The 5-HT1A-R Knockout Mouse as a Model of Later Life Anxiety Disorders: Implications for Sex Differences

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The $5-HT_{1A}$-R Knockout Mouse as a Model of Later Life Anxiety Disorders: Implications for Sex Differences

By

Tatyana B. Budylin

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2019
This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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By
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Advisor: Probal Banerjee

Anxiety affects nearly twice as many women as it affects men across all cultures and economic groups. Importantly, girls have a higher chance of inheriting anxiety disorders than boys, and many anxiety disorders appear at a very young age. However, little is known about sex differences in brain and behavioral development and how they relate to anxiety in adulthood. Serotonin 1A receptor ($5-HT_{1A}$-R) mediated signaling has been implicated in depression and anxiety, however most studies that focus on the involvement of the $5-HT_{1A}$-R have been conducted in adults. Little is known about how the $5-HT_{1A}$-R might be linked to behavioral and brain development and if sex differences play an important role in this process. A possible time window during which brain development might be vulnerable to aberrant signaling is during the first few weeks of life in the mouse, which may correspond to the first 3-6 months in human infants. This critical period is characterized by a peak in neurogenesis at around postnatal day 8 (P8) in mice, and the beginning of synaptogenesis thereafter. Although neurogenesis has been found to be decreased in the brains of depressed patients, and sex differences have been recorded, the link between postnatal neurogenesis and adult anxiety has not been investigated until now. In addition, the first few weeks of life in the mouse are also characterized by a series of behavioral milestones including ultrasonic vocalizations, righting, grasping and startle reflexes. The $5-HT_{1A}$-R knockout (KO) mouse model of anxiety has been used extensively to
investigate anxiety and depressive behaviors, but not in the context of brain and behavioral development. In this project, postnatal behavioral milestones were evaluated in relation to anxiety, and neurogenesis and synaptogenesis were also investigated. We observed sex differences in the production of ultrasonic vocalizations on P8, adult behavior, and postnatal neuroproliferation. KO female mice produced more vocalizations on P8 and, in adulthood, displayed elevated anxiety on the elevated plus maze, open field, and light dark chamber tests. In general, we observed a significant linear relationship between the number of calls at P8 and anxiety behavior in adulthood. The KO female mice also had lower levels of neuroproliferation, but higher amounts of post-mitotic cells in the dentate gyrus at P8. No significant differences were observed in synaptogenesis in the CA1 region of the hippocampus at P10. Together these results showed that the female mice were more prone to anxiety. Female-specific aberrances did appear postnatally in both behavioral features as well as brain development. These predictive phenotypes of anxiety can be used in future rescue studies to investigate early sex-specific interventions.
Acknowledgments

This project would not have been possible without the generous help of more than a few people. I appreciate the valuable help of Dr. Noboru Hiroi at the Albert Einstein College of Medicine in sharing his PLSDA code. Dr. Michael Beckert at the Albert Einstein College of Medicine helped us with his Markov model code. Drs. Tim Holy and Terra Barnes wrote and helped explain their USV detection code in MATLAB so that we would be able to adapt it for our model. We are also thankful for the undergraduate and high school students who manually detected USVs in the pilot studies, Sri Yalamanchi, Kelly Beharry, Maria Klauber, and Youstina Soliman. We would also like to thank Dr. Kathy K. Chadman, for helping us brainstorm for this project, and for allowing us to use her LD test. We would also like to thank everyone else on the neuroscience advisory committee at CUNY who gave valuable feedback for this project, Drs. Daniel P. McCloskey, Greg R. Phillips, Kathy Chadman, Sara Guariglia, Lorenz S. Neuwirth, Jeffrey Goodman, and Probal Banerjee. Dr. Abdeslem El Idrissi allowed us to use his AnyMaze software, and helped make the behavioral testing possible by providing the necessary recording tools and software. And an extra word of thanks will have to go out to my mentor, Dr. Banerjee, as without his support this would not have been possible.
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1. General Information

1.1 The Nature of Anxiety

The Diagnostic and Statistical Manual of Mental Disorders (DSM)-V defines anxiety disorders as sharing features of excessive fear and anxiety [1]. Whether fear and anxiety are the same behavioral expression of a underlying psychological phenomena has remained open to debate [2, 3]. Mainly, Freud represented a group of theorists that hold the position that fear is a reaction to a specific observable dangerous cue, whereas anxiety is necessarily a non-cue induced apprehensiveness [2]. Anxiety has also been termed as “unresolved fear,” in which avoidance of a fear stimulus is disrupted [3]. As mentioned before, in situations that lack a cue, or rather an obvious cue, anxiety may be the result of overestimating the threat in such an ambiguous situation. Epstein [3] further elaborated on this theory that this anxiety inducing event, in combination with a lack of avoidance options, could lead to apprehension and indecision.

Fear is often characterized as involving the bodily “fight or flight response,” or a heightened autonomic arousal [4] largely involving the cardiovascular system [2]. This leads to peripheral blood vessel constriction, which in turn, raises arterial pressure and decreases blood flow to the extremities [2]. Accompanying these cardiovascular events, the salivary glands loose blood supply, leading to dry mouth, and as a result an increased muscle tension in the laryngeal muscles produces a high-pitched voice [4]. Heart rate and respiration also increase, and blood flow is redirected to skeletal muscles in preparation for defense [2, 4]. However, fear and anxiety could be defined by their different durations in arousal, with fear having an acute and anxiety having a chronic duration typically characterized as a state of hypervigilance [3, 5]. Fear
can also be characterized as a defense mechanism used to deal with the present, as opposed to anxiety, which is a defense in response to future circumstances [3]. Gray described anxiety as a “behavioral inhibition system,” with the purpose of regulating inhibition of ongoing behavior; by which, the increase in vigilance and arousal, are caused by painful, punishing, novel, and uncertain stimuli [6].

The DSM-V lists separation anxiety disorder, selective mutism, specific phobias, social anxiety disorder (i.e., social phobia), panic disorder, agoraphobia, and generalized anxiety disorder as the main anxiety disorders [1]. The symptoms of generalized anxiety disorder that are used as diagnostic criteria in humans include: restlessness or feeling on edge, being easily fatigued, difficulty concentrating, irritability, muscle tension, and/or sleep disturbance [1]. These symptoms fall in line with the hypervigilance that Gray, Epstein, and Barlow [2, 3, 6] have characterized as being an important part of anxiety. Interestingly, Gray [6] argued that phobias should be considered separate from the anxiety system as in experiments, phobic behavior is insensitive to anti-anxiety medication and seemingly relies on specific fear learning circuitry centers, such as the amygdala (as opposed to the hippocampus, which they suggested was central to anxiety). Taken together, these range of symptoms provide a unified concept of the anxiety experience, in that it

Figure 1. Well-known people with anxiety. These people have/had anxiety with another mental disorder. (a) comedian Maria Bamford (b) author and journalist Hunter S. Thompson, a suicide victim (c) famous physicist Ludwig Boltzman, suicide victim (d) artist Edvard Munch.
stems from an apprehension of an unavoidable future threat rather than an immediate danger.

1.2 Prevalence and Burden

A select few of celebrity and/or historical figures with anxiety and depression are shown in Figure 1. Steel and colleagues [7] conducted a meta-analysis of epidemiological studies of common mental health disorders including those conducted from 1980 – 2013. What they found was that for the most part, there was a high degree of heterogeneity between many of the survey responses. The findings could have been either due to a limited sample size and/or the type(s) of questions that were asked in the survey [2, 7]. Pooling from data across many studies, what they found was that about 1 in 5 (17.6%) adults had met the criteria for a common mental health disorder during a 12-month period [7]. However, the lifetime prevalence, which is a more robust expression of the prevalence of mood disorders, as it surpasses a period of 1 year was nearly twice as high (i.e., ~ 30%) [7]. Importantly, this meta-analysis was more informative than many independent research studies, as it took into account cross-cultural surveys from international origins [7]. For anxiety disorders, the pooled period prevalence was about 7%, whereas the lifetime prevalence was approximately 13% [7]. These estimates of mental health disorders seem dramatically lower than Kessler’s [8] commonly cited 1994 study, where he found that the 1-year period prevalence, at least among the U.S. population was 17% and the lifetime prevalence was 20%. However, it is noteworthy that among the many mental health disorders, anxiety had the highest prevalence, corroborating with the rates reported by Steel [7]. Considering that the research conducted on the anxiety prevalence was quite fragmented, Olivia Remes and colleagues [9] later conducted a systematic review on prior reports regarding the prevalence of anxiety disorders in adults to try to resolve the gaps in the literature. The pooled year and lifetime prevalence from Remes et al. [9] were estimated to be 11% and 17%, respectively.
Given these more conservative results, there appears to be a 1 in 10 chance that an adult could be affected by anxiety in a given year, and further, a 1 in 5 chance that they could also be affected by anxiety at some point during their lifetime.

Despite the fragmentary results regarding the epidemiology of anxiety, what the above-listed studies all conceded to was that females were almost twice as likely to be affected by anxiety than their male counterparts [7-10]. The estimated prevalence for the 1-year period offered by Steel [7] was 5.2-8.7% for women and 3.7-4.9% for men, whereas Remes et al. [9] suggested that the lifetime prevalence was 16.2-20.4% in women and 8.8-11.6% in men. Moreover, these alarming sex differences were also shown to be prevalent on a global level [9].

The annual economic costs to address anxiety disorders within the US is estimated to be $42.3 billion [11]. These economic costs were from values estimated on 1990 prevalence data, and would come out to approximately $81.3 billion in 2018. The estimate given by the Global Burden of Disease is that 26.8 million disability-adjusted life years was attributed to anxiety disorders [9]. In addition, the remission rate for Major Depressive Disorder (MDD) is fairly high, whereas anxiety disorders tend to persist for long periods of time [2, 12]. Recurrence rates over a long-term period for adult patients with MDD have been found to be 35% compared to 39-58% for anxiety disorders [12]. Together these mental health issues, their prevalence, and their remission or recurrence rates underscore the clear need for better understanding of the sex-based neurobiological differences in order to establish better treatments.

Even more alarming are the statistics on children and adolescents that are diagnosed with anxiety. The median age of onset for an anxiety disorder in children, including separation anxiety and phobias, ranges from 7-14 years of age [13]. Separation anxiety disorder is known to affect 4% of children and adolescents [14]. According to Wehry [15] the age of onset of any
anxiety disorder starts as early as two years of age. According to Beesdo [16] at 6-8 months, shyness and anxiety with a stranger correspond to separation anxiety disorder, whereas toddlerhood (i.e., 12-18 months and 2-3 years) is when separation anxiety, as well as an emergence of specific phobia, can be observed. Moreover, the mean age of onset for overanxious disorder (also known as Generalized Anxiety Disorder [GAD]) is 5-10 years [17]. Although sex differences as a function of anxiety are enhanced with age [17-19], cumulative incidences of anxiety are reported to be higher in girls than in boys starting in early childhood, as shown by Breslau and Merikangas [20, 21]. Separation anxiety disorder is also specifically known to be more frequent in females than males, and shares comorbidity with GAD, depression, and somatic complaints [14]. The early emergence of anxiety disorders is rather serious, as it highlights that early life prevention and proper treatment interventions for anxiety are warranted.

Anxiety disorders are usually observed to be comorbid with 60% of depressed patients [22]. Anxiety symptoms are also known to precede the onset of depressive symptoms [20]. Intriguingly, Breslau [20] found that preexisting anxiety increased the chances of acquiring depression and that this observation was sex-dependent. Furthermore, their research emphasized that sex differences in MDD were specifically due to sex differences in preexisting anxiety conditions and that any sex differences that might have been observed in MDD could perhaps be explained by these findings [20]. The correlation between MDD and sex was reduced by 50% when anxiety was taken into account; therefore, suggesting that the relationship was mainly due to sex differences in anxiety [20].

1.3 Behavioral Predictors of Anxiety
Predictors of anxiety can be summarized as a set of personality traits that appear at various developmental stages across the lifespan. Typically, these involve a blend of theories that include Eysenck’s neuroticism, Gray’s trait-anxiety, and Kagan’s behavioral inhibition [16].

Briefly, Eisenck [2] rated personality in terms of extrovertedness and introvertedness, as well as neuroticism and stability. In this model, neurotic individuals tend to have a high level of autonomic arousal and consequently, are highly reactive [2]. Gray [2] described an active and sensitive behavioral inhibition system in play, which establishes the role of suppressing behavior as a reaction to novel and punishing stimuli. Finally, Kagan’s theory of behavioral inhibition more specifically applies to behavioral predictors in children[2]. What he observed was that a certain number of children would have a consistently “stable” characteristic of interacting with novel stimuli or strangers [23]. The inhibitory aspect of their behavior was seen in their predilection to withdraw from unfamiliar people, as well as would appear fearful or distressed, and would suppress ongoing social behavior [23]. About 70% of these children who would go on and be tested for anxiety levels at 4 years of age, would consistently be diagnosed with an anxiety disorder. In addition, behaviorally inhibited children have been selected for as early as the age of 4 months, in which their right frontal cortex had higher electroencephalograph (EEG) activity, a consistent signature of highly reactive children at risk of developing anxiety disorders [24, 25].

Indeed, signs of emotional development seem to emerge early on in infancy. Innate emotional responses seem to emerge at 3-months of age when facial expressions of infants in various emotional states start to show consistency with those of adults [2]. Importantly, these facial expressions exist across cultures, and communicate to parents the needs of the infant, and therefore how to interact with them [2]. Other compounded emotional displays and expressions
such as crying, have also been a focus of investigation, especially as it can signal stranger distress, a type of anxiety present in early infancy [2]. Infant crying has been increasingly investigated, whereby spectrograms have been an area of current research directed towards analyzing the predictive qualities of phonation on present or future illnesses [26-29]. Another active area of research is the response and perception of adults to infant crying [30, 31]. In summary, there are various predictors of anxiety that rely on the range of emotional expression(s) that are quantifiable at different developmental stages of development.

1.4 Genetic Predictors of Anxiety: The Serotonin 1A Receptor Gene

A seminal study by Avshalom Caspi [32] sought to investigate the extent to which the interplay between genes and the environment affected stress and depression. The study concluded that a polymorphism within the serotonin transporter promoter modulated the influence of stress and subsequently depression [32]. In a similar study, Caspi and colleagues [33] also showed that children who grew up to develop antisocial behavior were more likely to do so if they had a polymorphism in the Monoamine Oxidase A (MAO) gene. In addition to the serotonin transporter gene, the serotonin 1A receptor (5-HT1A-R) gene has also been implicated in depression [34]. Since Selective Serotonin Reuptake Inhibitors (SSRIs) are effective antidepressants, as well as anti-anxiety medication, and anxiety is comorbid in 60% of depressed patients, many of the theories on the interaction between the 5-HT1A-R and depression have also been extended to anxiety disorders [35]. The 5-HT1A-R gene has been observed to have a polymorphism in suicide patients, which could confer a biomarker of suicide susceptibility [36]. This potential suicide biomarker was later discovered to be a C-1018G single base-pair change in the promoter region of the 5-HT1A-R gene [37]. What was most remarkable was that Lemonde and Albert [38] found that the G/G phenotype had a much stronger ($p < 0.01$) association with
depressed females than males ($p = 0.0574$). Notably, this particular allele has also been linked to the decrease in SSRI responsivity seen in patients and the direct agonist of the 5-HT$_{1A}$-R, flibanserin [36].

1.5 Distribution of 5-HT$_{1A}$-R in the Brain and Corresponding Molecular Signaling Pathways

The neurons that produce serotonin (i.e., serotonergic neurons) are part of the raphe nuclei and are distributed along the entire length of the brainstem [39]. Through the neuraxis, the caudal raphe nuclei project their efferent axonal pathways that descend through the brainstem, whereas the dorsal raphe nuclei (DNR) project their efferent axons dorsally throughout the rest of the forebrain and cortex (Figure 2) [39]. The 5-HT$_{1A}$-R is found in both the presynaptic serotonergic neurons of the midbrain dorsal raphe nucleus as somatodendritic autoreceptors and in postsynaptic targets as heteroreceptors [40, 41]. Mainly, the heteroreceptors are found throughout most of the brain including the hippocampus, septum, cortex, thalamus, and hypothalamus [34, 40, 42]. Levels of mRNA have also been localized to 60% of glutamatergic and 25% of GAD-expressing cells, and are even observed in glial cells [42]. 5-HT$_{1A}$-R labeling with [³H]8-OH-DPAT has been found in the CA1 region of the hippocampus stratum oriens and stratum radiatum [42]. Importantly, 5-HT$_{1A}$-R have also been found in the Dentate Gyrus (DG) region of the hippocampus [43]. As a result of the widespread presence of the receptor, aberrations in 5-HT$_{1A}$-R function would likely
affect various facets of brain activity, from emotional regulation to learning. Intriguingly, the levels of autoreceptor binding have been shown to be increased in the dorsal rostral raphe of depressed suicide victims, and this was more pronounced among women [44].

In establishing the early serotonergic systems, the 5-HT$_{1A}$-Rs are expressed early during embryonic development and their appearance in different cells is dynamic during postnatal stages [45]. Initially, 5-HT$_{1A}$-R transcripts can be detected within the rodent brain on Embryonic Day 12 (E12), and continually increase until they reach their maximal level at E15, before reducing to low levels before birth [45]. The receptors are first seen in the hippocampus on E16 after mitosis completion, and then on interneurons in the stratum radiatum and stratum oriens on E18 [42, 45]. After birth, on Postnatal Day 5 (P5) the 5-HT$_{1A}$-Rs receptor can be localized to cell bodies, whereas on P10 they appear proximal apical dendrites of pyramidal and granule cells[42]. At the start of rodent maturation on P21, the distribution of 5-HT$_{1A}$-R on pyramidal hippocampal neurons in the stratum radiatum of the hippocampus decreases [42].

1.6 Implications of molecular disfunction in 5-HT$_{1A}$-R: From Genes to Proteins

The 5-HT$_{1A}$-R genes is intronless, like the rest of the 5-HT$_1$ receptor genes, and is localized to the chromosome 5q11.2-q13 [46, 47] (Figure 3). Transcriptional repressors of the

![Figure 3. Chromosome 5. Arrow is pointing to location of 5-HT$_{1A}$-R gene. Image credit: Ensembl.](image-url)
gene are known to be Freud-1/CC2D1A and Freud-2/CC2D1B[36, 48]. Transcription factors, Deaf1 and Hes1/Hes5 also act as repressors at the polymorphism site (C(-1019)G) on the 5-HT\textsubscript{1A}-R gene [36]. The G-allele, however, lacks this repression by Deaf1 thereby leading to over-transcription of the gene [49]. Alternative splicing of the receptor has been a mechanism recently implicated in MDD as well [50]. The 3'-UTR is alternatively spliced yielding two splice variants, which do not appear in mice [50]. These variants have a greater translational output than those, which are unspliced and were found to be reduced in patients with MDD[50]. This novel mechanism significantly changes the conceptual framework by which, the expression of the gene was once thought to be regulated. The implications of this new construct for this genes regulation will be addressed and further described in the section that follows.

The 5-HT\textsubscript{1A}-R may represent an ancestral archetypical 5-HT receptor, being that evolutionarily conserved similarities have been found in 5-HT receptors between invertebrates and nematodes [46]. The 5-HT receptor has been implicated to regulate a large range of physiological and behavioral functions including: neuroendocrine function, thermoregulation, vasoreactive headaches, sexual behavior, food intake, tooth-germ morphogenesis, memory, immune function, aggression, depression, and most importantly anxiety [47]. The 5-HT\textsubscript{1A}-R is a heptahelical G-protein coupled-receptor (Figure 4) and was the first of the 14 known receptors to be cloned [51]. The amino terminus of the 5-HT receptor is located on the outside of the cell, while the carboxyl terminus is located on the inside [52]. The lipophilic transmembrane regions sit

![Figure 4. 5-HT\textsubscript{1A}-R structure.](image)
within the plasma membrane, while the hydrophilic residues form the 5-HT receptor core [52].

Mutations in the 5-HT receptor transmembrane domains 2, 3, 5, and 7 have all been shown to influence and/or directly affect ligand binding [51]. The third intracellular loop is involved in G-protein coupling and is likely the site of Protein Kinase C (PKC) phosphorylation of the 5-HT receptor [51]. Some of the ligands that bind the 5-HT$_{1A}$-R are (±)-8-hydroxy-2-(di-N-propylamino)tetrinal (8-OH-DPAT), (R)-(−)-10-methyl-11-hydroxyaporphine (MHA), buspirone and derivatives (i.e., gepirone, ipsapirone, tanospirone, and zalospirone), WAY 100635 (WAY), BMY 8227, NAN-190, RK-153, flesinoxan, propranolol, MEP 125, MPPF, S14063, binospirone, and finally serotonin (5-hydroxytryptamine; 5-HT) [52, 53]. Among these, WAY, binospirone, and S14063 are 5-HT receptor antagonists, with WAY having the strongest affinity for the receptor [52, 54, 55]. Many of these 5-HT receptor ligands have been used to explore 5-HT$_{1A}$-R function in their radioactive forms.

Serotonin (5-HTP), discovered in 1948 [47], is a monoamine neurotransmitter ligand of 5-HT$_{1A}$-R, which is synthesized in a two-step reaction from tryptophan [56]. The first synthetic step is catalyzed by the enzyme tryptophan hydroxylase (TPH), while the second is catalyzed by aromatic amino acid decarboxylase (AADC) with TPH being the rate-limiting enzyme [56]. In children, the synthesis of serotonin has been found to be 200% of that of adults until 5 years of age and interestingly declines earlier in girls than in boys [57]. Serotonin is known to be involved in many processes, including the regulation of feeding behavior, mood, aggression, and pain [51]. Lacking sufficient amounts of serotonin developmentally have been associated with various behavioral abnormalities such as hyperactivity, imbalanced circadian rhythms, [58] and anxiety [34]. Tryptophan hydroxylase 2, the neuronal isoform of TPH, has also been implicated in depression, with increasing levels found in depressed humans that committed suicide [59].
1.7 5-HT₁A-R Signaling: Different Brain Regions

The main type of G-protein signaling linked with the 5-HT₁A-R is mainly inhibitory, and this is accomplished usually through the \( G_{i/o} \) mediated inhibition of adenylyl cyclase (AC) [47]. The release of \( \beta\gamma \)-subunits causes an interaction between G-protein-gated inwardly rectifying K⁺ (GIRK) channels, thereby releasing intracellular K⁺ out into the extracellular space, and hyperpolarizing the cell [47]. This can occur in both hippocampal and cortical neurons. One important exception to this is the dorsal raphe nuclei, where the 5-HT₁A-R coupling to G-proteins utilize a different mechanism[42]. The 5-HT₁A-R in the dorsal raphe nuclei may signal preferentially through Gi₃ G-protein leading to partial inhibition of AC [60]. The 5-HT₁A-R can also couple to phosphatidylinositol-specific (PI)-PLC, resulting in the generation of secondary messengers inositol trisphosphate (IP₃), and diacylglycerol (DAG)[47]. Thus, IP₃ regulates intracellular Ca²⁺ release, thereby hyperpolarizing the cell. Additionally, DAG binds and activates PKC [47]. Other mechanisms of signaling between the 5-HT₁A R and G-proteins can cause cell proliferation through the activation of Erk (Figure 5)[47]. Banerjee [42, 61] also proposed that an alternative pathway by which a specific isoform of PKC, PKC Epsilon, can signal through the secondary messengers Raf1, MEK, and lastly Erk, that in turn would also stimulate cellular proliferation (Figure 5).
The dorsal raphe 5-HT<sub>1A</sub>-R autoreceptors also become inhibited following the release of 5-HT [42]. The first few weeks of SSRI treatment, by which 5-HTT blockade prevents the removal of 5-HT from the synaptic cleft and as a result, causes inhibition of the raphe autoreceptors [62]. Temporarily, this causes a delay in treatment because of a decrease in serotonin release from the dorsal raphe neurons [63, 64]. However, after a period of approximately two weeks the 5-HT<sub>1A</sub>-R desensitize at their presynaptic sites, causing a temporary delay in treatment due to decrease in serotonin release from the dorsal raphe neurons [63, 64].

**Figure 5. Theorized mechanism of SSRI induced desensitization of 5-HT<sub>1A</sub>-R autoreceptors on dorsal raphe serotonin-producing neurons.** Adapted from Banerjee 2007.

**Figure 6. Inhibitory signaling pathways of the 5-HT<sub>1A</sub>-R.** The 7 transmembrane G-protein coupled 5-HT<sub>1A</sub>-R targets inhibitory messenger pathways. Ultimately this results in hyperpolarization of the cell through the expulsion of K<sup>+</sup> ions. Adapted from Adayev & Banerjee 2005.
disinhibition of the DNR and subsequent increase in 5-HT release onto postsynaptic heteroreceptors (Figure 6)[64]. This has also been demonstrated by siRNA mediated inhibition of autoreceptors in mice by Bortolozzi’s group [65]. Interestingly Bouali’s group found that autoreceptor desensitization was sensitive to sex hormones in 5-HTT knockout mice [66] - another possible mechanism of the sex-specific anxiety and depression risk. Desensitization would presumably lead to the anxiety alleviating actions of SSRIs, which is intriguingly the opposite effect seen in the early-life blockade of the 5-HTT [67]. Together, these enlightening studies highlight and point to both sex- and age-dependent effects of autoreceptor desensitization and emphasize the important role of studying 5-HT\textsubscript{1A}-R signaling throughout development to elucidate anxiety and depression problems across the lifespan.

The polymorphism site (C(-1019)G) on the 5-HT\textsubscript{1A}-R gene leads to a decrease in transcriptional repressor binding, subsequently leading to an increase in 5-HT\textsubscript{1A}-R transcription [36]. This is theorized to be one mechanism in support of increased 5-HT\textsubscript{1A}-R transcription and protein in the dorsal raphe nuclei, which in turn, leads to a decrease in 5-HT being released at postsynaptic sites [36]. For emphasis, the presence of these transcriptional repressors is sex-dependent, adding to sex-dependent depression susceptibility mechanisms involving presynaptic serotonergic neurons [36]. However, other studies point to the ultimate function of heteroreceptors as being essential in the success of SSRI treatment [68]. Additionally, it was shown by Garcia-Garcia’s group that heteroreceptor loss in adolescents leads to a depression-like behavioral syndrome [68]. This is not too surprising, as these heteroreceptors ultimately would lead to serotonergic signaling pathways that would alter mood and behavior [62]. Also, it is known that the heteroreceptors themselves do not desensitize unlike the autoreceptors, which
would eliminate the possibility of there being a similar inhibitory mechanism postsynaptically, that would provide an alternative explanation for counteracting anxiolytic effects [69].

### 1.8 Neurogenesis and the 5-HT1A-R: An essential means of treating depression and anxiety

Much of the SSRI action, which has been previously explained on a molecular level above, also has important effects on cell proliferation [70]. The hippocampus DG region, is one of few brain regions that continually generate new neurons throughout life, and as such, has proven to be an essential target of SSRI treatment [71]. Depressed patients have been shown to have lower numbers of proliferating cells within the neurogenic subgranular zone region of the DG, and this has been corrected by SSRI treatment [72]. Since SSRIs are also used to treat anxiety, the *neurogenic theory of depression* has also been extended to patients with anxiety. This topic will be discussed more extensively within the neurogenesis chapter.

### 1.9 Synaptogenesis and the 5-HT1A-R: Determinants of Connectivity essential for mood

Does the 5-HT1A-R play a role in synaptogenesis related to early or late brain connectivity? Mehta and Banerjee [61] showed the importance of 5-HT1A-R in the establishment of normal brain connectivity early on in life. Using organotypic cultures of hippocampal slices, they demonstrated a transition in the function of 5-HT1A-R signaling from promoting neuroproliferation at P6 to primarily promoting synaptogenesis at P15 [61]. Yan and his colleagues [73, 74] demonstrated that in early postnatal development, on P3, the depletion of 5-HT reduced the dendritic length and spine density in hippocampal granule neurons. Recently, Rojas and Fiedler [73] had also found that serotonin regulates neurite outgrowth and that this is specifically accomplished through 5-HT1A-R in cultured hippocampal neurons. The timing and establishment of hippocampal neurocircuitry in early development will be further discussed in
the last chapter of this thesis. In short, the development of the hippocampus being protracted, the CA1 region does not fully connect with the rest of the hippocampus until at least 2-weeks after birth in the mouse [35].

1.10 Animal Models of Anxiety

Animal models of anxiety concerning with 5-HT receptor signaling include the 5-HTT and the $5-HT_{1A}$ knockout mouse models [35]. Tecott and colleagues [75] developed the $5-HT_{1A}$-R mutant mouse, which lacks functional $5-HT_{1A}$-Rs. On behavioral tests of anxiety, the mice displayed anxiety-like behavior in the open field test, elevated zero maze, and the novel object assays [75-77]. No sex differences were found in the anxiety-behavior of this model in the labs that engineered these mice [76]. However, since experimental methods in anxiety behavioral tests vary from laboratory-to-laboratory, and with the recently discovered effect of the sex of an experimenter on experimental results, these results are likely to change.

1.11 Goals of the study

The goals of the present study were to examine whether Sex and Genotype would interact in the postnatal behavioral and brain development of the $5-HT_{1A}$-R KO mouse model of anxiety. Furthermore, along with testing these hypothesized effects, it was of interest to examine if early developmental behavioral milestones could be used as an ontogenic screening predictor for later life anxiety disorder susceptibility. These hypotheses were carefully approached through the use of experiments involving behavioral milestone testing, correlates of adult behavior, as well as examination of postnatal neuroproliferation and synaptogenesis within the hippocampus of wild type (WT) and $5-HT_{1A}$-R KO mice. The hypothesized effects in behavioral milestone changes are in the first few weeks of life ($i.e.$ P6, P8, and P10). The hypothesis regarding synaptogenesis
in the first few weeks was that genotype and sex differences would likely be observed on P10, corresponding to the beginning of synaptogenesis. Since behavioral milestones would be examined at P10, any differences observed would be examined in relation to synaptogenesis at this age as well. We also expected that any sex-specific aberrance in synaptogenesis in the 5-\textit{HT}_{1A}-R KO mice would be further accentuated during the peak of synaptogenesis around P14-16.
1.12 Specific Aims

**Aim 1.** Investigate the postnatal behavioral development in $5-HT_{1A}$-R knockout and wildtype male and female mice in order to assess if early developmental postnatal behavioral milestones could predict later life anxiety-like behaviors.

**Aim 2.** Study postnatal neuroproliferation in the Dentate Gyrus in the $5-HT_{1A}$-R knockout and wild type mice with special attention to possible genotypic and sex differences.

**Aim 3.** Investigate the ultrastructure hippocampal *stratum radiatum* in the $5-HT_{1A}$-R knockout and wild type mice with special attention to possible genotypic and sex differences...
2.0 Aim 1: Investigate the postnatal behavioral development in $5-HT_{1A}$-R knockout and wildtype male and female mice in order to assess if early developmental postnatal behavioral milestones could predict later life anxiety-like behaviors.

2.1 Introduction

Anxiety disorders have an economic burden of $40 billion in the U.S. [78], but will affect one in ten adults across the globe this year, and 1 in 5 adults at some point during their lifetime [9]. Anxiety disorders have the earliest onset of any other psychiatric disorder at a median age of 7-14 years old [21]. Women are twice as likely to develop and to be diagnosed with an anxiety disorder throughout their lifetime [79] and are the first to show symptoms in childhood compared to males [16, 19, 80]. Some types of anxiety disorder symptoms can emerge from as early as 6-8 months, consistent with stranger or separation anxiety[16]. Anxiety generally involves a chronic state of agitation and hypervigilance, but can be specific for different subtypes of anxiety disorders, such as specific phobias [1-3, 6]. Hirshfeld and Kagan [81] were able to identify a reliable set of traits in children as young as 21 months, which would predict anxiety when they were older. The set of symptoms they observed were not far from symptoms seen in adults, and included “inhibited” behavior that was characterized as quiet, withdrawn, and hyperreactive [81].

In the human population, the C-1018G single base-pair change in the promoter region of serotonin 1A receptor ($5-HT_{1A}$-R) [41] gene, as well as the reduction of serotonin metabolites [34, 82], have been implicated in anxiety and depression. Lemonde and Albert found that the homozygous G/G genotype had a stronger ($p < 0.01$) association with females than males [38]. The $5-HT_{1A}$-R is found in both the presynaptic serotonin-producing neurons of the midbrain.
DNR as somatodendritic autoreceptors and in postsynaptic targets as heteroreceptors [40, 41]. Since the receptor has an inhibitory effect, the combination of high levels of autoreceptors and low levels of heteroreceptors are thought to lead to a decreased level of serotonergic signaling [36, 44, 60, 63, 64, 83]. Therefore, SSRIs which act through 5-HTT, are thought to ultimately have an effect on 5-HT$_{1A}$-R signaling after a few weeks of treatment, in which the autoreceptors desensitize and disinhibit presynaptic serotonergic raphe neurons [63]. Boldrini [36] found that females have higher levels of 5-HT$_{1A}$-R binding, while Lemonde and Albert [38] found a decrease in responsiveness to SSRI treatment that was more significant in females with the G/G genotype. Intriguingly Lemonde and Albert also showed that RNA levels of Deaf1, a transcription factor that represses 5-HT$_{1A}$-R activity in the dorsal raphe nuclei, is present in higher levels in female serotonergic neurons [36]. This could be a sex-based compensatory mechanism for the higher levels of 5-HT$_{1A}$-R in female dorsal raphe neurons [36]. Furthermore, autoreceptor functions have also been shown to be sensitive to sex hormones [66]. Taken together there seem to be multiple sex-dependent mechanisms that involve the 5-HT$_{1A}$-R that could predispose women more to anxiety than men.

Gross and Hen [40] showed that the presence of the 5-HT$_{1A}$-R in the postnatal mouse brain is crucial for the establishment of normal anxiety-like behavior in adulthood. The expression levels of the 5-HT$_{1A}$-R during development are dynamic with transcripts being detected in the rodent brain on E12 and decreasing before birth [45]. On E16 they are detectable within the hippocampus [42, 45]. However, on P21, the distribution of 5-HT$_{1A}$-R on pyramidal hippocampal neurons in the stratum radiatum of the hippocampus decreases, coinciding with Gross’s study [42] as being the time during which the expression of the 5-HT$_{1A}$-R begins to have less of an effect on the establishment of normal anxiety-like behavior. These studies provide
evidence that the 5-HT_{1A}-R is crucial during development, and other studies show that it is
crucial for synaptogenesis as well as neurogenesis [43, 61, 71, 74, 84, 85].

The 5-HT_{1A}-R knockout (KO) mouse model of anxiety has been used extensively to study
behavioral, molecular and cellular phenotypes of anxiety[75, 76, 86-88]. On different behavioral
tests, including the open field (OF), elevated plus maze (EPM), elevated zero maze, and novelty-
suppressed feeding test, KO mice have consistently been shown to exhibit anxiety-like behaviors
[75, 76, 86]. In addition, KO mice have been shown to have elevated autonomic responses, such
as an elevated heart rate, to novel environments, as well as to various stressors, and aversive
stimuli such as the electric footshock [86]. In addition, KO mice also exhibit enhanced fear
conditioning to ambiguous conditioned stimuli, reminiscent of GAD in humans [89]. Taken
together, the 5-HT_{1A}-R KO mouse model is an effective model for studying anxiety.

In analyzing early life behavior, the critical period characterized by a sharp increase in
the birth of new neurons and synapses occurs in the first few weeks of life in the mouse [90].
During this time, mouse pups go through a series of developmental behavioral milestones [91].
Aside from the motor and reflex related developmental milestones such as the righting and
grasping reflexes, mice also emit ultrasonic vocalizations (USVs). Produced by the regulation of
laryngeal muscles [92], whistles may function in postnatal mice to attract the attention of their
mothers in conjunction with odor [93] upon separation. Human infants also vocalize to attract
the attention of their mothers, which is thought to be an innate behavior as it does not require
auditory feedback [94]. Mouse USVs range from 20 kHz to above 125 kHz [95] and their
spectral and semantic properties have been characterized and categorized in different mouse
models [96]. Interestingly, separation USVs are emitted in the critical first few weeks, but later
play a role in social communication in mice and fear in rats as well [97-99]. They are of
increasing interest to translational research as they have led to predictive phenotypes of a variety of later life brain disorders, such as autism [96, 100-104].

The involvement of the serotonergic system in regulating the emission of USVs in rodents has been shown. The depletion of 5-HT with PCPA or 5,7-DHT caused a decrease in USVs in rat pups [105]. In addition, the tryptophan hydroxylase 2 knockout mouse model also produced a lower number of USVs than their wild-type counterparts [106]. However, in a mouse model of autism that had hyperserotonemia, the number of calls was found to be reduced [107].

The 5-HT1A-R has been shown to be directly as well as indirectly implicated in USV production as SSRI treatment and direct stimulation with The 5-HT1A-R agonist (±)-8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) attenuated USV duration in the adult rat [108], and pups [109-114]. In addition, the 5-HT1A-R KO mouse model was also used to study isolation induced USVs in pups, as well as the effect of maternal care and handling [115-118]. In these studies, mixed results have been obtained on the effect of the lack or blockade of 5-HT1A-R on USV production, and sex-dependent effects on USVs were not found [115, 118, 119]. However, Bowers and his group [120] were able to demonstrate that there were sex differences in USV production in that depended on the presence of Foxp2 protein. The rate of calling has been explored in the 5-HT1A-R KO mouse model, but different subtypes of calls, have not, although they have been implicated in other brain diseases such as autism [103, 121]. Takahashi [94] showed that the TbxI heterozygous mutant mouse model of autism had a reduced number of complex type calls, which are characterized by higher waveform modulation. These types of calls are less likely to be perceived as aversive [94]. In addition, entropy analysis has rarely been conducted on the sequence structures of USVs in the 5-HT1A-R KO mouse model. This sort of analysis can be useful in describing the range of call types in the repertoire [94, 95]. The
quantitative methods of analysis of structure and function of ultrasonic vocalizations have evolved in recent years [122], and are now needed to provide more meaningful knowledge of the vocal repertoire of different mouse models. The present study reports for the first time that 5-
$HT_{1A-R}$ KO pups’ vocal repertoire differs in a sex- and age-dependent manner. The current data herein suggest that 5-$HT_{1A-R}$ KO USV call frequency, as well as spectrographic structures and sequences are indeed predictive of later life anxiety.
2.2 Methods

2.2.1 Mice

Procedures were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and The City University of New York College of Staten Island. The 5-HT1A-R KO mice were obtained from Dr. Laurence Tecott from the University of California San Francisco.

2.2.2 Husbandry

The C57BL/6J mice as well as 5-HT1A-R knockout mice were housed in groups containing a maximum of 3-5 same sex mice per cage with food and water available ad libitum. The light cycle was 12:12-h light/dark with all behavioral testing was conducted during the light period. The 5-HT1A-R KO mice were back-crossed with wild-type C57BL/6J mice. The KO pups were randomly selected to breed the F2 generation mice, which were then used for later experiments. At least 4 breeding pairs were used per genotype for all behavioral tests that follow.

2.2.3 Experimental Mice

Pups were tattooed on P5 [123]. Pups that were used for USVs were randomly selected [116]. The dams for which the simulated maternal approach test was conducted, were the dams of the pups that were used in the USV experiment [94]. Litter sizes ranged from 7-11, with larger litters containing at least 1 underweight runt, which was excluded from the analysis. On P10, one to two mice were randomly selected for ultrastructural analyses for a different study.

2.2.3 Postnatal Developmental Milestone Behavioral Testing
2.2.3.1 Ultrasonic Vocalizations (USVs)

The USVs were recorded on P6, P8, and P10 within a 12-hour time-window consistent with other reports [94, 96, 101]. Following one hour of habituation, pups were randomly chosen and placed into a temperature-controlled room that was maintained at 70 ± 2 °F [116]. Then 3-minute USV recordings were conducted using a Noldus microphone (Wageningen, The Netherlands) with a built-in analog-digital converter, and Noldus Ultravox 4.1 software (Wageningen, The Netherlands) consistent with procedures from other reports [101]. The microphone was then inserted into a sound attenuating Styrofoam box (17 x 10 x 10 1/2”) 6 inches from where the pup was situated in a pre-alcohol wiped beaker directly below the microphone in order to conduct the recordings[101]. After the recording, the pups were placed on a heating pad (37 °C), to prevent hypothermia [116]. The 3-minute recordings were then analyzed using Ultravox and MATLAB using a 250 kHz sampling rate and a maximum frequency of 125 kHz [101, 121]. Further, within the Ultravox analysis fast Fourier transformations of 250 point spectrogram lengths were generated and converted into “.WAV” file format [121]. USVs were first selected manually using the Ultravox software [101] by a researcher blind to the treatment groups and were later automatically selected using MATLAB code. The MATLAB code for detection and characterization of USVs was adapted from a prior established USV code written by Holy, Guo, & Barnes [94, 121] to fit the unique spectrographic profiles of the 5-HT1A-R KO and WT mice. Therefore, USV recordings were first filtered for white noise and other noise using the Audacity software spectral edit multitool and a High Pass Filter set to 80 kHz. The MATLAB code was then imported, filtered, and was used to further analyze the “.WAV” files, and was finally compared to the manually selected USVs from the Ultravox software with a USV detection rate averaging above 90% [121].
Twelve different USVs were analyzed based on the qualitative and quantitative characterizations reported by Scattoni et al. [96] with the addition of two USVs observed in the experimental 5-HT_{1A}-R KO and WT mice. A Downward (D) type USV had a downward inflection of the fundamental or formant frequency of $\geq 5$ kHz; an upward (U) type USV had an upward inflection $\geq 5$ kHz; a Chevron (CH) type USV had an upward followed by a downward inflection; a Short (S) type USV had a length of less than 5 ms; and a Flat (F) type USV had inflections less than 5 kHz. Further, a Two-Syllable (TS) type USV had 2 frequencies that were continuous in time; a Frequency-Step (FS) type USV had greater than 2 frequencies that were continuous in time; a Composite (C) type USV had two harmonic frequencies; a Harmonic (H) type USV had more than 2 harmonic frequencies; a Harmonic Two-Syllable (TS-H) type USV had multi-syllabic harmonics; a Complex (CX) type USV had multiple inflections; a Complex Two-Syllable (CX-TS) type USV had two-syllables with multiple inflections.

An Entropy Analysis was performed using a custom-written MATLAB code that is available upon request. A second code was written to analyze the results obtained from the Markov Chain Modeling code [94]. The $H_0$ was dependent only on the number of USV types that occurred. For zero-order entropy regarding the $H_0$, the equation used was $H_0 = \log_2 m$ where “m” is the number of USV types used. For the first-order model regarding the $H_1$, it assumes the USVs are statistically independent of each other and is defined as

$$H_1 = -\sum_{i=1}^{m} p_i \log_2 p_i.$$ 

Thus, $H_1$ is a weighted sum of $\log_2 p_i$, which is weighted by the probability, $p_i$, of the occurrence of each call USV type. For the entropy for the second-order entropy regarding the
H₂, it assumes that there is a conditional probability in observing the USV type j following call type i, and is defined as

$$H₂ = - \sum_{i=1}^{m} p_i \sum_{j=1}^{m} p_{j|i} \log_2 p_{j|i}$$

### 2.2.3.2 Real Pup Retrieval

Following the USV and behavioral milestone experiments, the dams were removed from their cages for at least 5 minutes. The pups were then returned to the side of the cage opposite that of the dam’s nest location consistent with other reports [124]. The dam’s pup retrieval latency was recorded immediately upon reintroduction of the dams to the cage facing opposite the pups [124]. The time to retrieve each pup (measured in seconds) was recorded by an experimenter blind to the treatment groups.

### 2.2.3.3 Simulated Pup Retrieval

Nesting material were saved from the real pup retrieval study so that they could be used later during the simulated pup retrieval experiments [94]. An apparatus was built using standard size (28.5 cm × 24 cm) rat cages with a middle compartment that had two (12.5 cm long) PVC tubes on each end. A mesh barrier was secured to the tube endings to prevent mice from escaping. Pup odor, in the form of bits of bedding from the original real pup retrieval litter, was used as another associative stimulus to evoke the contrived pup retrieval behavior in the dams and was placed at the end of each tube [94]. A digital-analog converter was used to convert the original USVs to be played back through a 5-125 kHz range Pioneer PT-R7III Beryllium ribbon tweeter (Bunkyo, Tokyo, Japan) as simulated USVs. Before the start of the experiment, the audio from the tweeter was recorded using Ultravox to ensure that the simulated USVs were as
similar as possible to the original USVs. The simulated USVs were chosen by playing a sequence from a real USV recording that was processed through a high-pass filter at 20 kHz using the Audacity software. A video camera was placed overhead that recorded the dams’ behavior for 10 minutes. The amount of time the dam spent in each tube, as well as interacting with each tube end in response to the odor and simulated USV was recorded. The simulated USV types that were chosen for this contrived experiment were intentionally discriminant from one other; therefore, D, H, and syllable USVs were used. The H type USVs also had multiple deflections in the formant frequency, which was consistent with a CX.

2.2.3.4 Behavioral Milestones

All behavioral milestone measurements were repeated 3 times on each day of testing. Weights were obtained following the USV recordings. To measure the righting reflex, pups were placed in the supine position and a stopwatch was used to measure the time it took for them to right themselves on all four paws (i.e., maximum: 40s). The Grasping reflex and overall strength was measured by gauging how strongly the pups grasped the wooden bar of a Q-tip (i.e., scored on a scale of 0-3) consistent with prior reports[102]. A Hand Clap-Elicited Startle response was measured by the presence of a whole-body startle response immediately following the stimulus (i.e., scored as 0 or 1 for absence or presence of a response) consistent with other reports [125].

2.2.4 Adult Assessment of Anxiety-like Behaviors

2.2.4.1 Open Field (OF)

The Open Field Test (OF) (Figure 7a) was used to assess preliminary anxiety-like behavior, as well as locomotor activity behavior in the tattoo-tracked adult mice[126, 127]. The
open field test dimensions were 92 cm × by 62 cm [128]. The lighting used in the experiment was based on other experiments performed in the lab and was made by a dim overhead green light of 40 lux [128]. Adult offspring mice were age-matched to 9-10 weeks old and were habituated in the behavior testing room for an hour in dim lighting prior to testing [128]. They were then carried in a pre-alcohol washed, perforated metal container and were placed in the center of the OF apparatus where their behaviors were recorded for 600 s using the CapWiz software and Panasonic Color Video wv3400 overhead camera (Kadoma, Japan) [128]. Videos were post-analyzed using a blinding procedure by a researcher trained on scoring behaviors through the AnyMaze software [128]. The Time Spent in the Outer (¼ of the arena) and Center, including the combined Inner and Middle Zones (inner ¾), were used for assessing anxiety-like behaviors [128]. Exploratory Activity was also measured by the ratio of Distance Traveled in the Center to the Total Distance Traveled. Locomotor Activity was assessed by the Total Distance Traveled to eliminate the potential confounding factor of hyperactivity [128].

2.2.4.2 Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) (Figure 7b) was used to further assess and confirm the presence of anxiety-like behavior in tracked in the OF from the adult mice. A 50 cm tall plus-shaped maze with two alleys containing walled arms (i.e., Closed Arms [CA]) 36 cm L x 7 cm W, and two alleys containing no walled arms (i.e., Open Arms [OA]) 36 cm L x 7 cm W [128] was used for the EPM. Mice were placed in the Center Zone (CZ) 5 cm L x 5 cm W that adjoined the OA and CA at the beginning of the experiment and were behaviorally monitored for 10 minutes. The Time Spent in the OA was associated with Exploratory Activity, and therefore less anxious-like behavior, as opposed to the CA [129]. The Time Spent in the CA was measured
to assess anxiety-like behaviors, despite the lighting conditions that were used for the experiment.

2.2.4.3 Light Dark Chamber Test

The Light-Dark Chamber (LD) (Figure 7c) test was used to assess for any anxiety-like behaviors that would corroborate with the OF and EPM, yet they would be based on the innate nocturnal preference in mice for dark spaces [130]. The LD test chamber was made out of plastic, and the dimensions were 50 cm L x 25 cm H x 40 cm W. The LD test chamber has two 25-cm compartments, one with an opaque black appearance (i.e., dark chamber), and the other with a clear appearance (i.e., light chamber). A little archway was present for the mouse to be able to traverse between both chambers. The dark chamber was unlit (< 5 lux), while the light compartment was transparent and illuminated by an overhead fluorescent light. At the start of each test, the mouse was placed in the light chamber and their behaviors were video recorded for 10 minutes using the CapWiz software. The Number of Entries (i.e., transitions) into the light chamber was recorded as the anxiety-like measure for the LD test.
2.2.5 Statistical Analyses

R software was used for the organization and analysis of data [94]. A Partial least squares Discriminant Analysis of the USV classification system was performed using the code provided by Dr. Hiroi from Albert Einstein College of Medicine and was further incorporated with mixOmics and DiscrMiner add-on packages, that were compatible with R versions 3.3.1 and 3.3.3., respectively [94]. An Analysis of Variance (ANOVA) was performed using TIBCO Statistica 13.3 software (Palo Alto, CA) [131]. A Repeated Measures ANOVA was used to analyze the $2 \times 2$ factorial design that contained 3 levels Postnatal days: P6, P8, and P10. These

Figure 7. Adult anxiety-like behavioral test screen shots. (a) Open Field (OF) (b) Elevated Plus Maze (EPM) (c) Light Dark Chamber Test (LD).
statistical calculations were followed by a *Newman-Keuls* planned posthoc comparisons. For comparing distributions *Mann-Whitney U* test was used.
2.3 RESULTS

2.3.1 Sex Differences in Adult Anxiety-like Behaviors

Behavioral testing followed an experimental study timeline (Figure 8), with adults being tested at P60.

![Figure 8. Experimental Study Timeline](image)

*Figure 8. Experimental Study Timeline.* A timeline of the present study showing the postnatal ages at which mice behavioral milestones were assayed, and brains were extracted for an ultrastructure study. *Note:* (P) = postnatal day; (USV) = ultrasonic vocalizations; and vertical cross lines infer chronological developmental timelines.

2.3.1.1 Open Field Preliminary Anxiety-like Behavioral Screenings

Locomotor activity (LMA) was assessed as the Total Distance Traveled within the OF test, to evaluate the potential for any confounds in LMA that may be misappropriated as anxiety-like behaviors in later tests. There was no significant effect of Genotype ($F_{(1, 36)} = 1.594, p = 0.214$ (n/s)), but there was a significant effect of Sex ($F_{(1, 36)} = 24.6, p < 0.001$, $\eta^2 = 0.400$) on LMA, with females showing more LMA than males (Figure 9a). There was no significant interaction observed between Genotype × Sex, nor were there any significant differences observed between KOF and WTF mice in LMA ($F_{(1, 36)} = 0.025, p = 0.876$ n/s; nor were there any significant differences observed between KOM and WTM mice (Figure 9a). However, there was a significant interaction between Genotype × Sex on Time Spent in the Center Zone of
the OF ($F_{(1, 52)} = 9.78, p < 0.001^{***}, \eta^2_p = 0.16$) (Figure 9b). The KOF mice spent less Time in the Center Zone than WTF mice in the OF ($p < 0.05^*$) (Figure 9b). There were no significant differences observed between KOM and WTM mice in Time Spent in the Center Zone of the OF. Notably, a significant Genotype × Sex interaction ($F_{(1, 48)} = 16.5, p < 0.001^{***}, \eta^2_p = 0.26$) was observed for Distance Traveled In The Center. The sex differences showed that KOF mice explored the center of the OF less than the WTF mice ($p < 0.001^{***}$), whereas there was no significant differences observed between the WTM and KOM mice (Figure 9c).

### 2.3.1.2 Elevated Plus Maze Corroborating Anxiety-like Behavioral Phenotypes

Additionally, a significant Genotype × Sex interaction ($F_{(1, 34)} = 5.56, p < 0.01^{**}, \eta^2_p = 0.14$) was observed for Open Arm Entries in the EPM. The KOF mice entered the Open Arm Entries less frequently than the WTF mice ($p < 0.05^*$), whereas the male mice showed no significant differences (Figure 9d). Similarly, the ANOVA revealed a significant Genotype × Sex interaction in Time Spent in the Closed Arms of the EPM ($F_{(1, 42)} = 6.64, p < 0.05^*, \eta^2_p = 0.14$). The KOF mice spent more time in the closed arms of the elevated plus maze than the WTF mice ($p < 0.05^*$), while the males showed no significant differences (Figure 9e).

### 2.3.1.3 Light-Dark Chamber Test Assessment of Inherent Anxiety-like Traits

In the LD chamber test, a significant Genotype × Sex interaction was observed ($F_{(1, 40)} = 7.52, p < .01^{**}, \eta^2_p = 0.16$), whereby the KOF mice entered the light chamber less than the WTF ($p < 0.001^{***}$) mice and the KOM mice entered the light chamber less than the WTM mice ($p < .001^{**}$)(Figure 9f).
Figure 9. As adults, the KOF mice displayed elevated levels of anxiety-like behavior. (a) No significant differences in locomotor activity on the open field test were found. KOF mice spent less time in the center of the open field test less than WT female mice. (d) KOF mice explored the center of the open field test less than WT female mice. (e) KOF mice entered the open arms less than WT male mice. (f) KOF mice spent more time in the closed arms compared to WT male mice. KOM mice entered the light chamber in the light-dark chamber test less than WT male mice. Two-way ANOVAs were followed by Newman-Keuls post-hoc tests, $p < 0.05^a$, $p < 0.001^{**}$, $p < 0.001^{***}$. Genotype effect $p < 0.05^a$, $p < 0.001^{ab}$; Sex effect $p < 0.01^d$, Sex effect $p < 0.001^e$, Genotype × Sex interaction, $p < 0.05^a$, $p < 0.01^{**}$, $p < 0.001^{***}$. The n-sizes per group are denoted inside the bars in white font. Data are presented as the mean ± SEM.
2.3.2 Later-life Anxiety Behavior Correlates with P8 USVs

A representative filtered USV recording is shown in Figure 10. A Repeated Measures ANOVA, with Age as the within-subject factor, and Genotype and Sex as the between-subject factors, revealed a significant effect of Age ($F(2,56) = 8.80, p < 0.001^h, \eta^2_p = 0.136$) and Genotype ($F(1,56) = 11.883, p < 0.001^c, \eta^2_p = 0.175$), but not Sex on the total number of USVs produced. Furthermore, a significant interaction was observed between Age $\times$ Genotype $\times$ Sex ($F(2,112) = 5.81, p < 0.001^{xy}, \eta^2_p = 0.094$) (Figure 11a). Planned post hoc comparisons revealed that the KOF mice produced significantly more USV calls than WTF mice on P8 ($p < 0.01^{**}$).

In order to assess the potential relationships between early P8 USV differences with later life anxiety-like behaviors in adult mice, a series of correlations were conducted using the dependent measures from Figure 9 from the OF, EPM, and LD tests, respectively. The total number of USVs on P8, across all mice, showed a significant moderate negative USV-OF linear relationship with Time Spent in The Center Zone ($r^2 = 0.390, p < 0.05^*, d = 0.152$) (Figure 11b). The KOM ($r = 0.853, p < 0.01^{**}, d = 0.728$) and KOF mice showed a strong negative linear USV-OF correlation ($r = 0.688, p < 0.05^*, d = 0.473$) (Figure 11b). There was a moderate negative linear relationship across all mice with the total number of P8 USVs and Distance Travelled in the Center of the OF ($r = 0.453, p < 0.01^{**}, d = 0.205$). Of the KO mice, the KOM mice exhibited a strong negative linear relationship ($r = 0.85, p < 0.01^{**}, d = 0.720$) (Figure 11c). There was a moderate positive linear relationship with P8 USV calls across all mice with Closed Arm Time on the EPM ($r = 0.420, p < 0.05^*, d = 0.176$). Among the KO mice, the KOF mice showed a strong positive linear correlation ($r = 0.681, p < 0.05^*, d = 0.464$) (Figure 11d). There was a moderate negative linear correlation with P8 USVs and Open Arm Entries across all
mice \( (r = 0.558, p < 0.01^{**}, d = 0.311) \) (Figure 11e). Of the KO mice, the KOF mice showed a significantly strong negative linear relationship \( (r = 0.768, p < 0.05^{*}, d = 0.589) \). The P8 USVs also shared a moderate positive linear correlation with the number of Light Entries in the LD test across all mice \( (r = 0.448, p < 0.05^{*}, d = 0.201) \) (Figure 11f).
Figure 10. A representative section of recording opened in MATLAB software. The recordings were filtered and analyzed in their post-filtered formats.
Figure 11. P8 USV number to adult anxiety behavior assessment revealed Sex-specific differences that originated in early development and related with persistent anxiety-like behavioral phenotypes across the lifespan. (a) At P6 in KOM exhibited elevated USVs when compared to WTM. KOF mice produced significantly more USVs than WTF mice, while no significant differences were observed between groups at P10. A Repeated Measures ANOVA was followed by Newman-Keuls post-hoc test. \( p < 0.05^*, p < 0.001^{**} \), Genotype effect \( p < 0.001^{b} \); Sex effect \( p < 0.001^{h} \); Genotype \( \times \) Sex \( \times \) Age interaction \( p < 0.01^{yy} \). The n-sizes for each groups are denoted inside the bars in white font. Data are presented as the mean ± SEM. Correlations between the P8 USVs and adult Open Field (OF) (b-c), Elevated Plus Maze (EPM) (d-f), and the Light Dark test (LD) (g) were evaluated to assess anxiety-like predictable relationships across the lifespan.
2.3.3 Specific USVs can Predict Later-life Anxiety-like Behaviors with Some Sex-dependent Specificity

A Partial Least Squares Discriminant Analysis (PLSDA) was performed to analyze the modified USV classification scheme in relation to the Treatment groups at all three postnatal ages recorded: P6, P8, and P10 (Figure 12a). Only the first two statistical components are graphed in the correlation circle plot to provide a clear visual comparison of the dataset obtained. Component 1 shows the variance due to Age differences, with P10 mice clustered within the right quadrants, whereas the P6 and P8 mice are clustered closer to the left quadrants and center, respectively. Component 2 shows the variance due to Genotype, with most of the WT mice grouped within the lower two quadrants. The D USVs were most associated with P6 WTF mice, whereas the CX USVs were most associated with P10 WTF mice. Additionally, the PLSDA revealed that the simpler USV types (i.e., S, F, and D USVs) were observed most in P6 mice, as opposed to CX USVs. The frequency steps stood out as the most discriminant variable under study and were therefore the farthest dataset observed within the PLDSA correlation circle from the P6 WTF mice. The discriminant PLDSA scores of the different USVs are listed in Table 1.

Since the total number of USVs differed between groups, a Repeated Measures ANOVA was performed on the USVs type expressed as a percentage of total USVs obtained, with Age as a within-subject factor, and Sex × Genotype as between-subject factors (Figure 12b). There was a significant main effect of Genotype ($F_{(1,53)} = 31.9, p < 0.001$, $\eta^2_p = 0.38$), a significant interaction between Genotype × Sex ($F_{(1,53)} = 7.18, p < 0.01$, $\eta^2_p = 0.119$), as well as a significant interaction between Age × Genotype ($F_{(2,106)} = 4.332, p < 0.05$, $\eta^2_p = 0.075$), but there was no significant interaction between Age × Genotype × Sex ($F_{(2,106)} = 0.120, p = 0.89$ ns) on frequency step type USVs. The post hoc multiple comparisons test revealed that KO mice
produced more frequency step type USVs on P6 ($p < 0.001^{***}$) and P10 ($p < 0.001^{***}$), respectively. Further, the KOF mice produced more frequency step type USVs than the WTF mice ($p < 0.001^{***}$). Similarly, the KOM mice produced more frequency step USVs, when compared to the WTM mice ($p < 0.05^*$). A significant interaction between Age $\times$ Genotype $\times$ Sex influenced D type USV production ($F(2,82) = 5.16, p < 0.01^{\text{IY}}, \eta_p^2 = 0.11$) (Figure 12b, middle left). The KOF mice produced a significantly lower number of downward type USVs than the WTF mice on P6 ($p < 0.001^{***}$). On P6, there was also a significant main effect of Genotype ($F(1,39) = 5.16, p < 0.001^c, \eta_p^2 = 0.49$), as well as, a significant interaction between Age $\times$ Genotype ($F(2,78) = 5.02, p < 0.01^{##}, \eta_p^2 = 0.11$) on short type USV production, with the KO mice producing a lower number on P6 ($p < 0.001^{***}$), P8 ($p < 0.01^{**}$), and also trending lower on P10 ($p = 0.055$) (Figure 12b, middle right). A significant Age $\times$ Genotype interaction ($F(2,84) = 3.5, p < 0.05^*, \eta_p^2 = 0.078$) was observed in upward type USV production was seen on P10 with a lower number produced by KO mice.

The linear relationships between specific USVs and later-life anxiety-like behaviors were analyzed and the results are presented in Table 2. The findings revealed that harmonic type USVs, as well as multi-syllabic USVs, tended to correlate with anxiety-like behaviors more than other USV types. Most of the USV-Anxiety-like behavioral correlations were observed developmentally on P8, with the exception of the P10 two-syllable-harmonic type USV being a developmental milestone predictor of later-life adult anxiety-like behaviors regulated by 5HT-1AR expression and activity or lack thereof.
Figure 12. Circle correlation of 12 USV call types as a function of Age × Genotype × Sex and a sub-analysis of 6 USV call types quantitative differences. The 12 different call types (black font) whereas the color codes for Genotype and Sex are consistent between correlation circle (a) & 6 USV call types (b). In (b) the 6 USV types are as a percentage of total number of USVs. Legend (C = composites, F = flats, TS = two-syllable, CX-TS = complex two-syllable, H = harmonic, TS-H = multisyllabic harmonic, FS = frequency steps; CX = complex; D = downward; S = shorts; CH = chevron; U = upwards). A Repeated Measures ANOVAs were followed by Newman-Keuls post-hoc tests. Genotype effects $p < 0.001^{**}$; Sex effects $p < 0.001^{**}$; Age effect $p < 0.01^*$, $p < 0.001^{**}$; Age × Genotype interaction $p < 0.05^*$, $p < 0.001^{***}$, Age × Sex interaction $p < 0.01^*$, Genotype × Sex × Age interaction $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$; $p < 0.05^*$, $p < 0.001^{**}$, $p < 0.0001^{***}$. The n-sizes for each group are denoted inside the bars in white font. Data are presented as the mean ± SEM.
<table>
<thead>
<tr>
<th>USV Type</th>
<th>Correlation ratio</th>
<th>Wilk’s Lambda</th>
<th>F-statistic</th>
<th>p-value</th>
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<tr>
<td>C</td>
<td>0.139</td>
<td>0.861</td>
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<td>CX</td>
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<tr>
<td>CX-TS</td>
<td>0.045</td>
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<td>0.391</td>
<td>&gt; 0.05 ns</td>
</tr>
<tr>
<td>D</td>
<td>0.310</td>
<td>0.690</td>
<td>3.716</td>
<td>&lt; 0.001 ***</td>
</tr>
<tr>
<td>F</td>
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<tr>
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<tr>
<td>S</td>
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<td>U</td>
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<td>0.630</td>
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Table 2. Correlation statistics between post-natal USV subtypes and different measures of anxiety in adulthood

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<tr>
<th>Group</th>
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<th>P8</th>
<th>P10</th>
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<th>OF</th>
<th>OA-E</th>
<th>O-Imm</th>
<th>CAT</th>
<th>r</th>
<th>d</th>
<th>t</th>
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<tr>
<td>All</td>
<td>H</td>
<td>×</td>
<td>×</td>
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<td>-0.5624</td>
<td>0.3163</td>
<td>-3.0420</td>
<td>0.01**</td>
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</tr>
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<td>H</td>
<td>×</td>
<td>×</td>
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<tr>
<td>All</td>
<td>TS-H</td>
<td>×</td>
<td>×</td>
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<td></td>
<td></td>
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<td>21</td>
</tr>
<tr>
<td>All</td>
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<td>×</td>
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<td>All</td>
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<tr>
<td>All</td>
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<td>×</td>
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<tr>
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<tr>
<td>KOF</td>
<td>H</td>
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<td>×</td>
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<tr>
<td>KOF</td>
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<td>×</td>
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<td>KOF</td>
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<td>×</td>
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<td>0.8745</td>
<td>-5.9016</td>
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<td>7</td>
</tr>
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</table>

**Abbreviations:** L E; Light Entries (Light-Dark Box); IM; OF; Open Field (Center Zone); OA-E; Open Arm Entries (Elevated Plus Maze); O-Imm; Outer Zone Immobility (Open Field); TS; Two-Syllable; CX-TS; Complex Two-Syllable; H; Harmonic; TS-H; Two-Syllable Harmonic; KOF; Knock-out Female.
2.3.4 Specific USV types fail to influence maternal pup retrieval behaviors

During the simulated pup retrieval, in which pups were absent from the contrived test, the dams did not significantly respond to any of the different USV types that were played back to them in their own hearing range (i.e., D, TS, or H USV types) (Figure 13). The harmonic calls which were chosen also had a complex formant frequency. A Repeated Measures ANOVA revealed an Age × Genotype interaction ($F_{(2,18)} = 5.76, p < 0.01^{**}, \eta^2_p = 0.39$) on the real maternal pups retrieval when the pups were present. The KO dams retrieved their pups more often than the WT dams ($p < 0.01^{**}$) on P10
Figure 13. Simulated pup retrievals revealed no specific USV type preference. (a) Illustration of simulated retrieval set-up. (b) Graph showing the time spent by the mothers in the “Sound” tube. A one-way ANOVA revealed that there was no difference between the treatment group of the dam and time spent in the sound tube when playing back in the mouse hearing range a Downward, Two-Syllable or Harmonic type USV. (c) Real maternal pup retrieval test showing an increase in pup retrievals by KO dams on P10. Data are represented as mean ± SEM. The n-sizes for each group are denoted inside the bars graphs in white font.
2.3.5 Markov Analysis Reveals Sex-dependent Differences in USV Sequences

Since USV durations were significantly longer on P6 in the KO mice (Age × Genotype interaction: $F_{(2,74)} = 7.74$, $p < 0.001^{###}$, $\eta^2_p = 0.17$), it was appropriate that Inter-Call-Intervals (ICI) were found to be significantly shorter in the KO mice (Genotype: $F_{(2,96)} = 15.566$, $p < 0.001^{c}$, $\eta^2_p = 0.245$) (Figure 14a & d). The observed and theoretical distribution cross-points (Figure 14e & f) were used to set a “time window” at which two calls would be considered distinct from any other string of USVs. The observed ICI distributions were significantly different between KO and WT mice, as determined by using the Mann-Whitney U test ($p < 0.001^{***}$).

Strings of USVs were defined as USVS that had an ICI lower than the intersection of the observed and expected distribution curves of intervals, consistent with previously published reports (Figure 14b) [94]. It was hypothesized that there would be a difference in the diversity of USVs, as well as in their temporal sequences. To answer this question, strings of USVs were analyzed for zero-, first-, and second-order entropy, respectively (Figure 15a). A Repeated Measures ANOVA with Entropy as the repeated measure, and Genotype and Sex as between-subject factors, revealed a significant effect of Entropy ($F_{(2,254)} = 1266.93$, $p < 0.001^{h}$, $\eta^2_p = 0.9$) and Genotype ($F_{(1,127)} = 7.04$, $p < 0.01^{b}$, $\eta^2_p = 0.052$). An interesting phenomenon was observed that higher orders of entropy were significantly decreased and inversely proportional in comparison to lower orders of entropy ($p < 0.001^{***}$). In addition, KO mice had higher entropy than WT mice ($p < 0.001^{***}$) (Figure 15a).

As another logical step, it was further hypothesized that the differences in entropy might be observed in specific USV sequence structures. A first-order Markov Chain Analysis was modeled and performed on the USV strings using the ICIs obtained (Figure 14f). The PLSDA
revealed that the KO female mice produced fewer sequences with complex type USVs, and had less sequences ending with complex type USVs than the WT female mice on P10 (**Figure 15b**). These results were similar to those obtained in the PLSDA of single USVs where the WT female mice were more likely to be associated with complex type USVs on P10. A possible sex-dependent difference was observed in which P6 KO male mice produced many more complex-two-syllable or other two-syllable type containing USV sequences. For the USV classification system, the error rate plateaued at the 19 components to 20%. This was in contrast to the PLSDA that was performed on the individual USVs, which had a higher error rate at 52% with 11 components. Moreover, the *leave-one-out cross-validation* control did not alter the error rate significantly. Therefore, the discriminant PLSDA scores of the different USV sequence combinations showed a sex-dependent effect and are listed in **Table 3**.
Figure 14. Sex-dependent differences in USV ICI intervals and durations. (a) KO mice had longer USVs on P6, and KOM had longer USVs than WTM. (b) Bar graphs depict the means ± SEM. (c) Correlation of P8 average USV duration to Closed Arm Time. (d) KO mice had shorter ICIs than WT mice. (e) Observed and expected distributions of ICIs in WT and KO mice. A Two-way Repeated Measures ANOVA was followed by Newman-Keuls post-hoc analysis. Genotype effects $p < 0.001$; Age × Genotype interactions $p < 0.01$##, $p < 0.001$###, $p < 0.05$, $p < 0.01$**, $p < 0.001$***. (f) Cross-points of ICI curves used to detect strings of calls for Markov Chain Analysis. The n-sizes for each group are denoted inside bars in white font.
Figure 15. Entropy and first-order Markov analysis of 5-HT1A-R KO pups compared to WT pups. (a) Analysis of zero-order (H₀), first-order (H₁) and second-order (H₂) entropy. A Repeated Measures ANOVA with Entropy as a with-in subject factor, Genotype, and Sex as between-subject factors, was followed by Newman-Keuls post-hoc test. Genotype effect p < 0.01b; Entropy effect p < 0.001h; p < 0.001***. Data are presented as the mean ± SEM. (b) PLSDA of first-order Markov Chain Analysis. The USV sequences with significant discriminant scores are in black bold typeface.
### Table 3 Discriminant scores of call sequences

<table>
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<tr>
<th>USV Sequence</th>
<th>Correlation Ratio</th>
<th>Wilks' Lambda</th>
<th>F-statistic</th>
<th>p-value</th>
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<tbody>
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<td>U-CX</td>
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<td>3.686</td>
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</tr>
<tr>
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<td>0.870</td>
<td>3.282</td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>CXTS-H</td>
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<td>0.879</td>
<td>3.041</td>
<td>&lt;0.001 ***</td>
</tr>
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<td>0.900</td>
<td>2.443</td>
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<tr>
<td>CH-U</td>
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<td>0.902</td>
<td>2.394</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
<td>TS-TSH</td>
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<td>0.903</td>
<td>2.359</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
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### 3.6 Developmental Behavioral Milestones Differ in a Genotype-dependent Manner

It was hypothesized whether there would be any sex-dependent effects on postnatal behavioral milestones in the KO mice. A *Repeated Measures ANOVA* revealed a significant
effect of Genotype on startle response ($F_{(1,52)} = 9.948, p < 0.01^b, \eta^2_p = 0.16$) with KO mice having a higher likelihood to startle than WT mice (Figure 16a). Moreover, a significant effect of Genotype ($F_{(1,48)} = 7.821, p < 0.01^b, \eta^2_p = 0.14$), Sex ($F_{(1,48)} = 7.244, p < 0.01^d, \eta^2_p = 0.13$), Age ($F_{(2,96)} = 49.32, p < 0.001^h, \eta^2_p = 0.51$) was observed. A significant interaction between Genotype and Age ($F_{(2,96)} = 5.832, p < 0.01^{##}, \eta^2_p = 0.11$) was observed, as well as a significant Genotype × Sex × Age interaction ($F_{(2,96)} = 3.13, p < 0.05^\gamma, \eta^2_p = 0.06$) in grasping reflex strength. The KOF had a stronger grasp than the WTF mice on P6 ($p < 0.05^*$), whereas the KOM mice were relative to WTM mice on P6 ($p < 0.01^{**}$). However, no significant interaction between Genotype, Sex, or Age was observed for grasping reflex strength (Figure 16b). Additionally, there were no significant differences observed between groups in the righting reflex (Figure 16c). A Repeated Measures ANOVA revealed a significant effect of Genotype ($F_{(1,45)} = 121.4, p < 0.001^c, \eta^2_p = 0.73$), Age ($F_{(2,90)} = 786.2, p < 0.001^h, \eta^2_p = 0.95$), and a significant interaction between Genotype × Sex ($F_{(1,45)} = 6.158, p < 0.05^\varepsilon, \eta^2_p = 0.12$), a significant Genotype × Age interaction ($F_{(2,90)} = 30.49, p < 0.001^{###}, \eta^2_p = 0.40$), as well as a significant Genotype × Sex × Age interaction ($F_{(2,96)} = 3.583, p < 0.05^\gamma, \eta^2_p = 0.07$) on weight (Figure 16d). The KO mice weighed significantly less than the WT mice at all ages ($p < 0.001^{***}$). The mice weight were tested to determine whether their developmental weight could have had a relationship in predicting adult anxiety-like behaviors using a mixed model variance component selection approach. The mouse weight was determined to have a significant effect on complex USV production; however, the interaction between Genotype × Age still existed. Therefore the significant differences observed between WTF and KOF remained.
Figure 16. Behavioral milestones of 5-HT1A-R KO pups compared to WT. (a) There was a Genotype effect on startle response with KO pups being more likely to startle than WT pups. (b) There were effects of Genotype, Sex, and Age, as well as a Genotype × Age interaction and an interaction of Genotype × Sex × Age on the grasping reflex strength. (c) There was an effect of age on the righting reflex latency. (d) There were significant main effects of Genotype, Sex, and Age and interaction between Genotype × Age and Genotype × Sex × Age on pup weight. A Repeated Measures ANOVAs were followed by Newman-Keuls post-hoc tests. Genotype effect \( p < 0.01^b, p < 0.001^c \); Sex effect \( p < 0.01^d \); Age effects \( p < 0.01^g, p < 0.001^h \); Age × Genotype interactions \( p < 0.01##, p < 0.001### \); Genotype × Sex interaction, \( p < 0.05^e \); Genotype × Sex × Age \( p < 0.05^¥; p < 0.001***, p < 0.05^€, p < 0.001** \). The n-sizes for each group are denoted inside the bars in white font. Data are presented as the mean ± SEM.
2.4 Discussion

Kagan and his colleagues, Fox, Snidman, and Biederman [23, 25, 132] have contributed significantly to the central role in establishing early childhood behavioral predictors of anxiety. Indeed, emotionality seems to emerge as early as 3-months of age in human infants as they start to show facial expressions consistent with emotional states that persist well into adulthood [2]. Kagan [24] defined a series of behaviors that indicate “behavioral inhibition” that include social withdrawal and higher levels of physical arousal and reactivity that were initially detected at 2-years of age, and later on were detected as early as 4-months of age. Importantly, these traits were shown to be predictive of later-life anxiety and had shown right frontal EEG activation, as well as increased amygdalar activity [24, 133]. This earlier age emergence of an anxiety phenotype in humans also corresponds to those that equivocally emerge in 8 to 10-day old mice and further falls well into the postnatal brain developmental critical period [134].

Most of the negative emotional affect that can be communicated in human infancy and childhood is conveyed mainly through crying or gesturing [120]. Similar to mouse USVs, infant cries have also been analyzed for their spectrographic properties [26, 135]. One example is that studies in human infants show that abrupt frequency switches, also known as “shifts”[27] could be indicative of overexcitability in prenatally cocaine-exposed, asphyxiated, and asthmatic infants [26, 31, 136]. This may be analogous to the correlations seen between subtypes of USVs and anxiety-like behaviors observed in this study. However, this relationship would need to be explored further; especially, as the analyses of infant crying and rodent USVs tend to diverge between species rather than converge creating limitations and warranting further study [31, 137]. In addition, the number of utterances in human infants [31, 136] has also been associated with prenatal cocaine exposure, which may be similar to the elevated USV numbers seen in postnatal
KO mice in this study. More studies need to be conducted to determine whether or not a connection between human infant utterances and anxiety in adulthood exists, and whether or not these predictions are in-line with the present study, as female infants are more prone to anxiety and produce a higher number of utterances. Although it is a challenge to study human infant crying in relation to adult anxiety for obvious longitudinal study and varying environmental/demographic factors, existing longitudinal studies have fortunately been able to connect adult anxiety to infant temperament, as rated by the infants’ subjective parents and teachers reports [137, 138]. Despite these studies limitations and equal contributions, not much has been done to study infant crying acoustics or frequency in humans or rodents and their relationship to later-life behaviors, except mainly for the select populations of drug-exposed infants [136] or populations which were exposed to environmentally induced prenatal stress [139]. The present study results are, however, similar to other rodent studies that have already established the relationship between USV duration with later-life anxiety [109] as well as USV numbers in serotonin-deficient adult and neonatal rats [118].

The brain pathways and corresponding neurotransmitter systems that regulate USVs have also been related to separation anxiety disorder in humans infants [140]. In general, many studies do corroborate with or support the finding that USVs are modulated by the same neurological systems that are involved in stress and fear regulation[141-145]. One brain region that is involved in modulating both fear responses to aversive stimuli as well as USV production is the periaqueductal gray (PAG) region of the midbrain[141-145]. Across various species, including rodents, cats, monkeys, and humans, the stimulation of the PAG produces species-specific vocalizations, and contrastingly, lesions of the PAG cause mutism, which is also classified as an anxiety disorder [1, 141, 146]. The serotonergic system has been shown to be
involved in the phasic modulation of the PAG in augmenting fear [147]. For example, opioids have been found to interact with the 5-HT$_{1A}$-Rs, specifically, to mediate escape related behavior [147]. The lack of 5-HT$_{1A}$-R in this mouse model could, therefore, have a direct/indirect effect on opioid-5-HT$_{1A}$-R mediated PAG activity [147]. One proposed mechanism is that the increase in 5-HT$_{1A}$-R autoreceptors binding in the DNR could further decrease the release of serotonin and inhibition of the PAG leading to overexcitation of the PAG and increased vocalizing [148-150].

When stimulated, the hippocampus also has been shown to be involved in USV production in rats and their USVs decrease upon treatment with the 5-HT$_{1A}$-R agonist ipsapirone [151, 152]. This is relevant to the present mouse model because neurogenesis in the subgranular zone of the dentate gyrus is lowered in anxious and depressed patients, which is corrected with 5-HT$_{1A}$-R agonism and SSRIs [43, 70, 72, 84]. Hippocampal excitability has also been found to be altered in 5-HT$_{1A}$-R KO mice [61, 77, 153]. Together these studies suggest that there is a strong link between the 5-HT$_{1A}$-R and its involvement in both anxiety and USV production mechanisms. Since there is a sex-specific effect in 5-HT$_{1A}$-R binding, RNA, and protein levels in the human brain, it is not surprising that there would be a difference between WTF and KOF regarding USV production [36, 44, 154, 155]. There is also evidence that there are sex-dependent differences that mediate the hippocampal control of the hypothalamic pituitary adrenal axis (HPA) through serotonergic mechanisms that are sensitive to androgens [156]. Sex hormones could, therefore, play a unique role in the developmental and pubescent programming of USV production in this mouse model; however, more studies would be needed to investigate this link fully.
The current finding that WTF mice had more complex USVs and complex USVs containing sequences is similar to those found in the control group of the *Tbx1* heterozygous mutant mouse model of autism [94]. Others have also found that complex USVs increase with age in rodents, as they did in this study [157, 158]. Studies in infants have also found an increase in complexly modulated by multiple-arc melodic crying with age [159]. Reductions in complex type USVs have also been found in the Parkinson’s disease rat model by Ciucci’s group [160], as well as unenriched environments by others [160, 161]. Frequency modulated USVs are also associated with appetitive and pleasurable affective states such as play [162]. Sometimes also referred to as a “trill” type USVs, it is also possible that this type of USV requires more active oscillatory movements of the laryngeal muscles [163, 164]. This could suggest that the KOF lacked the ability to produce complex type USVs. In addition, it is conceivable that the KOF were better able to produce flat and short type USVs due to the same reason that they might have not had the ability to produce downward type USVs on P6 as these would require more vocal modulation [158]. Two-syllable type USVs have also been associated with increased anxiety in mice born to Rapid Eye Movement (REM) sleep deprived mothers [158], as well as repetitive behavior in Fragile-X mice [103]. The present study did find that tw-syllable-harmonic type USV containing sequences were more associated with KOM mice on P6 and that they correlated with anxiety-like behavior in adulthood.

In the “real maternal retrieval” experiment, there were no significant differences found in the percentage of pups retrieved except on P10, when USV numbers were not significantly different between groups. Therefore, this difference may have been due to the behavioral phenotypes of the dams, and not that of the pups. It is possible that the KO dams had elevated levels of anxiety, which may have caused them to retrieve their pups more often. Furthermore,
in the “simulated retrieval experiment,” in which pups were absent, the types of USVs, limited to (but covering a broad range of USV types) downward, two-syllable, and harmonics type USVs did not have an effect on the time the dams spent in the “Sound” localized tube. This may suggest that the dams did not prefer certain USVs over others, as this covered a wide range of USV types. Alternatively, the dams may either require additional visual feedback of an actual pup to evoke the pup retrieval behavior or they require the exact HPA hormonal synchronization with the timing of their programmed maternal behaviors to engage in pup retrieval behaviors. However, one lacking variable in this study that should be noted is the pitch and amplitude characteristics of USVs, as these are both useful predictors of brain diseases in other rodent [95] and human studies [135]. Future studies will be aimed to evaluate whether or not these other spectral properties define USV developmental differences between the groups and whether they can be pharmacologically rescued to wild-type levels.

Unique to studies on USVs in the 5-HT₁A-R KO mouse model was the Markov Chain Model Analysis used in this study. The PLSDA of the Markov Chain Model Analysis had a lower classification error rate than the PLSDA of single USV analysis. This may be due to the fact that the PLSDA of non-sequence USV analysis involved a much lower number of variables [165] in proportion to the number of groups. Previous studies with lower error rates had a higher ratio of variables to groups [94], which can reduce error rates as a result. For this reason, another quantitative analysis, by way of a Repeated Measures ANOVA was paired with PLSDA to analyze the Genotype × Sex interactions as a function of Age. The results indeed did reveal that Age was a major factor in USV call repertoire, as was also suggested by the pattern of ages across “Component 2” of the correlation circles to be significant. Although the KO mice had higher entropy levels overall than the WT mice, the WT mice did have a higher tendency to
produce sequences of USVs that had complex type USVs, which would involve more vocal modulation. In this case, the lower level of entropy seen in WT mice might not be attributable to repetitiveness or monotony in the mouse vocal repertoire, but rather to an ability to produce USVs that are in themselves less monotonous and require more vocal control. Moreover, this might also suggest that there is less randomness in the USV sequence structure produced by the WT mice.

Finally, behavioral milestone tests used in this study may suggest differences in growth and motor development in the KO mice relative to their WT counterparts. In human infants, differences in motor development have been related to anxious and inhibited temperaments, hypothesized to be a result of amygdalofugal overactivity [23]. The enhanced startle response seen in KO mice may also be indicative of overexcitability, a trait that has been noted in other studies focusing on this mouse model [77]. The increase in grasping strength in KO pups early on might suggest a difference in motor development. In future studies, pharmacological activation of 5-HT1A-R signaling can be used to further explore neonatal motor development.

5. Conclusion

In this study, the adult KO female mice exhibited elevated anxiety-like behaviors compared to the WT female mice. It was also observed that adult anxiety-like behaviors, as measured across different behavioral tests involving the OF, EPM, and LD tests, can be predicted by early postnatal developmental USV numbers; especially, on P8. In addition, patterns of vocal communication differ between KO and WT mice and are Age and Sex-dependent. For example, complex USV containing sequences, are less likely to be seen in KOF mice on P10. This is the first study to find a unique early behavioral developmental milestone that is predictive of adult anxiety-like behavioral phenotypes in KO female mice, and this
phenotype can be targeted in future studies to correct anxiety-like behavior through behavioral pharmacology.
3 **Aim 2.** Study postnatal neuroproliferation in the Dentate Gyrus in the 5-HT<sub>1A</sub>-R knockout and wild type mice with special attention to possible genotypic and sex differences.

3.1 **Introduction**

According to the World Health Organization (WHO), the global prevalence of anxiety in 2015 was 3.6% of the population. Importantly, almost twice as many females *(i.e., 4.6%)* compared to males *(i.e., 2.6%)* were affected by some form of anxiety disorder[166]. However, the age of onset for an anxiety disorder occurs early in development [15], yet during early childhood and adolescence, girls have a higher heritability estimate for developing and/or acquiring anxiety disorders [167]. Notably, of the two sexes, girls have an earlier onset of anxiety disorders, when compared to boys [15, 21]. It is well-established that childhood trauma [168] and stress [169, 170] can negatively affect a child’s brain development resulting in a range of biopsychological disorders. Still, the relationship between brain development, susceptibilities resulting from such aberrant developmental process, and the compounding sex-dependent differences, that could in part or in cooperation, result in adult anxiety disorders remain to be elucidated [171].

A small number of studies have focused on brain activity in children and adolescents who are predisposed to anxiety disorders or depression. Children who are at risk of developing an anxiety disorder have been found to have a comorbid increase in activity in frontal and limbic brain regions when exposed to emotional stimuli *(e.g., angry or happy faces)* [172]. These emotionally susceptible neural structures are tightly associated with the brain’s emotion-modulating centers that comprise the limbic system [173, 174]. Amongst the limbic system, the hippocampus, anterior cingulate, prefrontal cortex, and insula had the greatest change in activity in response to emotionally charged stimuli [172].
The hippocampus has been considered as a part of the limbic system since James Papez formulated the limbic network model in the 1930s, which since has been referred to as the Papez circuit [175]. As a part of the Papez circuit, the hippocampus plays a critical role in the initiation of cognitive activity that would give rise to emotion, by way of signaling the fornix, and further relayed to the mammillary bodies [175]. From the mammillary bodies, these signals would ultimately be sent to the thalamus, which would then communicate with the cingulate gyrus [175]. The final receptive cortical structures, such as the cingulate cortex, would then be responsible for the perception of one’s emotional experience [175].
The hippocampus itself may be subdivided into dorsal or rostral and ventral or caudal regions, or neuraxes [176] (Figure 17). It is a more popular theory that the ventral hippocampus plays a central role in emotional regulation [177, 178], while the dorsal section is mainly involved in “cold” cognitive processes such as spatial learning [178]. The ventral hippocampus, for example, is thought to mainly signal to regions of the brain that are involved in emotional regulation (e.g., amygdala and medial prefrontal cortex [mPFC]) [5]. However, there are studies that suggest that the dorsal hippocampus, is in fact, equally important in regulating fearful emotions in concert with the amygdala [179-182]. Mainly, these studies focused on fear learning and memory, but there is a certain degree of overlap between the neural circuits that are activated during fear learning and anxiety symptoms [5]. Further, dorsal complexities are also encountered in this theoretical model by the fact that the ventral hippocampus has been shown to also be crucial for spatial learning [183]. Furthermore, Swanson and colleagues [184] traced populations of ventral hippocampal neurons and showed that there is distinct neural connectivity within the hippocampal formation along the dorsal-ventral axes. As an analogy to the dorsal hippocampus, for which prior evidence has suggested a wide-variety of functions, it is to be contested whether or not the number of a specific cell type (i.e., a place cell), can dictate the function and strength of neural connectivity along the hippocampal dorsal-ventral (DV) neuraxis. In regards to the DV neural connectivity of the hippocampus, this can be further complicated by the fact that the hippocampal CA3 region has a
large number of collaterals that have a very broad range of connections; some of which, feed back to the hippocampus along the DV neuraxis [173]. It might, therefore, be presumptuous to assume that the hippocampus can truly be divided into two separate yet distinct regions with separate functions.

The hippocampal formation is a cortical structure buried deep within the temporal lobe [185]. Briefly, hippocampal neurocircuitry, also known as the trisynaptic loop of the hippocampus, involves the perforant pathway of efferent projections to the entorhinal cortical axons directed towards the dentate gyrus (DG) granule cells, which in turn, send efferents to CA3 pyramidal cells via the mossy fiber pathway, and finally, the CA3 efferents that project to the CA1 pyramidal cells, which then send their axons back to the entorhinal cortex [185]. One process that could, therefore, have an effect on hippocampal function starting at the perforant path, is the birth of new neurons in the DG, which are thought to play a key role in pattern separation and extinction learning, an important part of regulating fear and anxiety [89, 186, 187]. Although recently contradicted by one study [188], it has been well accepted that neurogenesis continues throughout adulthood in the human DG, among a few other regions in the brain (i.e., olfactory bulb, amygdala, hypothalamus, and visual cortex) [186, 189, 190].

The neurogenic region of the adult DG is the subgranular zone (SGZ), located beneath the layers of spherical granule neurons [191-194]. Notably, 85% of granular neurons are produced after birth, making it an active region of neurogenesis throughout life [195]. The neurons that are produced within the SGZ become incorporated into the DG granule cell layer in an outside-in pattern of development[196]. The newly incorporated cells eventually become integrated into the already established DG circuitry [197, 198]. It is known that granule cells undergoing maturation are able to control learning and memory within the brain [199].
neurogenesis can be affected by hormones, neurotransmitter balancing, environmental enrichment, neurotoxicants, and in response to seizures [200]. The SSRIs, which are used to treat depression and anxiety, have also been known to increase neurogenesis in the human and murine hippocampus [43, 70, 72]. The neurogenic theory of depression posits that impaired adult hippocampal neurogenesis may trigger depression and by restoring neurogenesis to normal levels can produce a form of stabilization that might lead to recovery [71, 201]. Since anxiety disorders are highly comorbid with depression, and serotonergic psychotropic medications are effective in treating anxiety, the neurogenic theory has been extended from depression to also include anxiety disorders [201].

Specifically, serotonergic signaling via the $5\text{-HT}_{1A}$-R has been implicated in major depression [41, 155, 202-205] and anxiety [205-208]. Moreover, SSRIs are known to indirectly cause an increase in serotonin signaling via $5\text{-HT}_{1A}$-R [62]. This process relies on the desensitization of presynaptic autoreceptor $5\text{-HT}_{1A}$-Rs on the DRN, which eventually leads to their disinhibition and ability to release more serotonin onto postsynaptic heteroreceptors [60, 65]. Furthermore, it has been shown that the presence of $5\text{-HT}_{1A}$-Rs on mature granule cells is crucial for the behavioral effects of antidepressants [43]. However, the presence of $5\text{-HT}_{1A}$-Rs from birth up to P21 have been shown to be crucial for normal establishment of mood in adult mice [40]. Despite these data, there has been little research into the effects of $5\text{-HT}_{1A}$-Rs on postnatal neuroproliferation. In addition, sex-dependent differences have yet to be examined in the postnatal mouse within the hippocampal neurogenic zone constituting the $5\text{-HT}_{1A}$-Rs within the DG SGZ [209].

Benerjee et al. [210] has previously shown that in postnatal development the $5\text{-HT}_{1A}$-R molecular signaling pathways are important for normal hippocampal function and plasticity. The
current study sought to examine whether or not sex-dependent differences in neuroproliferation in an established mouse model of anxiety exist, and whether the 5-HT$_{1A}$-R KO on P8, would be equivalent to the data reported on newly born human infants [86, 211]. Doublecortin (DCX) is a microtubule-associated protein involved in neuronal migration and is important in early development [212]. Newly born neurons are marked with Ki67, a protein found in the nuclei of dividing cells, and Doublecortin (DCX), a protein found in dendrites as well as in neuroblasts [213, 214], and the colocalization of the two biomarkers were used to positively identify new neurons that would allow for accurate visualization and counting of neuroblasts.
3.2 Methods

3.2.1 Subjects

Procedures were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and City University of New York College of Staten Island. 5-HT$_{1A}$-R knockout mice were obtained from Dr. Laurence Tecott from the University of California San Francisco. Four to six P8 mice were used per group (i.e. WTF, KOF, WTM, KOM).

3.2.2 Animal Husbandry

The C57BL/6J WT mice, as well as 5-HT$_{1A}$-R knockout mice on the same background were housed in groups of a maximum of 3-5 per cage with food and water available ad libitum. The light cycle was 12:12-h light/dark with all behavioral testing being conducted during the light period.

3.2.3 Immunohistochemistry (IHC)

Four to six mice were used for each treatment group. Mice were randomly selected in groups of two from a given litter. Subsequently, mice were then anesthetized with a Ketamine/Xylazine cocktail (30 mg/10kg s.c.) and were transcardially perfused with physiological buffered saline (PBS) pH 7.4 first to exsanguinate the mice prior to fixing them with 4% paraformaldehyde (PFA) in PBS. The right cerebral hemisphere was used for all IHC methods that follow. Next, 1 hr later, the fixed hemi-sected brains were then transferred to a 30% sucrose in PBS cryoprotectant solution and stored for 48 hours. Serial coronal sections (i.e., 30 μm thick) were cut using a Bright Instruments OTF5000 cryostat (Bedfordshire, UK) and stored as free-floating sections in PBS at 4 °C. When sectioning, each hemi-sected brain was started at a random point, and every 6th section was collected for IHC staining. The random
unbiased sections were then rinsed 3X in PBS and were then immune-blocked for at least 30 min (i.e., 0.5% Tween, 10% normal goat serum (NGS) in PBS) at room temperature. The sections remained in the immune-blocking solution and were then incubated with the primary antibody overnight at 4 °C. The following day, sections were then rinsed 3X in 0.5% PBS tween solution and then were incubated in immune-blocking solution with the secondary antibody overnight at 4 °C. Lastly, after rinsing off the secondary antibody, a fluorescent cell body dye (i.e., Hoeschst) was diluted in PBS (1:500) and was added to the sections and incubated for 30 min at room temperature. The concentrations for the antibodies were as follows: anti-Ki67 (1:200, Mouse, Santa Cruz Biotechnology), anti-DCX (1:250, Goat, Santa Cruz Biotechnology), Hoechst (1:1000). Slices were then mounted onto slides, and Gold-AntiFade Reagent was added, they were coverslipped, and allowed to dry for at least 48 hours in a dark place.

3.2.4 Confocal Imaging and Counting

A Leica SP2 AOBS Confocal microscope (Wetzlar, Germany) was used to image the sections. Both the 5X reference images and 40X Z-stack images were acquired with the purpose of imaging the full length of the superior and inferior blades of the DG. The Z-stacks were reconstructed using Imaris 7.6.4 and Imaris 7.6.7. (Oxford Instruments, Abingdon, UK). An observer blind to the treatment groups was trained to be able to competently count colocalized Ki67/DCX ++ cells. First, an overlay of colocalized red channel (Ki67) and blue channel (Hoechst) was made using the “Coloc” tool in Imaris 7.6.4. Then the newly generated “Coloc” layer was colocalized with the green channel (DCX). Additionally, the background selection was avoided as much as possible using the thresholding aspect of the tool. Imaris 7.6.7 had the more advanced rendering and was used for the 3D analysis of cells. Once the colocalized channels were constructed, they were opened in Imaris 7.6.7. The “Surface” 3D tool was used to
delineate the region of interest from which the cells would be counted. This included the entire granular area, hilus, and 4 cell thicknesses deep below the granular zone. This would be used to “mask” these layers so that a new channel would be formed including the colocalized channels only. From here, the “Spots” tool was used to count the colocalized cells, with an input diameter of 6 μm. The Section tool was then used to confirm the colocalized cells in the X-, Y-, and Z-axes (Figure 18). If cells were over-counted, they would be manually removed using the “Spots” tool. Subsequent Z-stacks that covered the rest of the DG blade volumes were analyzed in the same manner; however, overlapping regions in the X-axis were excluded to avoid double counting. To avoid double-counting of cells in the upper and lower parts of the section, the upper and lower 2 μm of the Z-stack slices were excluded.

Further, the DCX + cells with dendritic morphologies representing more mature neurons were counted separately. The mask generated in the first half of counting was reused to delineate the green channel (DCX+), and cells were counted using an input diameter of 10 μm.
Figure 18 Images taken from Imaris “Section” view showing colocalized cells in the X-, Y-, and Z-dimensions. (a) Image showing colocalized cell. Scale bar = 50 μm (b) Zoomed in image showing colocalization of Ki67 and DCX expression in the same cell. Scale bar = 15 μm.

3.2.5 Statistics

A 2×2 ANOVA was used to analyze the cell counts. Tukey’s post-hoc test was applied when the ANOVA revealed a significant effect.
3.3 Results

Levels of neuroproliferation were assessed in the DG of P-8 mice, since significant behavioral differences had been previously found at this age, and furthermore this age corresponded to human newborns [134]. Representative images were taken from 40X Z-stacks are shown in Figure 19. A 2x2 factorial ANOVA revealed that there was a significant main effect of Genotype ($F_{(1,15)} = 18.45, p < 0.01^*$, $\eta^2_p = 0.56$), and a trending Genotype × Sex interaction ($F_{(1,14)} = 3.83, p = 0.07, \eta^2_p = 0.21$) on the number of neuroblasts in the Dorsal DG. The Tukey’s post-hoc comparisons showed that the KOF mice had a significantly lower number of cells compared to the WTF mice ($p < 0.001^{***}$) (Figure 20a). A significant Genotype×Sex interaction ($F_{(1,19)} = 4.32, p < 0.051, \eta^2_p = 0.19$) was observed in Ventral DG neuroproliferation as well. However, the Tukey’s post-hoc comparisons did not reveal significant differences (Figure 20b). A significant main effect of Genotype ($F_{(1,15)} = 14.43, p < 0.01^*$, $\eta^2_p = 0.49$) on the total neuroproliferation in the DG was observed. A trending Genotype × Sex ($F_{(1,15)} = 3.74, p = 0.07, \eta^2_p = 0.20$) interaction was observed, and the Tukey’s post-hoc test revealed that the KOF mice had a significantly lower number of cells than the WTF mice ($p < 0.01^{**}$) (Figure 20c). Interestingly, a certain number of samples had a higher number of large post-mitotic DCX + cells. A 2x2 factorial ANOVA revealed that there was a significant Genotype×Sex interaction ($F_{(1,15)} = 6.23, p < 0.05^*, \eta^2_p = 0.27$) on the number of DCX+ postmitotic granule cells in the DG. The Tukey’s post-hoc comparisons revealed that the KOF mice had a significantly larger number of DCX+ cells than the WTF mice ($p < 0.05^*$) (Figure 20d). In addition, a significant Genotype×Sex ($F_{(1,15)} = 6.23, p < 0.05^*, \eta^2_p = 0.27$) interaction was observed regarding the total number of DCX+ postmitotic cells in the Dorsal DG. The Tukey’s post-hoc comparisons
revealed a significantly higher number in the KOF mice compared with the WTF mice ($p < 0.05^*$) (Figure 20e).
Figure 19. Representative 40X Z-stack volume renderings of dorsal region of the DG tips taken from P8 WT and KO mice. (a) WTF mouse DG (b) KOF mouse DG (c) WTM mouse DG (d) KOM mouse DG. Notice that the WTF mice have an increase in Ki67/ DCX++ expressing cells. Scale bar: 50 μm. Blue: Hoechst, Red: Ki67, Green: DCX.
Figure 20. **Sex differences in dorsal DG neuroproliferation.** (a) The number of cells in the dorsal DG of the KOF mice was significantly lower than the WTF mice. (b) There were no significant differences in the number of cells between groups. (c) The total number of proliferating cells in the DG was significantly higher in the WTF mice than the KOF mice. (d) The total number of mature DCX+ cells in the DG was significantly higher in the KOF mice than the WTF mice. (e) The total number of postmitotic cells in the DG was significantly higher in the KOF mice compared with the WTF mice. Significant Differences are illustrated as: $p < 0.05^*$ and $p < 0.01^{**}$. Data are presented as the mean ± SEM. The n-sizes for each group are denoted in white numbers inside the bars.
3.4 Discussion

This is the first study to discover sex-dependent differences in neuroproliferation in a mouse model of anxiety disorders. Admittedly, studies on neurogenesis in the postnatal rat hippocampus have already been conducted by Joseph Altman, the discoverer of neurogenesis, in the 1970s, and then by others starting in the 1980s [192, 195, 209, 214-224]. In 1975 Altman and Bayer [216] irradiated granule cells in the postnatal rat DG, and they found that this had a significant negative effect on DG granule cell numbers when the rats were adults. Altman and Bayer’s study underscored the importance of postnatal granule cell proliferation on hippocampal development.

The present study also found that postmitotic DCX positive neurons were increased in the DG of the KOF mice. This may suggest a compensatory effect, which may be related to the low amount of neuroproliferation in the KOF mice, when compared to the WTF mice. There are theories regarding the relationship between the level of granule cell dematuration and depression, rather than neuroproliferation [34]. At the state when the WTF mouse DG has cell had undergone mitosis, the KOF mice already had postmitotic cells and a low number of neuroblasts. This might indicate a different/aberrant developmental trajectory of the hippocampus in the KOF mice, when compared to the WTF mice [212]. The combined effect of attenuated neuroproliferation and augmented speed of maturation of granule cells could cause aberrations in DG circuitry, that in turn, may contribute to anxiety-like behaviors that might manifest in early adolescence and persist well into adult hood.

Muramatsu and colleagues [222] showed that the majority of adult DG cells come from those that were born postnatally, suggesting that this period of neurogenesis is crucial for the establishment of later life DG connectivity. Linda Overstreet Wadiche and Liu [225-227] were
able to show that 1-week old postmitotic granule cells exclusively demonstrate tonic GABA firing in the postnatal mouse DG. Wadiche’s group [226] had also shown that basket cell synapses were present near the newly born granule cells. Ge and colleagues [228] found that the tonic current was enhanced by stimulation of basket cells. Admittedly, it has already been evident that basket cells arborize and establish their connections by P7 [225, 229, 230]. However, tonic GABA firing is not enough for the newly born granule cells to fully integrate into the hippocampal circuitry, as synaptic responses start to occur in the second week of their maturation. Certainly, one-week-old granule cells do not have excitatory input from the entorhinal cortex either [227]. Most of the above-mentioned studies focused on “immature” granule neurons, which were 1-week old at the time that they showed GABA tonic currents. The tonic GABA currents were followed by a synaptic response to GABA, and later on to glutamate [228]. Showing that even neuroblasts contain GABA tonic currents, and further nestin+ neuroblasts had been shown to have tonic GABA currents by Bhattacharyya’s group [231]. The GABA activity in both postnatal and adult newly born granule cells, however, is depolarizing and excitatory [225, 227, 232]. Ge [228] went on further to show that the reversal of the depolarizing excitatory currents to shift to hyperpolarizing inhibitory currents (i.e., the GABA-shift) at this point was detrimental to the integration of the newly born granule cells with the rest of the DG circuitry. Taken together, these studies demonstrated that the developing granule cells, starting from neuroblasts to one-week after birth, undergo maturation rapidly through a tightly regulated developmental program. In fact, the postnatal granule cells matured at a rate of 1-week faster than adult-born granule cells [227]. The implication of this study was that within a very short period of time, the newly born granule cells would already begin to be integrated into the hippocampal circuitry. The more advanced postmitotic cells, would, however, form
GABAergic and sequentially, glutamatergic synapses sooner than neuroblasts [228], but there would be fewer potential immature granule cells to be born, according to the current study and data described herein.

An explanation for the consequence of having more postmitotic DCX+ cells than neuroblasts could potentially begin deriving from the evidence that newly born granule neurons may function in replacing old memories by replacing old granule neurons through the process of neurogenesis [233]. This important process is a part of extinction learning, in which experiments in fear conditioning are able to abolish or reduce conditioned fear responses [233]. This process is also very relevant to infantile amnesia, in which infants are prone to a period of forgetting, up until they are approximately 3-years old [233, 234]. Furthermore, the cause of infantile amnesia has been attributed to the rapid neurogenesis that occurs during infancy [233]. Akers and colleagues [233] were able to demonstrate that infantile amnesia positively affects forgetting during adulthood through the process of neurogenesis. Therefore, if the rate of neurogenesis is lower in KOF mice, but the presence of post-mitotic cells is higher, this could suggest a few important findings to carefully consider. First, the neuroblasts present in the WTF mice might suggest that there is a potential for more granule cells that can be replaced than is possible in the KOF mice; thus, making the forgetting process easier in the WTF mice. Therefore, the KOF mice, might have impaired infantile amnesia and this could affect the establishment of a normal emotional-behavioral system and contribute to the development of anxiety-like behavioral phenotypes in these mice. In support of this theory and it’s effect on the 5-HT_{1A}-R KO mice model have been shown to overgeneralize in contextual fear learning [235]. Second, the immature granule cells have higher input resistance, fire more spontaneously, and are able to better express long-term potentiation (LTP) than older granule cells [236]. If the higher number
of neuroblasts in the WTF mice eventually become immature granule neurons, they will have a greater potential in mediating the plasticity of the mossy fiber pathway, which the KOF mice would have difficulty since they have fewer newly born cells [236, 237]. The KOF mice, therefore, present with a unique neurodevelopmental phenotype that could prove useful in predicting later-life anxiety-like disorders as an important model system for anxiety and depression clinical and neuropsychopharmacological research.

As a predictive phenotype of anxiety, this \( 5-HT_{1A} \)-R KO mouse model can be pharmacologically targeted in future rescue studies to aid in developing better translational biomedical treatment approaches. Banerjee and colleagues [85] had shown possible downstream mechanisms of activation of \( 5-HT_{1A} \)-R mediated signaling, in the absence of the receptor. Intra-hippocampal injections of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid DCP-LA, a drug that targets the downstream signaling messenger, PKC Epsilon, can be used to assess its ability to rescue neuroproliferation. Since neuroproliferation sex-dependent differences were observed at P8, this may translate into human newborn infants and may also suggest future preventative treatment approaches for humans.
4 Aim 3. Investigate the ultrastructure hippocampal stratum radiatum in the 5-HT1A-R knockout and wild type mice with special attention to possible genotypic and sex differences.

4.1 Introduction

Anxiety disorders are the most common of mental disorders, especially among children [21, 238]. However, girls have a higher chance of inheriting anxiety disorders than boys [239]. Despite the growing research and clinical reports on sex-dependent differences observed in children, far less is known of any neurodevelopmental differences that could be attributed to this sex-based disparity [238, 239]. However, the biological vulnerabilities that comprise anxiety disorders, may be logically expected to involve neuroconnectivity and synaptic connections that would best explain the emotional dysregulation observed in the clinical manifestation of the disorder as a consequence of a developmental miss-programming [34, 238]. The critical periods regarding brain growth and their programmed phasic spurts, including the surge in new neurons and synapses (i.e., neurogenesis and synaptogenesis) have been previously described and there conserved comparative psychological and neurobiological traits proposed between humans and rodents [240, 241]. Thus, critical periods may further serve to allow human infants and rodent pups to acquire knowledge from key stimuli or cues within their surrounding sensory environments for the appropriate defensive and/or exploratory responses as a key feature of postnatal experiential learning [35].

In human infants, the earliest diagnosable form of anxiety is separation anxiety disorder, which can appear as early as 6-months of age [16]. The manifestation of this form of anxiety is apparent in fearfulness and shyness with new people and/or strangers [16] and further can be seen behaviorally, as a way to cautiously interact and learn about the environment for the purpose of self-preservation and safety through their behavioral inactivation systems at the
cortical level[35]. Shyness and timidness in children are traits that have been connected to what is termed “behavioral inhibition” that is directed by the cortical behavioral inactivation system[81]. This anxious phenotype starts to become detectable at 2-years of age, and it’s persistence from 2-5 years of age have been shown to be predictive of anxiety at later adolescent and adult ages [23, 81]. Since the timing of these events is contemporaneous with the critical periods that occur in distinct phases during brain development, they offer a precise and yet unique opportunity for investigating how sex and the anxiety/depression mediated 5-HT1A-R might interact between the environment to assess the level of vulnerability that these neural circuits endure during key neural plasticity events during development [35]. The corresponding time-period that parallels that of a 6-12 month newborn human infant brain in the developing rodent brain is reported to be equivalent as the first few postnatal weeks [240]. During this critical postnatal developmental period within the analogous mouse model, the present study sought to investigate the sex-dependent and genotype differences that could be detected at the hippocampal stratum radiatum ultrastructural levels to better understand its contribution to the alterations in the behavioral (Aim 1) and neuroproliferative (Aim 2) differences previously observed as described in Chapters 1 and 2, respectively.

The hippocampal trisynaptic circuitry has been extensively studied within postnatal rodents [242], and in adults the 5-HT1A-R has been implicated in the regulation of glutamate sensitivity within the CA1 area [153]. However, until now hippocampal ultrastructural studies related to the CA1 region have yet to be performed. The present study sought to examine the hippocampal stratum radiatum ultrastructure within the P10 5-HT1A-R KO mouse model to elucidate its contribution to the manifestation of anxiety-like behavior as a structure-function relationship. Synaptogenesis at this particular age (i.e., P10), precedes but is near the critical
period for peak neurogenesis and is hypothesized to contribute to hippocampal-dependent serotonergic short-term and/or lasting changes in brain connectivity that may underlie emotional learning, memory consolidation, retrieval, updating and re-updating, respectively. This is important as these serotonergic neurobiological processes may be at the clinical core of both anxiety and depressive disorders. Since the expression of the 5-HT\textsubscript{1A}-R appears in the first week after birth in rodents [40] and is involved in actin dynamics [243], the present study sought to assess whether or not KO mice had a different number of serotonergic synapses during the early part of peak synaptogenesis, when compared to WT mice.
4.2 Methods

4.2.1 Subjects

Procedures were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and City University of New York College of Staten Island. 5HT1A-R knockout mice were obtained from Dr. Laurence Tecott from the University of California San Francisco. Three mice were used per treatment group (i.e. WTF, KOF, WTM, KOM).

4.2.2 Husbandry

The C57BL/6J mice, as well as 5HT1A-R knockout mice bred from the same background were housed in groups of a maximum of 3-5 per cage with food and water available ad libitum. The light cycle was 12:12-h light/dark with all behavioral testing being conducted during the light period.

4.2.3 Specimen Preparation

Mice were anesthetized with a ketamine/xylazine mixture of 100 mg/kg and were perfused exsanguinated followed by transcardial perfusion with 2.5% glutaraldehyde: 2 % paraformaldehyde in PB. Brains were then extracted and postfixed in the same perfusion solution overnight. The next day, the brains were transferred into 1X PBS and 300 μm thick coronal sections were cut using a vibratome. The brain sections were then incubated in the same fixative solution overnight. The brain sections were reviewed and only sections that contained the dorsal hippocampus were selected and were then washed with 0.1 M PBS for a total of 30 min. The entire dorsal hippocampal sections were post-fixed in 1% osmium tetra-oxide in 1X PBS for 60 min. The brain sections were then rinsed again with 0.1M PBS for 30 min and serially dehydrated with graded acetones (i.e., 50%, 70%, 85%, 95%, 2 X with 100%) for 10 min.
each step, then pre-infiltrated with a 1:1 acetone: Spurr medium, and followed by a 100% Spurr medium overnight at room temperature. The processed dorsal hippocampal sections were then embedded with Spurr medium in Beem capsules and polymerized in an oven at 70 ℃ overnight to embed the hippocampi into a resin block. The Beem capsules were then thick-sectioned until the hippocampus exposed the *stratum radiatum* and was then further isolated by chipping off the rest of the embedded section with a razor (Figure 21). The resin block containing the hippocampal *stratum radiatum* was then thick-sectioned again to remove jagged edges, and then thin-sectioned with a diamond knife. Ultrathin hippocampal *stratum radiatum* sections (70–100 nm) were placed on uncoated 200 mesh copper grids, stained with saturated uranyl acetate in 50% ethanol for 30 min, rinsed with 0.22 mM Millipore-filtered distilled water for 2 min to obtain clean sections without any trace of uranyl acetate residue. These hippocampal *stratum radiatum* sections were then stained with Reynolds’s lead citrate for 10 min, and rinsed again with Millipore-filtered distilled water for 90 s. The resulting hippocampal *stratum radiatum* sections were then examined and imaged with a Hitachi 7500 electron microscope (Chiyoda, Tokyo, Japan) operated at 80kV. Quantification was achieved at 23,000x, using a minimum of 3 sections per mouse hippocampal *stratum radiatum* with three mice were used for each treatment set.
Figure 21. Resin block showing Stratum Radiatum region that was isolated during block trimming.
4.3 Results

Representative hippocampal *stratum radiatum* micrographs at 23,000x are presented in Figure 22. The present study assessed whether or not the KO and the WT mice would have a difference in their numbers of synapses on P10. The data revealed that there were no significant differences observed in the number of symmetric ($F_{(1,8)} = 0.005, p = 0.95, \eta^2_p = 0.006$) nor asymmetric ($F_{(1,8)} = 1.21, p = 0.37, \eta^2_p = 0.10$) synapses between the KO and the WT mice (Figure 23a). From these observations, it was then hypothesized whether or not there were any differences that could be observed in vesicle numbers within the presynaptic terminals. The data revealed that there were no significant differences ($F_{(1,8)} = 0.169, p = 0.691, \eta^2_p = 0.02$) observed in the number of vesicles between the KO and the WT mice (Figure 23b).
Figure 22. Transmission electron microscope images from (a) WTF (b) KOF (c) WTM and (d) KOM. Scale bar = 500 nm.
Figure 23. Synapse and vesicle counts in the CA1 region of age P10 WT and 5-HT1A-R KO mice. (a) There were no significant differences were observed between the WT and the KO mice in the numbers of symmetric and asymmetric synapses on P10. (b) There were no significant differences observed between the WT and the KO mice in the number of vesicles in presynaptic terminals of P10 mice. Data are presented as the mean ± SEM. The n-sizes for each group are denoted in white numbers inside bars.
4.4 Discussion

The results of the hippocampal *stratum radiatum* ultrastructure study regarding the numbers of excitatory and inhibitory synapses in the CA1 region of the developing WT and KO mice at P10 showed no significant structural changes that could be detected through TEM imaging techniques. These results are consistent with reports regarding the timing of the “brain growth spurt,” a term coined by Dobbing and his group [240, 244]. The timeline of neurodevelopment as a whole has been elucidated over the years as critical periods are of great interest to those studying this labile period of connectivity for its vulnerability to environmental insults [245]. However, more recent research has contended that specific brain regions have their own unique pattern of developmental trajectories (*e.g.*, the GABA-shift) and may suggest that there are many brain region specific critical periods that have yet to be fully resolved in understanding susceptibilities for acquiring neurodevelopmental pathologies. Some neurotoxicologists have prefer to study the effects of environmental toxins (*i.e.*, neurotoxicants) on neurodevelopment at P10 [246], but others agree that where synaptogenesis is concerned, P14-P16 is the critical period for synaptogenesis [35, 247]. Many events that occur in the development of the infant hippocampus can explain this particular window of time as being crucial for establishing hippocampal circuitry.

In the adult brain, the hippocampal CA1 region plays an active role in the output signaling from the hippocampus to the cortex; however, the same cannot be said about the CA1 region in infant brains [247]. The reason is that the time-period of postnatal hippocampal development is highly protracted, in humans with different parts of the trisynaptic circuitry fully developing at different time-points [247]. At least in humans, infantile amnesia prevents the formation of long-lasting memories for at least the first 18-24 months [247]. Notably,
complications also arise in the fact that even between subregions of the hippocampus, specific types of memory formation have been shown to vary [247]. The particular role of pattern separation of the hippocampal DG does not necessarily lead to the CA1 also being involved in this particular type of learning at the same time; especially, as connectivity between the two regions takes time to become established [247]. However, studies show that the hippocampal CA1 region is also more likely to be involved in spatial and temporal learning after repetitive training, and this has been shown in infants who learn to remember after repeated trial and error exposures [247]. In addition, memories formed by the hippocampal DG and CA3 are formed at a much faster rate than those formed by the CA1 region through repeated trial and error exposures [247]. As for volumes of these hippocampal subregions, the developmental trajectories also differs, with the DG and CA3 continuing to develop long after the CA1 has completed its developmental program [247]. This critical period phenomena also decreases the likelihood that the trisynaptic circuitry is established early on, as these regions are still not active to allow concerted activity at key points in early development.

However, even if pattern separation is unlikely to take place this early in human infants and perhaps as well in postnatal mice, the neural circuits responsible for such critical anxiety and/or depression programming through the hippocampal trisynaptic circuitry could still develop abnormally, form fully, but function aberrantly fully formed [247]. Therefore, the results obtained earlier in the present study could still have a major impact on the developmental pattern separation and its relationship to the neural connectivity due to the ongoing integration and maturation of the newly born cells during aging [209, 227, 233, 248]. If one were to look specifically at the age during which changes are known to occur as a function of neuroproliferation (i.e., P8) then the integration and connectivity of the newly born neurons at
this developmental age would occur within the second to third week in the postnatal mouse [221, 248]. Future studies on hippocampal ultrastructure within the CA1 region of P14-16 day old mice should be conducted to study the possibility of any difference in brain development between the WT and the 5-HT_{1A}-R KO mice during this period of heightened synaptogenesis in the hippocampus. Mogha, Guariglia, & Banerjee [85] had previously discovered that 5-HT_{1A}-R-linked activation of the MAP kinase pathway caused PKCα activation, which in turn boosted synaptogenesis in the P15 hippocampal CA1 region in the WT mice. Furthermore, activation of the downstream molecule PKCα in the 5-HT_{1A}-R KO mice also augmented synaptogenesis. These KO mice otherwise showed suppressed synaptogenesis at P15. Therefore, further studies are required to evaluate if the observed suppression of synaptogenesis in the 5-HT_{1A}-R KO mice at P15 was sex-dependent.
5 General Discussion

The present study sought to investigate and answer three experimentally driven questions on the relationship of the role of early neurodevelopmental serotonergic driven structure-function relationships as potential predictors for the manifestation of later-life anxiety and depressive behaviors between the 5-HT-1A-R WT and KO mice model. The first question hypothesized whether or not later-life anxiety disorders could be predicted by early behavioral milestones. The second question hypothesized whether or not postnatal neurogenesis could be a reliable predictor of later-life anxiety-like behaviors. The third question hypothesized whether or not postnatal synaptogenesis could be a reliable predictor of later-life anxiety-like behaviors. As the study was being conducted, it raised more complex questions to be considered. For example, sex differences were found in the adult anxiety-like mouse behaviors, and the first question that was hypothesized had evolved into: Are there sex differences in postnatal behavior as well? If so, do these sex-dependent differences correlate with the anxiety-like behavior that was observed later on in life in the 5-HT-1A-R mouse model? The second question also evolved into: Are there any sex-dependent differences in neuroproliferation that reflect those seen in postnatal mice behavior? It then became further complicated upon the observation that there was another type of proliferating cell present in certain samples, which turned out to have the appearance of postmitotic neurons. How do these postmitotic neurons add to the story about sex-dependent differences in early brain development? Finally, the third question did not raise too many additional questions to be considered. No significant differences were found in synaptogenesis, and there were no significant differences signaling related changes in brain connectivity that could be shown by vesicle numbers either. However, the lack of significant differences in
synaptogenesis still added an interesting insight into postnatal brain development, furthering research into the hippocampal trisynaptic circuit connectivity early on in life.

These studies were conducted on 5-HT-1A-R WT and KO mice during the first few postnatal weeks of life; a period that corresponds to a 3-6 month-old infant’s brain development [245]. Indeed most of the growth spurts in the rat and human infants begin in utero, including the development of gray and white matter, myelination, synaptogenesis, pruning, and synaptic modifications, respectively [249]. By the time human infants are born, their brains have 100 billion neurons [250]. At 2-4 weeks of age, the newborn human brain is about 36% of the size of an adult brain [249]. More primary sensory cortical areas than associational cortical areas can be identified at this point of neurodevelopment [249]. Although beginning prenatally, the most robust growth of cortical gray matter occurs during the first postnatal year in infants [249]. This corresponding cortical growth of gray matter in the rat also coincides with peaks in growth factor expression that appears developmentally in a regionally specific manner [251]. An example of this would be the mRNA for BDNF, which reaches adult levels on P7 within the hippocampus, but differentially expressed at adult levels on P14 with in the cortex [251]. After this point when the initial phase of innervation has passed, half of the neurons are eliminated by programmed cell death via synaptic pruning to allow stronger connections to thrive at the expense of intentionally eliminating weaker connections to conserve energy and neurobiological “real-estate” [251]. Synaptic transmission efficiency depends on morphological rearrangements that occur during this developmental time-period [251]. In humans, synaptic elimination and pruning occur between the 1st year after birth all the way up to periadolescence, around the 12th year [251]. Interestingly, sex differences in gray matter overproduction have been reported [251]. Girls reach peak levels of gray matter earlier than boys at 11.6 versus 12.8 years [251]. However,
males have an overall 8-9% larger total cerebral and cerebellar volume than females [251]. Interestingly, whereas the left amygdala increases significantly in males, the right hippocampus increases significantly in females [252]. Other sex-dependent differences that have been observed are that the relative volume of the putamen and globus pallidum are larger in males, whereas the caudate is larger in females [251]. Many of the sex differences that are observed are in either primarily dopamine- innervated areas (i.e., striatum) vs. serotonin innervated areas (i.e., hippocampus) that can be used to facilitate an explanation regarding the preponderance of aberrant dopamine transmission in disorders more commonly found in males such as Attention Deficit Hyperactivity Disorder (ADHD) type of disorders and substance abuse, as opposed to the higher levels of depression and anxiety in women [251].

The development of the hippocampus can be described as depending on three phases: 1) neurogenesis, 2) synaptogenesis, and 3) myelination. Since neurogenesis was discussed extensively early on, synaptogenesis and myelination will be the main focus of this part of the discussion. Where the hippocampus is concerned, in some ways the DG and the CA3 regions undergo a more protracted development from the rest of the hippocampus [250]. However, it is consistent and appears to be conserved between humans and rodents, that the peak of neurogenesis within the DG occurs at approximately 3 months in humans and 7-8 days in rodents, respectively[245, 253]. From a behavioral perspective, during this time, human infants are capable of exhibiting facial expressions consistent with human adults [2], as well as behavior that is predictive of later-life anxiety [24]. However, the hippocampal CA1 region has been formed at birth, and connections between the CA1 and the entorhinal cortex have also started to form as Lavenex had shown [253]. However, Levenex [253] also showed that human infant hippocampal CA3 connections with the CA1 stratum radiatum are sparse this early on in infancy
(i.e., 3 months), which would make fully formed trisynaptic connections unlikely at this point. According to their data, however, the human infant hippocampal CA1 region reaches 90% of adult volume by the age of 9 months [253]. However, according to Levenex [253], the human infant hippocampal CA3 mossy fiber terminals become more heavily stained by Timm’s stain at 3-months, when oligodendrocytes appear, and progressively increase after 6-months of age. Taken together, these findings suggest that even though the mossy fiber connectivity may be immature at 3-months of age in the human infant, a bit of connectivity does, in fact, exist [253]. This is corroborated by evidence from Zimmer and Haug [254], who in 1978 used Timm staining to show that mossy fibers start to appear at P3 in the rat, and continue to increase and appear adult-like on P12, but that is not suggest that is the full number of fibers. However, the hippocampal DG takes up till P21 in the rat to gain adult level connections from the entorhinal cortex, consistent with data that it takes 5-months in monkeys and humans [255]. It takes 3-4 weeks in adults for hippocampal granule cells to be able to receive glutamatergic input, presumably from the entorhinal cortex [236], however, this could happen a week earlier due to the quick development of newly born granule neurons in the postnatal DG [227]. However, the myelination of pathways is also of importance for hippocampal trisynaptic connectivity. It was observed that in the rat hippocampal slice culture, that myelination of the perforant path actually occurs at around P10 [256]. This would mean that the hippocampal DG would at least have limited connectivity from the entorhinal cortex at this point, which corresponds to about 5-month-old infants [245]. However, the P21 age of full hippocampal connectivity would make sense as the myelination of the entorhinal cortex connections are about 2-weeks from when the DG is thought to receive input from the entorhinal cortex, and that also coincides with about how long it would take for newly formed granule cells to become integrated into the circuitry as well.
With the peak of neurogenesis occurring at around P8 in the mouse, the neurons newly generated in the SGZ of the DG in our mice would have at least 14 days to be able to mature, which would place them at around the P21 mark for being potentially integrated into the DG circuit [236]. Others have used hippocampal ultrastructure studies to show that in fact, the time course of synaptogenesis is quite similar across both DG and CA1 regions, and seems to reach adult levels at around P21 in the DG and CA1 [257]. Kristen Harris [253, 258], the leading researcher in hippocampal ultrastructure, found that the largest increase in \textit{stratum radiatum} synapses occurs between P15 and adulthood in the rat, corresponding to 6-month old to 5-year old human infants. In terms of behavior at this time point, P21 would correspond to at least 9-month-old human infants, during which time they are able to remember episodic events, after a period of 5-weeks [250]. At this age, infants are also able to imitate an action for a period of 24 hours [250]. Thereafter, infants are able to remember single episodic events for period delays of at least 3-months [250]. However, by 17-23 months, is when infants are better able to remember temporally ordered sequences of actions [250]. In other words, 17-23-month old infants are fully capable of retaining episodic memories. According to Gomez [247, 259], this would involve the full support of the hippocampal trisynaptic circuit, which would be required for the pattern separation function of the DG to be able to fully be integrated. Since hippocampal pattern separation is thought to play an essential role in anxiety, the time-period during which the trisynaptic circuit is being set up to when it becomes established, could be crucial for its normal functioning.

To elaborate on the hippocampal connectivity further, so far it has been simply described as a loop that connects the entorhinal cortex to the DG to the CA3 to the CA1. However, the common experimental method often chosen has been to take a bottom-up approach, as this seems
to be a logical neurobiological micro-macro systems level approach to assemble the entire story of the structure-function relationship between the developing hippocampal circuits and anxiety-like behaviors. Interestingly, the hippocampus has 10 billion synapses [260] and the perforant path fibers from the second and third layers of the entorhinal cortex project to dendrites of DG granule or CA3 and CA1 pyramidal cells, respectively [260]. This could potentially bypass the hippocampal DG, but since the DG is involved in pattern separation, this type of connection would be involved in other types of memory and learning (e.g., object and scene recognition) [185]. The granule cells’ mossy fibers terminate on about half of hippocampal pyramidal cells in the CA3 region [260]. Further, the hippocampal CA3 neurons in the hilar region send their main collaterals to the CA1 pyramidal cells, but the remaining CA3 and CA2 cells form a strongly recursive network [260]. These recurrent collaterals contact nearby cells as well as distally located cells in the hippocampal hilar region [260]. In addition, these hippocampal CA3 cells contact CA1 cells and even granule cells in the DG [260]. Additionally, the hippocampal CA1 cells send axons to the subiculum and deep layer cells of the entorhinal cortex [260]. Therefore, the above-mentioned intricate neural circuitry already surpasses the scope of the arguably over-simplified hippocampal trisynaptic loop. A most telling example is that the entorhinal cortex can also receive multimodal connections from the postrhinal and perirhinal cortices, which are involved in object and scene inputs, respectively [185]. The postrhinal and perirhinal cortices can also project to each other, and receive projections from the prefrontal cortex and olfactory cortex to guide episodic and odor associated/dependent memories. Most importantly for this study, the entorhinal cortex receives input from the DNR as well. Because of these distinct roles of the postrhinal and perirhinal cortices, it is theorized by some, that many of the types of memories (e.g., object recognition) that infants seem to have before 18-months are really a
product of the coordinated action of these two cortices [247]. Aberrations in neurogenesis and synaptogenesis could potentially have long-lasting effects on behavior and anxiety, as the evidence shown above suggests that the connectivity in the hippocampus feeds back and receives input from many emotional areas of the brain. Contextual learning and the ability to forget fearful stimuli in certain contexts can therefore easily be affected by changes in neurogenesis.

How does abnormal hippocampal development affect anxiety behavior? Anxiety behavior, as was defined before, is characterized by increased autonomic arousal. The autonomic nervous system is regulated by the HPA axis [156]. The limbic system, as described before, can be described as coordinating impulses leaving the hippocampus via the fornix, which are then sent to the mammillary bodies, which then signal to the hypothalamus [156, 175]. The activation of the HPA axis causes the secretion of glucocorticoids, which bind to glucocorticoid receptors in numerous organs [261]. Glucocorticoid receptor activation causes the modification of transcription of regulatory protein, and have rapid effects by way of energy mobilization [261]. This occurs through glycogenolysis in the liver, suppression of innate immunity, vasoconstriction, proteolysis, lipolysis, and behavioral depression [261]. This is initiated by the paraventricular nucleus of the hypothalamus (PVN), which produce corticotropin-releasing-factor (CRF), that in turn, drive the pituitary to release adrenocorticotropic releasing hormone (ACTH) [261]. What follows next, is that the ACTH then mediates the synthesis of corticosteroids in the adrenal glands [261]. Interestingly, studies in rodents have shown that females have greater HPA responses than males [156]. Serotonergic neurons project to and activate CRF neurons in the PVN [262]. In support of this study, Goel [156] found that females who were treated with SSRIs had a lower number of c-Fos-immunoreactive cells (c-Fos-IR) in the CA3 region of the hippocampus. What the group also found was that there was a negative
correlation with the production of the stress hormone, corticosterone, to c-Fos-IR in both the hippocampal CA3 and DG [156]. The hippocampus is thought to dampen the activity of the HPA axis [156]. Furthermore, it is the electrical stimulation of the dorsal hippocampus, which inhibits PVN cells that project to the median eminence, and stimulation of the dorsal CA3 and DG also cause a decrease in plasma corticosterone [156]. The stimulation of the dorsal hippocampus has also been shown to be involved in USV production in rats. In addition, the 5-HT\textsubscript{1A}-R has been shown to be involved in the alteration of glucocorticoid receptors in the hippocampus and CRF in the hypothalamus [262]. These findings fit together with the findings in the present study, as the observations herein found that the dorsal DG, specifically, had a lower neuroproliferation in the KOF mice. Taken together, it is possible that the HPA dysregulation seen in previous studies might actually be due to lower proliferative levels in the SGZ of the DG of female mice prone to anxiety or stress.

The evidence that already exists that sex should be taken into account in treating and preventing anxiety and depression is overwhelming. Women are more prone to depression and anxiety, and experience symptoms more severely than men [263]. Some have even argued that the experience of depression in men might not even be accurately captured using the current DSM-V criteria, but this might be a sign that men experience depression differently (i.e., aggression and impulsivity) [263]. Only a subpopulation of people experience alleviation of depression symptoms, and sex-dependent differences might be a factor [263]. There have been studies showing that women do not respond to SSRIs as effectively as men; although, this particular issue is complicated by the fact this was mainly seen in postmenopausal women [263]. This, of course, may be due to a synergism between androgens and serotonin, as was shown in Goel’s study[156]. Premenopausal women have been shown to also be more resistant to
hippocampal volume reductions with depression; however, other studies did show decreased
neurogenesis in the hippocampi of women and not of men [263]. Taken together, adult studies
mainly support the sex-dependent differences that were observed very early on in life consistent
with the present study.

What this study presents is a set of behavioral and brain developmental phenotypes that
could be used as potential pharmacotherapeutic targets in the future behavioral pharmacological
rescue studies. Furthermore, this study provides a possible window of time during which
preventative treatments could be further explored and potentially used, on P8, during peak
neurogenesis in the DG. Overall, studies on anxiety in infants are lacking due to obvious ethical
reasons, especially in terms of brain development, as certain phases of brain development are not
even very clearly defined. As better tools are emerging to learn about the brain at a very rapid
pace, it would be even more informative to be able to conduct studies that show a direct causal
effect. However, this may be difficult to do if one would want to draw a direct relationship with
early brain development and later-life anxiety. Other behavioral studies could also be done
during adulthood that tests more directly for depressive symptoms (e.g., the novelty suppressed
feeding test). Additionally, USVs are also informative in terms of infant communication and
more studies should be conducted to explore how they relate to human infant crying.

Banerjee [210, 264, 265] has been able to show that DCPLA can be used to target
downstream effectors (i.e. PKC Epsilon) of the 5-HT\textsubscript{1A}-R thereby leading to an increase in cell
division. DCPLA was also used to correct anxiety behavior in the 5-HT\textsubscript{1A}-R KO mice [265]. In
future studies DCPLA may serve as a useful pharmacotherapeutic treatment of anxiety disorders.
The current study found a connection between USVs and later life anxiety-like behavior in the 5-
\textit{HT1A-R} KO mouse. In addition, an interesting sex-dependent relationship was seen within USVs
and neuroproliferation on P8. Although no differences were observed in synaptogenesis on P10 during the early stage of peaking synaptogenesis, future studies will aim to examine synaptogenesis on P16. Synaptogenesis at this later stage will then be compared to the sex-dependent differences found in neurogenesis on P8 to elucidate whether a link between the two stages can be observed. DCPLA can serve as one potential treatment for rescuing the behavioral and biological phenotypes observed in this study to WT levels, as it has already shown to be useful in adult anxiety-like behavior [265]. Additionally a focus on maternal retrieval under conditions such as cross-fostering and other measures of maternal care such as nest-building will also be examined. Together there are many future avenues to build upon the findings of the current study.
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