Functional Studies of the 5-HT1A Autoreceptor in Mice

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Abstract

The serotonin system, tightly controlled by negative regulation by 5-HT1A autoreceptors, is involved in MDD since manipulating different aspects of the 5-HT systems produces behavioral changes akin to MDD symptoms. Deaf1 knockout mice, which have shown increased 5-HT1A autoreceptor levels, were examined. Conversely, the effect of loss of 5-HT1A autoreceptors in adulthood was studied in 5-HT1A^{autoKO} mice engineered for inducible knockout of the 5-HT1A autoreceptor. Anxiety and depression behavioural assays were performed on the Deaf1^{KO} and 5-HT1A^{autoKO} models. Deaf1^{KO} mice displayed an overall increase in anxious behaviour, yet no change in depressive behaviour relative to controls. Conditional 5-HT1A mice displayed no changes in anxiety or depressive behaviour. Compensation from remaining aspects of the serotonin system and/or other neurological systems may account for some of the behavioural results (or lack thereof). Additional testing that includes a stress paradigm is warranted in order to reveal any stress-dependent vulnerabilities to anxiety and depression.

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List of Abbreviations

+/+ containing no copies of the floxed gene

5-HIAA 5-hydroxyindoleacetic acid

5-HT serotonin; 5-hydroxytryptamine

5-HT1A serotonin 1A receptor

5-HTP 5-hydroxytryptophan

5-HTT serotonin transporter

8-OH-DPAT 8-hydroxy-N,N-dipropyl-2-aminotetralin

ACTH adrenocorticotropic hormone

ANOVA analysis of variance

BDNF brain-derived neurotrophic factor

Ca^{2+} calcium

cAMP cyclic adenosine monophosphate

CGB corticosterone binding globulin

CMS chronic mild stress

CRH corticotropin-releasing hormone

Deaf1 deformed epidermal autoregulatory factor 1

DRN dorsal raphe nucleus

DSM IV diagnostic and statistical manual of mental disorders, version 4

ECT electroconvulsive therapy

EPM elevated plus maze

ER estrogen receptor

fl/+ containing one copy of the floxed gene

fl/fl containing two copies of the floxed gene

flx floxed; flanked by loxP sites

Freud1 five-prime repressor element under dual repression 1

FS forced swim test

G protein guanosine nucleotide-binding protein

GFP green fluorescent protein

G_i G protein inhibitory

G_o G protein other

GR glucocorticoid receptor

GRE glucocorticoid response element

G_s G protein stimulatory

Hes5 hairy/enhancer of split 5

HET heterozygote

HPA hypothalamic pituitary adrenal

K^+ potassium

KO knockout

LD light dark paradigm

MAOI monoamine oxidase inhibitor

MDD major depressive disorder

MR mineralocorticoid receptor

mRNA messenger ribonucleic acid

ns not significant

NUDR nuclear Deaf1 related

OF open field

PET positron emission tomography

REST repressor element 1-silencing transcription factor

SD standard deviation

S.E.M. standard error of the mean

SERT serotonin transporter

siRNA small interfering ribonucleic acid

SSRI selective serotonin reuptake inhibitor

TAM tamoxifen

TCA tricyclic antidepressant

TPH tryptophan hydroxylase

TS tail suspension

WT wildtype

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Serotonin (5-HT) is a widely distributed neurotransmitter that is synthesized from the essential amino acid tryptophan by the enzyme tryptophan hydroxylase (TPH). Raphe serotonin neurons express the neuronal form, TPH2, while TPH1 is expressed in the periphery. Serotonergic cell bodies located in the raphe nuclei project throughout the central nervous system. The caudal raphe nuclei project down the brainstem into the spinal cord; the rostral raphe nuclei project to higher brain areas and innervate much of the cortex, forebrain, and limbic structures. One of the roles of serotonin is in regulating mood and emotion. Reduced activity of the serotonergic system has been implicated in depression and suicide (Mann, 1999). Depression and related depressive disorders are among the most common afflictions in developed nations. Despite the high prevalence of depression, the mechanisms that underlie its cause are yet unexplained (Albert et al., 2011; Celada et al., 2013).

Major Depressive Disorder in Humans

Depression is a ubiquitous disease, with global rates estimated as high as 1 in 7 (Lam, 2012). It is also projected to have one of the highest global burdens of disease by 2030 (Branchi et al., 2013; Celada et al., 2013; Lam et al., 2012).

Diagnosing depression in humans involves primarily a series of subjective, interview-style sessions. There is a wide range of symptoms associated with depression that can be physical (gain/loss of weight, insufficient/excess sleep, overall fatigue), cognitive (guilt, difficulty concentrating, suicidal ideation), or emotional (decreased mood, anxiety, anhedonia). Not only are there many symptoms of depression, but depression itself can be divided into several subtypes with their own identifying features. For instance, melancholic depression, atypical depression, catatonic depression, and

seasonal affective disorder are all categorized under major depressive disorder (MDD; Lam, 2012), yet have different symptoms and even different treatment regimens (Savitz et al., 2009).

Since traditional diagnosis is not always accurate, genetic biomarkers are an attractive method for identifying depression. Several studies have found gene variants within the serotonin system that are associated with increased depression, suicide (Kishi et al., 2013; Lemonde et al., 2004), and susceptibility to stress (Caspi et al., 2003). Brain-derived neurotrophic factor (BDNF) polymorphisms have been associated with abnormal anxiety (reviewed in Archer et al., 2013). Despite the promising results of these genetic studies, the effects can be difficult to reproduce and often require specific types of stress to bring about depressive symptoms (Caspi et al., 2010). In addition, depression is not linked to a single gene; it is a multifaceted and multi-genic disease that encompasses many circuits, receptors, and neurotransmitters within the brain. It also depends heavily on environmental factors for both the development of depression as well as its severity (Caspi et al., 2010).

Depression diagnoses have high rates of comorbidity with other mental illnesses. Almost 75% of MDD patients in a US National Comorbidity Survey Replication study had at least one other DSM IV-recognized disorder, the most common being anxiety, substance abuse, and impulsivity control (Kessler et al., 2003). Comorbid depression also runs a higher risk for suicide attempts than depression alone (Bronisch & Wittchen, 1994). Other common comorbid disorders include irritability, anger, and aggression (Lam, 2012). A high rate of comorbidity coupled with diverse symptoms and countless subtypes makes depression very difficult to properly diagnose and treat.

Standard treatments for depression are antidepressant medications that target the serotonin system. Tricyclic antidepressants (TCAs), monoamine oxidase A inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRIs) are three common classes of antidepressant used. SSRIs however are the most commonly prescribed since they have fewer side effects due to the specificity of their action (Hasselmann, 2014). Traditional antidepressants have several caveats, not the least of which being that 1 in 3 patients will not respond to treatment (Lam, 2012). Electroconvulsive therapy (ECT) is one of the most effective treatments for patients who have failed antidepressant therapy and is reserved for those with severe depression (Lam, 2012).

Depression and Serotonin

As mentioned, serotonin-producing neurons in the brainstem project rostrally to the limbic system where mood and emotion is regulated. Further links between depression and serotonin were made when serotonin metabolites found in the cerebrospinal fluid of depressed suicide attempters were significantly reduced (Brown et al., 1982). In another study that used diet to drastically reduce tryptophan levels in the brain, depressed patients in remission quickly relapsed into a depressive episode. After 48 hours and a normal diet, patients returned to their original remission state (Delago et al., 1990). PET imaging has also been used to trace metabolic activity of living brains. Numerous PET studies have focused on the serotonin system and, in particular, 5-HT1A receptor binding potential (Hesselgrave & Parsey, 2013; Savitz & Drevets, 2009). In one study, less 5-HTP (serotonin precursor) was taken up into the brains of depressed patients compared to healthy patients (Agren et al., 1991). Post-mortem analyses of the brains of suicide victims show an increased level of 5-HT1A autoreceptor (Albert & Lemonde,

2004). The 5-HT1A binding potential was also higher in living MDD patients compared to controls (Sargent et al., 2000) and in MDD patients that had no exposure to antidepressants (Parsey et al., 2006). Similarly, Stockmeier and colleagues (1998) found an increase in 5-HT1A receptor binding in the midbrain raphe of depressed suicide victims. Reduced 5-HT1A receptor binding potential, especially in postsynaptic regions such as prefrontal cortex, has been repeatedly found in depressed living patients, postmortem patients, and animal models (reviewed in Savitz & Drevets, 2009). These findings indicate there is a relationship between depression and suicide and the regulation of serotonin by the 5-HT1A autoreceptor.

The Serotonin System

Receptor Signaling

The actions of serotonin after release are mediated by the serotonin receptors. There are seven families of receptors, numbered 1 through 7, and are G-protein coupled receptors (with the exception of the 5-HT3 receptor, a ligand-gated ion channel). Receptors that are coupled to the G_i/G_o subunit, such as 5-HT1 receptors that include the 5-HT1A, 1B, 1D, 1E, and 1F, have inhibitory actions and decrease the excitability of the neuron. Activation of the G_i inhibits adenylyl cyclase, which decreases the production of cyclic AMP (cAMP). cAMP is important in multiple signaling cascades within the cell. The G_i-coupled Gbg subunit also changes the conductance of potassium (K^+) and calcium (Ca^{2+}) channels in which K^+ channels are opened and Ca^{2+} channels are closed. As a result, there is hyperpolarization of the cell and decreased neurotransmitter release (Mann, 1999). Altogether, activation of the 5-HT1A receptor negatively regulates the firing of the neuron.

Receptors that are coupled to the G_s subunit, such as 5-HT4, 6, & 7, are stimulatory and activation of these receptors increases the excitability of the neuron by increasing adenylyl cyclase activity. The 5-HT2 family of receptors are also stimulatory and are coupled to the G_q subunit, which increases phospholipase C (PLC) activity. Both of the second messenger cascades elicited by the G_s and G_q subunits eventually resulted in an increase in the intracellular calcium concentration. In the case of excitable cells such as neurons, elevated calcium ions can cause the release of neurotransmitter.

5-HT1A Receptor

Hetero- and auto-receptors

The 5-HT1A receptor is encoded by a single gene and is expressed both presynaptically as an autoreceptor and postsynaptically as a heteroreceptor. The heteroreceptor is found on non-serotonergic neurons in the hippocampus, septum, and prefrontal cortex (PFC; Celada et al., 2013). In the PFC, a large proportion of pyramidal neurons and inhibitory interneurons express 5-HT1A receptors (Celada et al., 2013). Thus the 5-HT1A receptor is thought to be integrally involved in cortical functioning. It has been suggested that increased pyramidal neuron activity resulting from reduced 5-HT1A expression and increased 5-HT release also increases anxiety behaviour (Albert et al., 2014).

The 5-HT1A autoreceptor is expressed on the cell body and dendrites of serotonin neurons in the raphe nuclei. The autoreceptors essentially limit the activity of serotonergic neurons and thus control the amount of serotonin being released in the brain (Albert & Lemonde, 2004).

The 5-HT1A receptor gene does not have any splice variants since there are no introns in the promotoer and coding portions of the gene (Celada et al., 2013). However, there are functional differences that have been observed between the presynaptic and postsynaptic receptors. For instance, prolonged stimulation of 5-HT1A autoreceptors results in desensitization and downregulation of the receptor (Rainer et al., 2012; Albert & Le Francois, 2010). Interestingly, postsynaptic 5-HT1A receptors do not desensitize as easily, although the mechanism by which this occurs is not yet known (Bouazziz et al., 2014; Le Poul et al., 2000).

Transcriptional Regulation

Several transcriptional regulators control the gene encoding the 5-HT1A receptor (Figure 1). Deformed epidermal autoregulatory factor 1 (Deaf1) is a regulator located upstream of the promoter. In the raphe, Deaf1 is partly responsible for repressing transcription of 5-HT1A receptors (Czesak et al., 2006). Freud1 (five-prime repressor element under dual repression) is another repressor located upstream of Deaf1 that regulates the transcription of 5-HT1A receptors. Glucocorticoid and mineralocorticoid receptors can also bind to a GRE (glucocorticoid response element) on the promoter and repress transcription under certain conditions, such as increased stress. Sites for other transcription factors also exist, such as REST, which functions to restrict gene expression in appropriate neurons, and Hes5, which regulates expression during development. Interestingly, some evidence suggests depressed suicide-completed patients have upregulated REST expression in the dorsal raphe, indicating differential expression may occur in abnormal conditions (Goswami et al., 2010).

The Role of Deaf1

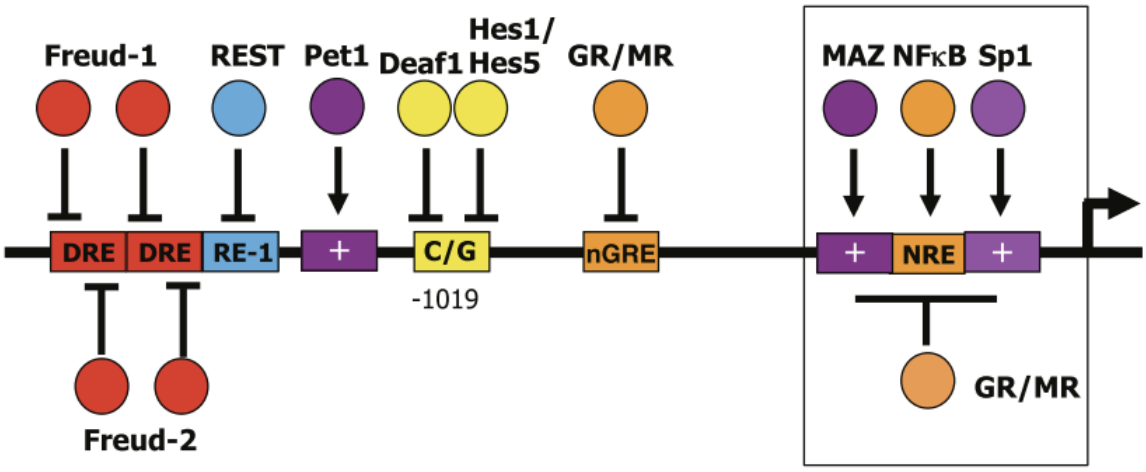


Figure 1. Human 5-HT1A receptor gene promoter elements

From Albert et al., 2011.Known promoter elements include Freud-1, Freud-2, and REST which repress 5-HT1A expression; Pet-1 which is a strong enhancer; Deaf1 and Hes1/5 which repress activity at the C(-1019) allele in serotonergic neurons. Glucocorticoid-mediated repression can also be found on the gene. MAZ, NFκB, and Sp1 elements are located in the minimal promoter (box). Activator (arrow) and repressor (bar) are shown.

As mentioned, the 5-HT1A receptor gene is controlled by several regulators, including Deaf1. Deaf1 was first identified as a gene regulator and DNA-binding protein in *Drosophila*. The human homolog (also referred to as nuclear Deaf1 related or NUDR) has been characterized by Huggenvik and colleagues (1998).

Several studies in humans have found a sequence variant in the promoter 5-HT1A receptor gene that is associated with depression and suicide. Deaf1 and Hes5 (another transcription repressor that is more active during development) bind the 5-HT1A sequence where a polymorphism has been identified in humans. The C(-1019)G polymorphism hinders the binding ability of the transcription repressors Deaf1 and Hes5, such that 5-HT1A autoreceptors are overexpressed. As a result, overall firing and serotonin release is reduced. In humans, the G(-1019) allele was found twice as frequently in depressed patients compared to the C(-1019) allele. As well, the G(-1019) allele was found four times more frequently in patients that completed suicide (Lemonde et al., 2003). In addition, patients homozygous for the G allele responded significantly less to chronic antidepressant treatment and were more likely to be non-responders than patients homozygous for the C allele (Lemonde et al., 2004). Carriers of the G allele also had higher 5-HT1A binding potential in the dorsal raphe in a PET study of MDD patients (Parsey et al., 2006). These results suggest proper expression of the 5-HT1A receptor and therefore proper regulation of serotonin is involved in normal mental functioning.

In the raphe, Deaf1 is partly responsible for repressing transcription of 5-HT1A receptors. In vitro studies show that Deaf1 binds to the C(-1019) site and functions as a repressor in RN46A cells, which are presynaptic serotonin cells. However in cell models of postsynaptic neuronal cells and glial cells, Deaf1 acts as an enhancer. Interestingly,

both repressor and enhancer activities were blocked when the G(-1019) mutation was used (Czesak et al., 2006).

In vivo, Deaf1 binds to the 5-HT1A promoter and therefore directly regulates 5-HT1A gene expression. Deaf1 knockout mice showed increased 5-HT1A mRNA in the dorsal raphe and reduced levels of raphe serotonin compared to wildtype controls. In the cortex, reduced levels of 5-HT1A mRNA were found. In addition, no changes in TPH2 mRNA or the number of serotonin cells were found throughout the brain, indicating that the changes in 5-HT1A mRNA that were found were due to Deaf1 itself, and not other components of the serotonin system (Czesak et al., 2012).

Antidepressant Action

SSRIs are a type of antidepressant that works by selectively blocking serotonin transporters located on the presynaptic terminal. Normally these transporters take excess serotonin back up into the terminal for recycling, which limits the amount of serotonin available in the synaptic cleft to stimulate postsynaptic receptors. In depressed patients, there is inadequate stimulation of the postsynaptic receptors, so by administering SSRIs, reuptake is prevented and more serotonin is left in the synaptic cleft. However the SSRIs do not exert an antidepressant effect immediately; it takes between two and four weeks for the depression symptoms to dissipate (Celada et al., 2013; Gray et al., 2013). Once the reuptake transporters are blocked, the 5-HT1A autoreceptor detects the excess serotonin and inhibits neuronal firing to decrease serotonin release. The effect of the SSRI to increase synaptic serotonin is essentially counteracted by the activation of the autoreceptors. After chronic SSRI treatment, the autoreceptors gradually desensitize to the excess serotonin and are downregulated. As mentioned, postsynaptic serotonin

receptors remain sensitive to serotonin and do not desensitize. Without the negative feedback from the 5-HT1A autoreceptor, the firing rate and serotonin release is increased such that the antidepressant effects of the SSRI can now be felt (Albert & Lemonde, 2004; Celada et al., 2013).

As mentioned, one theory for the delayed onset of antidepressant action is desensitization of 5-HT1A autoreceptors. Since they provide negative feedback to the neuron, reducing the amount of receptors will eventually increase the output of serotonin and help to alleviate depression (Gray et al., 2013). Several animal studies and animal models have examined the role of 5-HT1A receptors in anxiety- and depression-like behaviour and response to antidepressants (Table 1). In addition to desensitization, chronic treatment with SSRIs leads to a reduction in raphe 5-HT1A mRNA in rats (Le Poul et al., 2000) and reduced 5-HT1A binding in the raphe in human depressed subjects (Gray et al., 2013). These studies suggest that transcriptional mechanisms may be involved in long-term regulation of 5-HT1A autoreceptors upon chronic SSRI treatment. Stimulating the 5-HT1A receptor with agonists, such as buspirone, can have anxiolytic effects (Albert et al., 1999). Conversely, 5-HT1A antagonists can have anxiogenic effects. Animal models in which the 5-HT1A receptor is knocked out completely display anxiety-like behaviours and an increased stress response (Parks et al., 1998). In terms of SSRIs, the 5-HT1A autoreceptor is essential for antidepressant response and its downregulation is likely involved in alleviating symptoms.

The Influence of Stress

The serotonin system, in particular the 5-HT1A receptor, is heavily influenced by stress. Electrophysiological studies of the DRN have found that both in vivo stress

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 1. Summary of genetic 5-HT models.

From Albert et al., 2014. The effect of the indicated genetic model on target gene expression (−, none; +, normal; ++, increased; +X, increased by X%) at indicated developmental time [embryo, early post-natal (PN) or adult] and adult behavior phenotype or response to SSRI are indicated. Promoter used indicated in parentheses; rescue is expression in knockout background, over-expression (OE) is on wild-type background; auto, autoreceptor-specific; hetero, heteroreceptor-specific. *loss of 5-HT release; enhanced activity 5-HTT mutant.

paradigms and simulating stress with corticosterone injections can produce desensitization of the 5-HT1A receptors (Fairchild et al., 2003; Laaris et al., 1999). Additionally, even though the 5-HT1A receptors were desensitized, there was no change in 5-HT1A mRNA levels (Fairchild et al., 2003). Stress produces changes in the serotonin system at the molecular level and can this can lead to lasting changes.

Recall the 5-HT1A gene promoter region has binding sites for GR/MR dimers, which repress transcription (Albert et al., 2011; Ou et al., 2001). Two glucocorticoid receptor subtypes have been identified in the brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MRs have a high affinity for glucocorticoids, are tonically active, and inhibit serotonergic transmission. GRs respond to high levels of glucocorticoids following stress (Savitz & Drevets, 2009). Upon glucocorticoid release, GRs and MRs heterodimerize then are translocated into the nucleus and bind to a negative GRE on the 5-HT1A promoter, thereby inhibiting its transcription (Ou et al., 2001; Savitz & Drevets, 2009). As such, plasma cortisol levels are negatively correlated with 5-HT1A receptor levels (Albert et al., 2014; Meijer and de Kloet, 1995; Meijer et al., 1997).

The hippocampus, which is rich in 5-HT1A heteroreceptors, also has a high level of expression of glucocorticoid receptors (Owens et al., 1990; Sullivan Hanley et al., 2003) and, consequently, learning and memory are impaired when glucocorticoids are high (Frodl & O'Keane, 2013). Chronic stress (or simulated chronic stress via corticosterone) resulted in desensitization or downregulation of 5-HT1A receptors in the hippocampus, amygdala, and hypothalamus in rodents. Conversely, adrenalectomy,

which reduces glucocorticoids, prevented the effects of the stress on 5-HT1A receptors (Laaris et al., 1999; Meijer & de Kloet, 1995).

Several studies have found that glucocorticoid receptors are also expressed in the DRN (Harfstrand et al., 1986; reviewed in Frodl & O'Keane, 2013). Simulating stress by administering chronic corticosterone resulted in desensitized 5-HT1A autoreceptors, yet caused no change in mRNA (Fairchild et al., 2003). Similarly, pretreating adrenalectomized rat brain slices with corticosterone reduced the efficacy of a 5-HT1A agonist from inhibiting 5-HT release (Laaris et al., 1995). These studies all demonstrate a strong link between stress and the serotonin system, in particular the 5-HT1A receptors. Not only does the serotonin system have strong effects on affective behaviour, the vast projections throughout the brain increases the likelihood that it is also connected to other systems, as is the case with the stress system.

Rodent Studies

Studying Mental Health in Animal Models

Depression in humans is often categorized by emotional symptoms such as lowered mood and feelings of worthlessness (Lam, 2012). In order to model depression in animal models, methods are needed that can turn these symptoms into an identifiable and quantifiable property. There are many advantages to using animal models for researching human diseases such as genetic manipulation, drug testing, and brain tissue analysis.

Many behavioural assays have been developed for depression-like symptoms. In rodents, depression behaviour is referred to as "behavioural despair". The tail suspension and forced swim tests are two methods for evaluating behavioural despair and are based

on the immobile posture rodents develop once they find themselves in an inescapable situation (Cryan et al., 2005). The forced swim test involves placing the rodent in a large cylinder of water. After a short period of habituation, the rodent will stop struggling and spend more time floating in the water. Initially the test was designed for rats and involved two days of testing in which the first day was a longer period of swimming (15 min.) and the second day consisted of a shorter (5-6 min.) probe test (Bogdanova et al., 2013; Porsolt, 2001). In mice, the test is usually only a single, six-minute test, although some researchers have used a two-day paradigm in which the first session acts as a stressor to induce immobility in the second session (Richardson-Jones et al., 2010). Parameters such as struggling and climbing are also measured and can sometimes yield differences that cannot be seen in only measuring immobility time (Bogdanova et al., 2013).

Another test of behavioural despair is the tail suspension test in which the mouse is suspended by its tail and its movements (or lack thereof) are recorded (Castagne et al., 2011; Cryan et al., 2005). In both the tail suspension and forced swim tests, acutely treating the mice with antidepressants decreases the amount of time spent immobile and so these tests have been employed as behavioural assays for depression (Bogdanova et al., 2013; Castagne et al., 2011; Cryan et al., 2005).

Anxiety is highly comorbid with depression (Lam 2012) and so to evaluate anxiety in mice, tests often involve bright lights and open spaces, to which rodents have a natural aversion. Rodents also have a natural curiosity and desire to explore when placed in a new environment and so anxiety paradigms attempt to expose this paradox. The light dark box, elevated plus maze, and open field are all tests of anxiety that consist of an area of bright, open space. The more time the animal spends in the bright or open area

indicates less anxiety (Borsini et al., 2002; Clement et al., 2007; Cryan & Holmes, 2005). Similar to the behavioural despair tests, antidepressants can increase the amount of time spent in the open spaces (Borsini et al., 2002; Cryan & Holmes, 2005).

Inescapable shock has commonly been used to study depression in rodents. Briefly, the procedure consists of subjecting the rodent first to a series of unpredictable foot shocks over several days from which the rodent cannot escape (termed inescapable shocks). On a separate testing day, the rodent is put back in the same box. Now when the shocks are given, a door opens that allows the rodent to escape to a section that has no shocks. Despite having an escape option, some rodents will remain in the shock-delivering section of the box and those rodents are grouped as having a depressive phenotype (Anisman & Merali, 2001; Pryce et al., 2012). Many researchers have used this paradigm to study not only the behavioural consequences of depression, but also the molecular mechanisms underlying depression. Other common methods of inducing depression include chronic stress paradigms that can involve physical and/or social stress. Interestingly, the serotonin system is not only involved in inducing depression in response to inescapable shock, but alterations in the serotonergic pathway, particularly the 5-HT1A autoreceptor regulation, is necessary and sufficient to produce a depressive phenotype (Maier et al., 1995a; 1995b). Microinjections of a 5-HT1A autoreceptor inverse agonist into the DRN successfully produced the behavioural effects of inescapable shock (such as increased latency to exit the shock-receiving section of the apparatus) without experiencing the inescapable shock training procedure described above. The same result could be obtained by lesioning the DRN (Maier et al., 1995a). Conversely, the behavioural effects of inescapable shock were blocked by microinjection

of a 5-HT1A autoreceptor agonist into the DRN, despite the rats having completed the training procedure (Maier et al., 1995a). Therefore decreasing serotonin transmission by activating 5-HT1A autoreceptors blocks the depression-like response to the inescapable shock paradigm. A similar result was found in hamsters in which microinjection of a 5-HT1A receptor agonist into the DRN reduced the amount of submissive behaviour in a social defeat paradigm. Conversely, injecting a 5-HT1A antagonist increased the effects of social defeat (Cooper et al., 2008). Thus the DRN is also involved in behavioural changes induced by social defeat stress as well.

It should be noted that, while they can be quite convincing, behavioural assays are susceptible to many external factors such as genetic strain, lighting conditions, and the time of day the test takes place (Cryan & Holmes, 2005; O'Leary et al., 2013). So it is important to consider these factors when evaluating the results of the tests.

Serotonin Rodent Models of Anxiety and Depression

Multiple studies focusing on the serotonergic system have resulted, in varying combinations, in changes in anxiety, depressive, and aggressive phenotypes. Several studies have focused on the serotonin transporter (5-HTT), which is responsible for recycling excess serotonin from the synaptic cleft back into the presynaptic terminal. A variant in the human 5-HTT gene was associated with elevated anxiety in both humans (Caspi et al., 2003; Lesch et al., 1996) and 5-HTT knockout mice (Holmes et al., 2002; 2003). Work by Holmes and colleagues (2002) also found 5-HTT knockout mice displayed higher immobility in tests of inescapable stress, reduced aggression in a resident-intruder test, and reduced firing of neurons in the dorsal raphe. Mosienko and colleagues (2012) used a TPH2 knockout mouse to reduce whole brain serotonin to less

than 2% of wildtype controls. In doing so, mice lacking the TPH2 enzyme display decreased anxiety, increased aggression, and increased depression-like behavior via decreased struggling in the forced swim test (Mosienko et al., 2012).

It has been repeatedly shown that mice lacking the 5-HT1A receptor gene display increased anxiety in several behavioural assays as well as no changes to serotonin itself or its metabolites (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). However it was shown that raphe serotonin neurons display increased firing activity in these 5-HT1A knockout mice (Richer et al., 2002). Knockout mice also displayed decreased immobility in both the forced swim test (Ramboz et al., 1998) and the tail suspension test (Heisler et al., 1998). The authors also suggest that the 5-HT1A receptor knockout mice have an altered response to stress, which may have produced the increase in mobility in the tail suspension and forced swim tests (Parks et al., 1998; Ramboz et al., 1998). However Ramboz and colleagues (1998) points out the models knock out both pre- and post-synaptic receptors and so the underlying cause of the behavioural outcomes cannot be fully known. Global 5-HT1A knockout models result in strong anxiety and depression phenotypes. Unfortunately the global model lacks specificity and makes it difficult to determine which population of 5-HT1A receptors mediates which behaviour.

Conditional Genetic Mouse Models

The serotonin system is quite complex and retains many compensatory mechanisms. In this way, results obtained from knockout models are often criticized for their global and potentially non-specific effects. Turning off certain genes, especially during development, often recruits other cellular processes that can result in unwanted and confounding behavioural phenotypes. Conditional mouse models allow for control of

both the tissue-specific location of the mutation within the brain as well as the time point during the life of the animal that the mutation is expressed. In this way, complex mechanisms can be teased apart and better understood (Dhaliwal & Lagace, 2011).

5-HT1A Rodent Models of Anxiety and Depression

Initial gene knockout studies of the 5-HT1A receptor involved generating a complete 5-HT1A receptor knockout mouse that displayed elevated anxiety and increased mobility in behavioural tests (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). However, since the behavioural changes could not be attributed to either pre- or postsynaptic 5-HT1A receptors, conditional mouse models have been developed that are able to target specific receptors in specific types of neurons. As mentioned, conditional models offer more control and thus more accurate investigations of a given system.

Gross and colleagues (2002) developed a conditional rescue mouse in which the autoreceptors in the raphe nuclei were knocked out, but heteroreceptors located in the forebrain were conditionally expressed. Presence of the forebrain receptors throughout life as well as a conditional knockout during adulthood produced anxiety behaviours similar to wildtype mice. However, when forebrain receptor expression was eliminated during early postnatal development, the mice performed similar to the 5-HT1A knockout mice and displayed increased anxiety behaviours (Gross et al., 2002). Thus Gross and colleagues (2002) began a trend to develop conditional 5-HT1A mouse models in order to dissociate autoreceptors and heteroreceptors from each other and their respective functions.

Other conditional knockouts were generated that preserve the 5-HT1A receptor during development in order to avoid an anxiety phenotype. One conditional method used

small interfering RNA (siRNA) to selectively reduce 5-HT1A autoreceptors in adult mice (Bortolozzi et al., 2012). siRNA-injected mice displayed reduced depression-like behaviour in the tail suspension and forced swim tests, but no anxiety behaviour in the elevated plus maze. In addition, siRNA-injected mice showed enhanced serotonin release produced by a SSRI (Bortolozzi et al., 2012).

Richardson-Jones and colleagues (2010) created a conditional 5-HT1A receptor knockdown mouse in which normal expression of the 5-HT1A autoreceptor was reduced by 30% in adulthood. The 30% knockdown mice performed similar to the control mice in several tests of anxiety, indicating that the conditional knockout was successful at avoiding the anxiety phenotype brought on by lack of 5-HT1A receptors during development (Richardson-Jones et al., 2010). Interestingly, Richardson-Jones and colleagues (2011) created two conditional 5-HT1A knockout models targeting either auto- or heteroreceptors. Contrary to Gross and colleagues (2002), early postnatal reduction of 5-HT1A heteroreceptors did not appear sufficient to induce anxiety behaviours, however autoreceptor knockout did (Richardson-Jones et al., 2011). Compared to controls, full life 5-HT1A heteroreceptor knockout mice showed decreased mobility on the second day of forced swim testing, suggesting a mild depression phenotype.

Piszcek and colleagues (2013) created 5-HT1A knockout, raphe-overexpressing, and raphe-rescue mice. Although the complete 5-HT1A knockouts, raphe-overexpressing, and the raphe-rescue mice all showed increased anxiety compared to wildtype, the raphe-rescue mice consistently showed the most anxiety. In addition, the raphe rescue mice also showed immobility (and therefore behavioural despair) similar to

the knockouts. This indicates that, contrary to the findings of Richardson-Jones and colleagues (2011), rescuing the 5-HT1A autoreceptors was not sufficient to rescue the anxiety or behavioural despair phenotype. The authors did note however that they used a 5-HT1A knockout background to create their mice, while Richardson-Jones and colleagues (2011) used a wildtype background. Thus it appears the autoreceptor is necessary but not sufficient for normal anxiety behaviour (Piszcek et al., 2013). Overall it is important to consider not only the temporal and spatial expression of the 5-HT1A receptor, but also the mouse background and the amount of receptor changes since all of these variables appear to play important roles.

Effect of Stress on Anxiety and Depression

Numerous studies have found correlations between stress, particularly early life stress, and adult mental illness (Hornung & Heim, 2014). Depression, anxiety, and suicidal behaviours are all increased in those with multiple maltreatment experiences in childhood (Heim et al., 2008). However, not every person who experiences stressful life events will develop depression.

Even though environmental influences are a strong predictor of depression, the difference between vulnerability and resilience could be genetic (Caspi & Moffitt, 2006). Several studies have found correlations between certain genetic polymorphisms and vulnerability to depression. For instance, a polymorphism in the 5-HTT gene has been associated with increased susceptibility to depression after, and only after, stressful life events. Caspi and colleagues (2003) studied the long versus short form of a region in the promoter of the 5-HTT gene (5-HTTLPR) in a prospective-longitudinal design and found that individuals having two copies of the short allele in conjunction with stressful life

events had more symptoms of depression and ideation of suicide compared to those with two copies of the long form. Kaufman and colleagues (2006) found an interaction between the 5-HTTLPR polymorphism and a BDNF polymorphism that predicted depression in maltreated children. That is, those with both polymorphisms and a history of maltreatment had the highest depression scores. Interestingly, these effects were reduced by proper social support (Kaufman et al., 2006). Other genetic variants have been found that may confer resilience to depression. For instance, polymorphisms in the corticotropin-releasing hormone receptor gene (CRHR1) have been associated with blunted depressive symptoms in adults exposed to childhood abuse (Bradley et al., 2008), indicating a possible genetic resiliency.

Effect of Gender in Depression

Lifetime prevalence for affective disorders is higher in women than it is in men, with women being diagnosed with major depressive disorder twice as frequently as men (Lasiuk & Hegadoren, 2007; Marcus et al., 2005). Women also display more atypical symptoms of depression, such as fatigue, increased eating, and sleep disturbances, compared to men who commonly report weight loss (Scheibe et al., 2003). Comorbid anxiety is more typical in women, while men typically have comorbid substance abuse (Breslau et al., 1995). In a study of human MDD patients, both Deaf1 and 5-HT1A protein levels were significantly reduced in the prefrontal cortex of female patients compared to matched controls. In contrast, male MDD patients did not show changes in either protein concentration (Szewcyk et al., 2009).

Not surprisingly, gonadal hormones have been found to greatly influence depression. The regular and rapid fluctuation of female hormones is correlated with

increased risk of depression (Keers & Aitchison, 2010). Interestingly, before the onset of puberty, depression rates between genders do not differ (Lasiuk & Hegadoren, 2007). Once in the age of childbearing, women have been found to have an overall lower serotonergic function that is correlated with changes in estrogen (Keers & Aitchison, 2010; Lasiuk & Hegadoren, 2007). Hormones are also believed to impact antidepressant efficacy between genders in that women tend to respond better to SSRIs while men respond better to TCAs (Keers & Aitchison, 2010) although there are still discrepancies as to the mechanism by which this occurs.

Estrogen has an excitatory effect on HPA axis function and increases corticosterone and glucocorticoid receptor binding, while androgens have the opposite effect (Kudielka & Kirschbaum 2005; McCormick et al., 2002). Females also have higher ACTH levels than males, possibly as a result of having higher levels of corticosterone binding globulin (CBG). CBG binds free corticosterone and may blunt its effects in females, thus requiring more ACTH for more corticosterone release (McCormick et al., 2002). In response to stress, ovariectomized rats given exogenous 17β-estradiol had elevated corticosterone concentrations compared to non-treated controls. In addition, treated rats failed to show reduced corticosterone levels over time (Lunga & Herbert, 2004).

Gender Differences in Animal Models of Depression

Animal studies have also shown relationships between gonadal hormones and depression. Ovariectomized macaques treated with estrogen and progesterone have reduced 5-HT1A receptor RNA and protein in the raphe (Pecins-Thompson & Bethea, 1999; Henderson & Bethea, 2008) and were downregulated in the dorsal raphe (Lu et al.,

2002). Estrogen and progesterone treatment was also found to increase TPH2 gene expression (Sanchez et al., 2005) and decrease 5-HT1A (Pecins-Thompson & Bethea, 1999) and SERT expression (Lu et al., 2002). Similarly, ovariectomized mice displayed more immobility in the tail suspension test compared to sham-operated mice. In addition, treating the ovariectomized mice with estradiol and progesterone rescued the behaviour to that of control mice (Bernardi et al., 1989). Properly controlled gonadal hormone concentrations are key to maintaining normal mental health since it is the fluctuations that correlate the strongest with depression.

In terms of stress, female cynomolgus monkeys that were stress sensitive had lower TPH2, SERT, and 5-HT1A mRNA in the raphe compared to highly stress resilient females after prolonged stress (Bethea et al., 2013; Lima et al., 2009). Stress sensitive females also failed to ovulate during the stress procedure, while the resilient females showed no changes (Lima et al., 2009).

Males and females also respond differently in stress-induced depression. Dalla and colleagues (2005) used a chronic mild stress (CMS) protocol on male and female rats and found that CMS caused more profound changes in females compared to males. Although CMS influenced both male and female behaviour in the open field and sucrose preference tests, females showed decreased open field activity after three weeks of CMS compared to males that showed the same behaviour after six weeks. Interestingly, over the course of the sucrose preference test, male CMS rats had a reduced rate of weight gain while females showed the same rate of weight gain as controls. Dalla and colleagues (2005) also found that CMS caused biochemical changes in females that were not present in males. Specifically, corticosterone levels were increased and serotonergic activity was

decreased in female, but not male, rats (decreased 5-HIAA/5-HT ratio in the hippocampus). In terms of the forced swim test, female rats spent less time immobile than males on the second day of testing, suggesting females have different coping mechanisms than males when it comes to repeated stressors (Alonso et al., 1991; Dalla et al., 2005).

Although it is well accepted that depression rates, symptoms, and treatments vary between the genders, few rodent studies directly compare males and females. However the abovementioned studies show differences in not only behaviour, but also at the biochemical level across different species and gender effects need to be considered especially in affective disorders.

Hypothesis and Objectives

Based on the evidence presented above, I used two different mouse lines to examine the effects of changing the level of 5-HT1A receptors on anxiety and depressive behaviour. The results of these experiments allowed me to observe the phenotypes resulting from the loss of 5-HT1A autoreceptors in the DRN and to further elucidate the role of the 5-HT1A receptor in depression and anxiety.

Given the correlation between the C(-1019)G polymorphism and depression rates, I used a complete Deaf1 knockout mouse model to reduce the transcriptional repression on the 5-HT1A receptor gene. In doing so, I increased the amount of 5-HT1A receptors in the raphe. Previous characterization of the mutation has already been completed, leaving my focus to the behavioural phenotype. I expect to confirm that Deaf1 knockout mice will have increased 5-HT1A receptor expression and mice are expected to show inherent anxiety- and depressive-like behaviours.

Previous work on 5-HT1A knockout mice report an anxiety phenotype that can be avoided if receptors are present during development. I used a conditional knockout approach to eliminate the 5-HT1A autoreceptor in the dorsal raphe in adult mice. By knocking out the 5-HT1A autoreceptor in adulthood and only in the raphe, I expect to avoid complex development effects such as the anxiety phenotype. I expect the conditional 5-HT1A knockout mice will have decreased 5-HT1A autoreceptor expression. Mice are expected to show reduced depressive-like behaviours, but no anxiety-like behaviours since knockout will be induced in adulthood.

Methods

General Animal Procedures

All animal studies were in accordance with the University of Ottawa Animal Care Committee guidelines. Animals were maintained on a 12-hour light/dark cycle with food and water ad libitum.

Deaf1

Deaf1 mice were received from Dr. Jane Visvader (Hahm et al., 2004) and rederived by embryo transfer (Taconic, Hudson, NY) on a mixed C57BL/6-BALB/c background.

flx5-HT1A-TPH2CreER^{T2}

The flx5-HT1A mice (C57BL/6 background) were obtained from Dr. Rene Hen, Columbia University and TPH2CreER^{T2} mice (mixed background) were obtained from Jackson Labs and bred together in the University of Ottawa Animal Facility. Mice containing the 5-HT1A receptor gene flanked by loxP sites (floxed) were be bred with mice containing a CreER^{T2} construct attached to the TPH2 promoter (5-HT1A-TPH2CreER^{T2}, henceforth referred to as conditional 5-HT1A or 5HT1A^{autoKO} line). The CreER^{T2} construct allows for time-specific induction of the Cre enzyme such that the enzyme cannot enter the nucleus and excise the desired gene until tamoxifen is added. Tamoxifen, but not estrogen, binds to the mutated form of the estrogen receptor (ER) and, once bound, can enter the nucleus. Since the CreER^{T2} construct is attached to the TPH2 promoter, recombination is restricted to serotonin-producing cells in the brain, which are exclusive to the raphe nuclei. By using this combination of gene constructs, knockout of the 5-HT1A receptor gene will be specific to the 5-HT1A autoreceptors in the raphe nuclei and expression will be turned off once the mice are fully developed. In

addition, the floxed 5-HT1A gene construct also contains a STOP codon in front of a GFP sequence. Once the recombination takes place, regions where the 5-HT1A gene has been excised will express GFP (Figure 2).

Genotyping

Deaf1

Ear tissue samples were taken and DNA was extracted (DNA extraction kit from the REDExtract-N-Amp Tissue PCR kit, Sigma). PCR was done using the following primers: 5' GGG CTT CCG GGT CAT TCT GT 3', 5' ACT AAG AGG GTC ACA CAA AAG AAC AAA 3', and 5' TGC ACC CAC CAC CAA GAT AAG AA 3'. PCR products at 267 base pairs (bp) represented wildtype, 421bp represented knockout, and both products represented heterozygote. Each mouse was genotyped at the time of weaning and again at sacrifice.

flx5-HT1A-TPH2CreER^{T2}

Ear tissue samples were taken and DNA was extracted (DNA extraction kit from the REDExtract-N-Amp Tissue PCR kit, Sigma). PCR was done using the following primers: flx1A: 5'-GGG CGT CCT CTT CAC GTA G-3' and
5'-CAG GGA CGT TGT GGT GTT GT-3' with 254bp wildtype and 292bp knockout products; TPH2-CreER^{T2}: 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', with 200bp wildtype and 300bp transgenic products. Each mouse was genotyped at the time of weaning and again at sacrifice.

Tamoxifen Injections

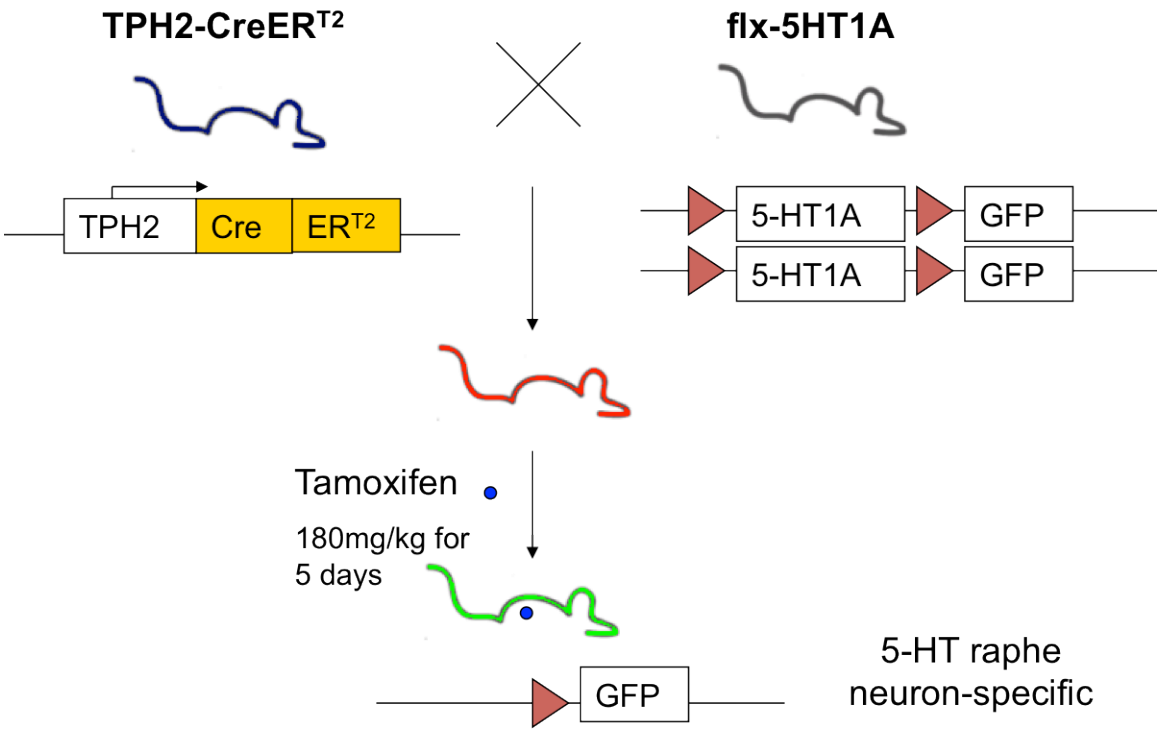


Figure 2. Conditional 5-HT1A Mouse Construct

The TPH2-CreER^{T2} line is bred with the floxed-5-HT1A line. At 6 weeks postnatal, mice are injected for 5 consecutive days with tamoxifen (180 mg/kg) which binds the ER^{T2} receptor and allows the Cre to enter the nucleus of the cell. In cells where the 5-HT1A gene has been excised, GFP will be expressed.

At six weeks postnatal, tamoxifen (180 mg/kg; Sigma) was injected intraperitoneally every day for five consecutive days. The mice were then left undisturbed for at least 14 days to allow for recombination.

Immunofluorescence Staining

Mice were anesthetized (Euthanyl; 0.01 mL/g) and perfused by cardiac infusion of PBS, then 4% paraformaldehyde. Whole brains were extracted and post-fixed overnight in 4% paraformaldehyde. Brains were then kept in 20% sucrose solution, changed daily, for five days and frozen. Coronal brain slices (12 μm) were taken from the raphe nuclei (Bregma -4.2 to -4.96), prefrontal cortex, and hippocampus (Bregma 1.54 to -2.80). Slices were thaw-mounted on Superfrost slides (Fisher) and kept at -80°C. Staining was done for GFP (chicken anti-GFP 1:2000, goat anti-chicken 1:250) and colocalized with TPH (mouse anti-TPH 1:500, donkey anti-mouse 1:250). Staining was also done for 5-HT1A receptors (homemade antibody raised in rabbit (Czesak et al., 2012) 1:50, donkey anti-rabbit 1:1000) and colocalized with TPH (sheep anti-TPH 1:100, donkey anti-sheep 1:200). Images were acquired using an Axiovert S100 Zeiss microscope. Positive-labeled cells were manually counted using ImageJ software.

Any positive-labeled cells falling on the far left or uppermost edge of the image were not counted, while any falling on the far right or bottom edge of the image were. Once all images were counted, randomly selected samples representing 10% of the total were re-counted, blind to the first values. All re-counted values fell within 10% of the first values. To calculate percent co-labeling, the TPH-positive and GFP-positive cells were first counted independently of one another. The percent co-localization represented the number of GFP-positive cells also positively expressing TPH out of the total number of

GFP-positive cells. At least three separate brain sections were counted from each animal and three animals per genotype were averaged to get the final graphed values.

8-OH-DPAT-Induced Hypothermia

The procedure was performed between 11am and 4pm. Mice were transported to the testing room and weighed. For the entirety of the procedure, internal temperature was taken once every 10 minutes using a rectal thermometer. Four baseline temperatures were taken followed by a single I.P. injection of 8-OH-DPAT (8-hydroxy-N,N-dipropyl-2-aminotetralin; 0.75mg/kg for conditional 5-HT1A mice and 0.25, 0.50, and 0.75 mg/kg for Deaf1 mice; Sigma) or an equivalent volume of 0.9% saline. Temperature was then measured 6 times over 60 minutes. Each mouse received the procedure with a saline injection on the first day, and the exact procedure with an 8-OH-DPAT injection on the third day (mice were given one day to rest in between procedures). Thus each mouse acted as its own control by received both the saline and 8-OH-DPAT injection. For analysis purposes, the first baseline temperature was discarded. The remaining three baselines were averaged together and the difference between the averaged baseline and recorded temperature was graphed across time.

Estrus Cycle Staining

Vaginal smears were taken from 20 female conditional 5-HT1A mice every day at 2pm for four consecutive days. Smears were dried overnight on Superfrost slides (Fisher) and stained with 0.1% cresyl violet (Sigma). Images were immediately taken using a Zeiss AxioImager M2 microscope. Samples were staged using the guidelines from McLean et al. (2012).

Behaviour testing

General Procedures

All tests were performed between 9am and 5pm. Mice were transported to the testing room and were left undisturbed for 1 hour of habituation with a white noise generator. Mice were 8-9 weeks of age at the beginning of testing. Behaviour tests were performed in the following order: Elevated Plus Maze, Open Field, Light Dark Paradigm, Tail Suspension, and Forced Swim. Beam Break was performed after Elevated Plus Maze and before Tail Suspension.

Elevated Plus Maze

The mouse was placed in the center of an elevated, two-arm maze measuring 6 cm wide and 75 cm long. The arms were crossed perpendicularly and were open where they met. The closed arm was enclosed by walls 20 cm tall while the open arms had no walls. The maze was raised 74 cm off of the floor with overhead illumination (100-110 Lux) and an overhead camera. The camera fed to a computer in a separate room and used Noldus Ethovision (version 10) to track the mouse during the test. After 10 minutes, the mouse was removed from the maze and returned to its home cage. The maze was cleaned after each trial.

Open Field Test

The mouse was placed in the corner of an opaque, white box measuring 45cm x 45cm x 45cm. The box was lit by overhead lighting (250-300 Lux) and an overhead camera fed to a computer in a separate room. Noldus Ethovision 10 was used to track the position of the mouse. Anxiety behaviour was measured by the amount of time spent outside of a 24cm x 24cm square in the center of the box. After 10 minutes, the mouse was removed from the maze and returned to its home cage. The maze was cleaned after each trial. If

the mouse had a white coat colour, a black insert was placed in the bottom of the box to allow the camera to recognize the mouse.

Light Dark Paradigm

The apparatus used in this test consisted of a squared chamber made of clear plastic and divided into two equal compartments. A covered, black, opaque insert divided the two compartments such that one was completely dark. An opening in the dark compartment allowed the mouse to move from one compartment to the other. The light compartment was lit by two bulbs at 390 Lux. The apparatus was equipped with infrared transmitters and receivers positioned around the periphery of the chamber. The infrared beam array defined an X and Y coordinate map system. At user defined intervals, the software scanned the environment for the presence of the beam at each receiver. If the beam failed to reach the receiver, the system registered this event as a broken beam and recorded the presence of the animal. During the test the entire apparatus was contained in a custom cabinet-type enclosure wherein the mouse was completely separated from the rest of the room. The enclosure was equipped with a fan to circulate air during the test. The mouse was placed in the corner of the lit compartment. After 10 minutes, the mouse was removed from the chamber and returned to its home cage. The chamber was cleaned after each trial.

Tail Suspension Test

The mouse was suspended by its tail via adhesive tape to an aluminum bar. The bar was attached to a transducer that measured the force of the mouse while it was moving. Above a set threshold, the computer registered this movement as mobility, while anything

below the threshold was immobility. After 6 minutes, the mouse was removed from the chamber and returned to its home cage. The chamber was cleaned after each trial.

Forced Swim Test

The mouse was placed in a clear, plexiglass cylinder (measuring 37cm high and 22cm across) filled 5-10cm from the top with 23-25°C tap water. A camera was placed in front of the cylinders and Noldus Ethovision 10 was used to track the mobile versus immobile movements. The test was performed under red light and the habituation periods were staggered such that mice did not spend more than 1 hour in the testing room in order to maintain normal circadian cycles. After 6 minutes, the mouse was removed from the cylinder and returned to its home cage. The water was changed after each trial.

Beam Break

Each mouse was placed in a separate cage with clean bedding. The cage itself was placed in a metal frame equipped with infrared receptors (Micromax). When the mouse breaks the infrared beam, the event is recorded and the number of breaks is tallied in a given amount of time. After two hours the mice were removed from their individual cages and returned to their home cages.

Learned Helplessness

The apparatus consisted of a sonically isolated shuttle box (18cm x 41cm), divided into two equal compartments by an automated door. The enclosure was equipped with a fan to circulate air during the test. For the present study, the first three days comprised of training sessions in which the mouse was placed in one half of the shuttle box and given 0.15 mA shocks of 2-4 seconds in duration for 70 trials. In the no-shock control group, mice were placed in the same conditions, except no shock was delivered. The automated

door remained closed during these three days. On the fourth day, all mice received the same procedure. Mice were placed in one half of the shuttle box and, once the shock was delivered, the automated door opened, thus allowing the mouse to escape to the other half of the chamber, which did not deliver a shock. The shock lasted a maximum of 30 seconds and, if the mouse did not move to the other chamber, it was recorded as a failure. Infrared beams within the chambers recorded the position of the mouse at all times. At the end of the test, the mouse was returned to its home cage and the apparatus was cleaned.

Statistical Analyses

Data represent averaged values with error bars reflecting the standard error of the mean (S.E.M.). For all statistical analyses, $p<0.05$ was the level of significance. Graphpad Prism 6 was used for statistical calculations and graphs.

Immunofluorescence Analyses

Only sections falling between Bregma -4.36 to -4.96 were included in analysis. Statistical analyses were performed using a student's t-test.

Behaviour Analyses

Data points that lay outside ±2 standard deviations (SD) from the mean were excluded. For all Tail Suspension and Forced Swim data, a column graph representing the total time immobile is also shown. The total time averaged in these graphs are only for the final four minutes of the test and exclude the first two minutes which are commonly viewed as habituation and not representative of the actual behaviour. Statistical analyses were performed using an analysis of variance (ANOVA). When comparing data across time or

across gender, a 2-way repeated measures ANOVA was used. Post hoc comparisons were made with Bonferroni's multiple comparisons test.

Results

Higher 5-HT1A autoreceptor expression in Deaf1^{KO} mice

As a transcription repressor, Deaf1 has a direct effect on 5-HT1A autoreceptor expression. In order to visualize and identify the cells affected by the knockout of the Deaf1gene, immunofluorescent staining for 5-HT1A receptors and serotonin cells (labeled using an antibody against the TPH enzyme) was performed on dorsal raphe sections (Figure 3A). 5-HT1A autoreceptor expression was increased in mice lacking one copy of Deaf1 (Deaf1^{HET}) and was further increased in the full knockout (Deaf1^{KO}). Quantification of the 5-HT1A-positive cells indicated approximately 2x as many positive cells in Deaf1^{HET} compared to Deaf1^{WT} mice and approximately 2.5x as many positive cells in Deaf1^{KO} compared to Deaf1^{WT} mice (Figure 3B). Although these results are promising, the low sensitivity of the 5-HT1A antibody to detect the receptor in wildtype raphe makes these results difficult to replicate in both the Deaf1 mice and 5-HT1A autoreceptor knockout mice.

The functionality of the 5-HT1A autoreceptors can be evaluated using 8-OH-DPAT, an agonist specific to the autoreceptors. The compound induces a temporary hypothermia that is dependent on functional 5-HT1A autoreceptors. Since the Deaf1^{KO} and Deaf1^{HET} mice express more autoreceptors, it was expected that the knockouts and heterozygotes would show a greater decrease in core body temperature compared to the wildtype controls.

Using a dose of 0.5 mg/kg resulted in a significant decrease in core body temperature in all three male Deaf1 genotypes at various time points. Deaf1^{WT} and Deaf1^{HET} males showed the greatest decrease in temperature when compared to their

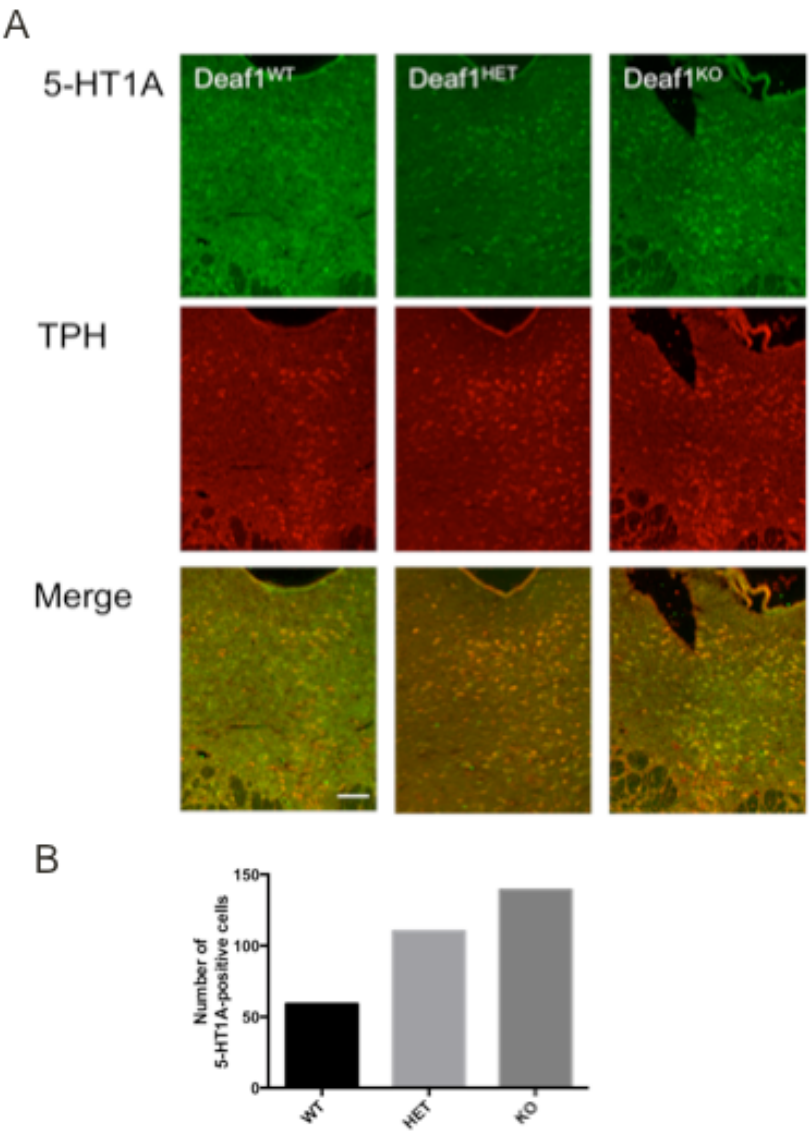


Figure 3. Immunofluorescent staining of dorsal raphe in Deaf1 mice

A. Immunofluorescent staining for TPH (sheep anti-TPH, 1:100) and 5-HT1A receptors (homemade antibody raised in rabbit, 1:50) was performed on dorsal raphe slices of Deaf1 mice. B. Quantification of 5-HT1A receptor staining. Scale bar indicates 100 μm.

respective saline controls. Deaf1^{WT} mice also showed a significantly larger decrease in temperature compared to Deaf1^{KO} mice when injected with 8-OH-DPAT, which is opposite of what was expected (Figure 4A).

Female Deaf1 mice all showed a similar decrease in temperature when injected with a 0.5 mg/kg dose of 8-OH-DPAT (Figure 4B). All three genotypes significantly differed from their respective saline controls. None of the three genotypes differed from one another in response to the 8-OH-DPAT injection.

Neither the male nor female Deaf1^{KO} or Deaf1^{HET} mice displayed the expected greater decrease in temperature following 8-OH-DPAT administration as would be expected from the greater level of 5-HT1A autoreceptor immunofluorescence. Using either a higher (0.75 mg/kg) or lower (0.25 mg/kg) dosage of 8-OH-DPAT also showed similar results to the 0.5 mg/kg dosage (Appendix Figure i). Overall, results of the agonist-induced hypothermia were not consistent with an increase in 5-HT1A autoreceptor function in the Deaf1 knockout mice. Therefore further experiments are warranted to confirm these findings.

Gender Comparisons

Gender comparisons were made between genotypes for all of the behavioural assays. In the Deaf1 anxiety tests, significant main effects of gender and/or significant gender x genotype interactions were found in numerous measurements (Appendix Figures ii, iii). Similar differences between genders were found for the locomoter assay as well as tests of depressive behaviour. Based on these results, it was determined that the gender data should be divided and analyzed separately instead of collapsed together. The same

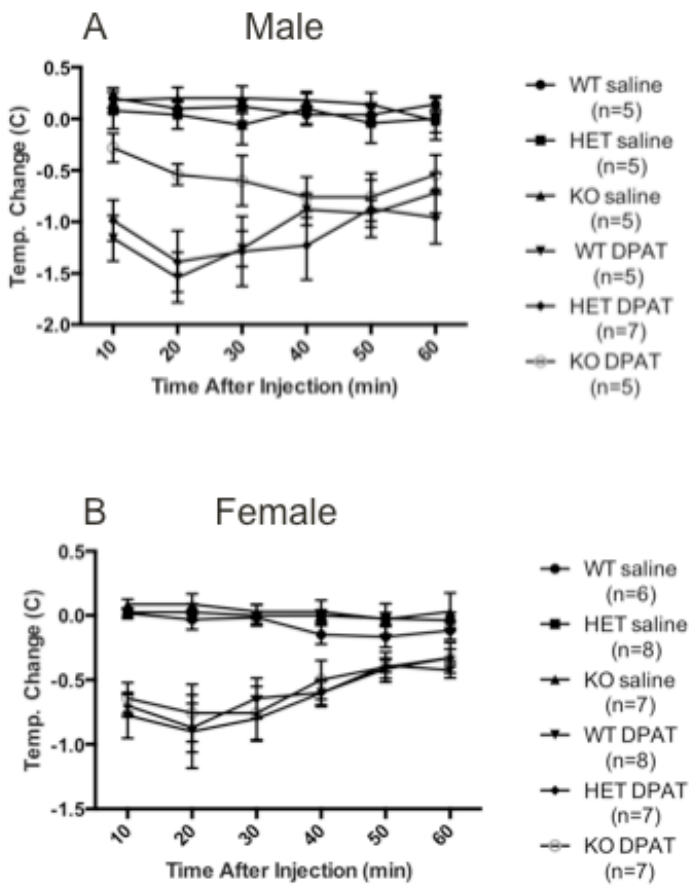


Figure 4. Agonist-induced hypothermia in Deaf1 mice

In order to test the functionality of the 5-HT1A autoreceptors, the 1A-specific agonist 8-OH-DPAT was used. A. Temperature changes in male mice using a dose of 0.5 mg/kg. B. Temperature changes in female mice using a dose of 0.5 mg/kg. Statistical effects are shown in Table 2.

Deaf1 Male 0.5 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction
F	3.26		10.49		2.88
df	5		5		25
p	**0.01**	*	**< 0.0001**	****	**< 0.0001**

post hoc

Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	**0.0002**	**< 0.0001**	**0.0002**	**0.041**	**0.027**	***0.076***
HET saline x HET DPAT	**0.003**	**< 0.0001**	**0.0003**	**0.022**	***0.052***	**0.012**
KO saline x KO DPAT	> 0.9999	0.232	0.134	**0.033**	***0.051***	> 0.9999
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT DPAT x KO DPAT	***0.062***	**0.018**	0.457	> 0.9999	> 0.9999	> 0.9999

Deaf1 Female 0.5 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction
F	5.58		11.58		2.72
df	5		5		25
p	**< 0.0001**	****	**< 0.0001**	****	**< 0.0001**

post hoc

Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	**0.0002**	**< 0.0001**	**0.0002**	0.163	> 0.9999	> 0.9999
HET saline x HET DPAT	**0.0004**	**< 0.0001**	**0.003**	**0.006**	0.319	> 0.9999
KO saline x KO DPAT	**0.001**	**< 0.0001**	**0.0001**	**0.039**	0.498	0.608
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT DPAT x KO DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999

Table 2. Agonist-induced hypothermia in Deaf1 mice

Statistical results of a two-way ANOVA comparing genotype and time for both male and female Deaf1 mice. Bold font indicates significance; bold italic indicates a non-significant trend.

statistical comparisons were made for the 5-HT1A autoreceptor knockout mice and that data was also separated by gender (Appendix Figures iv, v). Given the gender differences observed in the behavioural assays, all immunofluorescent and 8-OH-DPAT data was also reported separately for each gender in order to remain consistent throughout the text.

Increased Anxiety, No Depression Behaviour in Deaf1 mice

The anxiety phenotype was assessed using three tests: the Elevated Plus Maze (EPM), the Open Field (OF) test, and the Light Dark (LD) test. In all three tests, more time spent in the brightly lit areas indicated less anxiety.

In the EPM, $Deaf1^{HET}$ males spent more time in the open arms of the maze (Figure 5A), indicating reduced anxiety compared to $Deaf1^{WT}$ males. However this behaviour was not shown by $Deaf1^{HET}$ males in any measures taken from the OF or LD tests (Figure 5B-H). $Deaf1^{KO}$ males did not display significant differences from $Deaf1^{WT}$ males in either time spent in open arms or time spent in the center of the EPM, indicating no change in anxiety in that test (Figure 5A-C). However $Deaf1^{KO}$ males did show a significant reduction in distance traveled in the EPM compared to $Deaf1^{WT}$, which could indicate a higher level of freezing during the test. Since no differences were found between all three genotypes in the locomotor assay, the change in distance traveled is not likely due to changes in locomotor activity (Appendix Figure vi). In the OF, a near significant trend showed $Deaf1^{KO}$ males spent less time in the center of the arena compared to $Deaf1^{WT}$ males, which indicates a possible anxiety phenotype (Figure 5D-E). In the LD, $Deaf1^{KO}$ males spent significantly less time in the light side of the apparatus compared to $Deaf1^{WT}$ males, which indicates an anxiety phenotype (Figure 5F). Conversely, $Deaf1^{KO}$ males showed a significantly longer latency to escape the light side

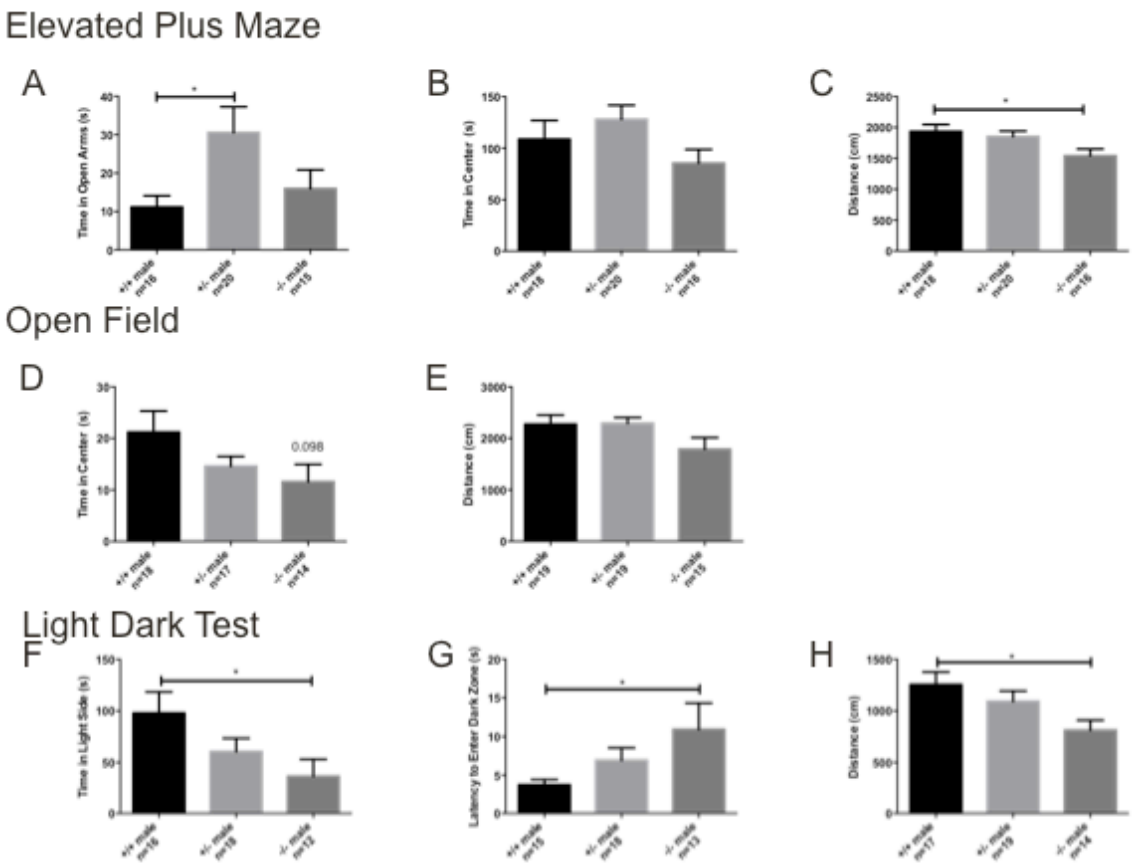


Figure 5. Deaf1: Male Anxiety Assays

Three assays were used to test for the presence of anxiety: EPM, OF, and LD. A. Time spent in the opens arms of the EPM. B. Time spent in the center of the EPM. C. Distance traveled in the EPM. D. Time spent in the center of the OF. E. Distance traveled in the OF. F. Time spent in the light side of the LD test. G. Time taken to enter the dark zone for the first time in the LD test. H. Distance traveled in the LD test. Statistical effects are shown in Table 3.

Deaf1 Male					post hoc	
EPM						
	Time in Open Arms	F	3.70		WT x HET	**0.026**
		df	2		WT x KO	> 0.9999
		p	**0.03**	*		
	Time in Center	F	1.93		WT x HET	0.725
		df	2		WT x KO	0.600
		p	0.16			
	Distance	F	3.91		WT x HET	> 0.9999
		df	2		WT x KO	**0.021**
		p	**0.03**	*		
OF						
	Time in Center	F	2.23		WT x HET	0.297
		df	2		WT x KO	0.098
		p	0.12			
	Distance	F	2.54		WT x HET	> 0.9999
		df	2		WT x KO	0.113
		p	***0.09***	#		
LD						
	Time in Light Side	F	3.12		WT x HET	0.213
		df	2		WT x KO	**0.038**
		p	***0.055***	#		
	Latency to Enter Dark Side	F	2.88		WT x HET	0.508
		df	2		WT x KO	**0.042**
		p	***0.07***	#		
	Distance	F	3.87		WT x HET	0.545
		df	2		WT x KO	**0.016**
		p	**0.03**	*		

Table 3. Deaf1: Male Anxiety Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

and enter the dark side of the box (Figure 5G). However, since Deaf1^{KO} males also traveled a significantly shorter distance than Deaf1^{WT} males (Figure 5H), the longer escape latency may reflect freezing behaviour in the light side at the beginning of the test. Overall, Deaf1^{KO} males appear to have a mild anxiety phenotype, while Deaf1^{HET} males show a mild reduced anxiety phenotype when compared to wildtype counterparts.

Female Deaf1^{HET} and Deaf1^{KO} mice show an anxiety phenotype in the EPM (Figure 6A-C). Deaf1^{KO} females spent significantly less time in the open arms compared to Deaf1^{WT} females, indicating higher anxiety. Deaf1^{KO} females also showed significantly lower distance traveled in the EPM, which may reflect freezing or hiding in the closed arms. Since no differences were found between all three genotypes in the locomotor assay, the change in distance traveled is not likely due to changes in locomotor activity (Appendix Figure vii). Deaf1^{HET} females spent significantly less time in the center of the EPM as well as show a trend towards less time spent in the open arms when compared to Deaf1^{WT} females. No significant differences were found in the OF for either genotype (Figure 6D-E). In the LD, no significant differences were found for Deaf1^{HET} females (Figure 6F-H). Deaf1^{KO} females did spend significantly more time in the light side compared to Deaf1^{WT} females, which indicates reduced anxiety. Although Deaf1^{KO} females had a shorter escape latency to the dark side compared to Deaf1^{WT} females which would indicate higher anxiety, it was not statistically significant. Overall Deaf1^{HET} and Deaf1^{KO} females both show an anxiety phenotype when compared to Deaf1^{WT} females in the EPM.

In order to assess the depression phenotype, the Tail Suspension (TS) and Forced Swim (FS) tests were used, each measuring the time spent immobile as an indicator of

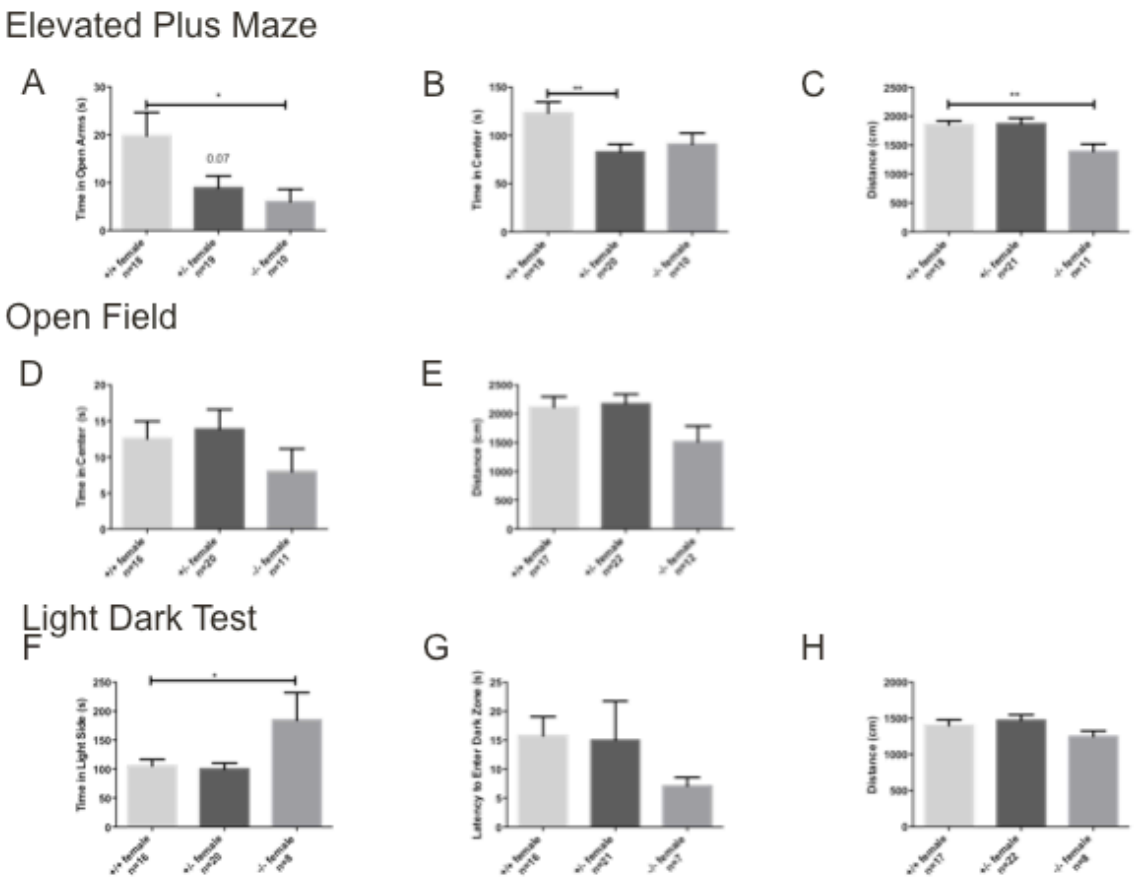


Figure 6. Deaf1: Female Anxiety Assays

Three assays were used to test for the presence of anxiety: EPM, OF, and LD. A. Time spent in the opens arms of the EPM. B. Time spent in the center of the EPM. C. Distance traveled in the EPM. D. Time spent in the center of the OF. E. Distance traveled in the OF. F. Time spent in the light side of the LD test. G. Time taken to enter the dark zone for the first time in the LD test. H. Distance traveled in the LD test. Statistical effects are shown in Table 4.

Deaf1 Female					post hoc	
EPM						
	Time in Open Arms	F	3.52		WT x HET	0.070
		df	2		WT x KO	0.052
		p	**0.04**	*		
	Time in Center	F	4.84		WT x HET	**0.008**
		df	2		WT x KO	0.104
		p	**0.01**	*		
	Distance	F	6.30		WT x HET	> 0.9999
		df	2		WT x KO	**0.007**
		p	**0.004**	**		
OF						
	Time in Center	F	1.04		WT x HET	> 0.9999
		df	2		WT x KO	0.592
		p	0.36			
	Distance	F	2.77		WT x HET	> 0.9999
		df	2		WT x KO	0.118
		p	***0.07***	#		
LD						
	Time in Light Side	F	4.76		WT x HET	> 0.9999
		df			WT x KO	**0.021**
		p	**0.01**	*		
	Latency to Enter Dark Side	F	0.38		WT x HET	> 0.9999
		df	2		WT x KO	0.826
		p	0.69			
	Distance	F	1.43		WT x HET	0.970
		df	2		WT x KO	0.560
		p	0.25			

Table 4. Deaf1: Female Anxiety Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

behavioural despair. Neither Deaf1 male (Figure 7) nor female (Figure 8) mice of either genotype displayed depression-like behaviour in either the TS or FS test.

A summary of all Deaf1 behaviour results is shown in Table 7.

Loss of 5-HT1A Autoreceptors and DPAT-Induced Hypothermic Response in Conditional 5-HT1A Mice

To avoid developmental effects of reducing 5-HT1A autoreceptors in early life, the 5-HT1A gene was knocked out in the raphe during adulthood only by using an inducible recombinase method. Cells in which the 5-HT1A receptor gene was excised subsequently expressed GFP, allowing for easy identification. Successful recombination of the floxed 5-HT1A gene in the dorsal raphe took place in both the 5-HT1A^{autoHET} and 5-HT1A^{autoKO} mice (referred to as fl/+ and fl/fl respectively in figures; Figure 9A), with a greater extent of recombination in serotonin cells in female 5-HT1A^{autoKO} compared to 5-HT1A^{autoHET} mice, as shown by immunofluorescent staining (Figure 9B). Interestingly, male 5-HT1A^{autoKO} mice did not show a significantly larger percentage of GFP staining on TPH-positive cells compared to 5-HT1A^{autoHET} males (Appendix Figure viii). In addition, female 5-HT1A^{autoKO} mice had a significantly larger percentage of GFP-positive cells that were not TPH-positive compared to 5-HT1A^{autoHET} (Figure 9C), while male 5-HT1A^{autoKO} and 5-HT1A^{autoHET} again showed no significant difference (Appendix Figure viii).

GFP staining was also performed in brains regions containing 5-HT1A heteroreceptors and did not show any specific GFP staining in male or female mice (Figure 10, Appendix Figure ix), which shows that there was no recombination detected

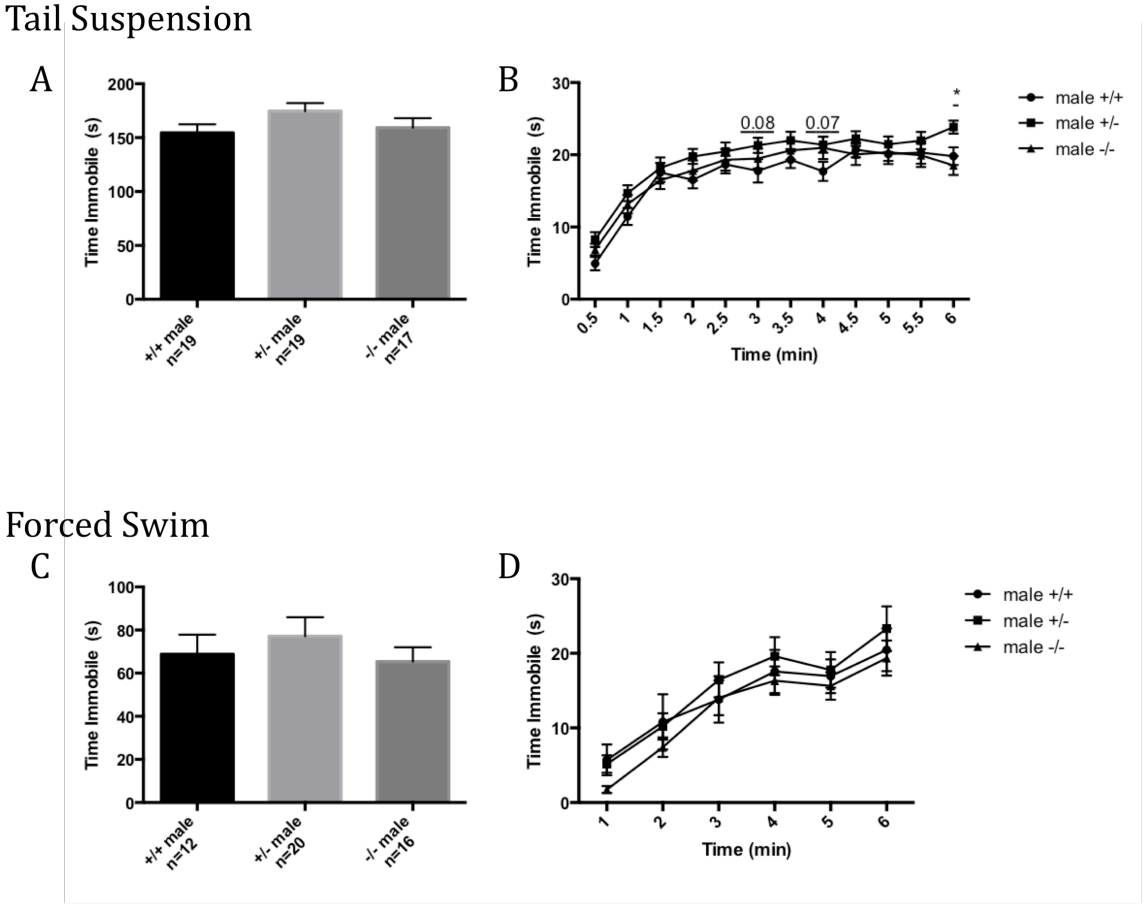


Figure 7. Deaf1: Male Depression Assays

Two assays were used to test for the presence of depression: TS and FS. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile shown in 30-second intervals across the full 6-minute TS test. C. Time spent immobile averaged across the final four minutes of the FS test. D. Time spent immobile shown in 30-second intervals across the full 6-minute FS test. Underlined symbols indicate significance for heterozygote mice. Statistical effects are shown in Table 5.

Deaf1 Male				post hoc	
TS					
	Time Immobile	F	1.78	WT x HET	0.151
		df	2	WT x KO	> 0.9999
		p	0.18		
FS					
	Time Immobile	F	0.58	WT x HET	> 0.9999
		df	2	WT x KO	> 0.9999
		p	0.56		

Table 5A. Deaf1: Male Depression Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

Deaf1 Male		Main Effect of Time		Main Effect of Genotype	Interaction		
TS							
Time Immobile	F	80.87		1.89	1.14		
	df	11		2	22		
	p	**< 0.0001**	****	0.16	0.30		
post hoc							
	Time	0.5	1	1.5	2	2.5	3
	WT x HET	0.113	0.126	> 0.9999	0.126	0.594	***0.084***
	WT x KO	0.546	0.682	> 0.9999	0.943	> 0.9999	0.681
	Time	3.5	4	4.5	5	5.5	6
	WT x HET	0.245	***0.067***	0.765	0.875	0.682	**0.042**
	WT x KO	0.921	0.137	> 0.9999	> 0.9999	> 0.9999	0.946
FS							
Time Immobile	F	41.56		0.79	0.30		
	df	5		2	10		
	p	**< 0.0001**	****	0.46	0.98		
post hoc							
	Time	1	2	3	4	5	6
	WT x HET	> 0.9999	> 0.9999	0.877	> 0.9999	> 0.9999	0.807
	WT x KO	0.523	0.677	> 0.9999	> 0.9999	> 0.9999	> 0.9999

Table 5B. Deaf1: Male Depression Assays

Statistical results of a two-way ANOVA comparing genotype across time for male Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

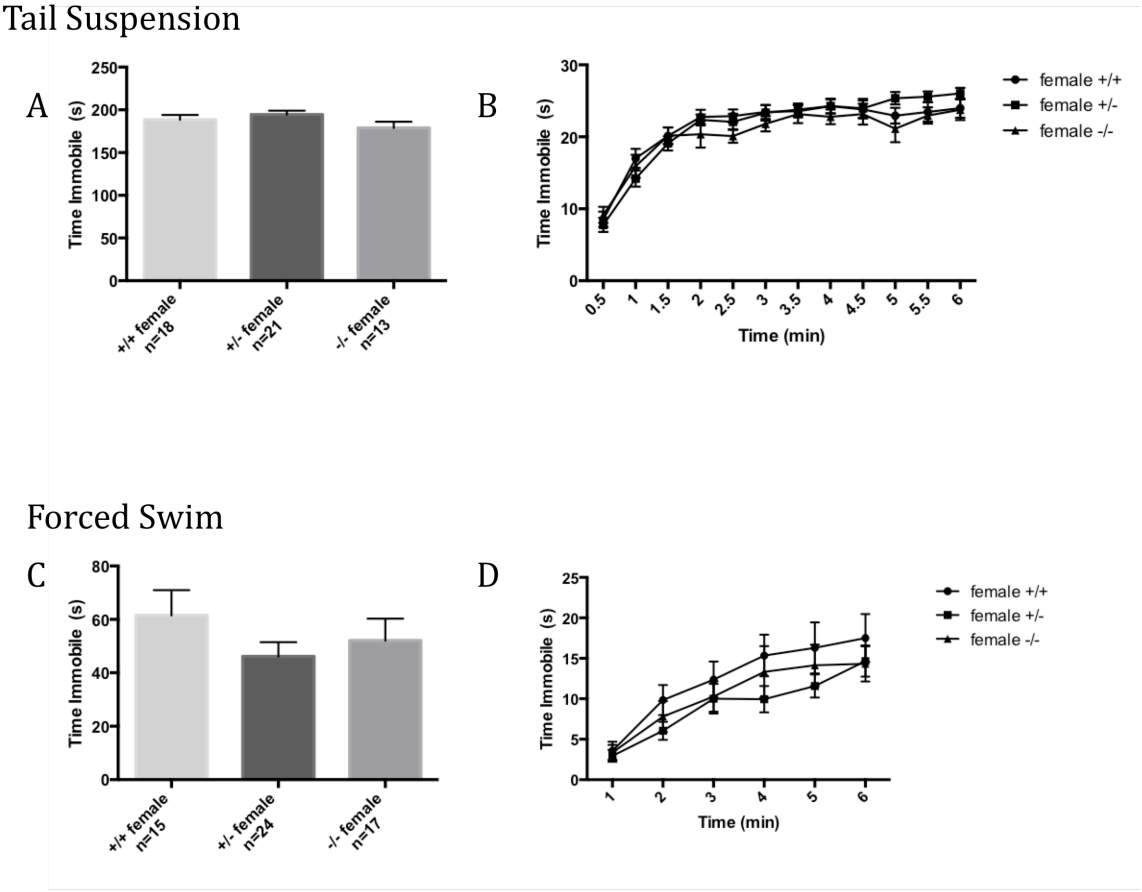


Figure 8. Deaf1: Female Depression Assays

Two assays were used to test for the presence of depression: TS and FS. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile shown in 30-second intervals across the full 6-minute TS test. C. Time spent immobile averaged across the final four minutes of the FS test. D. Time spent immobile shown in 30-second intervals across the full 6-minute FS test. Statistical effects are shown in Table 6.

Deaf1 Female				post hoc	
TS					
	Time Immobile	F	1.73	WT x HET	0.856
		df	2	WT x KO	0.555
		p	0.19		
FS					
	Time Immobile	F	1.10	WT x HET	0.289
		df	2	WT x KO	0.796
		p	0.34		

Table 6A. Deaf1: Female Depression Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

Deaf1 Female		Main Effect of Time		Main Effect of Genotype	Interaction		
TS							
Time Immobile	F	71.51		0.69	1.24		
	df	11		2	22		
	p	**< 0.0001**	****	0.51	0.21		
post hoc							
	Time	0.5	1	1.5	2	2.5	3
	WT x HET	> 0.9999	0.129	0.9903	> 0.9999	> 0.9999	> 0.9999
	WT x KO	> 0.9999	> 0.9999	> 0.9999	0.322	0.212	0.668
	Time	3.5	4	4.5	5	5.5	6
	WT x HET	> 0.9999	> 0.9999	> 0.9999	0.216	0.322	0.348
	WT x KO	> 0.9999	0.779	> 0.9999	0.581	> 0.9999	> 0.9999
FS							
Time Immobile	F	30.16		1.20	0.54		
	df	5		2	10		
	p	**< 0.0001**	****	0.31	0.86		
post hoc							
	Time	1	2	3	4	5	6
	WT x HET	> 0.9999	0.349	0.795	0.107	0.184	0.616
	WT x KO	> 0.9999	0.996	0.971	0.997	0.941	0.576

Table 6B. Deaf1: Female Depression Assays

Statistical results of a two-way ANOVA comparing genotype across time for male Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

DEAF1		Male		Female	
		HET	KO	HET	KO
Beam Break	Number of Movements	ns	ns	ns	ns
EPM	Time in Open Arms	↑	ns	↓ (trend)	↓
	Time in Center of Maze	ns	ns	↓	ns
	Distance	ns	↓	ns	↓
OF	Time in Center	ns	↓ (trend)	ns	ns
	Distance	ns	ns	ns	ns
LD	Time in Light Side	ns	↓	ns	↑
	Latency to Enter Dark Side	ns	↑	ns	ns
	Distance	ns	↓	ns	ns
TS	Time Immobile	ns	ns	ns	ns
FS	Time Immobile	ns	ns	ns	ns

Table 7. Summary of Deaf1 Behaviour Assays

Indicated changes are compared to wildtype controls.

↓- significant decrease; ↑- significant increase; ns- not significant

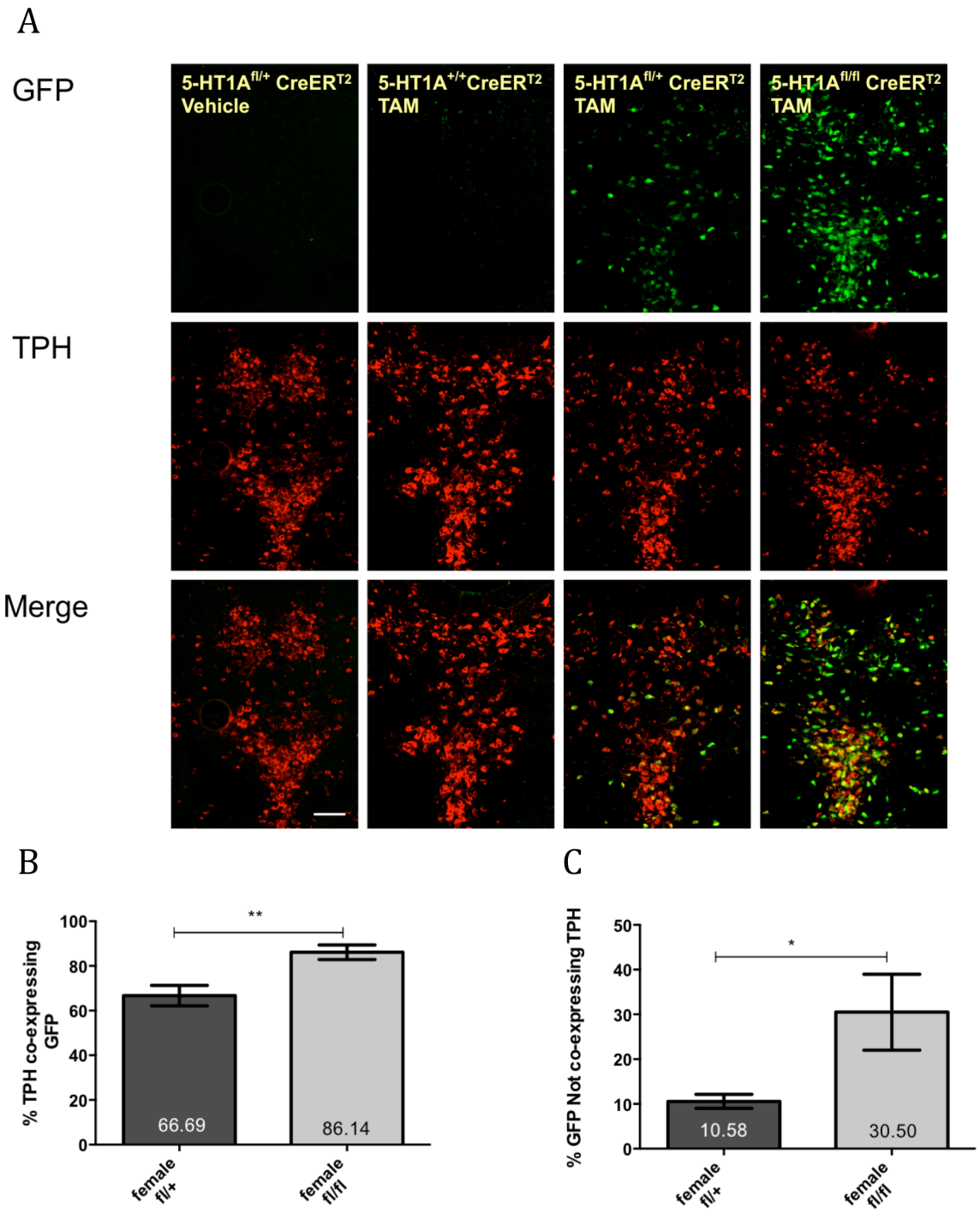

A
GFP
TPH
Merge
5-HT1A fl/+ CreER T2 Vehicle
5-HT1A +/+ CreER T2 TAM
5-HT1A fl/+ CreER T2 TAM
5-HT1A fl/fl CreER T2 TAM
B
% TPH co-expressing GFP
100
80
60
40
20
0
**
66.69
86.14
female fl/+
female fl/fl
C
% GFP Not co-expressing TPH
50
40
30
20
10
0
*
10.58
30.50
female fl/+
female fl/fl

Figure 9. Immunofluorescent staining of dorsal raphe in conditional 5-HT1A mice Female mice. A. Immunofluorescent staining for TPH (mouse anti-TPH, 1:500) and GFP (chicken anti-GFP, 1:2000) was performed on dorsal raphe slices of flx5-HT1A mice. B. Quantification of TPH-positive cells expressing GFP. C. Quantification of TPH-positive cells not expressing GFP. Scale bar indicates 100 μm.

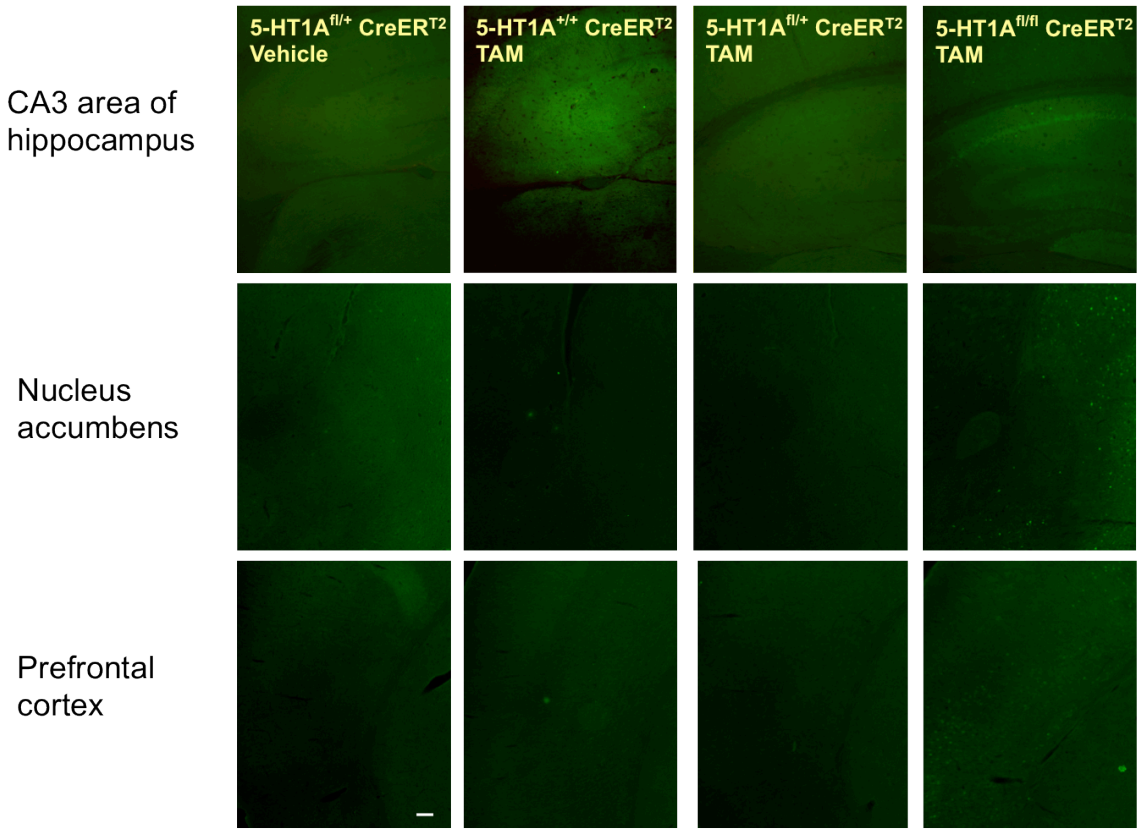


Figure 10. Immunofluorescent staining of 5-HT1A heteroreceptor-dense regions in conditional 5-HT1A mice

Female mice. Immunofluorescent staining for GFP (chicken anti-GFP, 1:2000) was performed on other brain regions known to be rich in 5-HT1A receptors. Scale bar indicates 100 μm.

in non-serotonergic neurons and hence the heteroreceptors remained expressed.

The functionality of the 5-HT1A receptors was tested using 8-OH-DPAT-induced hypothermia, which is mediated by 5-HT1A autoreceptors in mice. As expected, 5-HT1A^{autoWT} (referred to as +/+ in figures) mice showed a significantly greater drop in temperature following 8-OH-DPAT injection compared to both the saline injection and 8-OH-DPAT injection in the 5-HT1A^{autoKO} mice in both the male and female cohorts (Figure 11). These results further confirm the 5-HT1A autoreceptor has been greatly reduced in the 5-HT1A^{autoKO} mice, while it remained present and functional in the 5-HT1A^{autoWT} mice. Saline controls showed no change in temperature between genotypes and therefore indicated no effect of the injection on temperature change.

The hypothermic effect of 8-OH-DPAT injection was also tested on conditional heterozygous mice to examine any differences when a smaller portion of the autoreceptors was reduced. As expected, 5-HT1A^{autoWT} and 5-HT1A^{autoHET} mice showed a significantly greater drop in temperature compared to their saline controls in both the male and female cohorts (Figure 11). These results indicate that the 5-HT1A autoreceptor remains functional in both 5-HT1A^{autoWT} and 5-HT1A^{autoHET} mice. No differences in temperature were found between 5-HT1A^{autoWT} and 5-HT1A^{autoHET} mice, indicating either the remaining receptors in the 5-HT1A^{autoHET} mice are functionally sufficient to respond to the agonist or that the dose of 8-OH-DPAT was not low enough to detect any differences brought on by the change in 5-HT1A autoreceptor levels.

Overall the GFP immunofluorescent staining and lack of agonist-induced hypothermia in 5-HT1A^{autoKO} mice indicate a successful conditional 5-HT1A autoreceptor knockout model. The agonist-induced hypothermia in the 5-HT1A^{autoWT}

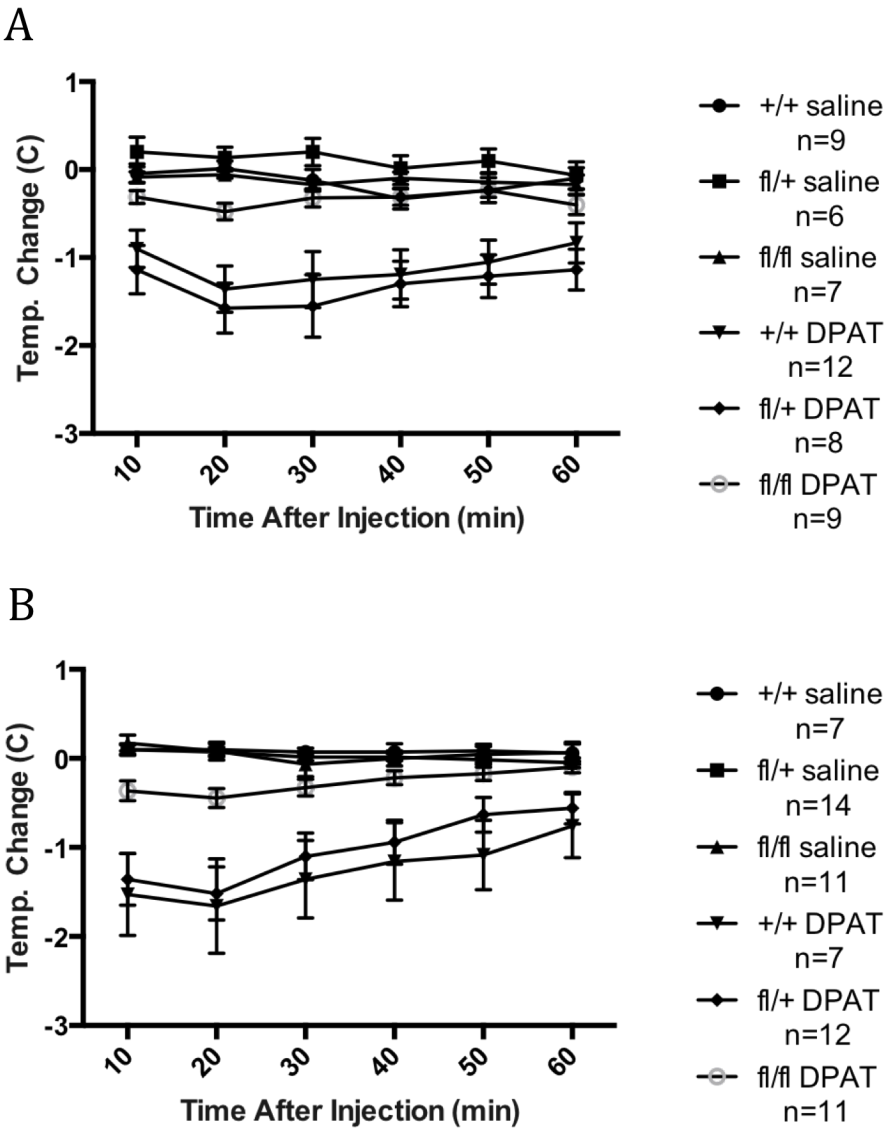


Figure 11. Agonist-induced hypothermia in conditional 5-HT1A mice

In order to test the functionality of the 5-HT1A autoreceptors, the 1A-specific agonist 8-OH-DPAT was used. A. Temperature changes in male mice using a dose of 0.75 mg/kg. B. Temperature changes in female mice using a dose of 0.75 mg/kg. Statistical effects are shown in Table 8.

flx1A Male 0.75 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	5.21		8.86		3.73	
df	5		5		25	
p	**0.0002**	***	**< 0.0001**	****	**< 0.0001**	

post hoc

Time	10	20	30	40	50	60
+/+ saline x fl/+ saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ saline x fl/fl saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ saline x +/+ DPAT	**0.018**	**< 0.0001**	**0.0003**	**0.018**	**0.030**	***0.082***
fl/+ saline x fl/+ DPAT	**0.001**	**< 0.0001**	**< 0.0001**	**0.001**	**0.001**	**0.014**
fl/fl saline x fl/fl DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ DPAT x fl/+ DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ DPAT x fl/fl DPAT	0.379	**0.013**	**0.007**	**0.013**	**0.030**	> 0.9999

flx1A Female 0.75 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	17.62		10.91		7.60	
df	5		5		25	
p	**< 0.0001**	****	**< 0.0001**	****	**< 0.0001**	****

post hoc

Time	10	20	30	40	50	60
+/+ saline x fl/+ saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ saline x fl/fl saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ saline x +/+ DPAT	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.002**	**0.003**	0.140
fl/+ saline x fl/+ DPAT	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.001**	0.109	0.409
fl/fl saline x fl/fl DPAT	0.474	0.518	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ DPAT x fl/+ DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
+/+ DPAT x fl/fl DPAT	**0.001**	**0.0003**	**0.005**	**0.014**	**0.020**	0.304

Table 8. Agonist-induced hypothermia in conditional 5-HT1A mice

Statistical results of a two-way ANOVA comparing genotype and time for both male and female conditional 5-HT1A mice. Bold font indicates significance; bold italic indicates a non-significant trend.

mice demonstrates that the 5-HT1A autoreceptors remain present and functional.

Changes in Anxiety, but not Depression, Behaviour in Conditional 5-HT1A Mice

Mice were tested for anxiety using the EPM, OF, and LD tests. Since the autoreceptors were only reduced in adulthood, no anxiety phenotype was expected as this phenotype is thought to be primarily elicited by by altered 5-HT1A receptor levels in the early postnatal period.

Male 5-HT1A^{autoHET} mice showed a significant reduction in time spent in the center of the OF compared to 5-HT1A^{autoWT}, indicating an anxiety phenotype (Figure 12D-E). However neither the EPM or LD tests indicated any changes in anxiety relative to wildtype controls (Figure 12A-C, F-H). No significant differences were found in any of the three anxiety paradigms comparing 5-HT1A^{autoKO} to 5-HT1A^{autoWT} males. Thus the 5-HT1A^{autoHET} males appear to show increased anxiety, while 5-HT1A^{autoKO} males showed no differences in anxiety behaviour.

Female 5-HT1A^{autoHET} mice did not show any differences in any of the three anxiety assays compared to 5-HT1A^{autoWT} females (Figure 13). 5-HT1A^{autoKO} females also show no significant changes in anxiety measures compared to 5-HT1A^{autoWT} females, however there is a trend for 5-HT1A^{autoKO} females to spend more time in the center of the OF compared to controls which suggests a reduced anxiety levels (Figure 13D). Overall 5-HT1A^{autoHET} and 5-HT1A^{autoKO} females displayed no significant changes in anxiety behaviour when compared to wildtype controls.

Similar to the Deaf1 mice, depression behaviour was also evaluated using the TS and FS tests. In the TS test, neither 5-HT1A^{autoHET} nor 5-HT1A^{autoKO} males displayed a

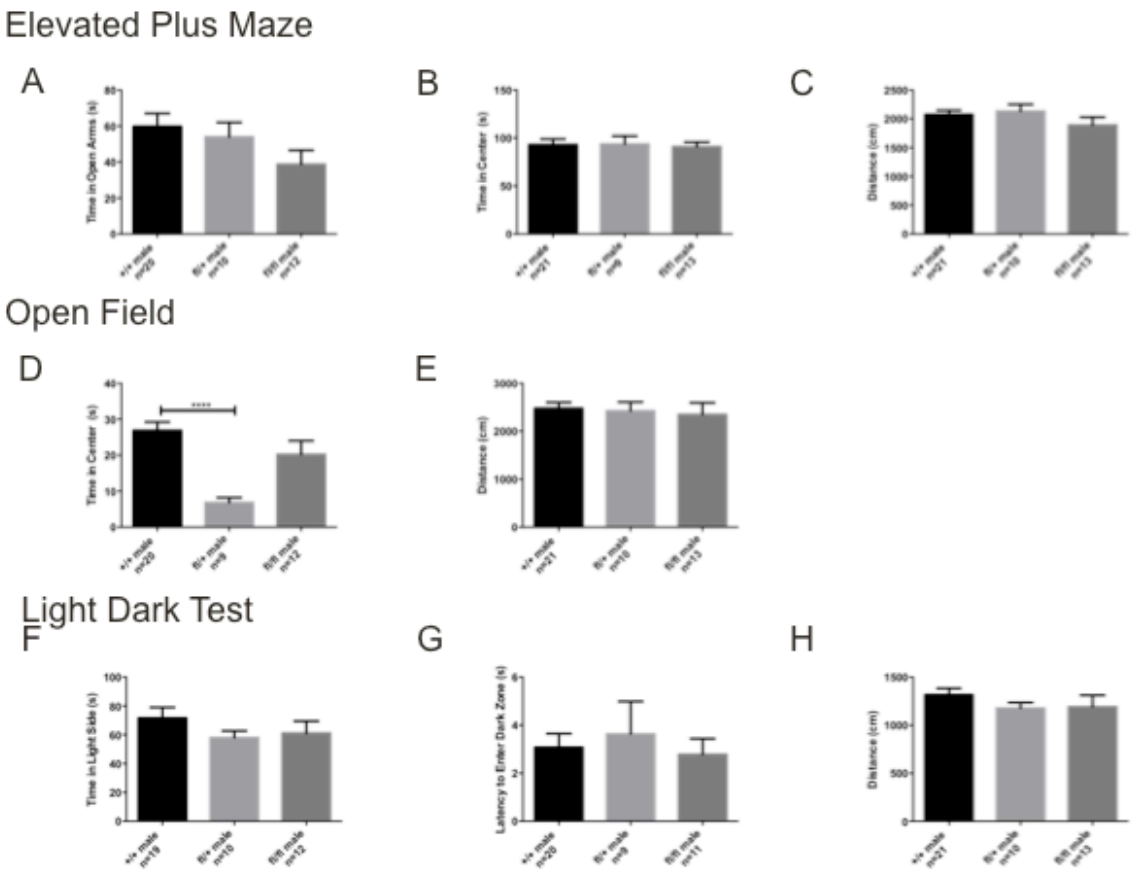


Figure 12. flx5-HT1A: Male Anxiety Assays

Three assays were used to test for the presence of anxiety: EPM, OF, and LD. A. Time spent in the opens arms of the EPM. B. Time spent in the center of the EPM. C. Distance traveled in the EPM. D. Time spent in the center of the OF. E. Distance traveled in the OF. F. Time spent in the light side of the LD test. G. Time taken to enter the dark zone for the first time in the LD test. H. Distance traveled in the LD test. Statistical effects are shown in Table 9.

flx1A Male					post hoc	
EPM						
	Time in Open Arms	F	1.92		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	0.118
		p	0.16			
	Time in Center	F	0.04		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.96			
	Distance	F	1.26		+/+ x fl/+	> 0.9999
		df	1		+/+ x fl/fl	0.398
		p	0.29			
OF						
	Time in Center	F	11.51		+/+ x fl/+	**< 0.0001**
		df	2		+/+ x fl/fl	0.177
		p	**0.0001**	***		
	Distance	F	0.14		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.87			
LD						
	Time in Light Side	F	0.97		+/+ x fl/+	0.434
		df	2		+/+ x fl/fl	0.624
		p	0.39			
	Latency to Enter Dark Side	F	0.22		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.80			
	Distance	F	0.87		+/+ x fl/+	0.564
		df	2		+/+ x fl/fl	0.580
		p	0.43			

Table 9. flx5-HT1A: Male Anxiety Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

Bold font indicates significance (*).

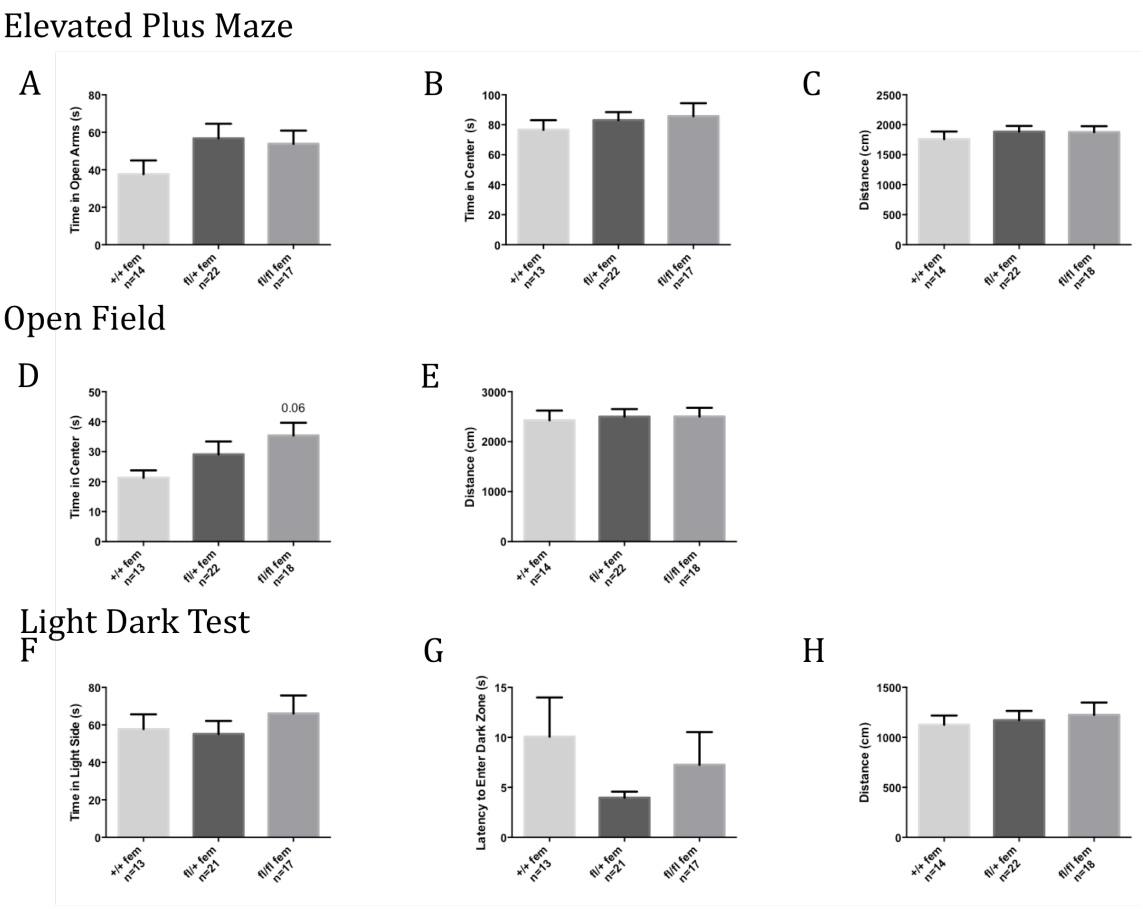


Figure 13. flx5-HT1A: Female Anxiety Assays

Three assays were used to test for the presence of anxiety: EPM, OF, and LD. A. Time spent in the opens arms of the EPM. B. Time spent in the center of the EPM. C. Distance traveled in the EPM. D. Time spent in the center of the OF. E. Distance traveled in the OF. F. Time spent in the light side of the LD test. G. Time taken to enter the dark zone for the first time in the LD test. H. Distance traveled in the LD test. Statistical effects are shown in Table 10.

flx1A Female					post hoc	
EPM						
	Time in Open Arms	F	1.63		+/+ x fl/+	0.176
		df	2		+/+ x fl/fl	0.335
		p	0.21			
	Time in Center	F	0.38		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	0.795
		p	0.69			
	Distance	F	0.39		+/+ x fl/+	0.827
		df	2		+/+ x fl/fl	0.934
		p	0.68			
OF						
	Time in Center	F	2.51		+/+ x fl/+	0.404
		df	2		+/+ x fl/fl	***0.059***
		p	***0.09***	*#*		
	Distance	F	0.05		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.95			
LD						
	Time in Light Side	F	0.51		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.60			
	Latency to Enter Dark Side	F	1.36		+/+ x fl/+	0.223
		df	2		+/+ x fl/fl	0.957
		p	0.27			
	Distance	F	0.20		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.82			

Table 10. flx5-HT1A: Female Anxiety Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

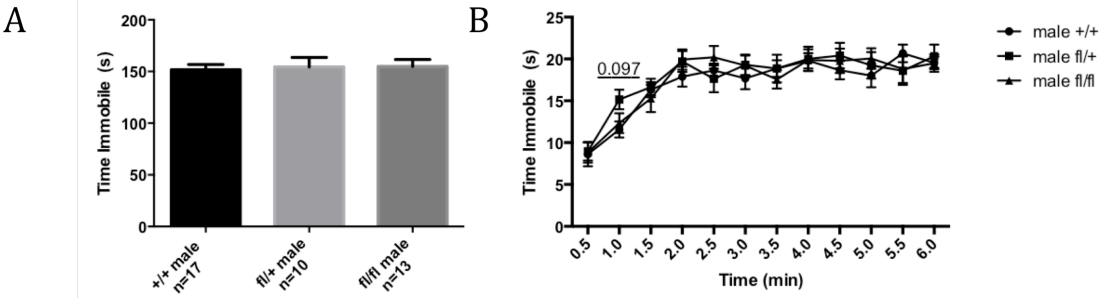
Bold italic indicates a non-significant trend (#).

change in immobile time compared to 5-HT1A^{autoWT} males, which indicates no differences in depressive behaviour (Figure 14A-B). In the FS test, 5-HT1A^{autoHET} males showed a significant decrease in time spent immobile compared to 5-HT1A^{autoWT} males, indicating a reduced depression phenotype (Figure 14C-D). A similar result was not found in 5-HT1A^{autoKO} males, which showed no differences in FS immobile time compared to controls. Overall 5-HT1A^{autoHET} males showed a reduced depressive phenotype while 5-HT1A^{autoKO} males show no differences in depression behaviour compared to wildtype controls.

Neither 5-HT1A^{autoHET} nor 5-HT1A^{autoKO} female mice showed any differences in immobile time for either TS or FS when compared to 5-HT1A^{autoWT} females (Figure 15). These results indicate that there were no changes in female autoreceptor knockout mice compared to controls.

Overall the lack of 5-HT1A autoreceptors in adulthood appeared to produce no change in anxiety or depression behaviour for both male and female transgenic mice, (with the exception of the male 5-HT1A^{autoHET} mice). Thus, despite the substantial change in autoreceptor levels, no strong change in depression behaviour was observed. No changes were found in locomotion for either males or females (Appendix Figures x, xi). A summary of all flx5-HT1A behaviour results is shown in Table 13.

Tail Suspension



Forced Swim

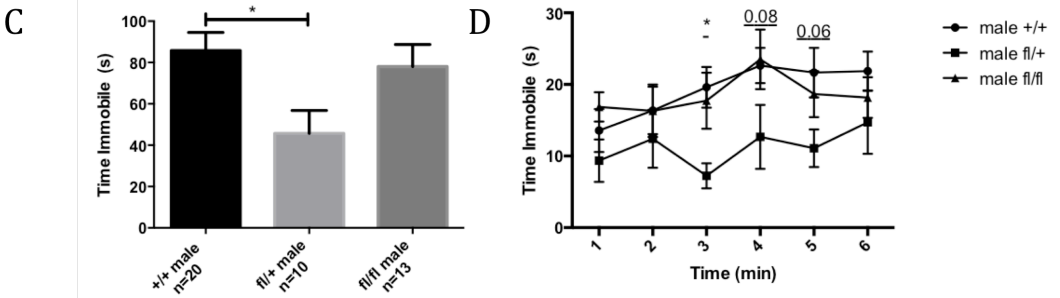


Figure 14. flx5-HT1A: Male Depression Assays

Two assays were used to test for the presence of depression: TS and FS. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile shown in 30-second intervals across the full 6-minute TS test. C. Time spent immobile averaged across the final four minutes of the FS test. D. Time spent immobile shown in 30-second intervals across the full 6-minute FS test. Underlined symbols indicate significance for heterozygote mice. Statistical effects are shown in Table 11.

flx1A Male					post hoc	
TS						
	Time Immobile	F	0.07		+/+ x fl/+	> 0.9999
		df	2		+/+ x fl/fl	> 0.9999
		p	0.93			
FS						
	Time Immobile	F	3.78		+/+ x fl/+	**0.020**
		df	2		+/+ x fl/fl	> 0.9999
		p	**0.03**	*		

Table 11A. flx5-HT1A: Male Depression Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

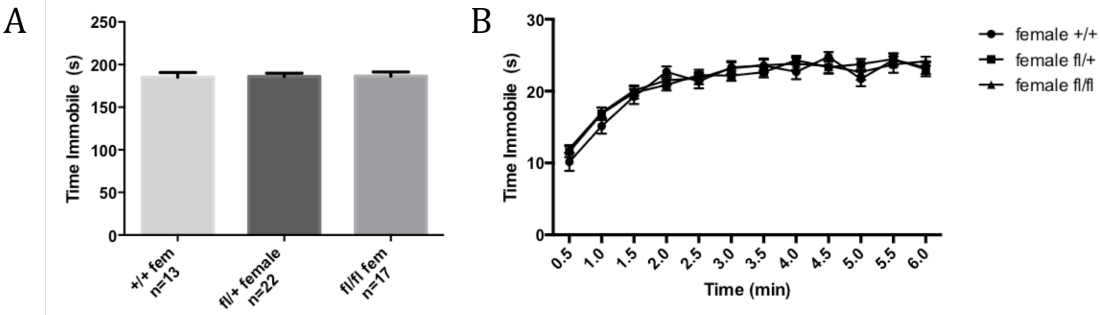
Bold font indicates significance (*).

flx1A Male		Main Effect of Time		Main Effect of Genotype		Interaction	
TS							
Time Immobile	F	31.88		0.21		0.88	
	df	2		11		22	
	p	**< 0.0001**	****	0.81		0.62	
post hoc							
	Time	0.5	1	1.5	2	2.5	3
	+/+ x fl/+	> 0.9999	***0.097***	> 0.9999	0.639	> 0.9999	0.745
	+/+ x fl/fl	> 0.9999	> 0.9999	> 0.9999	0.453	0.702	0.721
	Time	3.5	4	4.5	5	5.5	6
	+/+ x fl/+	> 0.9999	> 0.9999	0.675	0.988	0.508	> 0.9999
	+/+ x fl/fl	0.873	> 0.9999	> 0.9999	0.438	0.530	> 0.9999
FS							
Time Immobile	F	2.21		3.21		0.63	
	df	5		2		10	
	p	***0.054***	*** # ***	***0.051***	*** # ***	0.79	
post hoc	Time	1	2	3	4	5	6
	+/+ x fl/+	0.776	0.829	**0.023**	***0.083***	***0.061***	0.283
	+/+ x fl/fl	0.921	> 0.9999	> 0.9999	> 0.9999	> 0.9999	0.818

Table 11B. flx5-HT1A: Male Depression Assays

Statistical results of a two-way ANOVA comparing genotype across time for male Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

Tail Suspension



Forced Swim

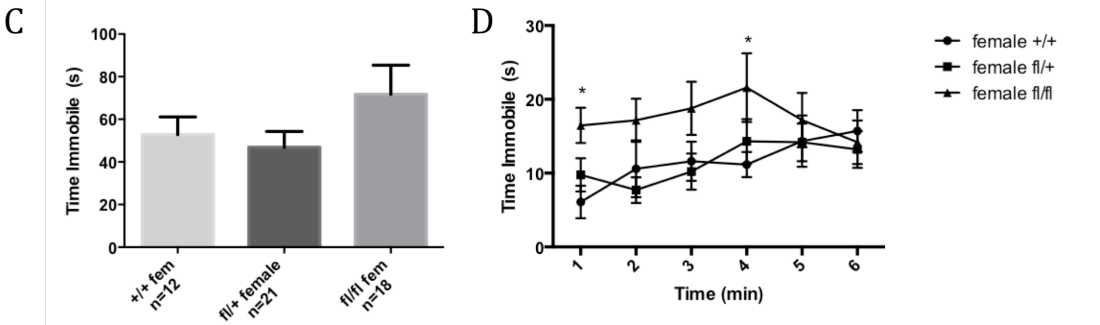


Figure 15. flx5-HT1A: Female Depression Assays

Two assays were used to test for the presence of depression: TS and FS. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile shown in 30-second intervals across the full 6-minute TS test. C. Time spent immobile averaged across the final four minutes of the FS test. D. Time spent immobile shown in 30-second intervals across the full 6-minute FS test. Not underlined symbols indicate significance for homozygote mice. Statistical effects are shown in Table 12.

flx1A Female				post hoc	
TS					
	Time Immobile	F	0.02	+/+ x fl/+	> 0.9999
		df	2	+/+ x fl/fl	> 0.9999
		p	0.98		
FS					
	Time Immobile	F	1.69	+/+ x fl/+	> 0.9999
		df	2	+/+ x fl/fl	0.489
		p	0.20		

Table 12A. flx5-HT1A: Female Depression Assays

Statistical results of a one-way ANOVA comparing genotype for male Deaf1 mice.

flx1A Female		Main Effect of Time		Main Effect of Genotype		Interaction	
TS							
Time Immobile	F	90.31		0.11		1.16	
	df	11		2		22	
	p	**< 0.0001**	****	0.89		0.28	
post hoc							
	Time	0.5	1	1.5	2	2.5	3
	+/+ x fl/+	0.502	0.336	> 0.9999	0.290	0.956	0.708
	+/+ x fl/fl	0.329	0.311	> 0.9999	0.753	> 0.9999	> 0.9999
	Time	3.5	4	4.5	5	5.5	6
	+/+ x fl/+	0.898	0.375	0.479	0.195	> 0.9999	> 0.9999
	+/+ x fl/fl	> 0.9999	0.773	0.629	0.921	> 0.9999	0.996
FS							
Time Immobile	F	2.21		2.53		1.29	
	df	5		2		10	
	p	***0.054***	*#*	***0.09***	*#*	0.24	
post hoc	Time	1	2	3	4	5	6
	+/+ x fl/+	0.813	> 0.9999	> 0.9999	0.958	> 0.9999	> 0.9999
	+/+ x fl/fl	**0.050**	0.309	0.241	**0.048**	> 0.9999	> 0.9999

Table 12B. flx5-HT1A: Female Depression Assays

Statistical results of a two-way ANOVA comparing genotype across time for male Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

flx-5-HT1A		Male		Female	
		autoHET	autoKO	autoHET	autoKO
Beam Break	Number of Movements	ns	ns	ns	ns
EPM	Time in Open Arms	ns	ns	ns	ns
	Time in Center of Maze	ns	ns	ns	ns
	Distance	ns	ns	ns	ns
OF	Time in Center	↓	ns	ns	↑ (trend)
	Distance	ns	ns	ns	ns
LD	Time in Light Side	ns	ns	ns	ns
	Latency to Enter Dark Side	ns	ns	ns	ns
	Distance	ns	ns	ns	ns
TS	Time Immobile	ns	ns	ns	ns
FS	Time Immobile	↓	ns	ns	ns

Table 13. Summary of flx5-HT1A Behaviour Assays

Indicated changes are compared to wildtype controls.

↓- significant decrease; ↑- significant increase; ns- not significant

Preliminary results of learned helplessness in 5-HT1A^{autoKO} mice

Preliminary studies were performed using the conditional 5-HT1A mouse line (mixed females and males, n=4-8 in each group) and a modified learned helplessness protocol (see Anisman & Merali, 2001). In general, shock-5-HT1A^{autoWT} mice displayed higher escape latencies across the testing blocks, as well as a higher number of failures to escape the shock when compared to the non-shocked-5-HT1A^{autoWT} mice (Appendix Figure xii). The shock-5-HT1A^{autoKO} mice also displayed higher escape latencies across blocks compared to no-shock-5-HT1A^{autoKO} mice, however the effect was more pronounced in the shock-5-HT1A^{autoWT} mice. Due to low n values, no statistical analyses were performed.

Overall, preliminary findings suggest a depression resilience in 5-HT1A^{autoKO} mice compared to 5-HT1A^{autoWT} mice, however more testing needs to be done in order to confirm these preliminary findings.

Discussion

In order to examine the effects of changing the levels of 5-HT1A autoreceptors on anxiety and depression, two different mouse lines were used. The Deaf1^{KO} line eliminated a portion of the repression on the 5-HT1A gene and resulted in more autoreceptor being expressed in the dorsal raphe. In this way, a genetic polymorphism previously reported in depressed humans (Lemonde et al., 2003) can be modeled. The Deaf1^{KO} mice were expected to have increased 5-HT1A receptor expression and to show anxiety and depression behaviour. In general, Deaf1^{KO} mice showed an anxiety phenotype, but did not show a depression phenotype (Table 7).

The conditional and inducible 5-HT1A autoreceptor knockout line was used to eliminate the 5-HT1A receptor gene only in serotonin neurons of the raphe nuclei and only in adulthood. In this way, anxiety behaviour that was previously reported to depend on early postnatal 5-HT1A receptor expression could be avoided and it was predicted that only reduced depression behaviour would be observed. Thus 5-HT1A ^{autoKO} mice were expected to show decreased 5-HT1A receptor expression that was specific to the autoreceptors in the dorsal raphe as well as the above-mentioned reduction in depression behaviour. In general, 5-HT1A ^{autoKO} mice showed no anxiety phenotype, with the exception of the male 5-HT1A^{autoHET} mice which showed increased anxiety. No clear depression phenotype was found, with the exception of the male 5-HT1A ^{autoHET} mice that showed anti-depressed behaviour (Table 13).

Immunofluorescent Staining in Deaf1 and Conditional 5-HT1A Mice

Immunofluorescent staining was used in both genetic mouse models to identify changes in 5-HT1A receptor levels. The conditional 5-HT1A knockout mouse contained

a GFP sequence that allowed for visualization of recombined cells and, although the majority of the GFP-positive cells were colocalized with serotonin cells, it was not a direct measurement of the 5-HT1A receptors themselves. In the Deaf1 mouse line, a 5-HT1A antibody was used to quantify the receptor levels. Unfortunately this antibody was not sensitive enough to use on the conditional 5-HT1A knockout mice and perhaps is more effective when there is an overexpression of 5-HT1A receptors (Czesak et al., 2012). Autoradiography using a 5-HT1A receptor-specific ligand is most commonly used to quantify 5-HT1A receptors (Bortolozzi et al., 2012; Ramboz et al., 1998; Richardson-Jones et al., 2010, 2011) and should be employed in future studies to accurately quantify the changes in 5-HT1A receptors that result from these genetic mouse models. As mentioned, electrophysiology is another method that would test the functionality of the receptors and give more insight as to how the cells behave in the absence of the autoreceptors.

Serotonin in Thermoregulation

Serotonin is involved in central thermoregulation. The raphe is believed to be a relay site that engages heat-generating pathways in the hypothalamus (Hodges & Richerson, 2010). Many serotonin-deficient mice display exaggerated decreases in core body temperature in response to a stressor, yet show no abnormalities under normal conditions (Hodges & Richerson, 2010). Using agonists that target the serotonin system and recording the subsequent change in core temperature has become a widely accepted method of measuring the functionality of a receptor.

In the present study, the agonist 8-OH-DPAT was used to temporarily induce hypothermia in both strains of mice and to demonstrate the functionality of the receptors.

5-HT1A ^{autoKO} Mice Show No Hypothermic Response

Mice lacking the 5-HT1A autoreceptor did not show any significant hypothermic response to the agonist 8-OH-DPAT, as was expected. The 8-OH-DPAT caused a significant decrease in core temperature of the 5-HT1A ^{autoWT} mice compared to the 5-HT1A ^{autoKO} mice in both males and females. This result seen in the 5-HT1A ^{autoWT} mice is consistently found with 8-OH-DPAT-induced hypothermia (Bortolozzi et al., 2012; Ferres-Coy et al., 2013; Gross et al., 2002; Hedlund et al., 2004; Millan et al., 1993; Richardson-Jones et al., 2010) and is dependent on 5-HT1A autoreceptor in mice (Piszczek et al., 2013). In both genders, the 8-OH-DPAT induced a decrease in temperature in the 5-HT1A ^{autoHET} mice that was very similar to the 5-HT1A ^{autoWT} mice. These results further show that functional 5-HT1A autoreceptors were not detected by this assay in the 5-HT1A ^{autoKO} mice.

Even though the female 5-HT1A ^{autoHET} mice had over 60% recombined serotonergic neurons (as evidenced by positive GFP expression; Figure 9B) compared to 5-HT1A ^{autoWT} females, both genotypes showed the same drop in core body temperature given the same dose of 8-OH-DPAT (0.75 mg/kg). It could be that the dose of 8-OH-DPAT was not low enough or that the assay was not sensitive enough to dissociate any differences between the two genotypes. However, since the 5-HT1A ^{autoHET} and 5-HT1A ^{autoKO} mice exhibited such a large difference in temperature having a difference in predicted 5-HT1A autoreceptors of roughly 20%, it is likely that further experiments testing other 8-OH-DPAT doses in 5-HT1A ^{autoHET} mice would reveal similar differences when compared to 5-HT1A ^{autoWT} mice. For instance, Richardson-Jones and colleagues

(2010) were able to detect a difference in temperature with a 30% knockdown model of adult 5-HT1A autoreceptors, albeit using a lesser dose (0.5 mg/kg) of 8-OH-DPAT.

Deaf1^{KO} Mice Do Not Show Enhanced Hypothermic Response

Since Deaf1, a transcriptional repressor of the 5-HT1A-receptor gene that is expressed in the raphe, was knocked out, Deaf1^{KO} mice were expected to show a greater drop in core body temperature compared to Deaf1^{WT}. In contrast, male Deaf1^{KO} mice showed a significantly smaller temperature decrease than Deaf1^{WT} mice and female Deaf1^{KO} did not show any significant temperature changes compared to their controls using a dose of 0.5 mg/kg 8-OH-DPAT. These results were not consistent with the immunofluorescent results in which a greater number of 5-HT1A autoreceptors were found in the raphe of Deaf1^{KO} mice compared to Deaf1^{WT}. The results are also inconsistent with previous literature in which upregulating the 5-HT1A receptor caused a greater hypothermic response (Piszczek et al., 2013).

The 0.5 mg/kg dosage produced similar temperature decreases across genotypes in female Deaf1 mice, which could indicate saturation of the receptors. Thus a lower dose (0.25 mg/kg) was used. However the low dosage resulted in smaller temperature changes that were not statistically different from the saline controls.

Another possibility was that the 0.5 mg/kg dosage saturated the Deaf1^{WT} receptors, but not the increased number of Deaf1^{KO} receptors. Thus a higher dose (0.75 mg/kg) was used. However at the high dose, the variability became too large for all genotypes and none were significant from one another. Since doses of 1.0 mg/kg or higher can affect other serotonin receptors, such as the 5-HT7 receptor (Naumenko et al., 2011), 0.75 mg/kg was the highest dosage used in the present study.

One possible explanation for these results is that, because the Deaf1 mice are a global knockout model, there may be other factors that were influencing their thermoregulation. For example, since Deaf1 acts as a repressor in the raphe and an enhancer in the frontal tissues, postsynaptic 5-HT1A receptors may be reduced as observed in the prefrontal cortex of Deaf1^{KO} mice (Czesak et al., 2012). As a result, the net effect could be no temperature differences between the Deaf1^{WT} and Deaf1^{KO} mice, which was evident in the female mice. Further experiments that are able to target the 5-HT1A receptors specifically are needed in order to confirm their presence and functionality, such as autoradiography as well as electrophysiological recordings to test functional properties of the receptors.

Anxiety Behaviour in Deaf1^{KO} Mice

Both male and female Deaf1^{KO} and female Deaf1^{HET} mice displayed an anxiety phenotype, while male Deaf1^{HET} mice displayed a reduced anxiety phenotype. Female Deaf1^{KO} and Deaf1^{HET} showed a prominent anxiety phenotype in the EPM, while male Deaf1^{KO} showed an anxiety phenotype in the OF and LD tests. Although there are not many studies that alter Deaf1 expression, the present results are consistent with a study that found increased anxiety behaviour in a model that greatly increased (300%) 5-HT1A autoreceptors were for the entire lifespan (Piszczek et al., 2013). The Deaf1 mice have been reported to have lower 5-HT levels in the dorsal raphe (Czesak et al., 2012). Other studies in which global alterations to the 5-HT system result in lower 5-HT also report an anxiety phenotype (Parks et al., 1998; Ramboz et al., 1998; Richardson-Jones et al., 2011). However the present results represent mutating a transcription repressor for the 5-HT1A gene and not the gene itself, which is likely why other researchers reported a more

robust anxiety phenotype in the global 5-HT1A knockout mice than what was found in the global Deaf1 knockout mice.

In addition, recall that Deaf1 is not the only transcription factor regulating the expression of the 5-HT1A gene and it is possible that the mutant mice had some type of ongoing compensatory mechanisms. For instance, Freud1 is another transcription repressor for the 5-HT1A gene and appears to be a stronger repressor than Deaf1, at least in a raphe cell line (Albert et al., 2011). Thus perhaps the Deaf1 mutant mice do not have a strong phenotype because Freud1 was able to adequately repress 5-HT1A transcription.

Further experiments such as measuring Freud1 levels in the Deaf1 mutants would show if Freud1 was indeed being upregulated. Recall that several other transcription factors also regulate 5-HT1A receptor expression such as REST and glucocorticoids, and there is evidence that REST is upregulated in depression (Goswami et al., 2010).

Thus due to the complications associated with global knockout models in addition to affecting a transcription regulator, other compensatory mechanisms may explain the moderate anxiety phenotype that was observed.

Depression Behaviour in Deaf1 Mice

It was hypothesized that the Deaf1^{KO}, which have reduced levels of serotonin in the raphe (Czesak et al., 2012), would have a depression phenotype due to reduced serotonin neurotransmission. Surprisingly, in the present study, male and female Deaf1^{KO} mice showed no differences in TS and FS tests compared to their respective controls, indicating no depression phenotype. Although these results are inconsistent with previous global models of 5-HT reduction, which found a prominent depressive phenotype (Parks et al., 1998; Ramboz et al., 1998; Richardson-Jones et al., 2011), the present model

knocked out a transcription factor for 5-HT1A receptor and not the 5-HT1A receptor itself. For this reason, the abovementioned compensatory mechanisms could account for the difference in depression phenotype.

There are not many studies that focus on behavioural effects of reducing Deaf1. However one study used a conditional Deaf1 knockout and found anxiety behaviour, but no depressive behaviour in either the FS, TS, or sucrose preference tests (Vulto-van Silfhout et al., 2014), which is consistent with the present results.

One explanation for the lack of depressive phenotype is the aforementioned compensation from other transcription regulators. Another possibility is that, as with other indirect manipulations of the serotonin system, the depression phenotype may not be detectable without a prominent stressor. Indeed, Caspi and colleagues (2003) reported that, in humans, a 5-HTT mutation only predicted depression when combined with stress. In addition, not every patient with the C(-1019)G polymorphism was diagnosed with depression (Lemonde et al., 2003), which means there are other mechanisms that contribute to a depression phenotype besides genetics. Further testing using a stress paradigm would determine if the Deaf1 mutation represents a genetic vulnerability to anxiety and/or depression rather than an innate behavioural change.

Anxiety Behaviour in Conditional 5-HT1A Mice

Inducing a knockout of the 5-HT1A autoreceptor during adulthood was expected to avoid an anxiety phenotype, which is thought to involve early postnatal 5-HT1A receptor expression (Gross et al., 2002). Although male 5-HT1A^{autoHET} mice displayed an anxiety phenotype in the OF, this behaviour was not observed in the other two anxiety assays.

No significant changes in anxiety behaviour were found in the remaining conditional knockout mice (male 5-HT1A^{autoKO}, female 5-HT1A^{autoKO}, and female 5-HT1A^{autoKO}), which is consistent with previously reported 5-HT1A knockdown models (Bortolozzi et al., 2012; Gross et al., 2002; Richardson-Jones et al., 2010). The present results indicate the adult knockout of the 5-HT1A autoreceptor was successful in avoiding an anxiety phenotype.

Depression Behaviour in Conditional 5-HT1A Mice

Despite a considerable decrease in 5-HT1A autoreceptors as shown by GFP-positive recombination staining, male 5-HT1A^{autoKO} as well as female 5-HT1A^{autoHET} or 5-HT1A^{autoKO} mice did not displayed any changes in depressive behaviour compared to wildtype controls.

The absence of a significant change in depressive behaviour could simply reflect a ceiling effect in the wildtype mice. Even though knocking down the 5-HT1A autoreceptors is expected to reduce depressive behaviour (Bortolozzi et al., 2012), it may not be evident if the wildtype mice are not already showing depressive behaviour. For this reason, a stress paradigm that induces depression such as the learned helplessness assay can be used to determine if the 5-HT1A^{autoKO} mice show reduced behavioural despair. The lack of a strong depression phenotype in the conditional 5-HT1A mice is consistent with the results of other partial 5-HT1A conditional receptor knockdown experiments, which failed to detect any depression phenotype for both autoreceptors (Richardson-Jones et al., 2010; 2011) and heteroreceptors (Richardson-Jones et al., 2011). Specifically, the 30% reduction in 1A autoreceptors did not produce a difference in behavioural despair in either the TS or the FS test. In fact, only after testing the mice

on two consecutive days in the FS test did the knockdown mice show a non-significant trend towards a difference in mobility, with the knockdown mice showing a slight increase in mobility (Richardson-Jones et al., 2010). According to the authors, this suggests a difference in susceptibility to stress between the two groups of mice. Consistent with Richardson-Jones and colleagues (2010), male 5-HT1A^{autoHET} mice, which represent approximately 75% 5-HT1A autoreceptor knockdown, also showed a decrease in immobility compared to 5-HT1A^{autoWT} in the FS test. These results indicate that a moderate decrease in 5-HT1A autoreceptor may cause a decrease in behavioural despair, at least in male mice. These results are also consistent with the anti-depressed phenotype observed by siRNA-mediated knockdown of 5-HT1A autoreceptors (Bortolozzi et al., 2012).

Given that, in the present study, conditional knockout mice failed to show any differences in either test of behavioural despair compared to wildtype controls and that stress was required to detect a phenotype in a other 5-HT1A autoreceptor mouse models (Richardson-Jones et al., 2010), perhaps the depression phenotype is not detectable until the mouse is stressed. This would be similar to human cases in which a stressful life event will trigger depression and some are more likely to succumb the stress than others (Caspi et al., 2010).

Future experiments may include a stress test, such as a hyperthermia test that measures the change in core body temperature in response to stress. Perhaps mice would respond differently to this type of acute stress (Richardson-Jones et al. 2010). In order to bring about a depression phenotype, a stress procedure could be used prior to testing,

such as chronic mild stress or chronic corticosterone treatment (Dalla et al., 2005; Fairchild et al., 2003; Laaris et al., 1999).

As mentioned, another potential future experiment would use a learned helplessness paradigm, which is a repeated stress procedure. Though used to induce depression in genetically wildtype mice, only 30% of mice in this paradigm actually show depressive behaviours (shown via a longer latency or failure to escape the shock; Chourbaji et al., 2005). By using mice with a genetically altered serotonin system, perhaps their increased susceptibility to stress (Richardson-Jones et al., 2010) would result in a larger percentage showing depressive behaviours. Indeed, in a 5-HTT mutation mouse model, serotonin deficient mice had significantly higher escape latencies in the learned helplessness paradigm than wildtype controls (Muller et al., 2011).

A modified learned helplessness procedure was performed on the present conditional 5-HT1A mouse line and these preliminary results are consistent with a depression resistant phenotype in the 5-HT1A^{autoKO} mice (Appendix Figure xii). Anisman and Merali (2001) found that mice under a similar procedure have higher escape latencies in uncontrollable shock conditions compared to non-shocked controls.

When conducted on wildtype C57BL/6 mice, approximately 30% of mice that undergo the learned helplessness procedure become depressed. For this reason, the learned helplessness procedure itself often involves large numbers of mice and thus many more mice are needed to confirm any present findings. In addition, the escape latencies reported here are much longer than those found previously (Anisman & Merali, 2001; Chourbaji et al., 2005). Thus the current protocol may benefit from a pain threshold test to determine the optimal amperage of the shock. Pain sensitivity varies across mouse

strains (Anisman & Merali, 2001) and most researchers will use a pain threshold test before conducting studies involving pain (Anisman & Merali, 2001; Muller et al., 2011; Pryce et al., 2012).

Although the conditional knockout mice did not display the expected changes in depressive behaviour, more extensive testing that utilizes higher levels of stress may dissociate changes in susceptibility to stress leading to depressive behaviour.

Anxiety Testing

In the present study, anxiety behaviour in Deaf1 mice in the EPM was not always shown in either the OF or LD tests. It should also be noted that testing mice on multiple anxiety tests could yield inconsistent results when the phenotype is weak. Anxiety tests use differing methods for inducing and measuring anxiety and it is now believed that different tests measure different types of anxiety (Belzung & Griebel, 2001). Even tests within the same class (ie utilizing natural fears, such as bright lights, versus using conditional fear responses) can yield different results and may target different underlying facets of anxiety. For instance, EPM combines rodents' fear of heights, lighting, and open areas, while OF has a larger open area to explore coupled with bright lighting. Some researchers argue OF is more a measure of activity and exploration in a novel environment and not a reliable measure of anxiety (O'Leary et al., 2013). However it is generally agreed that EPM is one of the most consistent and reliable anxiety tests used (Komada et al., 2008).

Gender Differences

Initially the behaviour results were compared across genders to determine if a gender effect was present (Appendix Figures ii-v). Indeed, several assays on both mouse

strains showed gender differences. From there, all of the results were divided by gender. Although gender differences in anxiety and depression behaviour were not the original purpose of this study, the fact that post-hoc differences were found needs to be addressed.

Despite the fact that depression is twice as common in women as in men (Lam, 2012; Lasiuk & Hegadoren, 2007) and that increased anxiety is typically seen in women with clinical depression (Gorman, 2006), very few studies have been done in rodents that focus on female anxiety and depressive behaviour. In order to minimize physiological variability, females are often excluded from animal studies.

In rodents, one study used a serotonin-deficient mouse model and found that females deficient in serotonin had a decreased preference for sucrose (i.e. increased anhedonia, which is characteristic of depression) while males with the same genetic mutation did not. Female mice in this study also displayed higher anxiety behaviours than males (Sachs et al., 2014). Interestingly, there is evidence that females have 5-HT1A autoreceptors that are more sensitive to 5-HT-induced down-regulation in 5-HTT knockdown and heterozygous mice (Li et al., 2000; Bouali et al., 2003). In addition, studies in female cynomolgus monkeys indicate that 5-HT1A autoreceptor expression is reduced by estrogen treatment (Pecins-Thompson & Bethea, 1999). Thus changes in 5-HT1A autoreceptor expression might be expected to have a greater effect in females.

As mentioned, a depression-inducing paradigm such as learned helplessness may reveal more profound depressive symptoms and could also further dissociate male and female susceptibility to behavioural despair. In addition, future studies may also use a non-stressful method to test for depression symptoms in addition to a high stress paradigm. For example, the sucrose preference test is a relatively non-stressful paradigm

that uses consumption of a sucrose solution as a measure of anhedonia (Dalla et al., 2010).

In the present study, both strains of mice show gender-dependent differences in many of the behavioural assays. In light of the gender differences already known in human depression, it is important to consider any potential gender effects when modeling depression in animal models. Future studies may also include measuring gonadal hormone and/or corticosteroid concentrations to further explore any biochemical differences between male and female susceptibility to depression.

Estrus Cycle

In order to determine if estrus cycles were influencing female behaviour in the present study, estrus cycle staging was performed (Appendix Figure xiii). Several studies have reported changes in serotonin-mediated behaviours when females are in different estrus cycles (Bodganova et al., 2013; Uphouse et al., 1991; Palanza et al., 2001).

In the present study, estrus cycle staging revealed a relatively similar distribution of each cycle (proestrus, estrus, metestrus/diestrus) between each genotype across the four days of testing. These results were unexpected given that, in mice, the entire cycle is four days long and the majority of that time is spent in the metestrus/disestrus phases (Byers et al., 2012). One would expect to see a higher proportion of females in the metestrus/diestrus phases across time.

One possible explanation for the unexpected cycling is that the procedure was performed after all of the behaviour tests were completed and after the females had been single-housed for approximately three weeks. The stress of the testing and individual housing may have contributed to the disrupted estrus cycling (Dalla et al., 2005; Lima et

al., 2009). Consistent with the present results, estrus and proestrus cycles were lengthened after stress in rats (Matysek 1989). Thus future estrus cycle staining should be performed before any behavioural testing, possibly on a separate cohort of females to avoid any stress-induced changes in cycling.

Rodent Models

There are many advantages to using rodents as models for various human diseases and disorders. Rodent models as well as behavioural testing of those models have become standard in identifying the etiology of disease. Unfortunately there are still a lot of confounding factors when using rodent models.

Effect of Background Strain

One limitation of using rodent, particularly mouse, models is the substantial influence of background strain (Crawley, 2008; O'Leary et al., 2013). Without any genetic manipulation, certain mouse strains are inherently more anxious than others. In general, BALB/c mice are regarded as more anxious; FVB/N mice show low anxiety, and C57BL/6 mice falls somewhere in between (Crawley, 2008).

In the present study, Deaf1 mice were bred on a BALB/c-C57BL/6 background. The Deaf1 mutation can be lethal when expressed throughout development (Hahm et al., 2004). For the present study, breeding the Deaf1 mice on the BALB/c-C57BL/6 background was preferred over a pure C57BL/6 background because a higher number of $Deaf1^{KO}$ mice survived. Future experiments may include a conditional Deaf1 model in which expression can be limited after the sensitive developmental period and allow for a greater number of surviving $Deaf1^{KO}$ mice.

Defining Depression

Possibly the greatest paradox in animal models of human disease is trying to develop a model that is specific enough to give conclusive findings about a particular gene, yet the model needs to be able to properly demonstrate human symptoms and behaviour. The common pitfall of a global knockout is that it is too broad of a model since the underlying developmental effects or how other parts of the brain uses that gene cannot be scientifically controlled and so any resulting behaviour is wrought with confounding factors. On the other hand, a conditional knockout model that targets a specific gene in specific brain region at a specific time-point is too restricted since human diseases are rarely carried on a single gene and any resulting behaviour cannot account for all of the symptoms and subtypes of a given disorder.

So how can both of these issues be addressed in order to develop a functional model of a multi-faceted disease such as depression? Perhaps by changing the definition of depression itself. Recall that human depression has such a wide variety of symptoms and can be classified into many subtypes. Some researchers are beginning to suggest that, instead of meeting a checklist of symptoms that funnels to a depression diagnosis, individual depressive symptoms should be addressed and treated accordingly (Anisman & Merali, 2001; Cryan & Holmes, 2005). Combined with more intensive behavioural tests (McIlwain et al., 2001; Milner & Crabbe, 2008; Rodgers & Johnson, 1995), this would allow animal models more reliability and be able to target a single symptom of depression, thus relating one symptom to potentially one gene.

Conclusion

Two genetically altered mouse models were used to study the effect of both mildly overexpressing (Deaf1) and greatly reducing (conditional 5-HT1A) 5-HT1A autoreceptors on anxiety and depression behaviour. 8-OH-DPAT induced hypothermia was used to assess function of 5-HT1A autoreceptors, which was blocked in 5-HT1A^{autoKO} mice, but not increased in Deaf1 knockout mice as expected.

In terms of behaviour, Deaf1 mutants showed an overall anxiety phenotype, yet no depression phenotype. The global Deaf1 knockout could have resulted in other compensatory mechanisms that lessened the behavioural phenotypes that are clearly shown in other published studies.

Conditional 5-HT1A mutants show an overall absence of an anxiety phenotype, which could be due to the presence of the autoreceptors during development. However the lack of depression phenotype may be due to a ceiling effect in the wildtype controls.

Further testing that involves a stress component may reveal genetic vulnerabilities to anxiety and/or depressive behaviour that may not be inherently present.

The results of these experiments add to the growing body of research pertaining to affective disorders and underscore the importance of gender in these studies. Although not always consistent with other published studies, the present results also illustrate the complexity of mental illness as well as the multitude of factors that influence such disorders.

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Appendix

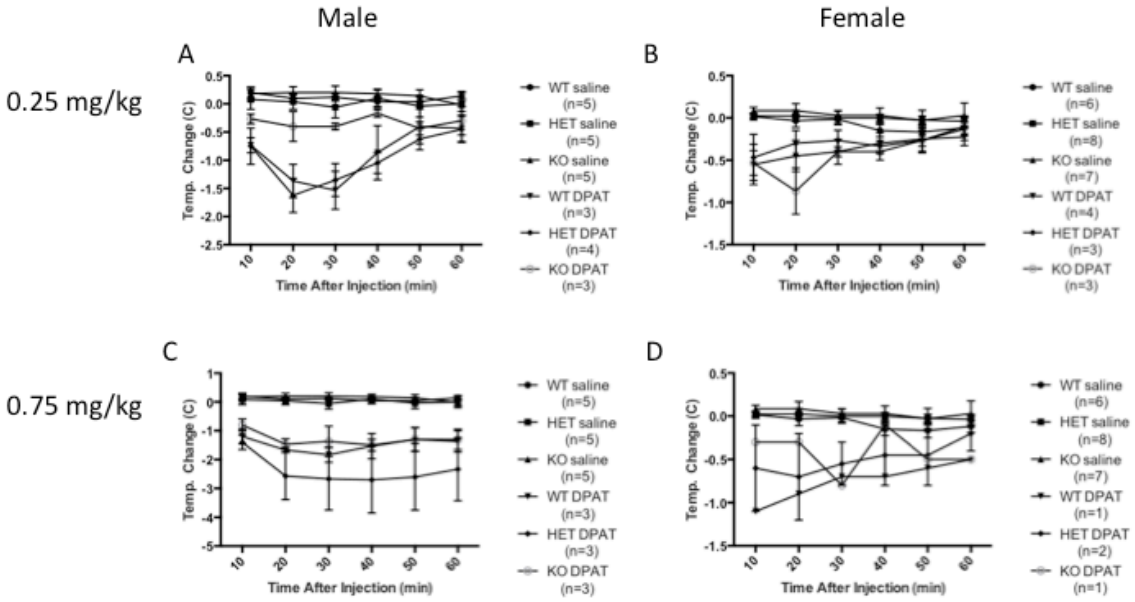


Figure i. Agonist-Induced Hypothermia in Deaf1 Mice: 0.25 mg/kg and 0.75 mg/kg

Given the unexpected results of 8-OH-DPAT-induced hypothermia in Deaf1 mice using the 0.5 mg/kg dosage, both a higher and a lower dose were used. A. A dose of 0.25 mg/kg of 8-OH-DPAT in male Deaf1 mice. B. A dose of 0.25 mg/kg in female Deaf1 mice. C. A dose of 0.75 mg/kg in males. D. A dose of 0.75 mg/kg in female mice. Statistical effects are shown in Table i.

male 0.25 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	11.55		10.62		5.30	
df	5		5		25	
p	**< 0.0001**	****	**< 0.0001**	****	**< 0.0001**	****

Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	**0.011**	**< 0.0001**	**< 0.0001**	**0.015**	> 0.9999	0.520
HET saline x HET DPAT	**0.015**	**< 0.0001**	**< 0.0001**	**0.0001**	0.288	> 0.9999
KO saline x KO DPAT	> 0.9999	0.408	0.408	> 0.9999	0.520	> 0.9999
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT DPAT x KO DPAT	> 0.9999	**0.025**	**0.004**	0.320	> 0.9999	> 0.9999

female 0.25 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	3.89		5.39		3.07	
df	5		5		25	
p	**0.003**	*	**0.002**	**	**< 0.0001**	****

Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	**0.003**	***0.078***	0.150	> 0.9999	> 0.9999	> 0.9999
HET saline x HET DPAT	**0.026**	0.550	> 0.9999	0.482	> 0.9999	> 0.9999
KO saline x KO DPAT	**0.002**	**< 0.0001**	0.1071	0.107	> 0.9999	> 0.9999
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT DPAT x KO DPAT	> 0.9999	0.27	> 0.9999	> 0.9999	> 0.9999	> 0.9999

male 0.75 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	6.69		10.99		1.72	
df	5		5		25	
p	**< 0.0001**	****	**< 0.0001**	****	**0.03**	*

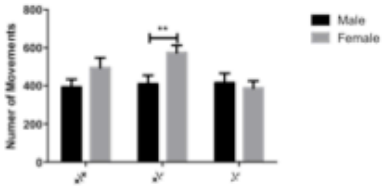
Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	***0.075***	**0.007**	**0.002**	**0.025**	0.107	**0.039**
HET saline x HET DPAT	**0.046**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
KO saline x KO DPAT	0.711	**0.014**	**0.027**	**0.013**	***0.059***	0.151
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	0.525	0.286	> 0.9999
WT DPAT x KO DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999

female 0.75 mg/kg	Main Effect of Time		Main Effect of Genotype		Interaction	
F	1.83		5.40		2.64	
df	5		5		25	
p	0.12		**0.003**	**	**0.0004**	***
Time	10	20	30	40	50	60
WT saline x HET saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x KO saline	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT saline x WT DPAT	**0.0002**	**0.009**	***0.093***	0.400	> 0.9999	> 0.9999
HET saline x HET DPAT	**0.010**	**0.001**	**0.040**	0.202	0.291	> 0.9999
KO saline x KO DPAT	> 0.9999	> 0.9999	**0.013**	> 0.9999	0.813	0.469
WT DPAT x HET DPAT	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999	> 0.9999
WT DPAT x KO DPAT	0.210	0.958	> 0.9999	0.958	> 0.9999	> 0.9999

Table i. Agonist-Induced Hypothermia in Deaf1 Mice: 0.25 mg/kg and 0.75 mg/kg Statistical results of a two-way ANOVA comparing genotype across time for male Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

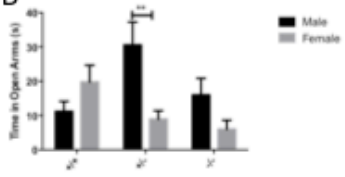
Beam Break

A



Elevated Plus Maze

B



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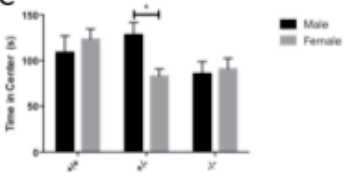

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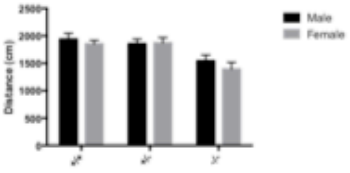
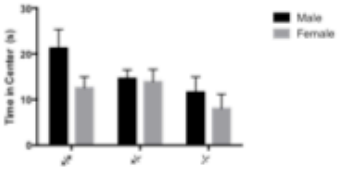


Open Field

E



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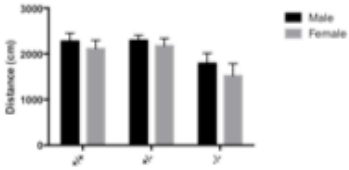
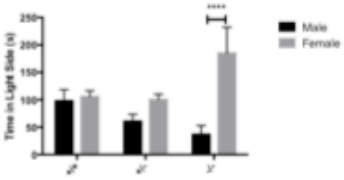


Light Dark Test

G



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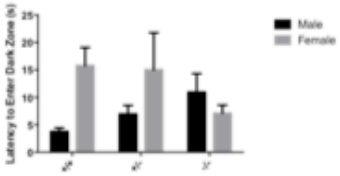

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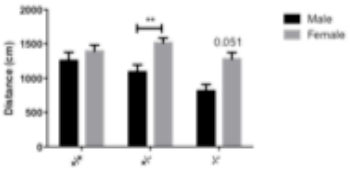


Figure ii. Gender Comparisons: Deaf1 Anxiety Assays

Comparisons between the gender and genotype were made for each anxiety measure to determine if the genders should be collapsed together for further statistical calculations. A. Number of movements in the Beam Break locomoter assay. B. Time spent in the opens arms of the EPM. C. Time spent in the center of the EPM. D. Distance traveled in the EPM. E. Time spent in the center of the OF. F. Distance traveled in the OF. G. Time spent in the light side of the LD test. H. Time taken to enter the dark zone for the first time in the LD test. I. Distance traveled in the LD test. Statistical effects are shown in Table ii.

			Main Effect of Gender		Main Effect of Genotype		Interaction		post hoc M x F		
BBK											
	Number of Movements	F	4.39		2.10		2.30		WT	0.455	ns
		df	1		2		2		HET	**0.009**	**
		p	**0.04**	*	0.13		0.11		KO	> 0.9999	ns
EPM											
	Time in Open Arms	F	3.69		1.55		5.60		WT	0.596	ns
		df	1		2		2		HET	**0.002**	**
		p	***0.06***	#	0.22		**0.01**	**	KO	0.625	ns
	Time in Center	F	0.58		1.88		3.18		WT	> 0.9999	ns
		df	1		2		2		HET	**0.032**	*
		p	0.45		0.16		**0.05**	*	KO	> 0.9999	ns
	Distance	F	0.80		9.77		0.37		WT	> 0.9999	ns
		df	1		2		2		HET	> 0.9999	ns
		p	0.37		**0.0001**	***	0.69		KO	> 0.9999	ns
OF											
	Time in Center	F	2.88		2.39		0.94		WT	0.127	ns
		df	1		2		2		HET	> 0.9999	ns
		p	***0.09***	#	0.10		0.39		KO	> 0.9999	ns
	Distance	F	1.46		5.31		0.08		WT	> 0.9999	ns
		df	1		2		2		HET	> 0.9999	ns
		p	0.23		**0.01**	**	0.92		KO	> 0.9999	ns
LD											
	Time in Light Side	F	19.32		1.58		6.83		WT	> 0.9999	ns
		df	1		2		2		HET	0.217	ns
		p	**< 0.0001**	****	0.21		**0.002**	**	KO	**< 0.0001**	****
	Latency to Enter Dark Side	F	1.99		0.09		1.26		WT	0.162	ns
		df	1		2		2		HET	0.439	ns
		p	0.16		0.91		0.29		KO	> 0.9999	ns
	Distance	F	14.75		3.18		1.51		WT	0.985	ns
		df	1		2		2		HET	**0.003**	**
		p	**0.0002**	***	**0.05**	*	0.23		KO	0.051	ns

Table ii. Gender Comparisons: Deaf1 Anxiety Assays

Statistical results of a two-way ANOVA comparing genotype and gender for Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

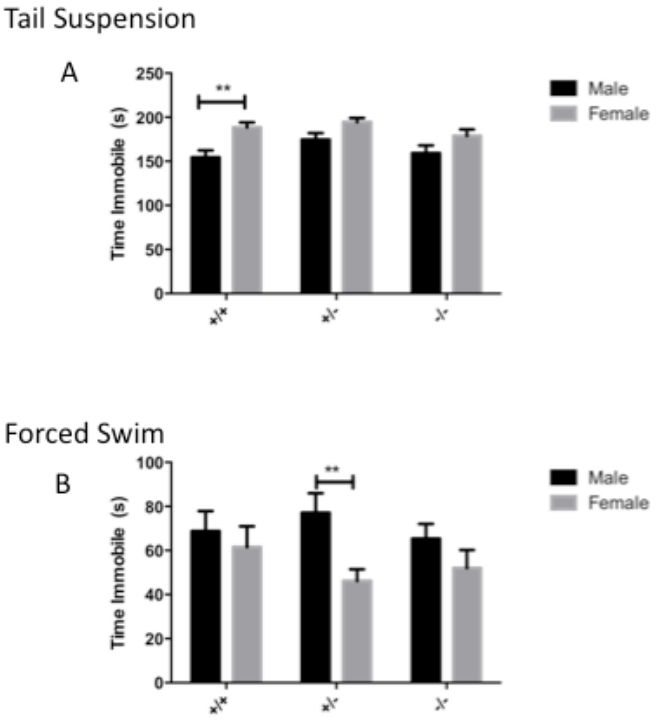


Figure iii. Gender Comparisons: Deaf1 Depression Assays

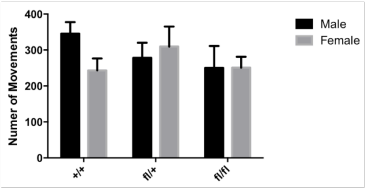
Comparisons between the gender and genotype were made for each depression measure to determine if the genders should be collapsed together for further statistical calculations. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile averaged across the final four minutes of the FS test. Statistical effects are shown in Table iii.

			Main Effect of Gender		Main Effect of Genotype		Interaction		post hoc M x F		
TS											
	Time Immobile	F	17.79		2.89		0.70		WT	**0.002**	**
		df	1		2		2		HET	0.114	ns
		p	**< 0.0001**	****	***0.06***	#	0.50		KO	0.225	ns
FS											
	Time Immobile	F	6.94		0.29		1.30		WT	> 0.9999	ns
		df	1		2		2		HET	**0.007**	**
		p	**0.01**		0.75		0.28		KO	0.723	ns

Table iii. Gender Comparisons: Deaf1 Depression Assays

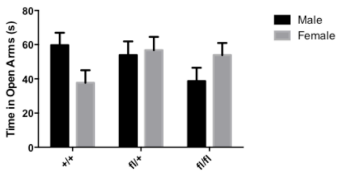
Statistical results of a two-way ANOVA comparing genotype and gender for Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

A Beam Break



Elevated Plus Maze

B



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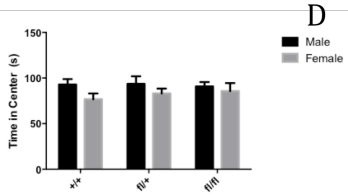

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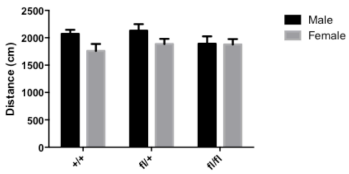
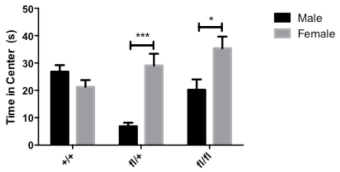
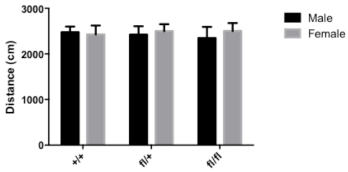


Open Field

E

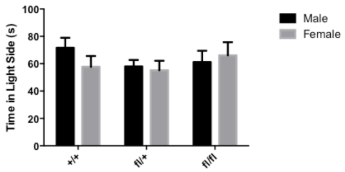


F

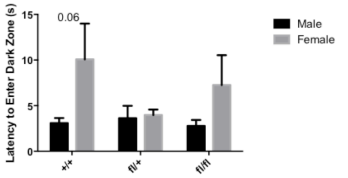


Light Dark Test

G



H



I

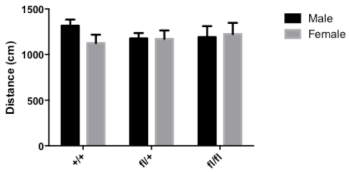


Figure iv. Gender Comparisons: flx5-HT1A Anxiety Assays

Comparisons between the gender and genotype were made for each anxiety measure to determine if the genders should be collapsed together for further statistical calculations. A. Number of movements in the Beam Break locomoter assay. B. Time spent in the opens arms of the EPM. C. Time spent in the center of the EPM. D. Distance traveled in the EPM. E. Time spent in the center of the OF. F. Distance traveled in the OF. G. Time spent in the light side of the LD test. H. Time taken to enter the dark zone for the first time in the LD test. I. Distance traveled in the LD test. Statistical effects are shown in Table iv.

			Main Effect of Gender		Main Effect of Genotype		Interaction		post hoc M x F		
BBK											
	Number of Movements	F	0.28		0.40		0.93		+/+	0.445	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.60		0.67		0.41		fl/fl	> 0.9999	ns
EPM											
	Time in Open Arms	F	0.04		0.64		2.88		+/+	0.132	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.84		0.53		***0.06***	#	fl/fl	0.595	ns
	Time in Center	F	3.33		0.17		0.33		+/+	0.278	ns
		df	1		2		2		fl/+	0.981	ns
		p	***0.07***	#	0.84		0.72		fl/fl	> 0.9999	ns
	Distance	F	4.62		0.67		1.06		+/+	0.105	ns
		df	1		2		2		fl/+	0.391	ns
		p	**0.03**	*	0.51		0.35		fl/fl	> 0.9999	ns
OF											
	Time in Center	F	11.05		3.05		7.06		+/+	0.869	ns
		df	1		2		2		fl/+	**0.001**	***
		p	**0.001**	***	**0.05**	*	**0.001**	***	fl/fl	**0.021**	*
	Distance	F	0.17		0.02		0.17		+/+	> 0.9999	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.69		0.98		0.84		fl/fl	> 0.9999	ns
LD											
	Time in Light Side	F	0.35		0.55		0.68		+/+	0.660	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.56		0.58		0.51		fl/fl	> 0.9999	ns
	Latency to Enter Dark Side	F	4.72		0.81		1.15		+/+	0.058	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	**0.03**	*	0.45		0.32		fl/fl	0.490	ns
	Distance	F	0.43		0.10		0.73		+/+	0.499	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.51		0.90		0.49		fl/fl	> 0.9999	ns

Table iv. Gender Comparisons: flx5-HT1A Anxiety Assays

Statistical results of a two-way ANOVA comparing genotype and gender for Deaf1 mice. Bold font indicates significance (*); bold italic indicates a non-significant trend (#).

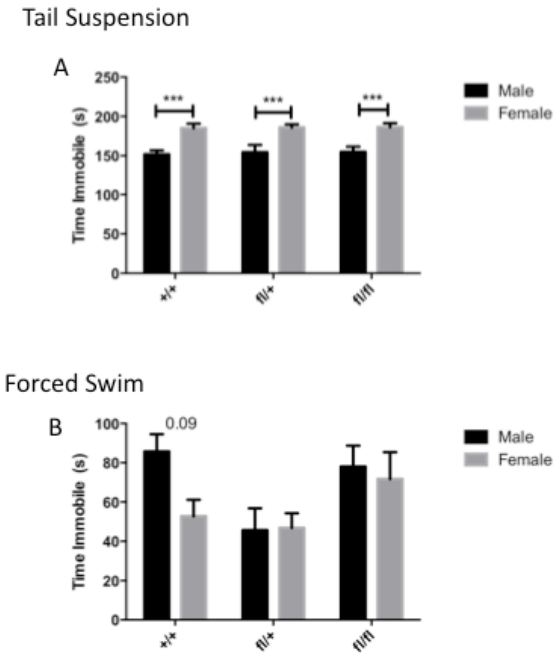


Figure v. Gender Comparisons: flx5-HT1A Depression Assays

Comparisons between the gender and genotype were made for each depression measure to determine if the genders should be collapsed together for further statistical calculations. A. Time spent immobile averaged across the final four minutes of the TS test. B. Time spent immobile averaged across the final four minutes of the FS test. Statistical effects are shown in Table v.

			Main Effect of Gender		Main Effect of Genotype		Interaction		post hoc M x F		
TS											
	Time Immobile	F	48.16		0.09		0.02		+/+	**0.0002**	***
		df	1		2		2		fl/+	**0.001**	***
		p	**< 0.0001**	****	0.91		0.98		fl/fl	**0.0004**	***
FS											
	Time Immobile	F	2.10		3.86		1.40		+/+	0.090	ns
		df	1		2		2		fl/+	> 0.9999	ns
		p	0.15		**0.02**	*	0.25		fl/fl	> 0.9999	ns

Table v. Gender Comparisons: flx5-HT1A Depression Assays

Statistical results of a two-way ANOVA comparing genotype and gender for Deaf1 mice. Bold font indicates significance (*****).

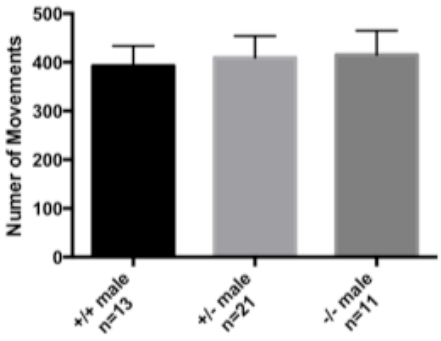


Figure vi. Deaf1: Male Locomotor Assay

The number of movements in the Beam Break test was used to evaluate locomotion in the mice. Statistical effects are shown in Table vi.

Deaf1 male				post hoc	
BBK					
	Number of Movements	F	0.05	WT x HET	> 0.9999
		df	2	WT x KO	> 0.9999
		p	0.95		

Table vi. Deaf1: Male Locomotor Assay

Statistical results of a one-way ANOVA comparing genotypes for male Deaf1 mice.

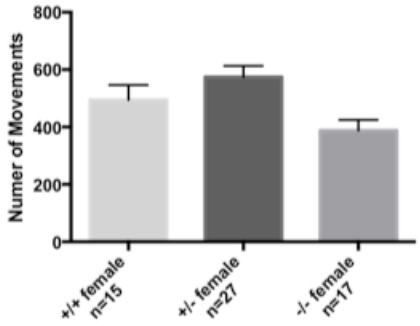


Figure vii. Deaf1: Female Locomotor Assay

The number of movements in the Beam Break test was used to evaluate locomotion in the mice. Statistical effects are shown in Table vii.

Deaf1 Female					post hoc	
BBK						
	Number of Movements	F	5.09		WT x HET	0.388
		df	2		WT x KO	0.235
		p	**0.01**	**		

Table vii. Deaf1: Female Locomotor Assay

Statistical results of a one-way ANOVA comparing genotypes for male Deaf1 mice. Bold font indicates significance (*).

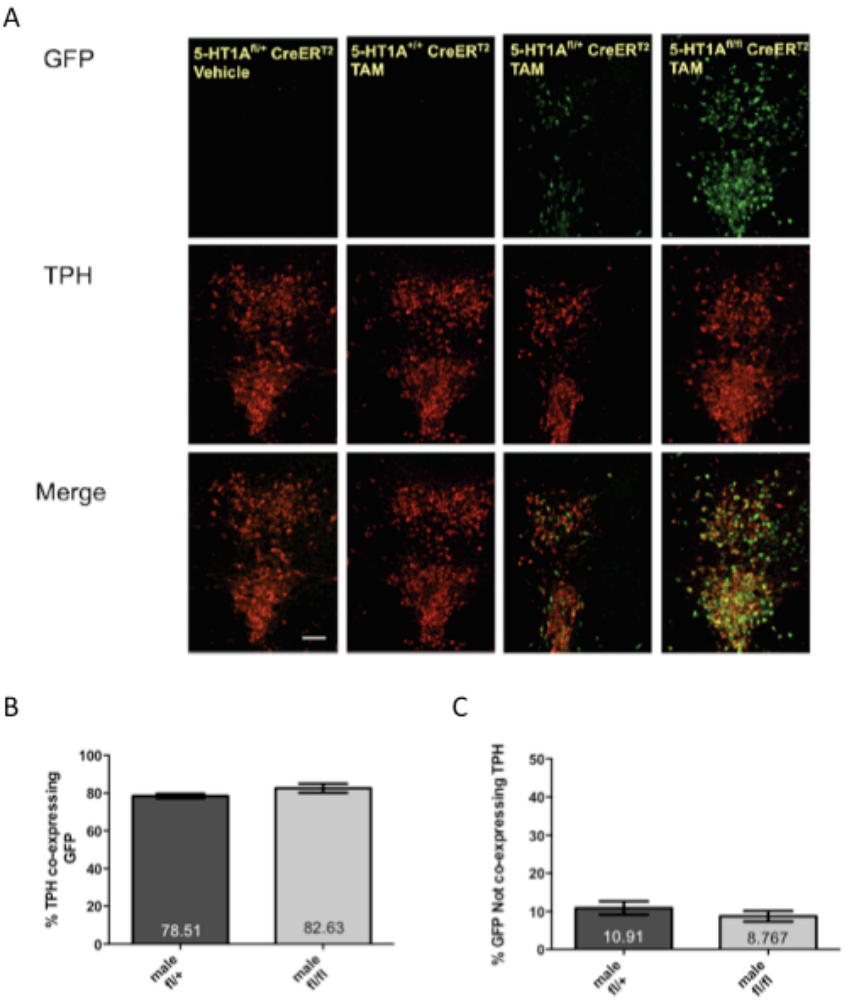


Figure viii. Immunofluorescent Staining of Dorsal Raphe in Conditional 5-HT1A Mice: Males

Male mice. A. Immunofluorescent staining for TPH (mouse anti-TPH, 1:500) and GFP (chicken anti-GFP, 1:2000) was performed on dorsal raphe slices of flx5-HT1A mice. B. Quantification of TPH-positive cells expressing GFP. C. Quantification of TPH-positive cells not expressing GFP. Scale bar indicates 100 μm.

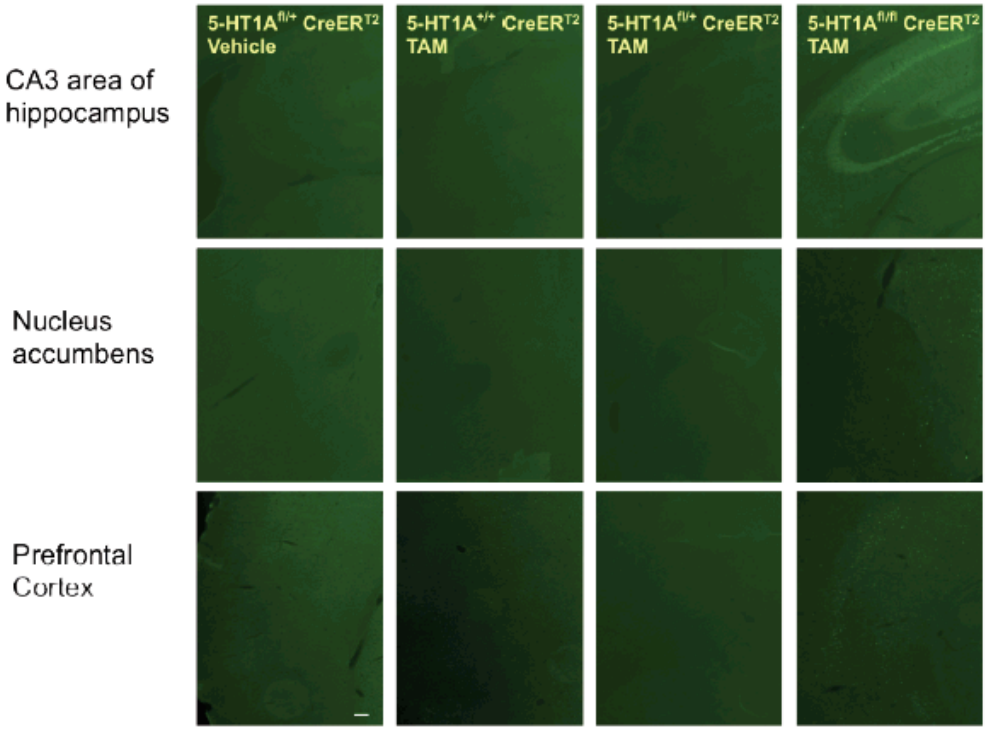


Figure ix. Immunofluorescent Staining of 5-HT1A Heteroreceptor-Dense Regions in Conditional 5-HT1A Mice: Males

Male mice. Immunofluorescent staining for GFP (chicken anti-GFP, 1:2000) was performed on other brain regions known to be rich in 5-HT1A receptors. Scale bar indicates 100 μm.

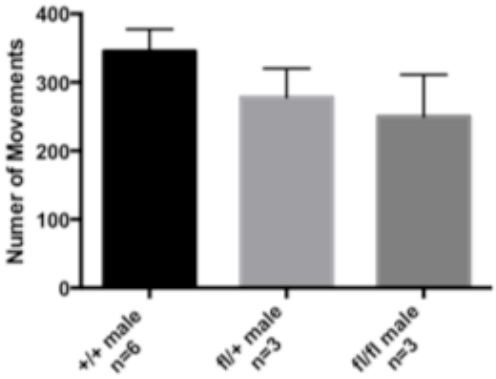


Figure x. flx5-HT1A: Male Locomotor Assay

The number of movements in the Beam Break test was used to evaluate locomotion in the mice. Statistical effects are shown in Table viii.

flx1A Male				post hoc	
BBK					
	Number of Movements	F	1.48	+/+ x fl/+	0.571
		df	2	+/+ x fl/fl	0.288
		p	0.28		

Table viii. flx5-HT1A: Male Locomotor Assay

Statistical results of a one-way ANOVA comparing genotypes for male Deaf1 mice.

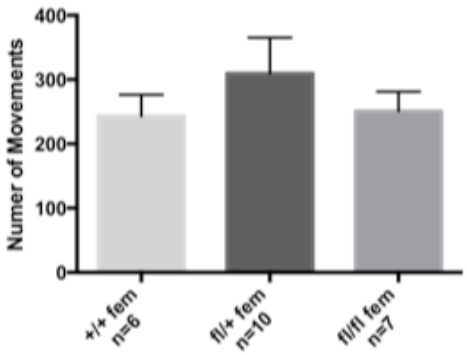


Figure xi. flx5-HT1A: Female Locomotor Assay

The number of movements in the Beam Break test was used to evaluate locomotion in the mice. Statistical effects are shown in Table ix.

flx1A Female				post hoc	
BBK					
	Number of Movements	F	0.64	+/+ x fl/+	0.685
		df	2	+/+ x fl/fl	> 0.9999
		p	0.54		

Table ix. flx5-HT1A: Female Locomotor Assay

Statistical results of a one-way ANOVA comparing genotypes for male Deaf1 mice.

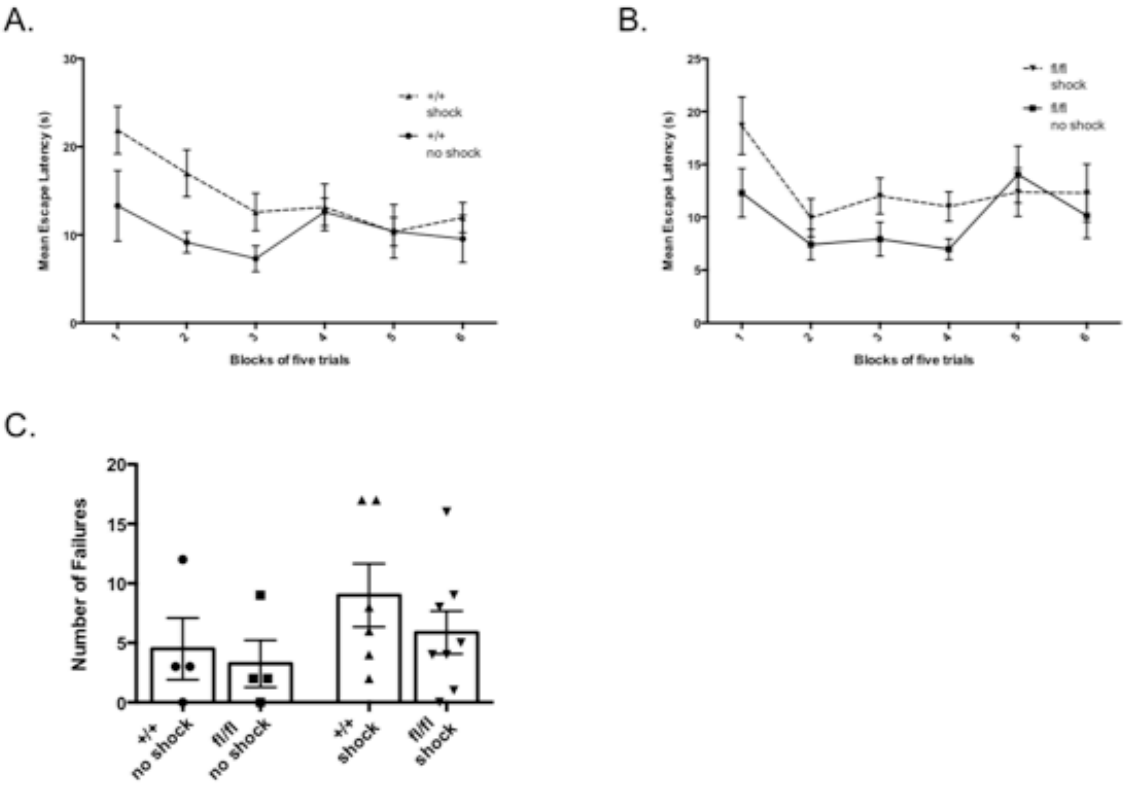


Figure xii. Preliminary Learned Helplessness Results on Conditional 5-HT1A Mice

A. Escape latency averaged in six blocks of five trials per block. Shock-5-HT1A^{autoWT} mice have longer latencies in the first three blocks than no-hock-5-HT1A^{autoWT} mice. B. Shock-5-HT1A^{autoKO} mice show similarly longer latencies than no-hock-5-HT1A^{autoKO} mice, however the difference appears greater in shock-5-HT1A^{autoWT} mice. C. Number of failures to escape the shock; shock-5-HT1A^{autoWT} mice have the highest number of failures. Shock-5-HT1A^{autoWT} mice have a larger number of mice with high frequencies of long latencies and failures.

Due to low n values, statistical analyses were not performed.

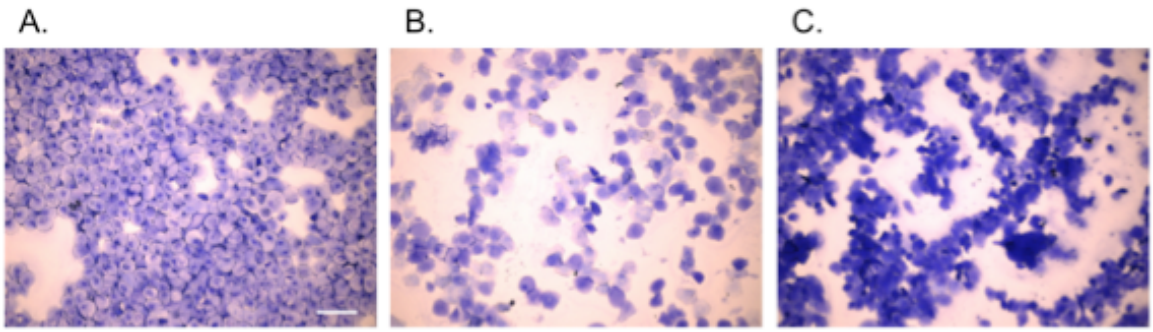


Figure xiii. Estrus Cycle Staining

Vaginal smears were collected over four days to evaluate the cycle distribution across females. Representative images are shown for proestrus (A), estrus (B), and metestrus/ diestrus (C) in flx-5-HT1A females. Slides were stained using 0.1% cresyl violet. Scale bar represents 100 μm.