, the mice were left undisturbed for two weeks for recovery and to allow time for complete down-regulation of 5-HT1A receptors.

Antidepressant treatment

At ten weeks postnatal, fluoxetine (80 mg/L in drinking water, 18 mg/kg/day total, Enzo)(Richardson-Jones, Craige et al. 2010) or escitalopram (225 mg/L in drinking water, 30mg/kg/day, Sigma) (Berger, Weber et al. 2012) was administered in the drinking water to the individually housed mice and changed every 3 days for a duration of 22 days. The dose for both drugs was chosen based on previous studies showing that they are clinically relevant doses (Santarelli, Saxe et al. 2003, Berger, Weber et al. 2012). The water consumption was monitor daily. The average water consumption was 4 ± 1 mL/mouse/day and was not different across the different groups. Mice were maintained under the treatment until they were sacrificed.

Perfusion

After having completed the behavior tests (see below), the mice were sacrificed at 14 weeks old. All mice were anesthetized using Euthanyl (0.01 ml/g) and perfused by cardiac infusion of PBS followed by 4% paraformaldehyde via the left ventricle. Whole brains were extracted and post-fixed in 4% paraformaldehyde for 1h. Brains were later transferred in 30% sucrose solution with 0.3% sodium azide and changed daily for four days to ensure the cryoprotection. They were fast frozen in methyl-butane at -60°C and stored at -80°C.

Cryostat sectioning

Perfused brains were transferred from -80°C to -20°C overnight to avoid temperature shock in the cryostat set at -25°C. The fresh frozen brains were kept in -80°C to avoid the protein degradation. Brains were cut at a thickness of 25 µm and only one slice of

specific regions was kept and put on a Superfrost slide (Fisher). The regions of interest (in mm) included the prefrontal cortex (Bregma +1.94), nucleus accumbens (Bregma +0.98), septum (Bregma +0.26), ventral hippocampus (Bregma -1.22), dorsal hippocampus (Bregma -1.94), basolateral amygdala (Bregma -1.94) and the complete raphe nuclei (Bregma -4.16 to -4.84). The sections were kept at -20°C if used within a week, otherwise they were stored at -80°C.

Immunofluorescence

Brain slides were thawed at room temperature for 1h and washed three times with PBS. The slides were incubated for 1h with blocking buffer (1% normal donkey serum, 0.3% Triton-X or 0.3% Tween 20 for 5-HT1A staining in PBS). Next, the liquid was tapped off and the slides incubated in the appropriate primary antibodies, including: sheep anti-TPH (1:100, Millipore, ab1541), chicken anti-GFP (1:500, Abcam, ab13970) or rabbit anti-5-HT1A (1:50, homemade designed for the i2 domain) (Czesak, Le Francois et al. 2012) for 2h or with rabbit anti-FosB (1:500, Santa Cruz, sc-48) overnight. After washing the slides in PBS, they were incubated for 1h in the appropriate secondary antibodies: donkey anti-sheep (1:200, Jackson, 713-165-003), goat anti-chicken (1:250, Jackson, 103-545-155), donkey anti-rabbit (1:1000, Life Technology, A-21206). Lastly, the slides are washed with PBS and protected by cover slip. The stained slides were stored at -20°C. All the pictures were taken using the inverted Zeiss AxioObserver.D1 Microscope and the Axiovision software. Each brain regions were outlined using Image J and the number of cells was manually scored. The genotype and the treatment were hidden.

Autoradiography

Flx1a WT and KO littermate mice were sacrificed at 14 weeks of age using CO_2 and decapitated. The brains were extracted and immediately frozen on dry ice and stored at -80°C until sectioning as described above. Sections were processed for ^{125}I-MPPI (Perkin Elmer, Boston, MA) autoradiography, incubated for 2 h at room temperature, washed and exposed to Kodak BioMax MR film (VWR) for 7 h (Donaldson et al. 2014, Luckhart et al. 2016). Films were scanned at 1200-dpi resolution using an Epson Perfection V500 Photo Scanner. To analyze the image, the DR and the MR were outlined using Image J creating a template. This template was used for all sections at the same Bregma coordinates. Sections between Bregma -4.36 mm and -4.60 mm were average for the DR and MR and Bregma -1.82 mm was used for the HPC. Next, the signal density was quantified using the mean luminosity function in ImageJ. To determine the level of 5-HT1A binding (μCi), the signal density of the region of interest was standardized using an adjacent background with non-specific binding. All the signals fell within the linear range of the film and were quantified following the standard curve using ARC146-F ^{14}C standard (American Radiochemicals Inc, St. Louis, MO).

Whole-cell electrophysiology

Mice were anesthetized at 15 weeks of age using a short exposure to liquid isoflurane and sacrificed by decapitation. Brains were removed and cut into coronal slices while immersed in an ice-cold cutting solution (119 mM choline-Cl, 2.5 mM KCl, 1 mM $CaCl_2$, 4.3 mM $MgSO_4$-$7H_2O$, 1 mM NaH_2PO_4, 1.3 mM sodium L-ascorbate, 26.2 mM

$NaHCO_3$, 11 mM glucose, pH ~7.3), and equilibrated with 95% O_2 and 5% CO_2. Next, the slices were transferred into a chamber bubbled with 95% O_2 and 5% CO_2 and containing standard Ringer's solution (119 mM NaCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$-$7H_2O$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose to recover for 1 h. Lastly, slices were equilibrated to a temperature of 25°C until recordings were performed. Brains slices were transferred to a recording chamber attached to an upright microscope equipped with differential interference contrast (DIC) optics (Olympus BX51W1; 40×/0.80 N.A. objective) and constantly perfused with standard Ringer's solution. Serotonin cells were identified using morphological and biophysical characteristics previously established (Calizo, Akanwa et al. 2011, Geddes, Assadzada et al. 2016). Whole-cell recordings were acquired using an Axon Multiclamp 700B amplifier and digitized with an Axon Digidata 1440A (or 1550) digitizer. The borosilicate glass patch electrodes were filled with an internal solution containing 115 mM potassium gluconate, 20 mM KCl, 10 mM sodium phosphocreatine, 10 mM Hepes, 4 mM ATP(Mg2+), and 0.5 mM GTP (pH 7.25) (adjusted with KOH; osmolarity, 280–290 mOsmol/L). To determine the functionality of the 5-HT1A autoreceptor, the non-selective 5-HT1A agonist 5-CT (100 nM) was applied to the slice creating a 5-HT1A–mediated outward current.

Behavior testing

General procedure

Behavior tests were done between 9 AM and 4 PM. Mice were transported to a holding room and habituated for at least 1 h. Mice were 11 weeks of age at the start of testing and were singly housed. The different behavior tests performed in order are: Novelty Suppressed Feeding test, Light-Dark, Elevated Plus Maze, Tail Suspension and Forced swim tests (Figure 3). Mice were kept under their respective antidepressant treatment during all the tests until they were sacrificed.

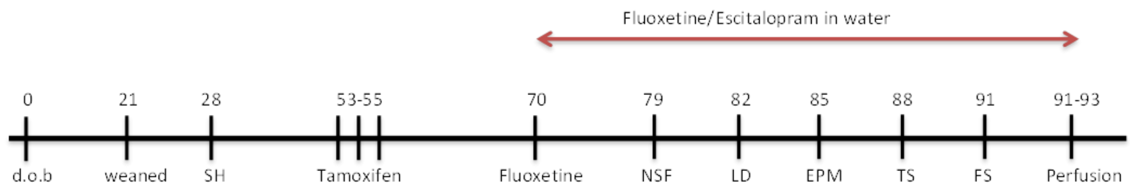

Figure 3: Experimental timeline for the flx1A mice

Novelty Suppressed Feeding test

At 24h prior to testing the cages were changed to ensure that there was no food remaining in the bedding and the mice were fasted for 16 h. The apparatus used in this test consisted of a 45 x 45 x 45 cm open box with bedding and overhead illumination (100 lux). A 10-cm petri dish flipped upside-down with a food pellet in the center was placed at the center of the arena. To start the test, the mouse was placed in a corner and the test was complete and time recorded when the mouse first chewed the food or after 600 s. Then, the mouse was placed back in the home cage with a weighted food pellet. Latency to feed was timed, and total food consumption after 5 min was determined by weighing the pellet. Food was added to the cages after the test.

Light-Dark box

The apparatus for this test of anxiety-like behavior consists of a sound-proof square chamber equally divided into two chambers measuring 27 × 13.5 cm. The dark compartment is covered in black plastic and the light compartment is transparent and uncovered (Med Associates, St Albans, VT, USA). The light side is lit with two bulbs at 390 lux. A wide opening in the opaque compartment allows the mouse to move between the dark and lit sides. At the periphery of the chambers, infrared transmitters and receivers at assigned X and Y coordinates map the chambers. When the chambers are scanned for the beam at each receiver and failed to reach the receiver, the software register this event as a broken beam indicating that the mouse was present at this specific coordinate. To start the assay, the mouse was placed in the light chamber and its

movement was recorded for 10 min. After the test, the mouse was returned to its home cage and both chambers were cleaned.

Elevated Plus Maze

This test is used to assess the anxiety-like phenotype. The apparatus consists of two 6- cm wide and 75 cm long arms crossed in the center at a perpendicular angle (Noldus, Wageningen, The Netherlands). One arm is an open platform and the other is closed with 20 cm high walls. The junction between the two arms is open to allow the mouse to freely move in the maze. An overhead illumination (100 lux) and a camera linked to the Ethovision tracking program (Ethovision 8, Noldus Information Technologies, Leesburg, VA, USA) records the mouse movements. To begin with, the mouse was placed in the center of the maze and the time spent in both closed and open arms was recorded for a 10 minutes' duration.

Tail suspension

This test of depression-like behavior involves suspending the mouse by its tail in a box made by Med Associates for 6 min. The tail was taped horizontally on an aluminum bar attached to a transducer that measured the force of movement. A lower threshold (3) is set in the automated detection device (ENV-505TS Load-Cell Amplifier, Med Associates, Fairfax, VT, USA), so that any force recorded below the threshold is considered immobility. The time spent immobile (below threshold) was recorded.

Forced swim

The equipment for this test consists of two clear Plexiglas cylinders with a diameter of 22 cm and a height of 37 cm. The cylinders were filled with 23-25°C tap water up to 5-10 cm from the top. A camera linked to video tracking software recorded the movement of the mouse. Mice were placed in the apparatus and recorded for 6 min (Ethovision 8) and the latency to immobility and time immobile were quantified.

<u>Statistical analyses</u>

Data are shown as mean ± standard error of the mean (S.E.M). All the statistical analyses and graphs were done using Graphpad Prism 6 (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA). For behavioral studies, outliers were identified using the ROUT method in Graphpad Prism 6 and were excluded from the analysis. There were 3 outliers in the NSF (2 WT vehicle, 1 KO vehicle), 1 in EPM (KO FLX) and 1 in FS (KO FLX). For comparing WT to KO, t-test was used. Two-way analysis of variance (ANOVA) was used and followed by post-hoc comparisons using Dunnett's test to compare to control animals, or Tukey's test for multiple comparisons, with $p<0.05$ considered statistically significant.

Results

Confirmation of the 5-HT1A knockout in the Flx1A mouse model

The 5-HT1A gene knockout was done during adulthood using the inducible recombinase method to avoid any developmental effects of altering the 5-HT system in early life. TPH2 positive cells in which the 5-HT1A autoreceptor was excised express the YFP reporter. Successful recombination occurred in the dorsal raphe nuclei (DRN) of both Flx1A +/- (HET) and -/- (KO) as identified with the YFP staining (Figure 4). In the HET, close to 60% of TPH-positive cells expressed the reporter and 89% in the KO mice. Furthermore, there was no expression of the YFP reporter in the Flx1A +/+ (WT) mouse. As a control, brain regions that are enriched in 5-HT1A heteroreceptors were assessed. DAPI was used to visualize the cells and the surrounding structures. As expected, there was no detectable specific YFP staining of the reporter in either the PFC or the NAcc of the Flx1A mice (Figure 5). Thus, the heteroreceptors were not recombined and remained expressed in those brain regions.

In order to have a direct measure of the presence of the 5-HT1A autoreceptors, immunofluorescence staining of the receptor and TPH, which was used to identify the 5-HT cells, was performed. In the DRN of the WT mice, the majority of TPH-positive cells (94%) were co-expressing the receptor (Figure 6). In the HET, 60% of 5-HT neurons were expressing the 5-HT1A receptor and only 7% in the KO mice. These results confirmed that the autoreceptors in the KO mice was significantly reduced in 5-HT cells by using the TPH2 promoter.

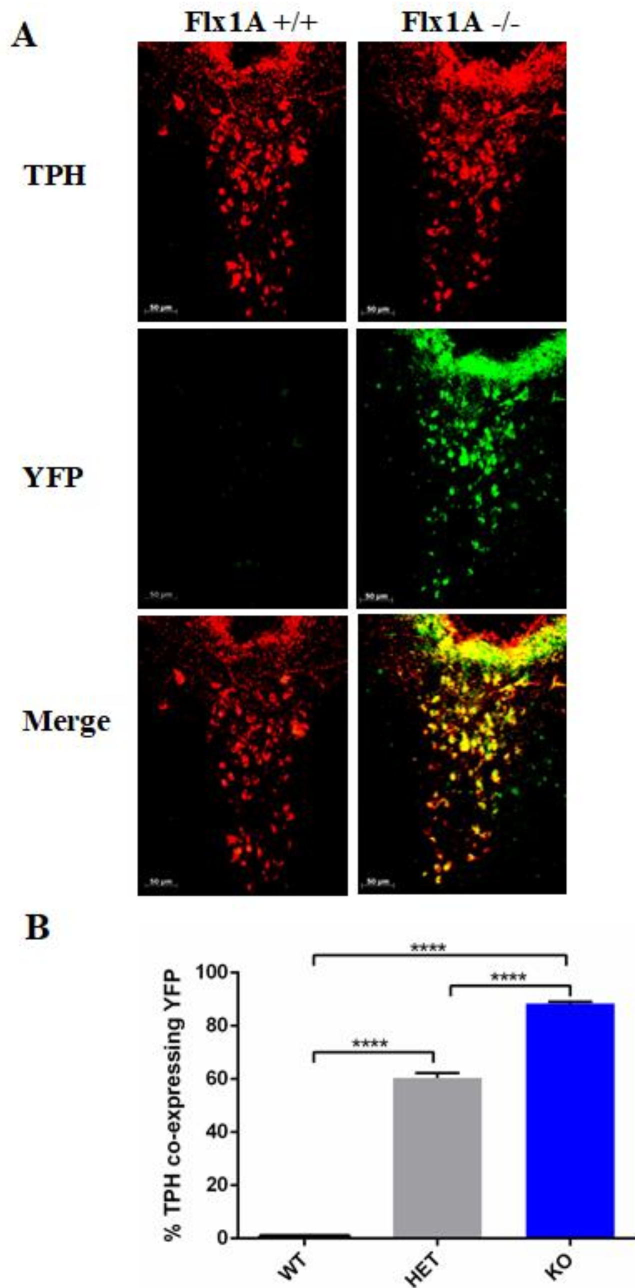

Figure 4: Robust augmentation of YFP immunofluorescence staining of the dorsal raphe of Flx1A KO mice. **a** Immunofluorescent staining for TPH (sheep anti-TPH, 1:100) and YFP (chicken anti-GFP, 1:500) was performed on dorsal raphe slices of Flx1A WT, Het and KO mice. Scale bar indicates 100 µm. **b** Quantification of TPH-positive cells with GFP staining. Data represent mean ± SEM (n=4), ****$p < 0.0001$.

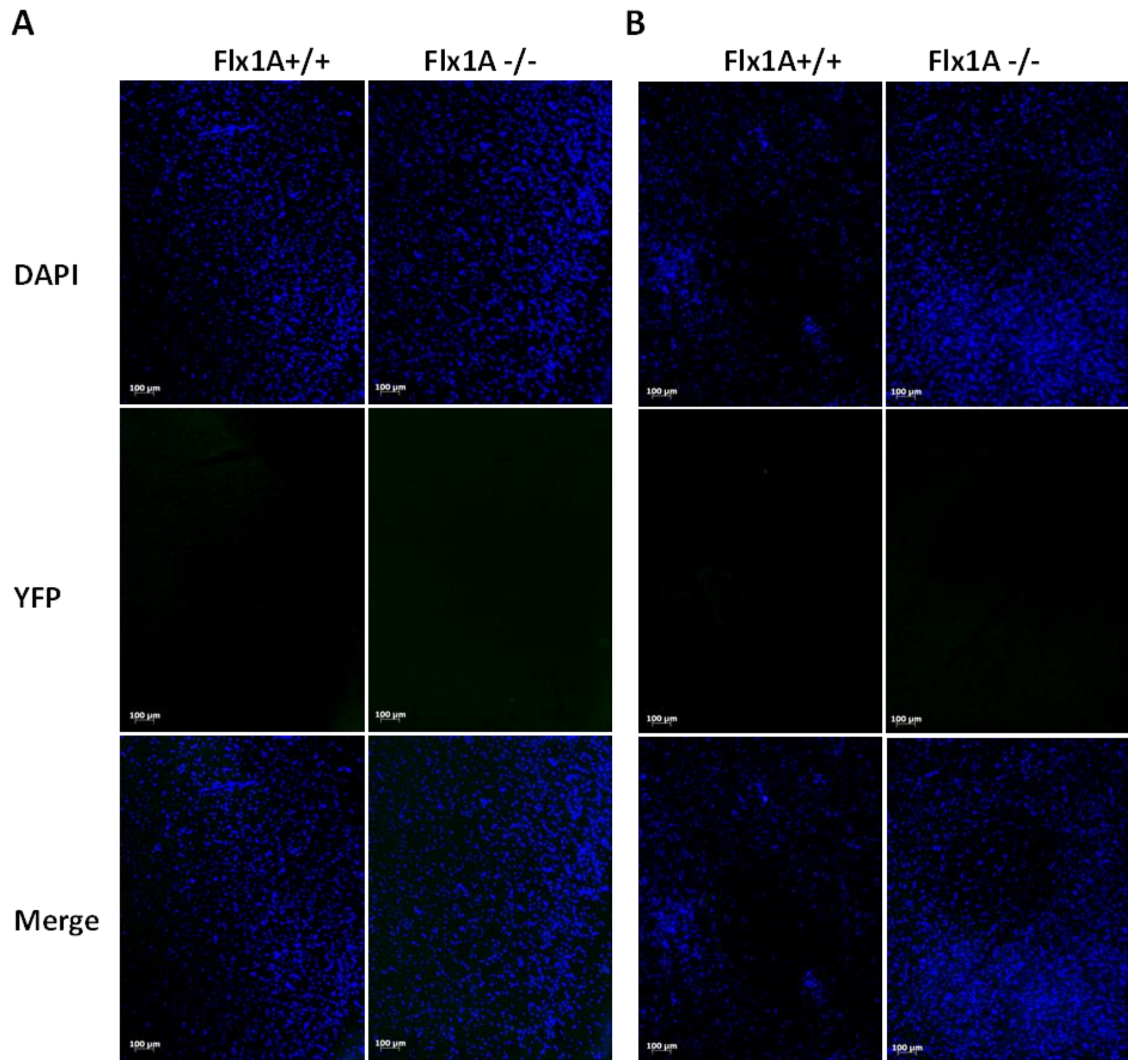

Figure 5: YFP immunofluorescence staining.
Immunofluorescent staining for YFP (chicken anti-GFP, 1:500) was performed on **a** prefrontal cortex and **b** nucleus accumbens of Flx1A mice. Scale bar indicates 100 µm (n=3).

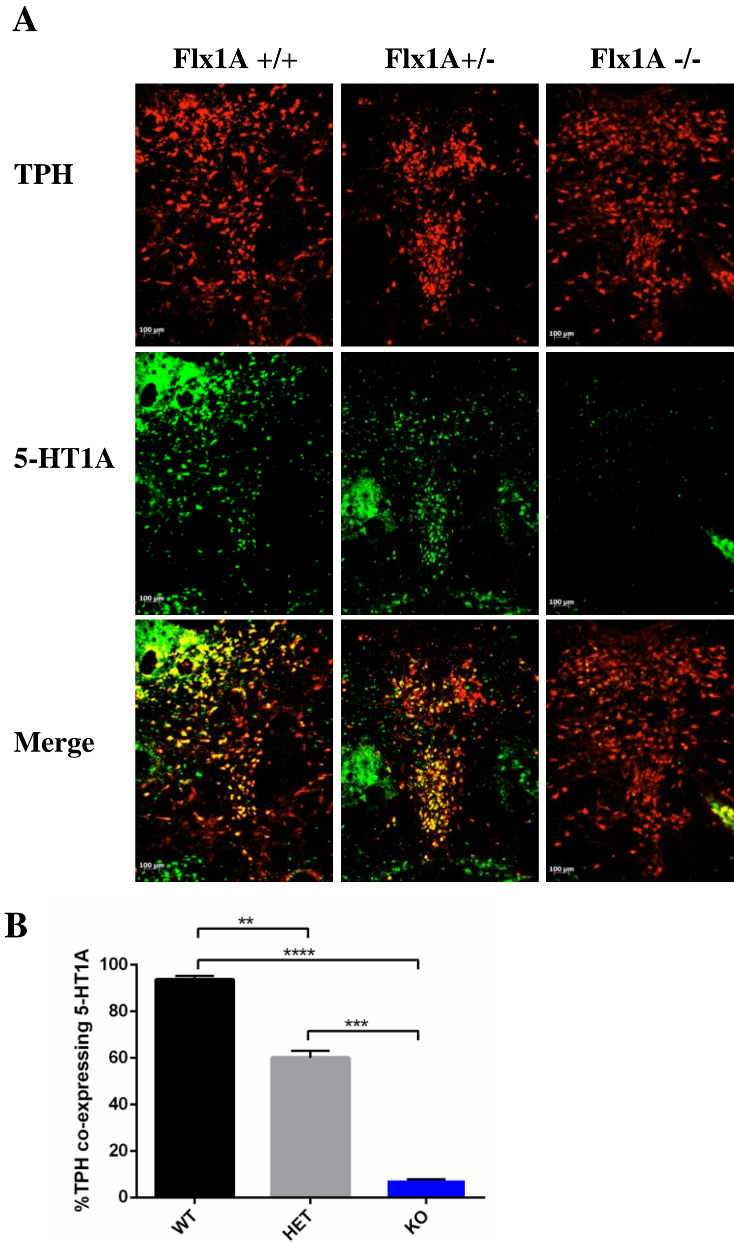

Figure 6: Robust reduction of 5-HT1A immunofluorescence staining of the dorsal raphe of Flx1A KO mice. **a** Immunofluorescent staining for TPH (sheep anti-TPH, 1:100) and 5-HT1A receptors (rabbit anti-5-HT1A, 1:50) was performed on dorsal raphe slices of Flx1A WT, Het and KO mice. Scale bar indicates 100 µm. **b** Quantification of TPH-positive cells with 5-HT1A staining. Data represent mean ± SEM (n=4), $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

The next experiment to detect the autoreceptor was by autoradiography using the 5-HT1AR radioligand ^{125}I-MPPI. This approach was used to quantitatively determine the reduction in 5-HT1A autoreceptors in the Flx1A KO mice 3 weeks after knockout induction by tamoxifen injection. The areas used to quantify the binding intensity of ^{125}I-MPPI are outlined by the yellow line and was used as a guide (Figure 7). In the DRN and MRN of the KO mice, there was a 50% reduction (10.38 ± 0.2829 for the WT and 5.424 ± 0.1240 for the KO) in the binding intensity of the 5-HT1A radioligand. As a positive control, the HPC was assessed due to its rich expression of 5-HT1A heteroreceptors. As expected, there was no significant difference in intensity between the WT and KO mice. These results further confirmed that the autoreceptor expression is reduced in the KO mice and not affected in the WT mice.

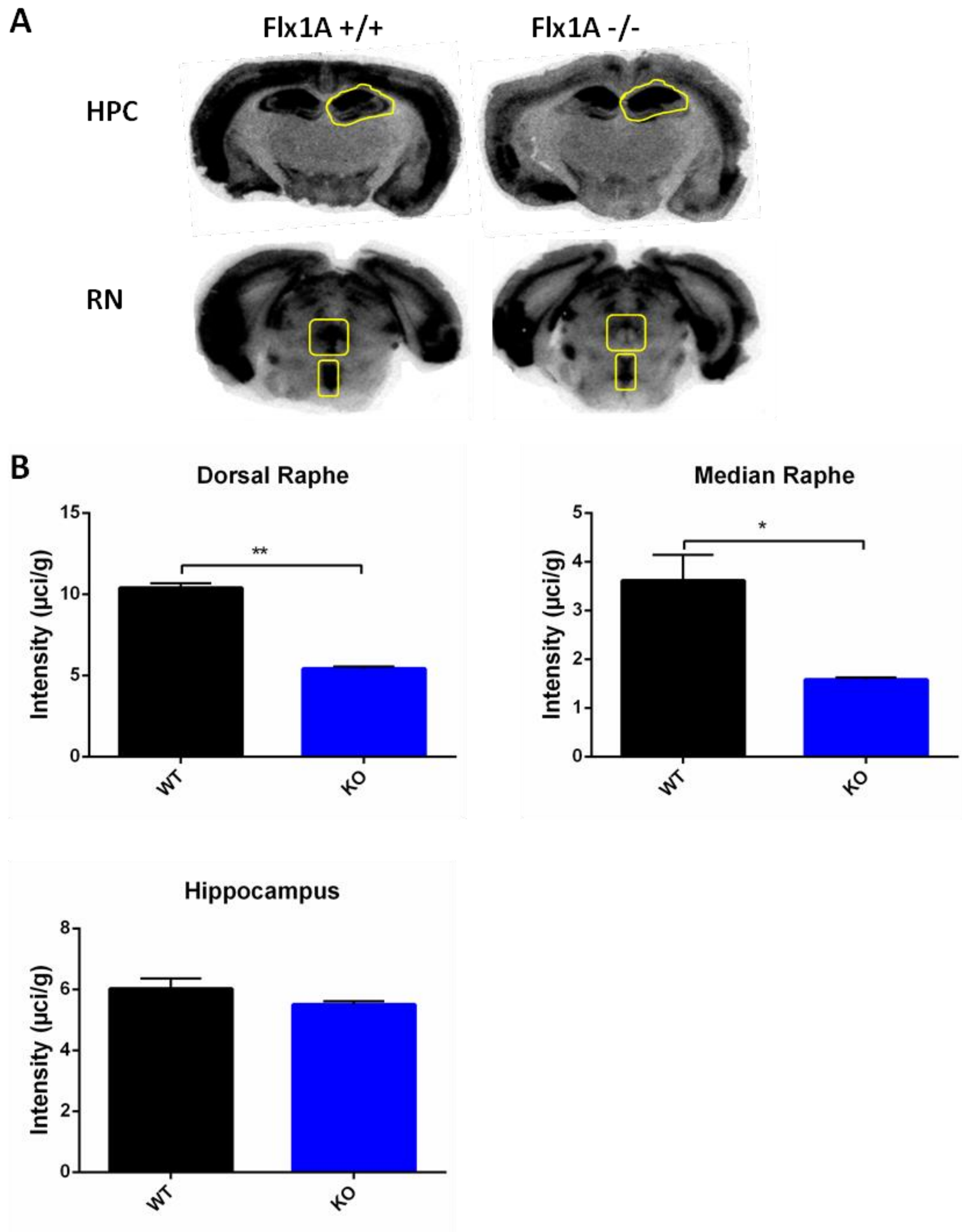

Figure 7: Decreased 5-HT1A receptor binding in Flx1A -/- raphe.
5-HT1A receptor autoradiography of raphe and hippocampus section from Flx1A +/+ (WT) and -/- (KO) mice was done using 25I-MPPI. **a** Representative section of raphe nuclei (RN), including median and dorsal raphe, and hippocampus (HPC) are shown. **b** Average intensity (µci/g) was quantified for HPC, MR and DR. Data represent mean ± SEM (n=3), *$p< 0.05$; **$p < 0.01$.

Whole-cell electrophysiology was the last experiment to confirm the mouse model and was performed by Sean Geddes from Dr. Jean-Claude Beique lab. The latter was performed 3 weeks post tamoxifen injection. This technique was used to assess the functionality of the 5-HT1A autoreceptors by the application of the agonist 5-CT. Results showed that application of 5-CT on the DRN of the WT mouse resulted in an outward current (Figure 8). In the KO mice, the 5-CT-induced outward current was abolished. Thus, the autoreceptors in the KO mice are not functional.

Overall, the YFP staining, the robust reduction in 5-HT1A staining and ^{125}I-MPPI binding intensity and the lack of 5-CT-induced outward current in the KO mice indicate a successful inducible conditional 5-HT1A knockout mouse model. The absence of YFP reporter, the robust 5-HT1A staining, ^{125}I-MPPI binding intensity and the 5-CT-induced outward current indicate that the autoreceptors in the WT mice remained intact.

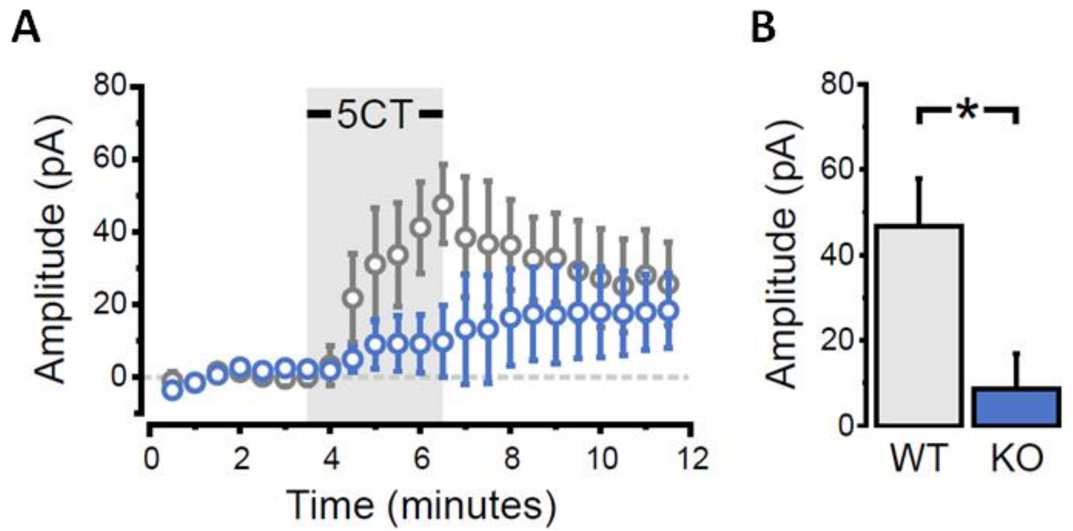

Figure 8: Reduced 5-HT1AR-mediated outward currents in Flx1A mice. Whole-cell voltage clamp recordings of DRN 5-HT neurons from wild-type (n = 6) and Flx1A (n =7) mice. **a** Time Course: 5-HT1AR-mediated outward current in response to 5-CT (100 nM; Vm = -55 mV). **b** 5-CT response: Average peak steady-state 5-HT1AR-mediated currents from recorded 5-HT neurons in wild-type and Flx1A tissues (WT, 46.82 +/- 11; Flx1A, 8.58 +/- 8.3; p = 0.02). Data are presented as mean ± SE. Performed by Sean Geddes from Dr. Jean Claude Beique lab.

Effect of SSRIs on the anxiety- and depression-related behavior

Flx1A mice were pre-treated for 9 days with Fluoxetine (FLX) or Escitalopram (ESC) in the drinking water and were maintained under treatment until sacrificed. After the pre-treatment, mice were tested for anxiety-like behavior using NSF, LD and EPM tests and for depression-like behavior using TS and FST. No anxiety phenotype was expected since the autoreceptor was removed during adulthood. Since the delay in SSRI action is thought to be due to desensitization of the 5-HT1A autoreceptors, a faster action of the SSRIs was expected in the Flx1A KO mice.

Flx1A WT and KO mice had a similar latency to feed under basal condition during the NSF test (Figure 8A-B) indicating no change in anxiety. However, their respective responses to FLX or ESC differed. Latency to feed in WT mice was not changed after 9 days of FLX or ESC treatment as expected for a sub-chronic treatment. Surprisingly, in the KO mice, both FLX and ESC increased the latency to feed, indicating an anxiety-like phenotype. As a control in a non-stressful environment, latency to feed in the home cage was recorded (Figure 8C-D). There was no significant difference across all groups indicating that the motivation to feed did not differ between groups. Food consumption was also measured for 5 minutes after the test and it was similar across all conditions and genotypes (Figure i). This suggests that the increase in anxiety-like phenotype is not due to a lack of appetite.

Flx1A mice were tested in the LD paradigm 12 days after their respective treatment. Time spent in light zone, number of entries in the light zone and latency to enter dark zone was not different between the vehicle-treated WT and KO (Figure 9) indicating no

change in anxiety in this test. Furthermore, SSRI-treated mice behaved similarly as their littermate control. Other parameters were analyzed including time spent in dark zone and number of entries in dark zone. There was no difference between the vehicle-treated and SSRI-treated mice (Figure ii). Lastly, WT mice treated with FLX displayed a reduction in total movement, whereas KO mice did not display any changes in ambulatory movement when treated with FLX or ESC. Therefore, the LD test was not sensitive to detect the SSRI-induced anxiety-like behavior seen in the NSF test.

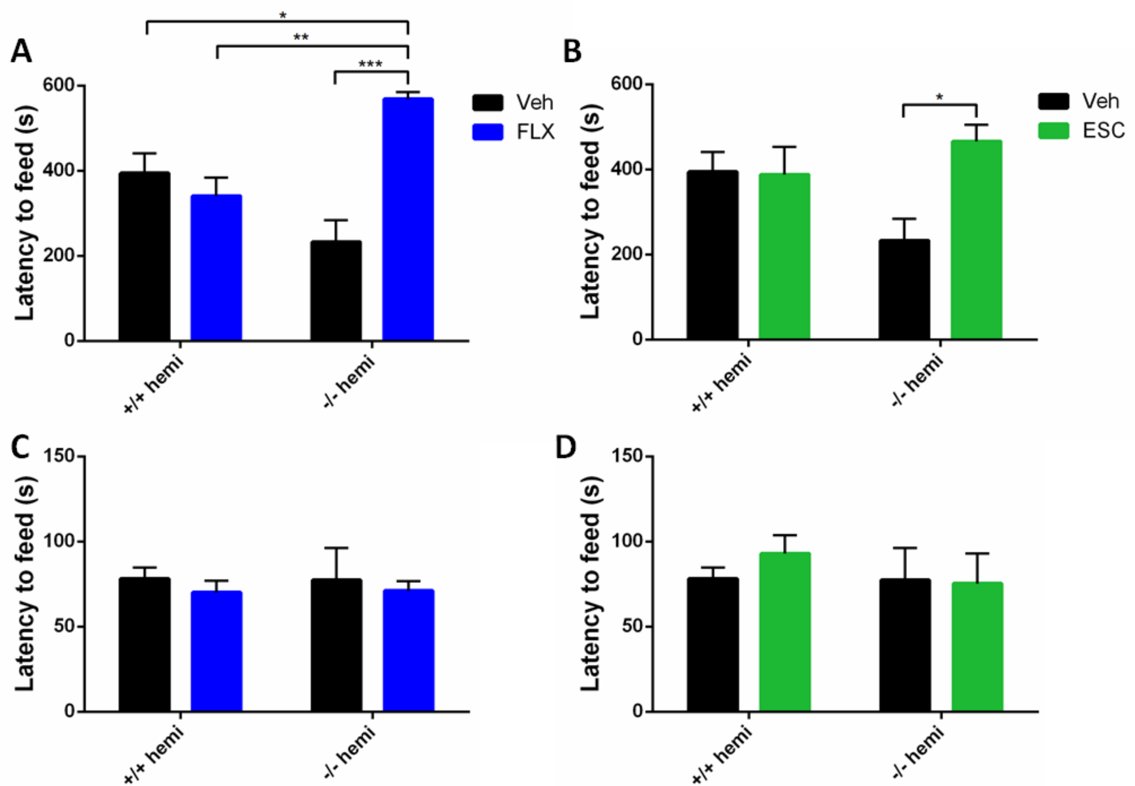

Figure 9: Robust effect of SSRIs during the NSF in Flx1A KO mice.
Vehicle, FLX and ESC were administered to Flx1A WT (n=17, 21, 7 respectively) and KO (n=11, 15, 8 respectively) for 9 days prior to testing. Latency to feed in **a, b** novel cage and **c, d** home cage was recorded. Data represent mean ± SEM, *p< 0.05; **p< 0.01; ***p< 0.001.

Light Dark Box

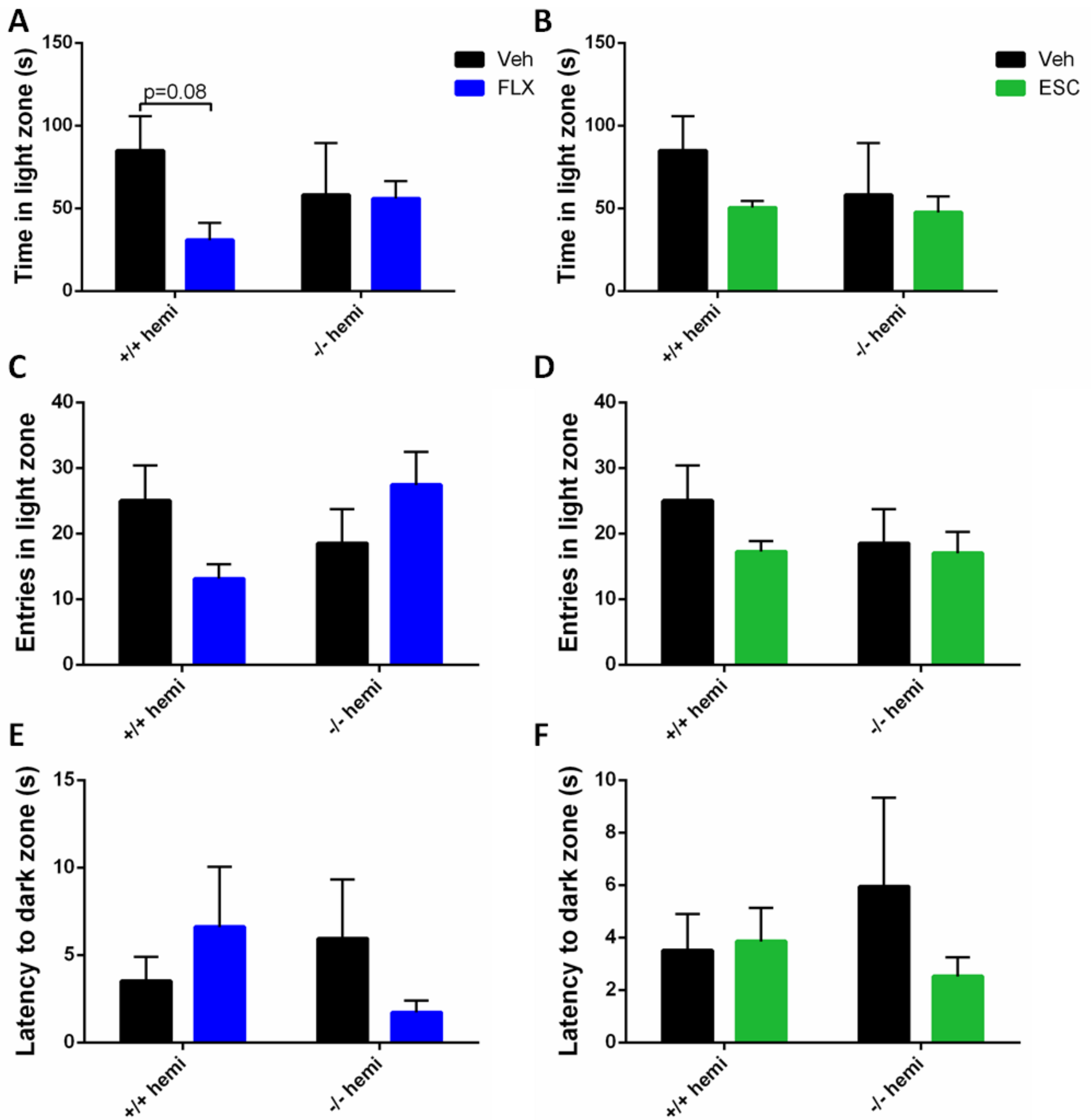

Figure 10: No effect of SSRIs during the LD test.
Vehicle, FLX and ESC were administered to Flx1A WT (n=8, 9, 8 respectively) and KO (n=5, 7, 8 respectively) for 12 days prior to testing. **a, b** Time spent in the light zone, **c, d** number of entries in the light zone and **e, f** latency to enter dark zone were recorded. Data represent mean ± SEM.

The last test used to assess the anxiety was the EPM and was performed after 15 days of treatment. Although there was no difference in time spent in the open arm in vehicle-treated mice, there was a robust reduction in the FLX-treated KO mice (Figure 10A). In fact, there was a 50% reduction in time spent in the open arm when the KO was treated with FLX. Interestingly, ESC did not induce the same extent of anxiety-like phenotype as FLX. Time spent in the closed arm was not changed by the genotype or the treatment (Figure 10C-D). Taken together, FLX induced an anxiety-like phenotype in the KO mice but not in the WT mice.

The next behavioral tests used (TS and FST) were to assess depression-like behavior. Under basal conditions, no depression phenotype was expected in the KO mice. However, an improvement in both tests was expected after the SSRIs treatment. Flx1A mice were tested in the TS following 18 days of vehicle, FLX or ESC. Time spent immobile was not changed in the vehicle-treated WT and KO mice (Figure 11). Mice treated with either FLX or ESC had a similar time spent immobile as their vehicle-treated littermate. Thus, SSRI treatment did not altered the behavior of WT nor KO mice.

The last behavioral test performed was the FST after 21 days of vehicle, FLX or ESC treatment. Time spent immobile was similar in the vehicle-treated WT and KO mice (Figure 12). Furthermore, SSRIs did not modify the immobility time in the WT and KO mice. The frequency immobile was not different between the WT and KO mice. Also, FLX- and ESC-treated mice had similar immobile times as their littermate vehicle-treated controls. Taken together, FLX and ESC did not improve or worsen the time spent immobile in both genotypes.

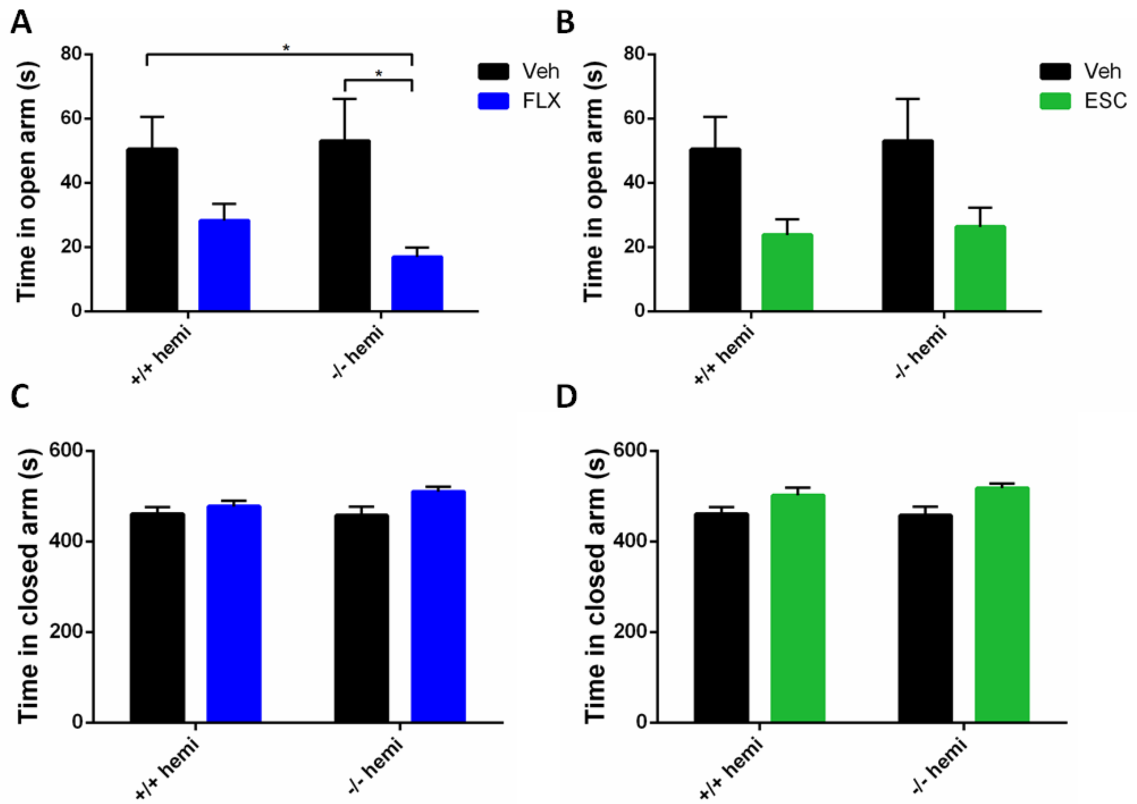

Figure 11: FLX-induced anxiety-like phenotype in the Flx1A KO mice during the EPM. Vehicle, FLX and ESC were administered to Flx1A WT (n=17, 20, 8 respectively) and KO (n=11, 13, 8 respectively) for 15 days prior to testing. **a, b** Time spent in the open arm and **c, d** time spent in the closed arm were recorded. Data represent mean ± SEM, *p< 0.05.

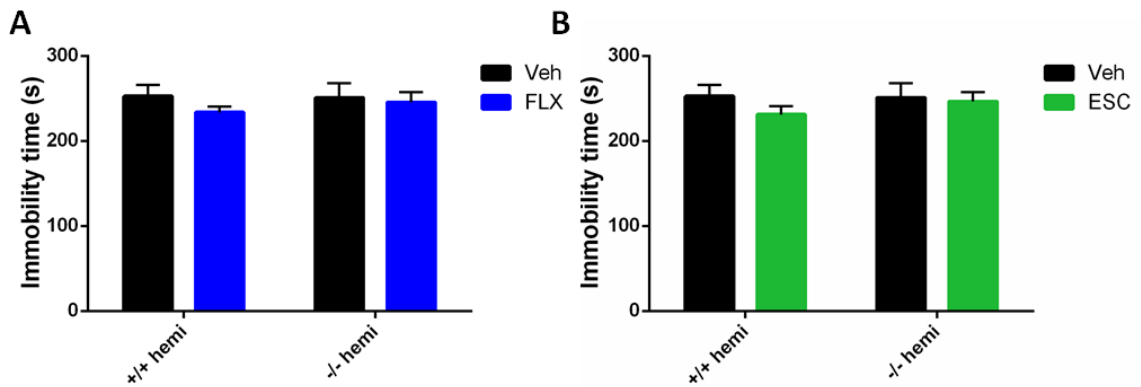

Figure 12: No antidepressant-like effect of SSRIs during the TS.
Vehicle, FLX and ESC were administered to Flx1A WT (n=6, 9, 8 respectively) and KO (n=5, 7, 8 respectively) for 19 days prior to testing. Immobility time was recorded. Data represent mean ± SEM.

Forced Swim

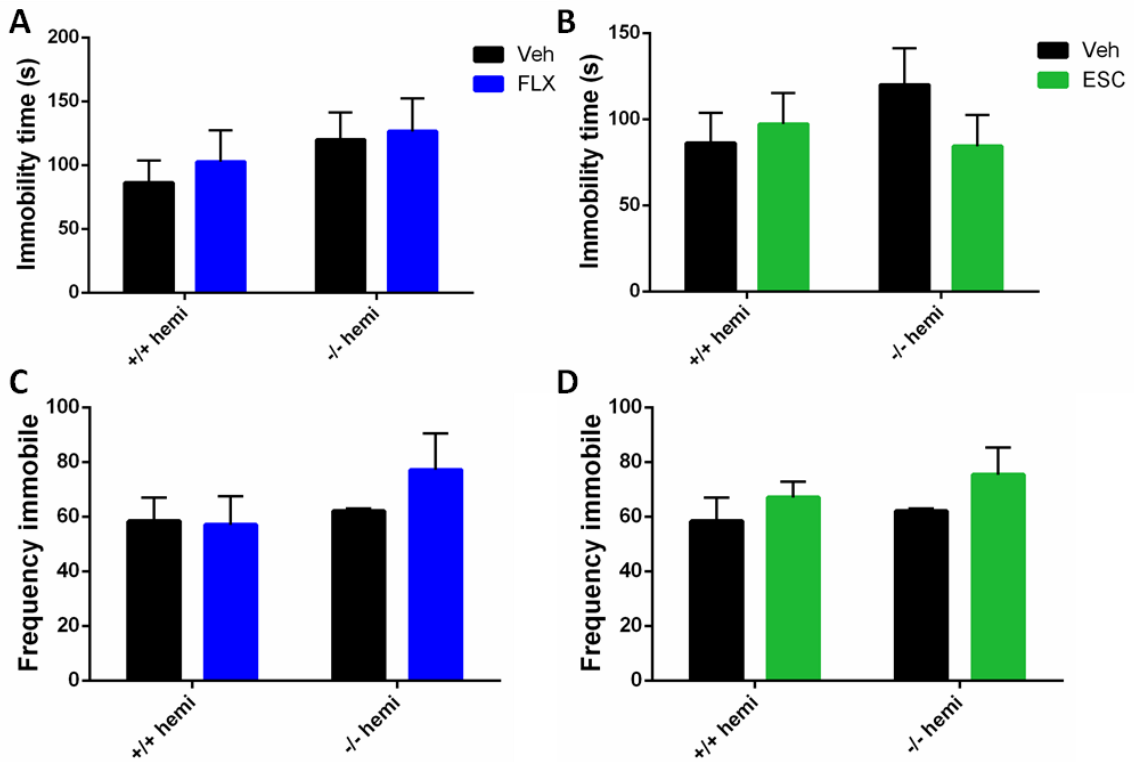

Figure 13: No antidepressant-like effect of SSRIs during the FST
Vehicle, FLX and ESC were administered to Flx1A WT (n=10, 13, 8 respectively) and KO (n=5, 6, 8 respectively) for 21 days prior to testing. **a, b** Immobility time and **c, d** frequency immobile were recorded. Data represent mean ± SEM.

The Flx1A HET mice were examined using the same battery of tests and FLX treatment. However, FLX did not influence the behavior of these mice in any of the tests (Figure iii-vii).

Overall, Flx1A WT and KO mice behaved similarly under basal condition (vehicle) and no anxiety- or depression-like phenotype was noted. Sub-chronic administration of FLX induced an anxiety-like phenotype in the KO mice that was detected in the NSF and EPM and ESC induced anxiety only during the NSF. Neither of the SSRIs displayed an antidepressant-like effect during the TS and the FST.

Effect of Fluoxetine on chronic brain activation patterns

As previously seen, Flx1A KO mice did not respond the same way to SSRIs as their littermate WT and since they have an altered 5-HT system, they might not process the neuronal information the same way. Therefore, chronic brain activation patterns were analyzed in specific regions involved in anxiety and depression to try to understand what might be happening. Mice were sacrificed the following day of the last behavior test, which corresponded to 24 days of treatment. Immunofluorescence staining was performed using FosB as a marker for chronic activation. The analysis was separate into forebrain regions, hippocampal regions and raphe nuclei.

The first 4 regions of the forebrain that were analyzed are the entorhinal cortex (EC), nucleus accumbens (Nacc), medial and lateral septum nuclei (MSN and LSN respectively). These brain regions were chosen based on the relevance to anxiety and their high levels of FosB expression. The EC is the region where the majority of changes

in the number of FosB-positive cells were observed (Figure 13A). A reduction was observed in the vehicle-treated KO mice compared to WT mice. FLX-treated WT or KO mice had a significantly lower expression of FosB when comparing to vehicle-treated WT or KO mice but this effect of FLX was blunted in the KO mice. Thus, the effect of FLX to reduce FosB cells in the EC was blunted in KO compared to WT mice, suggesting an impaired FLX response.

The next region analyzed was the Nacc (Figure 13B and Figure 14). There was a trend ($p<0.09$) for increased positive cells in the KO vs. WT mice, that became significant upon FLX treatment. Interestingly, the number of FosB-positive cells was slightly only in the WT mice after the FLX treatment. This suggests a basal activation of the Nacc KO mice, with a reduced responsiveness to FLX treatment in KO vs. WT mice.

The next two forebrain regions analyzed were the MSN and LSN (Figure 13C-D). There was no significant difference in the number of FosB-positive cells between the vehicle-treated WT and KO mice. However, there was a robust and significant reduction after FLX treatment in the WT mice, but not in the KO mice, suggesting a reduced responsiveness to FLX in the MSN of KO vs. WT mice. FLX-treated groups had similar levels of FosB-positive cells. In the LSN, there was no difference in FosB expression across all conditions.

Overall, FosB activation was changed in the EC when 5-HT1A autoreceptors are removed. Furthermore, the effect of FLX on the number of FosB-positive cells was different between the genotypes in all forebrain regions with the exception of LSN.

Forebrain regions

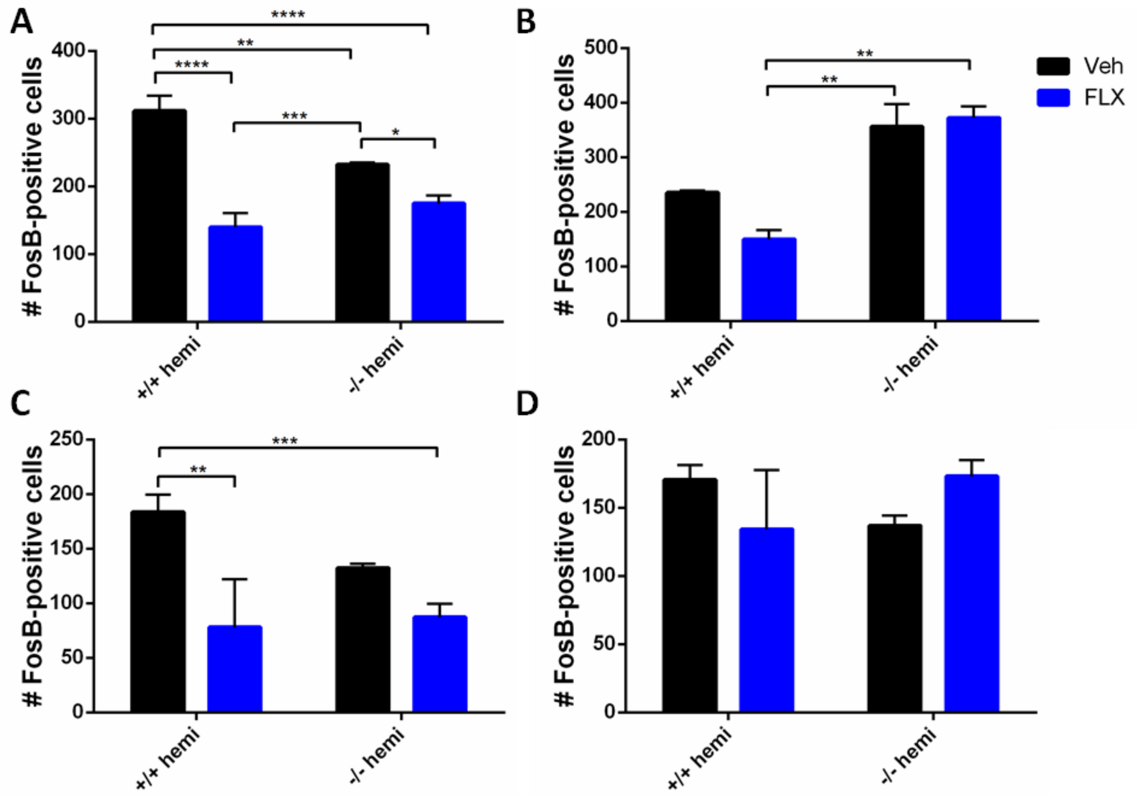

Figure 14: FosB-positive cells in forebrain regions.
Vehicle or FLX was administered to Flx1A mice for 24 days. Immunofluorescence staining using FosB on different brain regions including the **a** EC, **b** Nacc, **c** MSN and **d** LSN. Data represent mean ± SEM (n=4), *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

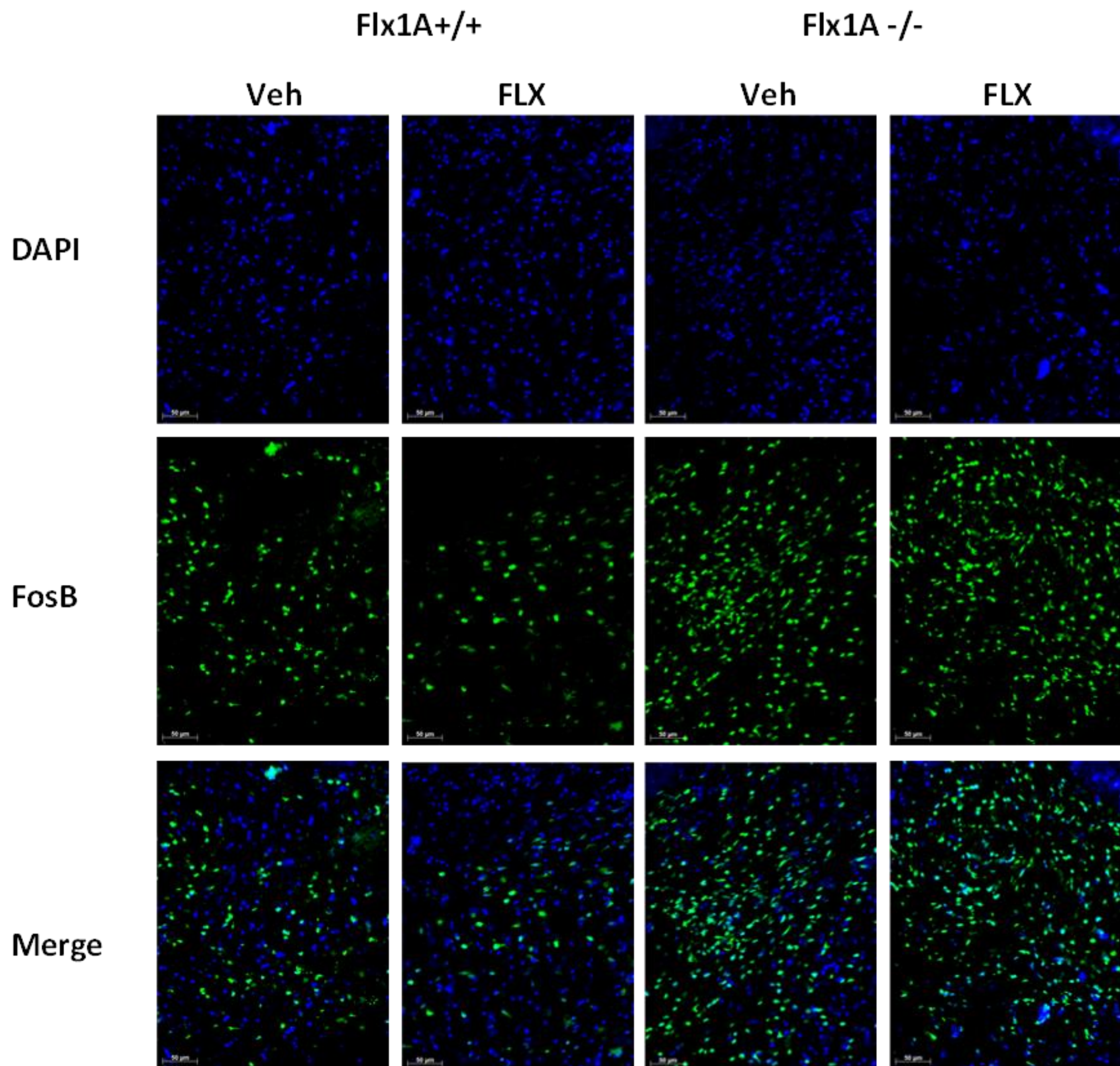

Figure 15: FosB- positive cells in the Nacc.
Immunofluorescent staining for FosB (rabbit anti-FosB, 1:500) and DAPI was performed on Nacc sections. Vehicle or FLX was administered to Flx1A mice for 24 days. Scale bar indicates 50 µm (n=3)

Interestingly, the Nacc is the only region where there is an increase in FosB expression in the KO compared to WT mice, particularly after FLX treatment.

The second region analyzed was the ventral hippocampus due to its implication in regulating anxiety and depression behaviors (Hill, Sahay et al. 2015) and the effect of SSRI to induce adult neurogenesis in this region. The latter slice was separated into smaller sections including the CA1, CA2/3 and dentate gyrus (DG). The basolateral amygdala (BLA) and the lateral habenula (LHB) were also analyzed (Figure 15). In the CA1, vehicle-treated WT and KO started with similar levels of FosB-positive cells (Figure 15A). In both genotypes, FLX significantly decreased the number of expressing cells to a similar extent.

The next sub-region analyzed was the CA2/3 (Figure 15B). The vehicle-treated KO displayed a 50% reduction in FosB-stained cells in comparison to its WT littermate. This may suggest reduced 5-HT activity in this region in the KO mice. Furthermore, FLX-treated WT mice had significantly lower levels of FosB expressing cells in comparison to their vehicle-treated WT littermates. Interestingly, FLX did not change the FosB expression in the KO mice, suggesting insensitivity to FLX treatment in this region.

The DG was the following brain region that was examined. The vehicle-treated WT and KO mice did not display any significant difference in the number of FosB-positive cells (Figure 15C). However, a significant reduction in FosB-cells in FLX-treated vs. vehicle was observed in the WT mice, but was blunted in KO mice. This suggests a reduced responsiveness of the DG to FLX in the KO mice.

Hippocampal regions

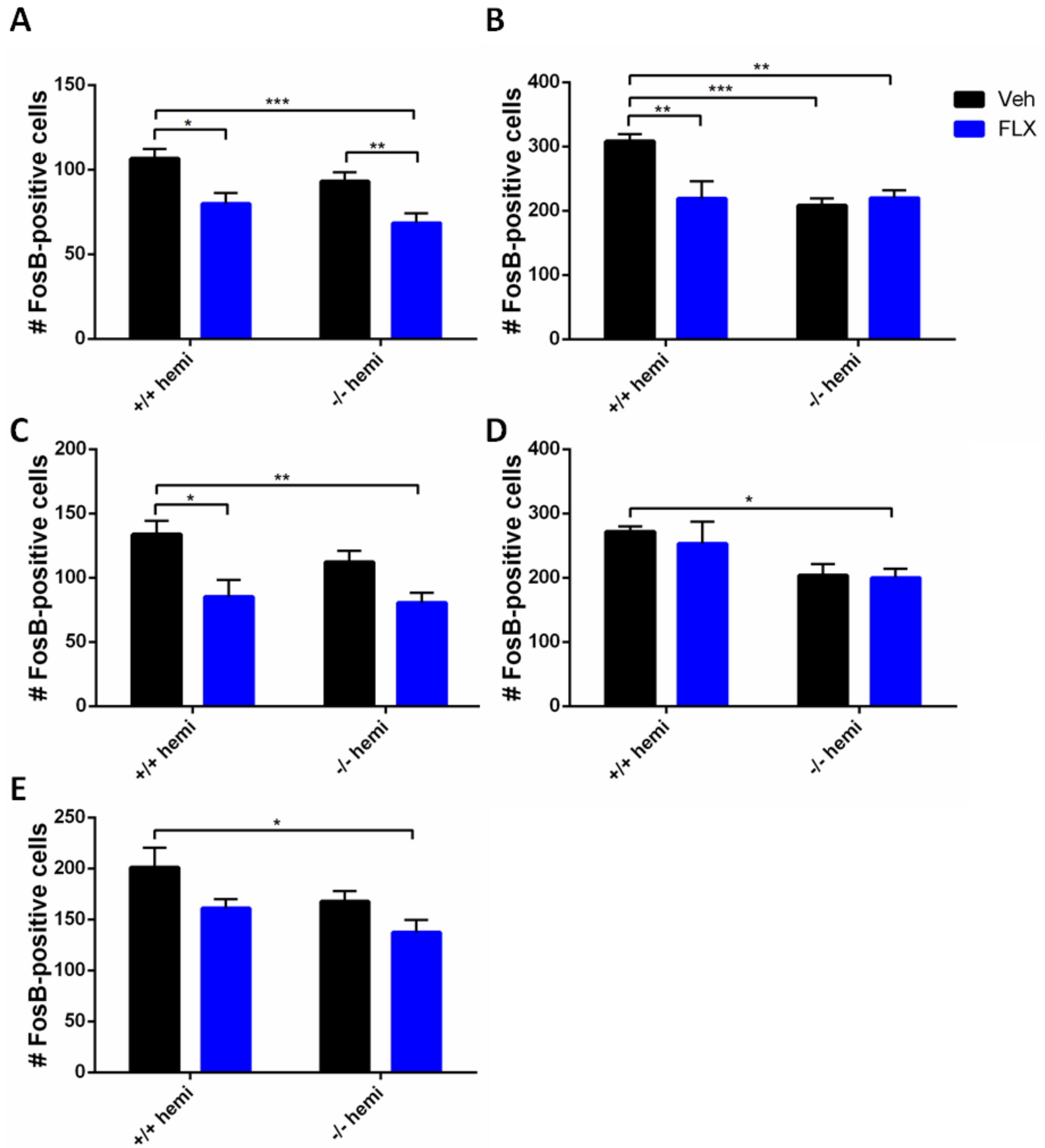

Figure 16: FosB-positive cells in hippocampal region.
Vehicle or FLX was administered to Flx1A mice for 24 days. Immunofluorescence staining using FosB on different brain regions including **a** CA1, **b** CA2 and CA3, **c** DG, **d** BLA and **e** LHB. Data represent mean ± SEM (n=4), *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The second to last region examined was the BLA due to its role in modulating anxiety-related behaviors (Felix-Ortiz, Beyeler et al. 2013). Both vehicle-treated groups showed similar levels of FosB expressing cells (Figure 15D). Similarly, both FLX-treated groups did not differ in their FosB-positive cells. A reduction was observed in the KO treated with FLX in comparison to the WT vehicle.

Similarly to the BLA, the FosB expression in the LHB remained constant with the exception of the KO treated with FLX that showed a decrease in comparison to the vehicle-treated WT mice (Figure 15E).

Overall, fewer changes occurred in the hippocampal-amygdala regions than in forebrain regions. The number of FosB-positive cells was similar in vehicle-treated WT and KO in all the regions excepting the CA2/3. Furthermore, FLX decreased FosB expression in both WT and KO mice.

The last brain region that was analyzed was the raphe nuclei, which was subdivided into the DR and MR (Figure 17and Figure 18). To identify 5-HT neurons, the TPH marker was used and co-stained with FosB. To begin with, the number of TPH-positive cells was assessed in both DR and MR (Figure 16A and D). Removal of 5-HT1A autoreceptors in DR and MR in the KO mice did not alter TPH-positive cell number. Also, FLX treatment in both WT and KO mice did not modify the number of TPH-positive cells.

In the DR, vehicle-treated WT and KO mice had similar total FosB expression levels (Figure 16B and E). Post FLX treatment, a significant reduction in the total number of FosB-positive cells was observed for the WT but not in the KO mice. When the

percentage of TPH cells co-expressing FosB was assessed (Figure 16C and F), the removal of the autoreceptor resulted in a significant increase in TPH/FosB co-expressing cells in the KO mice, which was reduced upon FLX treatment. By contrast in the WT DR, there was a slight increase in TPH/FosB positive cells. These results suggest that loss of the autoreceptor results in increase activity of 5-HT neurons in DR, but also increased sensitivity to chronic FLX-induced negative feedback.

In the MR, a different pattern was observed. The total FosB expressing cells in MR was unchanged in the 5-HT1A autoreceptor knockout compared to wild-type mice (Figure 16E) and chronic SSRI treatment did not alter the total FosB-positive cells in WT or KO. In contrast to DR, there was no change in TPH/FosB-stained cells in MR of KO vs. WT. Strikingly, FLX treatment reduced the number of TPH/FosB stained cells in the WT MR, but had no effect in the KO. Thus in MR, knockout of the 5-HT1A autoreceptor did affect FosB activation, but reduced the sensitivity to FLX-induced inactivation.

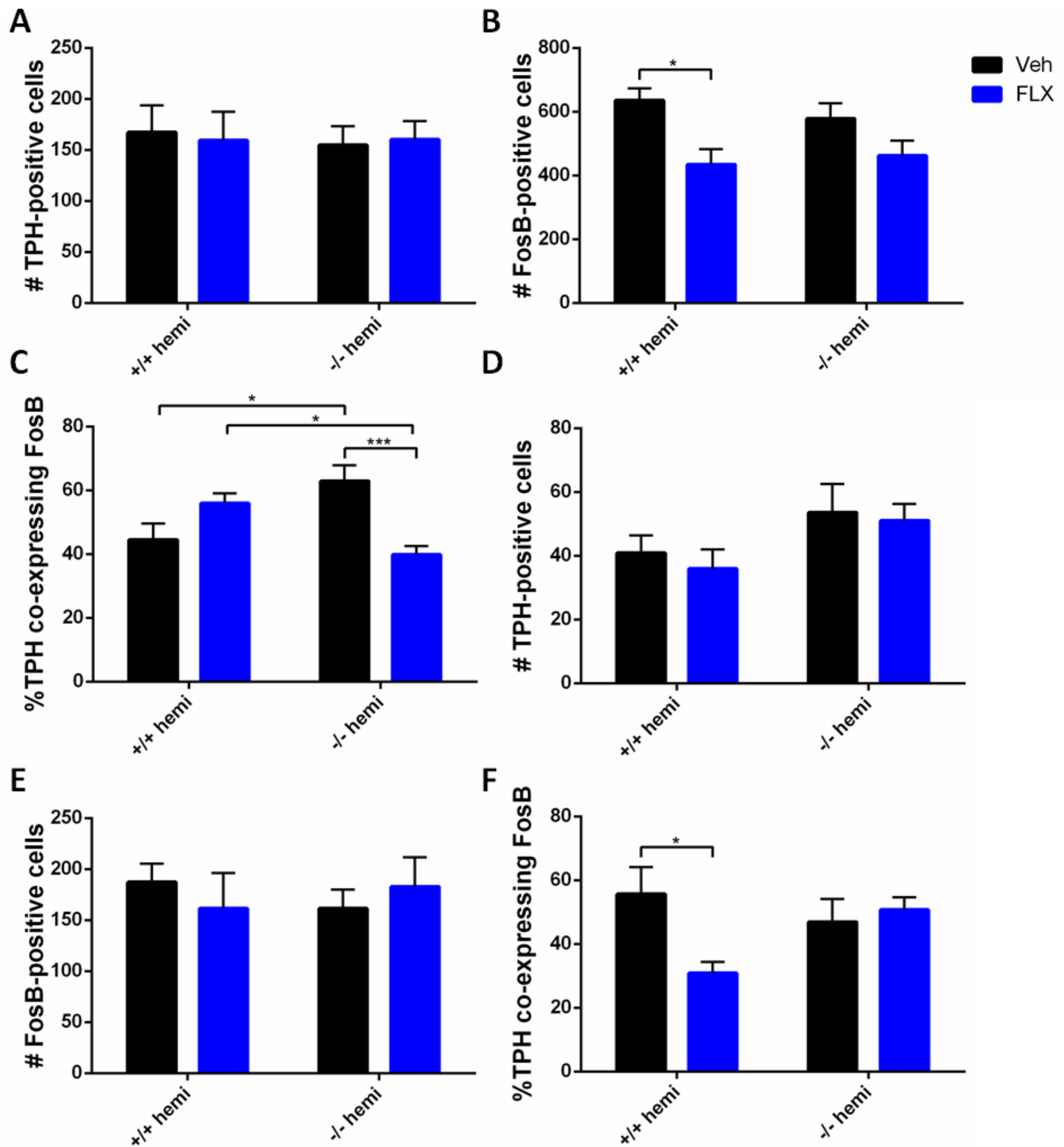

Figure 17: FosB-positive cells in the raphe nuclei.
Vehicle or FLX was administered to Flx1A mice for 24 days. Immunofluorescence staining using FosB and TPH on the **a, b, c** DR and **d, e, f** MR. Data represent mean ± SEM (n=4), *p< 0.05

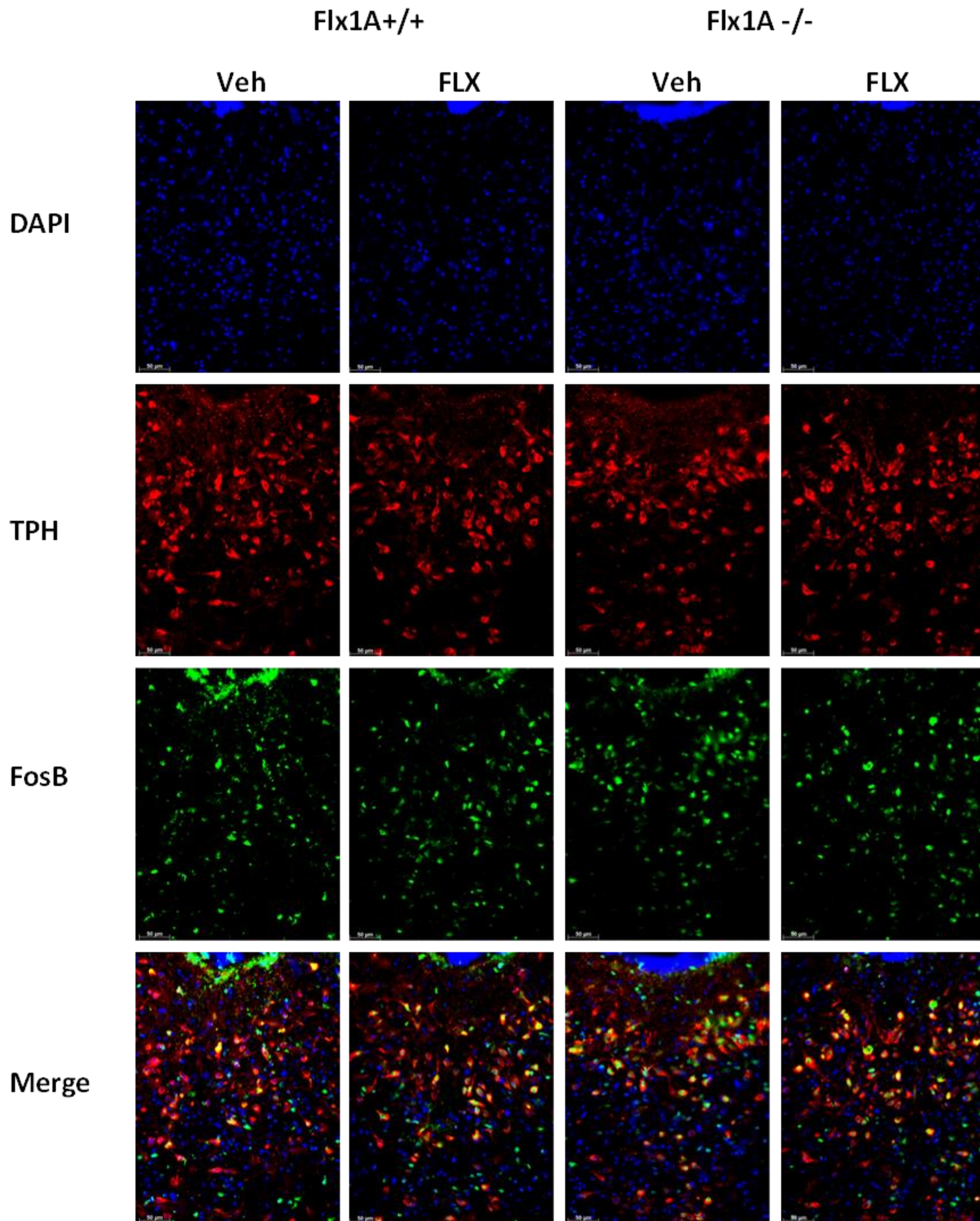

Figure 18: FosB-positive cells in the DR. Immunofluorescent staining for FosB (rabbit anti-FosB, 1:500) and DAPI was performed on DR sections. Vehicle or FLX was administered to Flx1A mice for 24 days. Scale bar indicates 50 μm (n=6).

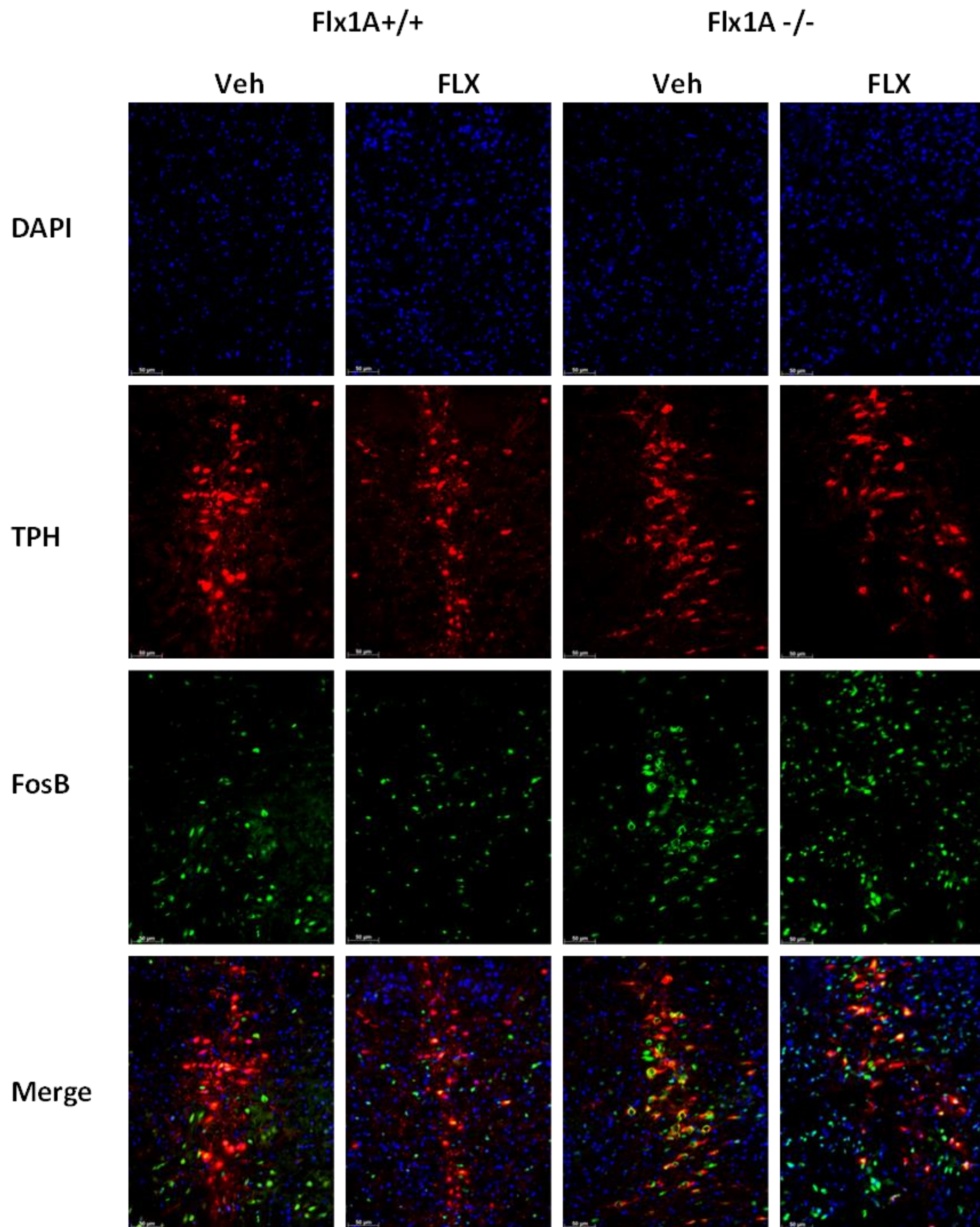

Figure 19: FosB-positive cells in the MR. Immunofluorescent staining for FosB (rabbit anti-FosB, 1:500) and DAPI was performed on DR sections. Vehicle or FLX was administered to Flx1A mice for 24 days. Scale bar indicates 50 µm (n=6).

Discussion

In order to examine the role of 5-HT1A autoreceptors in response to different SSRIs, here FLX and ESC, an inducible conditional 5-HT1A knockout was used to specifically remove the 5-HT1A autoreceptors in 5-HT neurons in the raphe nuclei only during adulthood. By doing so, the anxiety-like phenotype observed upon the postnatal removal of the 5-HT1A autoreceptor can be avoided (Richardson-Jones, Craige et al. 2011).

Immunofluorescence staining in Flx1A mouse

The behavioral phenotype of the Flx1A mouse was previously characterized in our lab. However, I wanted to further confirm the mouse model by using two other techniques to quantitatively and functionally examine the 5-HT1A autoreceptor knockout.

The inducible conditional 5-HT1A KO contains a YFP sequence adjacent to the flx-5HT1A gene, which allowed for the visualisation of the recombined cells. The strong expression of the reporter in 5-HT cells but not in non 5-HT cells suggests that the removal of the 5-HT1A receptor was specific to the autoreceptors. However, the YFP reporter only showed indirectly the effect on the 5-HT1A receptor. To address this caveat, the 5-HT1A antibody was used. The strong expression of the receptor in 5-HT positive cells in the raphe of WT mice confirmed that the TPH2 promoter was a valid choice. Also, the robust reduction in 5-HT1A expression in TPH-positive but not TPH-negative cells of the Flx1A mice indicates that the KO was efficient.

Quantitative analysis of 5-HT1A knockout

Autoradiography was used to accurately quantify the changes in 5-HT1AR expression resulting from this transgenic mouse. Although there was a 50% reduction in I^{125}-MPPI binding in the raphe, this number was lower than the 90% decrease previously saw in the immunofluorescence. However, the ligand-binding assay does not discriminate the cell type on which the 5-HT1AR is expressed and the activation state of the receptor. Thus, autoradiography quantifies the total 5-HT1AR whereas in the immunofluorescence, TPH marker was used to selectively assess the autoreceptors. As shown by immunostaining, TPH-negative cells that express 5-HT1A receptors were not affected by the knockout, and thus contribute to the 5-HT1A binding observed using autoradiography. Taken together our results indicate a substantial knockout of 5-HT1A autoreceptors in ~90% of 5-HT neurons in the DR and MR.

Functional analysis of 5-HT1A knockout

To determine whether the receptors detected in the Flx1A mice were functional, whole-cell electrophysiology in slices of the DR from WT vs. KO mice was done. Results show a lack of response to the 5-HT1A agonist in the KO, and thus confirm a lack of functional autoreceptors in the conditional 5-HT1A KO. To further examine whether 5-HT1A RNA levels are affected, RT-qPCR could be done to specifically measure the 5-HT1A RNA levels (Jacobsen, Czesak et al. 2011, Bortolozzi, Castane et al. 2012) and to precisely quantify the changes in the Flx1A genetic mouse model.

SSRIs induce pro-anxiety behavior in Flx1A mice

Excising the 5-HT1A gene during early adulthood was expected to avoid the anxiety phenotype, which was previously observed in mice with an early postnatal 5-HT1A knockout (Richardson-Jones, Craige et al. 2011). Under basal conditions (vehicle-treated group) Flx1A mice did not display a detectable anxiety phenotype in the NSF, LD and EPM. These results are in agreement with previously reported in adult 5-HT1A knockout mouse models (Richardson-Jones, Craige et al. 2010, Bortolozzi, Castane et al. 2012, Ferres-Coy, Santana et al. 2013). Thus, inducing the 5-HT1A knockout beginning at 7.5 weeks successfully avoided a developmental increase in anxiety phenotype.

Sub-chronic administration of FLX in conditional 5-HT1A knockout was expected to induce a stronger response to antidepressant than the response observed in mice with a 30% knockdown (Richardson-Jones, Craige et al. 2010). However, Flx1A mice displayed a strong anxiety-like phenotype in the NSF and EPM but not LD following FLX treatment. Furthermore, ESC also increased the anxiety level in the NSF, but not in LD and EPM. Perhaps the pro-anxiety phenotype is more readily detected using NSF test because it responds to chronic but not acute antidepressant treatment. This pro-anxiety effect is surprising considering that the desensitization of the 5-HT1A autoreceptors is thought to correlate with the improvement of the negative symptoms by the SSRI. Here, the 5-HT1A autoreceptors are eliminated to accelerate the effect of the treatment, but a worsening of the symptoms was observed when mice were treated with either SSRI.

Recall that the 5-HT1A autoreceptor is one of the main negative regulators of 5-HT release. In Flx1A mice, this regulatory mechanism is lost. Therefore, basal levels of 5-HT should increase and engender behavioral changes. This potential increase in 5-HT levels

in the Flx1A KO mice is based on the finding that adult mice with a 30% knockdown 5-HT1A autoreceptor showed elevated raphe 5-HT levels (Richardson-Jones, Craige et al. 2010). However, the basal anxiety levels remained stable even with the altered 5-HT system. This suggests that, under normal unstressful conditions, the 5-HT system was able to compensate and give a normal response following the removal of the 5-HT1AR gene.

The 5-HT transporter SERT is another crucial regulator of 5-HT release. Usually, an excessive amount of this monoamine in the synaptic cleft can rapidly be recaptured by SERT to reach homeostasis. Flx1A mice treated with SSRI have lost two major ‘brakes’. Therefore, 5-HT levels are poorly controlled and could increase locally but also at the periphery via the projections.

One explanation for the SSRI-induced increase in anxiety-like phenotype may reside within the neurons receiving the 5-HT projections. The affinity of 5-HT2A/2C for 5-HT is 10 times less than 5-HT1A (Albert, Vahid-Ansari et al. 2014). Thus, the possible increase in 5-HT after SSRI treatment in Flx1A mice could be soliciting more of those low affinity receptors. In a 5-HT2A KO mice model, the time spent in the open arm in the EPM was increased, as was the time spent in the light compartment of the LD box and they had a shorter latency to feed in a novel environment during the NSF (Weisstaub, Zhou et al. 2006). Also, upon reinsertion of the 5-HT2A receptor gene, the anxiolytic behavior observed was abolished. Thus, reinsertion of cortical 5-HT2A receptors modulates anxiety-like behavior in mice globally lacking the 5-HT2A receptor. Similarly but in a 5-HT2C receptor KO mouse, an anxiolytic behavior was observed during the

Elevated Zero Maze (EZM) and OF test (Heisler, Zhou et al. 2007). Furthermore, 5-HT release in BLA, which is enriched in 5-HT2C receptors, is enhanced in response to anxiogenic stimuli during the inescapable tailstock (IS), which enhanced BLA output and increased anxiety (Christianson, Ragole et al. 2010). These studies confirmed the implication of the 5-HT2C receptor in anxiety expression.

The implication of 5-HT2A and 2C receptors in a potential pro-anxiety phenotype following the SSRI treatment could be tested by using specific antagonists. Flx1A mice would be treated with SSRI using the same timeline and acutely injected with one of the 5-HT2A or 5-HT2C antagonists (e.g SB 242084 for 5-HT2C) prior to the behavior tests to block the pro-anxiety effect. As a more precise approach, a minipump could be inserted in brain regions enriched in these two receptors. The ligand could be slowly infused as a pre-treatment before the SSRI or infused simultaneously. A previous study has shown that local injection of 5-HT2C ligand in the amygdala in an ethanol-withdrawal mouse model was anxiolytic (Overstreet, Knapp et al. 2006). This experiment would give insight on the involvement of these two receptors in the establishment of the anxiety-like behavior in the 5-HT1A conditional KO mice.

Lastly, these findings may be relevant to adolescents since their 5-HT network differs from that in the adult (Murrin, Sanders et al. 2007) and they react differently to stress. Total 5-HT content, 5-HT uptake sites and receptors are higher in the developing brain in comparison to an adult (Murrin, Sanders et al. 2007). Interestingly, 5-HT binding level in the hippocampus and cortex are 75% of adult levels by 3 weeks of age. The 5-HT2 receptors are 8-fold higher between E17 and P13, thus showing that there are a lot of

changes in the 5-HT system occurring in an adolescent brain (Murrin, Sanders et al. 2007). Also, FLX administration in adolescents has been shown to worsen the depression symptoms, irritability, anger and anxiety acutely, but only in some cases (March, Silva et al. 2004). Similarly, SSRI treatment in juvenile mice induces a pro-anxiety response that can be normalized if treatment is discontinued (Oh, Zupan et al. 2009). It is important to note that a single dose of FLX is able to influence the emotions in young adults suggesting a greater benefit: risk ratio in this age range (Capitao, Murphy et al. 2015). Thus, in an immature or altered 5-HT system, the subjects are more vulnerable to the fluctuation in 5-HT concentration induced by SSRI treatment (Rahn, Cao et al. 2015). This vulnerability might be due to the fact that a developing brain has a very active 5-HT system. It may be that the 5-HT1A autoreceptor knockout is mimicking this juvenile vulnerability of the 5-HT system, although it is not known whether 5-HT1A autoreceptors are reduced in susceptible juveniles compare to adults. This knowledge could give clinicians better tools to appropriately treat adolescent suffering from depression.

<u>No antidepressant response after SSRI treatment in Flx1A mice</u>

Despite the fact that Flx1A mice have lost most of their 5-HT1A autoreceptors, the mice did not display any antidepressant-like phenotype under basal conditions (vehicle-treated) during the TS and FS tests. Also, Flx1A mice did showed an antidepressant-like phenotype following three weeks of FLX or ESC treatment.

The results under basal conditions are consistent with previous studies showing no changes in depressive behavior in both partial 5-HT1A auto- and heteroreceptors

knockout (Richardson-Jones, Craige et al. 2010, Richardson-Jones, Craige et al. 2011). One explanation would be that 5-HT levels reached a plateau upon removal of 5-HT1A autoreceptors, which does not affect the mice behavior. In order to detect any behavioral differences, the serotonergic system has to be pushed/stressed to tease out the effect of the excess of 5-HT (Ramboz, Oosting et al. 1998, Richardson-Jones, Craige et al. 2010). In mice with a 30% 5-HT1A autoreceptor knockdown (1A-low), no differences were observed during the TS or the FST (Richardson-Jones, Craige et al. 2010). However, 24 hours after the first FST, the mice were put through another FST and spent more time struggling/moving than the 1A-high (control). Thus, 1A-low mice showed a stronger respond to a repeated stress in a depression-related task. Therefore, perhaps the Flx1A mice need to be confronted with a repeated stressor in order to distinguish an alteration in their depression-related behavior. In the future, the FST should be repeated after 24 hours to determine if there is any disparity between the WT and KO littermates.

As proposed earlier, Flx1A mice may have a higher 5-HT levels and this could be an advantage in a prolonged stressful environment. In fact, they may be more resilient to different stress paradigms known to induce a depressive phenotype, by increasing 5-HT release throughout the brain. Chronic mild stress (CMS) is a validated tool to study depression that has been used for over 30 years. In this paradigm, the mouse is confronted to daily mild stressors for a prolonged period of time (Willner, Muscat et al. 1992). An advantage of CMS is that is it versatile in the sense of the choice and frequency of the stressors and the length of the experiment. Therefore, this tool can be manipulated to fit the specific conditions required for a specific experiment.

If Flx1A mice are more resilient, a control experiment could be done to assess how the KO mice are responding to the paradigm. After that, the frequency of the stressor daily could be increased to be able to observe a clear behavioral distinction between the KO and the WT mice. These results would give us more understanding of how the absence of the 5-HT1A autoreceptors is influencing the response to chronic stressful stimulus.

The lack of beneficial effect with chronic SSRIs on Flx1A mice was unexpected and could be due to multiple factors. The first one could be that the length of the treatment was not sufficient. In studies using rodents, the chronic antidepressant treatment ranges between 3-5 weeks. The second factor could be related to the mouse strain, since mouse strains can have different levels of baseline anxiety- or depression-like behavior, and respond differently to antidepressant treatment (Jiao, Nitzke et al. 2011). Flx1A mice are on a mixed background, thus it is more difficult to predict their response to chronic SSRI treatment. The last factor could be that related to the choice of the depression-related tests. Previous studies have used the FST following chronic antidepressant treatment and were able to detect the effect of the drug (Jiao, Nitzke et al. 2011). However, FST is mainly used to test the effect of acute antidepressant injection as an indicator of the potential clinical antidepressant activity of drugs (Cryan, Page et al. 2005, Holick, Lee et al. 2008). A previous study has shown that Flx1A KO mice acutely treated with zinc have an antidepressant response during the FST (Satala, Duszynska et al. 2015). Based on this finding, we wanted to determine if the effect of chronic SSRI administration could be detected using both FST and TS. As suggested earlier, a modified FST could be a better

choice to tease out the effect of FLX and ESC following three weeks (Richardson-Jones, Craige et al. 2010). These results could help us determine if SSRIs in the Flx1A mice are acting normally as antidepressant or if they have an unknown undesirable effect on depression similarly to the pro-anxiety effect.

Changes in chronic activation patterns in Flx1A mice

The aim of this experiment was to determine whether there were brain regions that are chronically less/more activated in the Flx1A KO mice, but also following the SSRI treatment. The post-synaptic regions examined were chosen because they are abundant in 5-HT1A heteroreceptors and they play a role in emotional states.

In the forebrain, chronic activation patterns were similar between the WT and KO mice in the medial and lateral septum. However, in the Nacc there was an increase in its chronic activity only in Flx1A KO mice and treated with FLX in comparison to the WT mice. This could indicate that this region plays a critical role in the development of the anxiety-like phenotype since it was the only regions that its activity differs from the control. The Nacc is part of the dopaminergic mesolimbic pathway and is enriched in GABAergic interneurons expressing 5-HT receptors. It was previously reported that 5-HT2C receptors mediate the inhibition of dopamine (DA) through GABA signaling (Alex and Pehek 2007, Dremencov, El Mansari et al. 2009). Also, chronic SSRI treatment causes a decrease in DA activity following the increase in synaptic 5-HT and this effect was abolished by treatment with a 5-HT2C antagonist. To explain how this phenomenon might be causing the increase in FosB staining in the Nacc, we need to look at the DA

receptors. In Flx1A mice treated with FLX, we are suggesting an increase in 5-HT levels that will reduce DA activity (Guiard, El Mansari et al. 2008) (Figure 20).

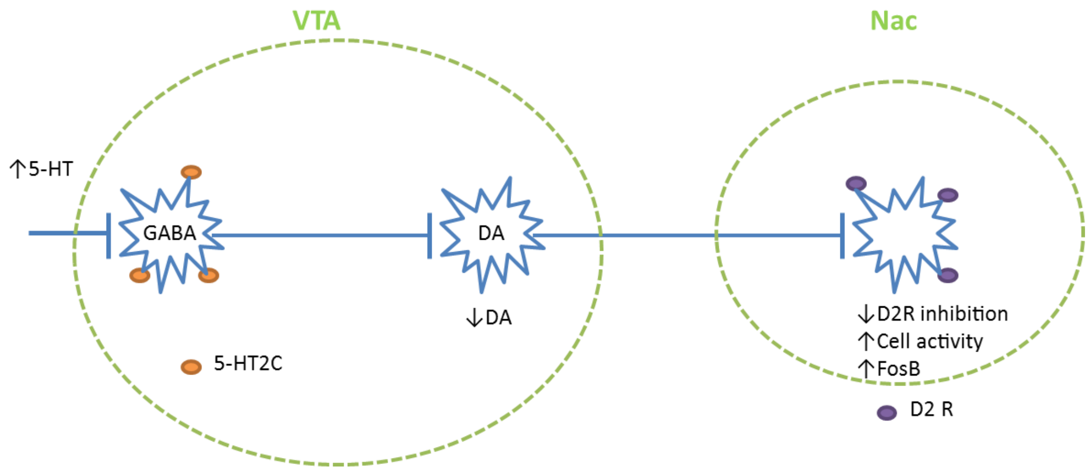

Figure 20: 5-HT increases the neuronal activity in the Nacc. The augmentation of 5-HT2C activity located on GABAergic cells is inhibiting dopaminergic neurons and decrease DA release. The decrease in D2R inhibition is increase the cell activity in the Nacc.

This reduction could lead to reduced D2 receptor inhibition in the Nacc and an enhanced FosB expression. In line with this idea, there is evidence in D2 knockout mice that they are more susceptible to stress-induced anxiety and they have altered Nacc activity (Sim, Choi et al. 2013). Furthermore, it was observed that fear activates ventral tegmental area (VTA) GABA interneurons to inhibit DA neurons (Tan, Yvon et al. 2012). Taken together, it suggests that the SSRI-induced anxiogenic phenotype could involve reduced DA activity, seen as an increase in FosB staining in Nacc.

In the hippocampal region, FosB expression in CA1, DG, as well as in LHB and BLA were not affected by the removal of the 5-HT1A autoreceptors during adulthood. However, the expression in CA2/3 region was reduced in the Flx1A KO to match the expression levels in the WT and KO treated with FLX. This could mean that the absence of 5-HT1A autoreceptors is causing a similar effect on CA2/3 cells to chronic treatment with FLX. It would be interesting for future studies to repeat the experiment and co-stained with a specific cell marker to determine if the cells population that is chronically active is the same in these three groups.

In the raphe, the dorsal and median regions have different chronic activation patterns. In the MR, the WT mice had a lower level of TPH cells co-expressing FosB following FLX treatment whereas the KO mice had similar levels of co-expression prior and after the treatment. The activation of 5-HT neurons in the MR has been shown to mediate anxiety-like behavior, hence loss of FLX-induced 5-HT neuron inactivation in the 5-HT1A autoreceptor knockout could impair the anti-anxiety effect of FLX in the NSF or EPM (Teissier, Chemiakine et al. 2015). In the DR, there was more co-expression of

TPH and FosB in the KO vehicle than the WT vehicle mice. Thus, under basal condition, the 5-HT cells appear to be more active in absence of the 5-HT1A autoreceptors, which is consistent with the hypothesis that they have become "dis-inhibited" due to reduced auto-inhibition in the absence of 5-HT1A autoreceptors. Following chronic FLX treatment, the level of TPH/FosB co-expressing cells in the DR was decreased in the KO mice, but not the total number of FosB positive cells. This could suggest that the 5-HT1A receptors located on non-serotonergic cells are being activated to decrease 5-HT neuron activity. To test whether GABA cells are activated, GAD67 or VGLUT3 (Hioki, Nakamura et al. 2010) could be used to in co-staining with FosB to identify the chronically active cells.

Thus, it is not surprising that both regions of the raphe have different activation patterns since they innervate different brain regions (Bang, Jensen et al. 2012) and their efferent projections are different (Szonyi, Mayer et al. 2016). It has been suggested that a low ratio of DR/MR activity results in increased depression-like behavioral response in a model of depression (Teissier, Chemiakine et al. 2015). We also observed that upon FLX treatment in the flx-1A KO mice the "anxiety-depression" behavior in the NSF correlates with reduced DR/MR activation in TPH-positive cells. Taken together, these results suggest that it is most probably the raphe that is responsible for the anxiogenic effect of SSRIs.

Conclusion

The Flx1A (inducible conditional 5-HT1A autoreceptor knockout) mouse model was used to specifically study the effect of SSRIs on the 5-HT1A autoreceptors. Immunofluorescence staining was used to assess the loss of the receptor on 5-HT neurons. Autoradiography was performed to qualitatively assess the decrease of the number of 5-HT1A receptors and the whole-cell electrophysiology to assess the functionality of the remaining autoreceptors.

Under basal conditions, the Flx1A KO mice did not show any change in anxiety or depression-related behavior compared to WT mice in the tests used. The absence of phenotypic differences is most probably due to compensatory mechanisms to rebalance the 5-HT system, as suggested by reduced activity in the hippocampal CA2/3 and entorhinal cortex, and no change 5-HT neuron activation of MR, but increased in DR.

Sub-chronic FLX and ESC treatment resulted in an overall increase in anxiety-like phenotype in the Flx1A KO mice and no antidepressant-like effect. This pro-anxiety effect could be due to a lack of FLX-induced inactivation of the MR in the KO, resulting in higher 5-HT levels at anxiogenic projection sites. There was also attenuated FLX-induced inactivation in the KO in the entorhinal cortex, median habenula, hippocampal CA2/3 and DG, areas implicated in anxiety and depression.

FosB immunofluorescence showed an increase in activity upon FLX treatment in the Nacc only in Flx1A KO mice, which might correspond to the anxiety-like phenotype. Thus, the higher 5-HT levels may lead to the overactivation of the 5-HT2C receptors in

the Nacc. The latter are inhibiting the DA activity which might correspond to the increase chronic activity observed due to the reduce activation of D2 receptors.

The results of these experiments contribute to understanding how SSRIs can mediate aversive effects in an altered serotonergic system. Also, it suggests a complex relation between the monoamine systems that needs to be further studied.

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Appendix

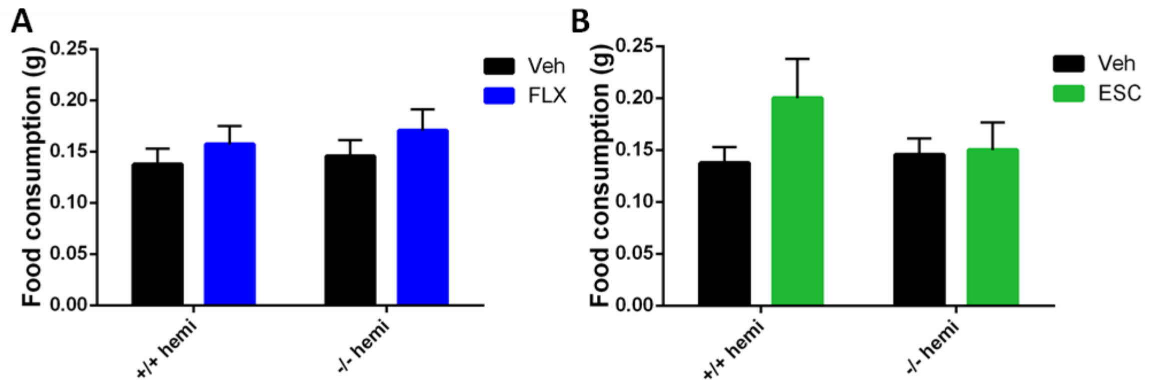

Figure i. Food consumption during the NSF.
Represent the amount of eaten food for mice treated with **a** FLX and **b** ESC. Data represent mean ± SEM.

Light Dark Box

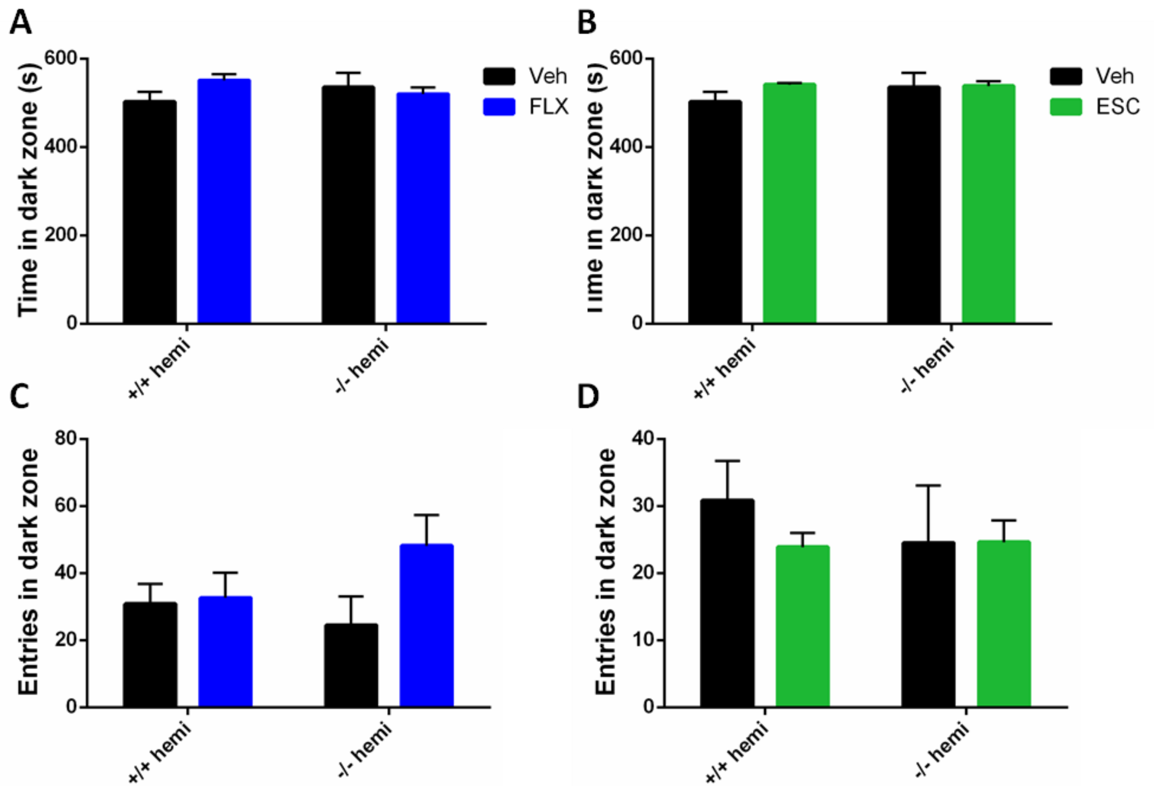

Figure ii. No effect of SSRIs during the LD test. Vehicle, FLX and ESC were administered to Flx1A WT (n=8, 9, 8 respectively) and KO (n=5, 7, 8 respectively) for 12 days prior to testing. **a, b** Time spent in the dark zone and **c, d** number of entries in the dark zone were recorded. Data represent mean ± SEM.

Novelty Suppressed Feeding test

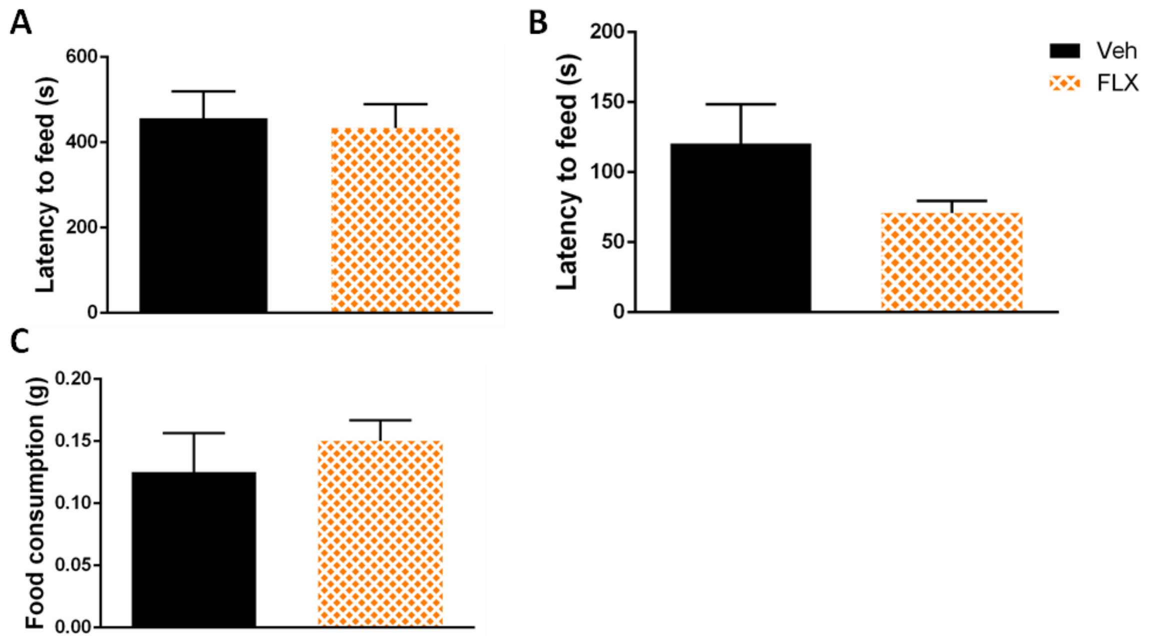

Figure iii: No effect of SSRIs during the NSF in Flx1A Het mice.
Vehicle (n=12) and FLX (n=12) were administered to Flx1A Het for 9 days prior to testing. Latency to feed in **a** novel cage and **b** home cage and **c** food consumption were recorded. Data represent mean ± SEM.

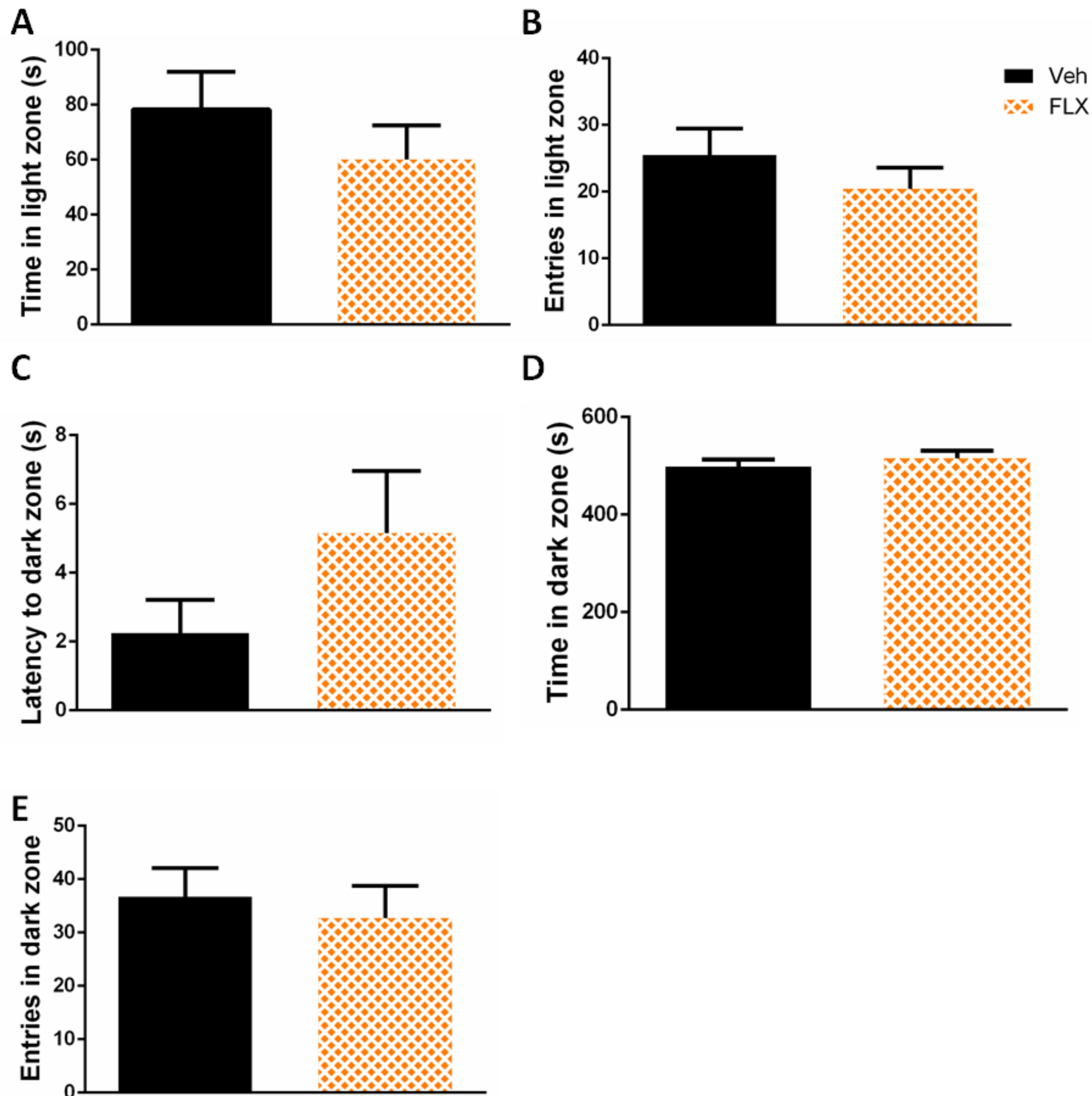

Figure iv: No effect of FLX on Flx1A Het mice during the LD.
Vehicle (n=12) and FLX (n=12) were administered to Flx1A Het for 12 days prior to testing. **a** Time spent in light zone, **b** number of entries in light zone, **c** latency to enter dark zone, **d** Time spent in dark zone and **e** number of entries in dark zone were recorded. Data represent mean ± SEM.

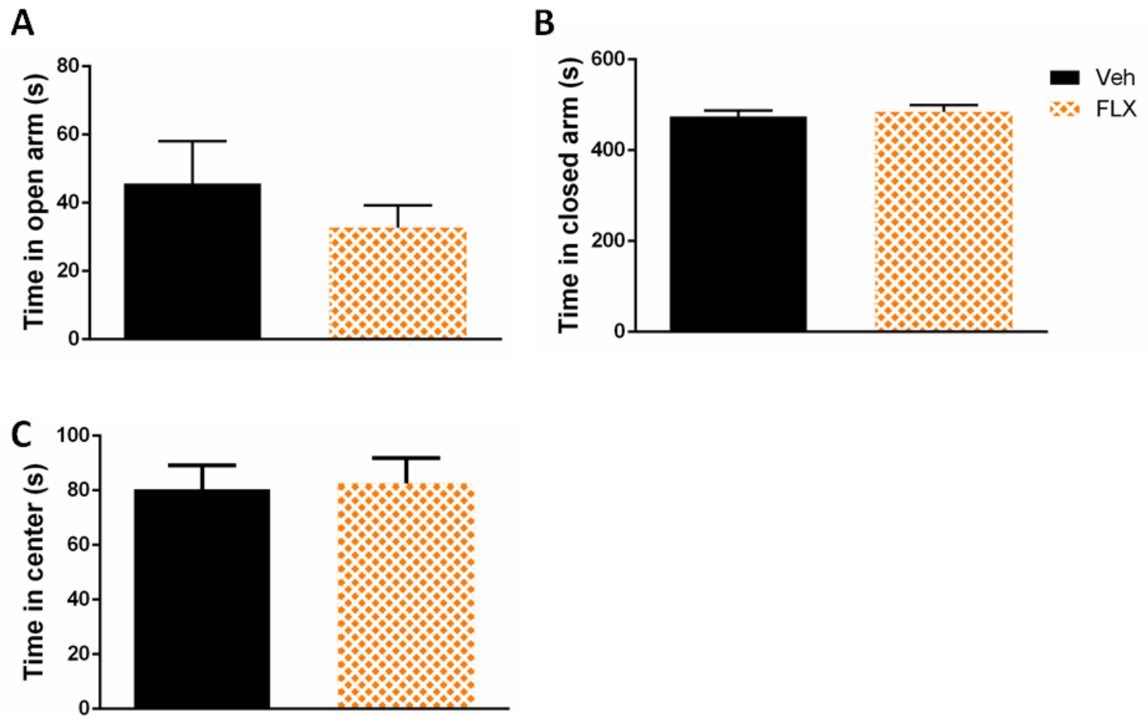

Figure v: No effect of FLX on Flx1A Het mice during the EPM.
Vehicle (n=12) and FLX (n=12) were administered to Flx1A Het for 15 days prior to testing. **a** Time spent in open arm, **b** closed arm and **c** time in the center were recorded. Data represent mean ± SEM.

Tail Suspension

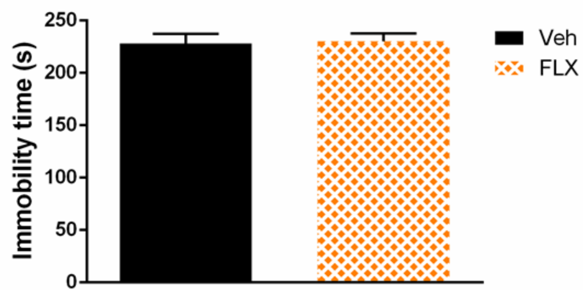

Figure vi: No effect of FLX on Flx1A Het mice during the TS.
Vehicle (n=12) and FLX (n=12) were administered to Flx1A Het for 19 days prior to testing. The time spent immobile was recorded. Data represent mean ± SEM.

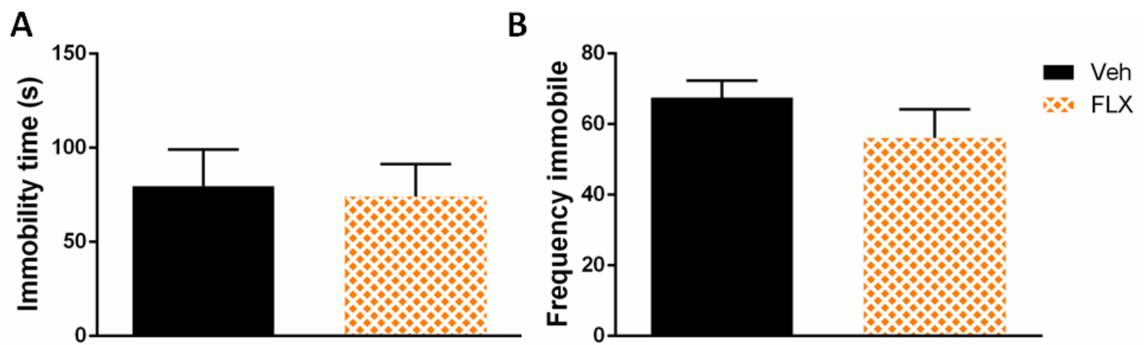

Figure vii: No effect of FLX on Flx1A Het mice during the FST.
Vehicle (n=12) and FLX (n=12) were administered to Flx1A Het for 21 days prior to testing. **a** Time spent immobile and **b** frequency immobile were recorded. Data represent mean ± SEM.