Developmental Exposure to a Serotonin Agonist Produces Subsequent Behavioral and Neurochemical Changes in the Adult Male Prairie Vole

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DEVELOPMENTAL EXPOSURE TO A SEROTONIN AGONIST PRODUCES
SUBSEQUENT BEHAVIORAL AND NEUROCHEMICAL CHANGES IN THE ADULT
MALE PRAIRIE VOLE

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I dedicate this thesis to my Mom and Dad, for their unwavering and unconditional love and support throughout the years, and to Jeffrey Arthur, for being the best brother in the world and always knowing how to make me laugh.
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ABSTRACT

Autistic spectrum disorders (ASDs) are classified as pervasive developmental disorders characterized by abnormalities in various cognitive and behavioral functions. Although exact underlying causes are still unknown, autism is thought to be caused by a complex combination of genetic and/or environmental factors. Interestingly, nearly 30% of autistic patients show elevated blood levels of serotonin (5-HT) and, therefore, certain genetic and environmental factors that are known to elevate 5-HT levels may play a role in the development of autism. It has previously been shown that serotonergic manipulation during early brain development promotes brain abnormalities in areas associated with social and anxiety-related behaviors. However, due to the lack of an appropriate animal model, the effect of this serotonergic manipulation on pro-social and anxiety-related behaviors has yet to be investigated. In the present study, we used the prairie vole (Microtus ochrogaster) as an animal model system. The prairie vole is a socially monogamous rodent that forms long-term pair bonds after mating and demonstrates an array of affiliative behaviors towards conspecifics. In these experiments, it was found that perinatal treatment with 5-methoxytryptamine, a non-selective serotonin agonist, impairs social affiliation and may increase anxiety-related behavior. It was also found that these behavioral changes correlate with a decrease in the number of 5-HT neurons in the dorsal raphe nucleus as well as a decrease in 5-HT fiber optic densities within four distinct amygdalar nuclei. Collectively, these data demonstrate the effects of neonatal exposure to 5-HT on pro-social and anxiety-like behaviors and the possible involvement of 5-HT in the regulation of these behaviors. Ultimately, the data obtained from these experiments may help to establish the prairie vole as an animal model of autism and also help to facilitate our understanding of ASDs and the neurobiological abnormalities that underlie such a complex neuropsychiatric disorder.
INTRODUCTION

Autistic spectrum disorder (ASD), or more commonly referred to as autism, is considered to be a developmental disease characterized by abnormalities in various cognitive and behavioral functions, including increased anxiety, stereotyped and repetitive behaviors, hypersensitivity to sensory stimuli, and deficits in communication and social interactions [1]. Although all of the contributing factors are not known and thought to be a complex combination of both endogenous and environmental factors, a few possible etiologies include genetic mutations and polymorphisms [2, 3], early environmental exposure to toxins and pharmacological agents [4, 5], and even maternal use of drugs [6]. Most relevant to my own experiments, genetic mutations in serotonin transporter (SERT) gene [7-12] and maternal use of monoamine oxidase inhibitors [13, 14], selective serotonin reuptake inhibitors (SSRI’s) [15-20], and even cocaine [21-26] - all of which, interestingly enough, are known to elevate serotonin (5-HT) levels - are thought to increase the risk of autism [27]. In addition, nearly 30% of patients with autism demonstrate elevated blood levels of 5-HT [28-32]. This condition, known as hyperserotonemia, is defined by a 50% increase in blood platelet 5-HT levels and is considered by many to be the most commonly observed and replicated change seen in patients with ASDs [33-37]. Therefore, a dysfunction in the 5-HT system may have large implications in the pathogenesis of ASDs.

Before assuming its role as a neurotransmitter, 5-HT acts as a developmental signal in the brain. It functions to promote neural organization and differentiation [38-41], neurite outgrowth [42], synaptogenesis [41, 43, 44], dendritic branching [43, 45-47], and neurogenesis [48-50] and also serves to autoregulate its own neuronal outgrowth via a negative feedback mechanism [51-56]. In regard to its autoregulatory function, previous studies in rat pups have shown that increasing or decreasing 5-HT levels during early brain development induces abnormal serotonergic development [14, 43, 57]. Specifically, it has been shown that exposure to 5-methoxytryptamine (5-MT), a non-selective 5-HT agonist, decreases serotonergic outgrowth [56] and prenatal depletion of 5-HT delays the onset of neurogenesis in 5-HT target brain regions [58]. Therefore, experiments like these provide evidence that 5-HT neurons act as a developmental signal and because 5-HT neurons also become apparent before other monoamines [59], it is thought that the 5-HT system thereby regulates the development of other
neurotransmitter systems, such as the oxytocin (OT) and dopaminergic systems [60-64]. In particular, McNamara et. al, demonstrated that rats which received 5-MT treatment during early development had fewer OT cells in the paraventricular nucleus (PVN) and an increased number of neurons in the amygdala (AMY) [64]. In addition to these neuroanatomical changes, this same study also investigated subsequent behavioral changes and found that perinatal 5-MT treated rats displayed a decreased habituation response to a novel stimulus (cardboard roll test) and decreased behavioral inhibition to a harmful stimulus [64]; it is thought that these behaviors may correlate with the stereotyped and self injurious behaviors typically seen in ASDs. In this same study, 5-MT-treated rat pups were also found to have decreased litter bonding behavior and also had reduced olfactory-based social interactions indicating that the neuroanatomical changes may underlie the behavioral deficits seen in 5-MT subjects [64].

Not only do patients with autism demonstrate changes in various neurotransmitter systems, but neuroanatomical abnormalities are also associated with this disease. Relevant to this present study, it has been well established that the dorsal raphe (DR) sends 5-HT projections ubiquitously throughout the brain, in particular, to regions such as the AMY that regulate social and anxiety-related behavior [65, 66]. Specifically, the AMY functions to integrate the autonomic and memory systems as well as various sensory modalities where it processes this information locally before sending dense efferent projections to areas such as the hypothalamus and brain stem nuclei [67-69]. Taking into consideration the fact that 5-HT neurons function as a developmental signal, disruptions in 5-HT development could potentially have a profound impact on normal amygdalar development and function. In fact, it has been shown that children with autism tend to show increased amygdalar volume [70-72] and post-mortem studies report that patients with autism had fewer neurons in the AMY [71, 73]. Lastly, brain imaging studies have shown that AMY function is dysregulated in patients with autism in that amygdalar activation was decreased during a social cognition test [74]. Collectively, these data suggest that disruptions in normal brain development, specifically in regions such as the AMY, may contribute to the behavioral deficits seen in ASDs.

Although various neuroanatomical and neurochemical abnormalities have been associated with ASDs, the specific neuromechanisms that underlie these behaviors are still unclear, mostly due to the lack of an appropriate animal model. The prairie vole (Microtus ochrogaster) is a highly social monogamous rodent that demonstrates an array of affiliative
behaviors towards conspecifics, behaviors that are not typically found in other rodent species [75-78]. Studies have also shown that male and female prairie voles form long lasting pair bonds following mating [79-81] and that these behaviors are regulated by a variety of neurochemicals including OT [81, 82], arginine vasopressin (AVP) [79, 83, 84], dopamine (DA) [85] and 5-HT [86]. Therefore, the prairie vole model provides an excellent opportunity to study the neurochemical mechanisms underlying a complex psychiatric disorder such as ASDs. Therefore, in the present study, I was able to use this excellent animal model to examine both the behavioral and neuroendocrine effects of perinatal exposure to 5-MT in male prairie voles.
MATERIALS AND METHODS

Animals

Capture-bred male and female prairie voles (*Microtus ochrogaster*) were housed in large polycarbonate cages (25x45x20 cm) with cedar chip bedding together and allowed to produce multiple litters to ensure breeding reliability. Upon weaning, male subjects were housed in same-sex sibling pairs (same treatment pairs) in small polycarbonate cages (18x29x13 cm). All cages were maintained under a 14L: 10D photoperiod and the room temperature was maintained at 21 ± 1°C. Food and water were provided *ad libitum*. Behavioral testing began once subjects reached adulthood at approximately PND80. The elevated plus maze and the open field tests were conducted beginning at 0900 whereas the social affiliation test was conducted beginning at 1300.

Injections

Throughout the perinatal injection period, 1mg/kg of 5-MT was used. Based on its pharmacokinetics, this dose of 5-MT will induce a 50% increase in blood 5-HT levels in rats [64] similar to that seen in hyperserotonemia and has been shown to do so without inducing signs of 5-HT syndrome [87]. 5-MT is a non-selective 5-HT agonist (chosen for its relatively high potency at all tested serotonin receptors [88]) and 1mg/kg of 5-MT has been shown to be sufficient in inducing neurochemical and behavioral changes in rats [56, 64, 89]. Prairie vole dams are immediately impregnated following a gestation period and birth of a new litter [90] and so pregnant-timed prairie vole dams received prenatal daily injections of either 5-MT (1mg/kg) or saline (control) from GD 12 (when 5-HT neurons first become evident [50]) to GD 21. It is important to note that post-partum Fluoxetine treatment in prairie voles dams does not affect maternal behavior [86] and, therefore, pre-partum 5-MT effects on post-partum maternal behavior was not expected. Beginning at parturition (PND0), due to the 4:1 male:female gender bias found in ASDs [91], male pups continued to receive daily injections of either saline or 5-MT (1mg/kg) until PND20 when peak 5-HT synaptogenesis typically ends [92]. Therefore, subjects were exposed to either 5-MT or saline during a time when 5-HT has its peak developmental influence [50].
**Social Affiliation Test**

The social affiliation test apparatus consists of two polycarbonate cages (28x16x12cm) connected via a hollow tube (7.5x16cm) and has been previously established to test social affiliation in prairie voles [93-95]. Subjects were first transferred to a holding room, however, remained in their home cages until testing occurred. To begin, an unfamiliar, same-sex, stimulus prairie vole was loosely tethered in the stimulus cage whereas the subject animal was placed into the empty, non-stimulus cage but allowed to move freely throughout the apparatus. The 30 minute test was video-recorded and behaviors such as the duration and frequency of cage entries and stimulus animal contacts were later scored and analyzed. After the completion of the test, subjects were returned to their home cages in the holding room where they remained until the completion of all social affiliation tests - this was done to ensure that all subjects remained in the holding room for equal amounts of time.

**Elevated Plus Maze Test**

The elevated plus maze (EPM) has been validated as a measure to test anxiety-related behavior in both rats [96], mice [97], and voles [98, 99] and consists of an elevated (45cm high) four arm maze consisting of two open arms (35×6.5 cm) and two closed arms (35×6.5×15 (H) cm). Subjects were first transferred to a holding room and remained in their home cages until the start of their individual EPM test. To begin, subjects were placed in the center of the EPM apparatus and were observed by the experimenter throughout the entire 5 minute test to ensure that the subject animal remained on the apparatus. If the animal were to fall off the apparatus, subjects were then placed back into the center of the EPM. This 5 minute test was also video-recorded so that anxiety-related behaviors as well as stereotyped/ritualistic behaviors could be later scored and analyzed using JWatcher; scored behaviors included the duration and frequency of open and closed arm entries in addition to defecation, grooming, chewing, and freezing behaviors. After the completion of the EPM test, subjects were returned to their home cages in the holding room. Between subjects, the apparatus was thoroughly cleaned and wiped dry with 75% ETOH and ddH$_2$O.

**Open Field Test**

The open field (OF) test has been validated as a measure to test locomotor activity and anxiety-related behavior in both rats and mice [100, 101] as well as voles [85, 95, 102] and consists of an open-field box (56×56×20 cm) in which the floor is equally divided into 16
squares (14x14 cm). Subjects were first transferred to a holding room and remained in their home cages until the start of their individual OF test. To begin, subjects were placed in the center of the OF test and the 10 minute test was video-recorded. Behaviors such as frequency of square crossings and the duration of time spent in the center, corner, and peripheral squares in addition to defecation, grooming, rearing, and freezing behaviors were then later scored and analyzed using JWatcher. After the completion of the individual OF test, subjects were returned to their home cages in the holding room. Between subjects, the apparatus was thoroughly cleaned and wiped dry with 75% ETOH and ddH$_2$O.

**Immunohistochemistry (IHC)**

60 minutes following the conclusion of the elevated plus maze, subjects were perfused with 4% paraformaldehyde (PFA) and their brains collected and post-fixed in the PFA solution for 2 hours before being stored in a solution containing 30% sucrose and 0.1M phosphate buffer (PB). Brains were sliced into 40 µm sections using a microtome and stored in a 0.1M PB solution containing 1.0% sodium azide. Free floating sections were then processed for 5-HT immunostaining using the following previously established protocol. First, sections were rinsed in 0.1M PB 5x5min; incubated in 1% NaBH$_4$ 1x10min; rinsed in 0.1M PB 5x5min; incubated in 0.5% H$_2$O$_2$ for 30 minutes; rinsed in 0.1M PB 5x5min; and then incubated in a solution containing 10% normal rabbit serum (NRS), 0.3% triton, and 0.1M PB for 1 hour. Sections were then incubated in a solution containing 5-HT polyclonal goat IgG antibody (1:5k, Thermo Scientific, Inc., Rockford, IL), 2% NRS, 0.3% triton, and 0.1M PB for 48 hours at 4°C followed by one hour at room temperature at the end of the incubation period. At the conclusion of the primary antibody incubation period, sections were rinsed with 0.3% triton 5x5min and then incubated in a solution containing biotinolated rabbit anti-goat secondary antibody (1:300 Vector Laboratories, Inc., Burlington, CA), 2% NRS, 0.3% triton, and 0.1M PB for two hours before being rinsed in 0.3% triton (3x5 min) and 0.1M PB (2x5min) and incubated in ABC complex (Vector Elite) in 0.1M PB for 90 minutes. Lastly, sections were rinsed with 0.1M PB 5x5min and then stained with Nickel-DAB (Vector Laboratories, Inc. Burlington, CA). Tissue sections were mounted on microscope slides and cover slipped using HistoChoice clearing agent to rid excess water and Permount medium as the cover slip adhesive. In order to control for variability, all sections were processed simultaneously. Once slides were completely dried, using a high magnification microscope and StereoInvestigator software, images were taken of the dorsal
raphe (DR) and amygdala (AMY) brain regions. The total number of 5-HT cells in the DR was manually quantified and the fiber optic densities in the central (CeA), basolateral (BLA), medial (MeA), and cortical (CoA) amygdala nuclei were determined using ImageJ software.

**Data Quantification and Analysis**

The social affiliation, elevated plus maze, and open field tests were scored by an observer blind to the treatment groups and subsequently analyzed. IHC staining was used to determine the total number of 5-HT cells from 4 consecutive DR-matched sections whereas the 5-HT fiber optic densities were determined using 3 consecutive bilateral AMY-matched sections from four AMY nuclei: the CeA, BLA, MeA, and CoA (these specific nuclei were chosen based on their involvement with regulating social and anxiety-related behaviors). The overall optic densities were determined by subtracting the background optic density from the optic density of the particular AMY subregion. Differences between the treatment groups in the behavioral and molecular assays were all analyzed using independent samples t-Tests.
EXPERIMENT 1: DOES PERINATAL 5-MT MANIPULATION ALTER THE BEHAVIORAL PHENOTYPE IN THE ADULT MALE PRAIRIE VOLE?

ASDs are typically characterized by increased anxiety, stereotypic and repetitive behaviors, impaired learning and memory, and deficits in communication and social behavior [1]. Studies in rodent species have previously been able to replicate some of the behavioral abnormalities typically seen in autism [103-110]. However, due to the lack of an appropriate animal model, testing for social behavior has been restricted to olfactory-based social investigation and pup play behavior [64, 103]. The prairie vole and social affiliation paradigms will thereby provide an excellent opportunity to examine the behavioral deficits in social bonding behavior which cannot be addressed by using traditional laboratory rodents. In addition, the elevated plus maze and open field tests will investigate anxiety-related behavior, locomotor activity, as well as motor idiosyncrasies. Therefore, these experiments will help provide a better overall understanding of the behavioral deficits that occur as a result of perinatal 5-MT treatment and will also help to establish the prairie vole as an animal model to study autism.

Design

Briefly, pregnant-timed prairie vole dams first received prenatal daily injections of either 5-MT (1mg/kg) or saline (control) from GD 12 to GD 21. Beginning at parturition (PND0), male pups (n=8 5-MT, n=5 saline) then continued to receive daily injections of either 5-MT (1mg/kg) or saline, respectively, until PND20. Subjects were weaned at PND 21 and housed in same-sex, sibling (same treatment) pairs under standard housing conditions. Beginning at approximately PND80, subjects were first tested for differences in anxiety-related, locomotor activity, and stereotyped/repetitive behaviors using the open field test. On the following consecutive days, the social affiliation test was used to determine deficits in social behavior whereas the EPM test investigated differences in anxiety-related and stereotyped/repetitive behaviors.
Results

Social Affiliation

Male prairie voles that received perinatal 5-MT treatment spent a significantly less time in the stimulus animal cage (Figure 1A) \( (t_{(1, 11)} = -2.64, p < 0.05) \) and spent less time in side-by-side contact with the stimulus animal (Figure 1C) \( (t_{(1, 11)} = -2.45, p < 0.05) \) compared to saline treated controls, demonstrating that perinatal 5-MT treatment produced deficits in social bonding behavior. It should be noted that no significant differences were found in the number of cage entries into either the empty or stimulus animal cage between treatment groups (Figure 1B), suggesting that locomotor activity was not affected by perinatal 5-MT treatment. In addition, even though the number of short contacts significantly differed in that 5-MT animals briefly contacted the stimulus animal more frequently compared to saline controls, the number long contacts did not significantly differ and neither did the total number of contacts (Figure 1D). This demonstrates that the significant difference in the time in side-by-contact was not a result of fewer contacts (i.e. locomotor activity and investigative behavior were not affected).

![Figure 1](image_url)

**Figure 1**: Examining the effects of perinatal 5-MT treatment on adult male prairie vole social behavior via the social affiliation test. 5-MT subjects spent less time in the stimulus animal cage compared to saline controls (a) whereas no differences were found in the number of cage entries (b). 5-MT subjects also spent less time in side-by-side contact with the stimulus animal (c) whereas no differences were found in the total number of contacts (d).
**Elevated Plus Maze**

The EPM test was used in order to determine any differences in anxiety-related behavior. Although near significance ($t_{(1, 11)} = -1.25, \ p > 0.05$), it was found that compared to saline controls 5-MT animals did not significantly differ in the amount of time spent in the open arm (Figure 2A) nor did 5-MT animals differ in frequency of entering the open arm (Figure 2B) ($t_{(1, 11)} = -1.84, \ p > 0.05$). Additionally, no significant differences were found in either the duration of grooming, freezing, and chewing behaviors (Figure 2C) nor in the frequency of grooming, defecation, freezing, chewing behaviors (Figure 2D).

![Figure 2: Examining the effects of perinatal 5-MT treatment on adult male prairie vole anxiety-related behavior via the elevated plus maze test. Although not significant, 5-MT subjects seemed to spend less time in the open of the elevated plus maze compared to saline controls (a) and also seemed to enter the open arm less (b). No differences were found in the duration of grooming, freezing or chewing behaviors (c) and, similarly, no differences were found in the frequency of grooming, defecation, freezing, and chewing behaviors (d).](image-url)
Open Field Test

The OF test was used in order to determine any differences in locomotor activity and anxiety-related behavior. It was found that 5-MT treated animals spent more time in the corners of the open field box compared to saline controls (Figure 3A) \( (t_{(1, 11)} = 3.09, p < 0.05) \), suggesting an increase in anxiety-related behavior. The two groups did not differ significantly in the frequency of center, corner, or peripheral crossing (Figure 3B) \( (t_{(1, 11)} = -1.60, p > 0.05) \), demonstrating again that locomotor activity was not affected by the perinatal 5-MT treatment. Similar to the EPM test, no significant differences were found in the duration of grooming, freezing, and rearing behaviors (Figure 3C) nor were there differences in the frequency of grooming, defecation, freezing, rearing behaviors (Figure 3D).

**Figure 3:** Examining the effects of perinatal 5-MT treatment on adult male prairie vole anxiety-related behavior and locomotor activity via the open field test. 5-MT subjects spent more time in the corners of the open field compared to saline controls (a) whereas no differences were found in the frequency of square crossings (b). Similar to the elevated plus maze, no differences were found neither in the duration of grooming, freezing or rearing behaviors (c) nor in the frequencies of grooming, defecation, freezing, and rearing behaviors (d).
EXPERIMENT 2: DOES PERINATAL 5-MT MANIPULATION ALTER NORMAL SEROTONERGIC DEVELOPMENT?

5-HT neurons first appear during early brain development and function to regulate outgrowth [42], neural organization and differentiation [38-41], synaptogenesis [41, 43, 44], dendritic branching [43, 45-47], and neurogenesis [48-50] among various brain regions and neurotransmitter systems. In particular, studies have demonstrated that 5-HT neurons project to areas important for social and anxiety-related behaviors such as the PVN and AMY [111]. The AMY is considered part of the limbic system and is considered an important region for emotional processing. It functions by integrating memory, autonomic, and behavioral information as well various sensory modalities. AMY activation, therefore, depends on an array of cortical, hypothalamic, thalamic, and brain stem afferent projections [69, 112]. Interestingly enough, the AMY receives dense serotonergic projections from the DR [66] and these afferent inputs are thought to provide inhibitory control within the AMY [113, 114]. Studies have also demonstrated that 5-HT plays a key role in the modulation of autonomic and behavioral responses in both humans [115-119] and animals [105, 120-122] and, therefore, 5-HT has been shown have effects on other brain regions both directly (via 5-HT projections) and indirectly (in that 5-HT is able to regulate brain development). In this present experiment, our aim was to investigate the effects of perinatal 5-MT exposure on 5-HT cell counts and fiber densities in the DR and AMY, respectively to then be able to predict how abnormalities in the 5-HT system may underlie the behavioral changes seen in 5-MT treated adult male prairie voles.

Design

Brain sections were selected from subjects used in Experiment 1 of which received daily injections of either 5-MT (1mg/kg) or saline (control) from GD 12 until PND20 and were processed for 5-HT-ir staining using IHC. The number of 5-HT cells in the DR was quantified and the fiber optic densities were determined in four sub-regions of the AMY for each subject animal.
Results

5-HT Cell Density

The number of 5-HT immunoreactive (ir)-labeled cells in the DR was quantified and data analysis revealed that 5-MT treated animals may have fewer 5-HT labeled cells in the DR (Figure 4B) ($t_{(1, 5)} = -1.64$, $p > 0.05$). We also found that 5-MT treated animals weighed significantly more compared to saline controls (Figure 4C) ($t_{(1, 8.203)} = 4.02$, $p < 0.05$) and when 5-HT cell counts were adjusted for body weight, data analysis indicated a significant group difference in which 5-MT treated animals have fewer 5-HT neurons in the DR/body weight (g) compared to saline controls (Figure 4D) ($t_{(1, 5)} = -2.68$, $p < 0.05$).

Figure 4: Body weight and quantitative measurements of 5-HT neurons in the dorsal raphe following perinatal 5-MT treatment. Both the photomicrograph (a) and cell counts (b) illustrate differences in the cell density between 5-MT subjects and saline controls (a). 5-MT subjects weighed significantly more than saline controls (c) and when body weight was taken into account, the number of cells per gram of body weight was significantly smaller in 5-MT subjects compared to saline controls (d).
5-HT Fiber Optic Density

5-HT, in part, functions to autoregulate its own serotonergic outgrowth via an inhibitory feedback mechanism and so after 5-MT manipulation we expected to find fewer 5-HT projections in the AMY. Indeed, 5-MT subjects had significantly decreased 5-HT fiber optic densities in the CeA ($t_{(1, 5)} = -5.34, p < 0.05$), BlA ($t_{(1, 5)} = -2.67, p < 0.05$), MeA ($t_{(1, 5)} = -3.37, p < 0.05$), and CoA ($t_{(1, 5)} = -2.59, p < 0.05$) nuclei when compared to saline controls (Figure 5).

**Figure 5:** Differences in the fiber optic densities of 5-HT innervations in four different amygdalar nuclei following perinatal 5-MT treatment. Photomicrograph of 5-HT-ir staining in the central (CeA), basolateral (BlA), medial (MeA), and cortical (CoA) amygdalar nuclei (a). Quantification of fiber optic densities in the CeA, BlA, MeA, and CoA nuclei indicate that 5-MT subjects had significantly fewer 5-HT projections compared to saline controls (b).
DISCUSSION

Autism is considered to be a pervasive developmental spectrum disorder and is characterized by various cognitive and behavioral abnormalities including deficits in social behavior and social communication, increased anxiety, stereotyped and repetitive behaviors, and hypersensitivity to sensory stimuli [1]. Most relevant to these current experiments, evidence suggests that disruptions in the 5-HT system, such as genetic mutations in the SERT gene or environmental exposure to elevated levels of 5-HT, may play an important role in pathogenesis of autism. In fact, hyperserotonemia is considered by many to be one of the most consistent changes seen in patients with autism [28, 30-32]. It has been previously established that 5-HT acts as an important developmental signal among various brain regions and neurotransmitter systems and that manipulation of the 5-HT system in rodents induces various neurochemical and behavioral changes [50, 64]. However, due to the lack of an appropriate animal model, the investigation of social behavior has been limited to behaviors such as pup play behavior and social interactions (i.e. sniffing, investigation) [64] [103]. The prairie vole is a rodent animal model that displays highly affiliative behavior towards conspecifics and forms long-lasting pair bonds after mating thereby providing a unique opportunity to investigate changes in true prosocial behaviors following 5-MT treatment. Overall, this current study demonstrates that perinatal 5-MT treatment is sufficient to disrupt the normal development of the 5-HT system and that these neurochemical abnormalities correlate with subsequent behavioral changes. Therefore, these results ultimately provide further evidence that a disruption in the 5-HT system may play a large role in the pathogenesis of autism and also help establish the prairie vole as an animal model to study both the behavioral phenotype as well as the underlying neuromechanisms in such a complex psychiatric disorder.

5-MT treatment alters social and anxiety-like behavior

Results in these experiments indicate that 5-MT treated animals spend significantly less time in the stimulus animal cage and less time in side-by-side contact with the stimulus animal, compared to saline controls. Therefore, these results provide evidence that 5-MT rat pups display deficits in social behavior [64]. In genetic animal models of autism, deficits in social interactions have also been found [103, 123]. Specifically, Neuroligin-4 is a cell adhesion
protein that, similar to 5-HT, promotes synaptogenesis and induces proper brain development [124]. In comparison to wild type (WT) mice, Neuroligin-4 knockout mice preferred to spend less time in a cage containing a stranger mouse and also failed to display an increased preference for a novel over a familiar mouse in a choice test [103]. Even more relevant to our findings, SERT gene mutations have been implicated in patients with autism [7-12] and such mutations are thought to affect brain development by increasing levels of 5-HT in the developing brain [125]. In fact, SERT null mutant male mice displayed decreased sociability compared to WT controls in a behavioral assay similar to our social affiliation test [123]. In these experiments that investigate social behavior, however, it is important to note that these studies are limited to olfactory-based social interactions and pup play behavior in rodent species with limited social behavior and therefore these social behavioral measurements may not properly correlate with human social behavior or for that matter lack of social behavior. Therefore, the prairie vole and social affiliation paradigms allow us to directly measure true pro-social behaviors; allowing us to better investigate social deficits in complex neuropsychiatric diseases such as autism.

5-MT treated animals also seemed to display increased anxiety-related behavior compared to saline controls. In the open field test, 5-MT-treated voles spent significantly more time in the corner squares of the open field (OF), thereby suggesting increased anxiety-like behavior. This increase in anxiety-related behavior is further supported by the tendency for 5-MT voles to enter the open arm fewer times and spend less overall time there. This data is consistent with the tendency for patients with autism to display increased anxiety. Although previous studies using the same 5-MT experimental design have been unable to demonstrate differences in anxiety-related behavior, other studies using genetic animal models of autism have, indeed, been able to show increased anxiety-related behavior using the elevated plus maze [126] and the light-dark box [127]. These discrepancies may result from the idea that anxiety-related behavior is considered to be a co-morbid behavioral characteristic and the severity of this feature is highly variable in patients with autism and unfortunately animal models have yet to replicate all the behavioral characteristics typically associated with autism. Lastly, behavioral tests such as the elevated plus maze and the open field box may not be suitable for testing the anxiety-related behavior found in patients with autism due to the complexity of this particular human psychiatric disorder.
Although 5-MT treated prairie voles displayed significantly decreased social affiliation and increased anxiety-related behavior, no significant differences were found in defecation, grooming, freezing, chewing and rearing behaviors; behaviors that are typically associated with the motor idiosyncrasies, i.e. stereotyped and ritualistic behaviors, typically seen in patients with autism. Due to the fact that these behaviors were observed during the 10 minute OF and 5 minute EPM tests this may be an insufficient amount of time to determine any differences between treatment groups when the baseline frequency of the behavior is already low. Therefore, in order to address this floor-effect, we may want to consider an open field test duration of 60 minutes, which has been previously established to better measure exploratory and stereotyped behaviors [128] and thus may be more appropriate under these circumstance.

*Neuroanatomical changes may correlate to the observed behavioral abnormalities*

Previous studies have demonstrated that 5-HT acts as a developmental signal during early brain development and that elevated levels of 5-HT during this critical developmental period is sufficient to induce abnormalities in various brain regions and disruptions in not only the serotonergic system, but other neurotransmitter systems as well [27, 50, 129]. Previous evidence has found that one way in which 5-HT acts as a developmental signal includes an inhibitory feedback mechanism such that 5-HT neurons contain autoreceptors that help regulate proper serotonergic outgrowth [130]. Therefore, high levels of 5-HT during early brain development, similar to that seen in 5-MT subjects in this present study, will consequently result in a reduction in 5-HT content and number of 5-HT neurons in the DR as well as fewer 5-HT projections to various brain regions [13, 22, 25, 52, 56]. The data presented in these experiments is consistent with these previous findings in that 5-MT treated male prairie voles had fewer 5-HT stained cells in the DR and also had fewer 5-HT fibers projecting into the AMY compared to saline controls. It was also found that 5-MT subjects weighed significantly more compared to saline controls. Previous evidence suggests a positive correlation with body mass and brain mass [131] and therefore it was determined appropriate to calculate an adjusted 5-HT cell count in which the total number 5-HT cells in the DR for each subject was divided by the subject’s body mass. Failure to reach significance in the total cell count data was therefore resolved when body weight was taken into consideration, thereby indicating that 5-MT treated animals had significantly fewer 5-HT cells in the DR per gram of body mass compared to saline controls.
The AMY has been shown to be a brain region important for the modulation of social and anxiety-related behaviors. For instance, fMRI imaging studies have demonstrated that the AMY has increased activation during fearful events [132] and when confronted with angry, sad, and fearful facial expressions [133-135] whereas bilateral damage to the AMY results in the inability to recognize facial expressions, in particular fearful expressions [136, 137]. Increased AMY activity has also been demonstrated in humans with a social phobia indicating its involvement in regulating not only anxiety-related behaviors but also social behavior [138]. In fact, human and rodent studies have been able to demonstrate the role of the AMY in social behavior. For instance, AMY activity in fMRI studies directly correlates with the ability to trust others and whether to approach or avoid social interactions [136] and, interestingly enough, patients with autism, of whom display an array of social deficits, tend to misjudge the trustworthiness of human facial expressions [136, 139]. Similarly, in an fMRI study investigating AMY activity during a social cognition test, patients with autism have decreased amygdalar activation in comparison to normal participants [74]. In addition to these changes in AMY activation, patients with autism reveal amygdalar neuroanatomical abnormalities. Specifically, children with autism tend to have increased amygdalar volume [70-72, 140]; however this effect does not seem to persist into adulthood as indicated by normal amygdalar volume in adolescents and adults with autism [141]. In fact, studies have shown that AMY enlargement have been associated with more severe anxiety [142] as well as deficits in social behavior and communication [143]. Post-mortem studies in adult patients with autism have also found neuroanatomical abnormalities in that they have fewer neurons in the AMY [71, 73]. Even though these results seem to contradict one another, the general consensus seems to be that the AMY initially overgrows in patients with autism however the fails to undergo the age-related increase in AMY volume that typically occurs during later stages of brain development [144]. Therefore, evidence suggests that 5-HT and its role as a developmental signal may very well play a role in the abnormal development of the AMY and subsequent irregular activation patterns of which is likely to contribute to the various behavioral deficits seen in patients with autism.

It has been previously established that 5-HT acts to autoregulate its own development via an inhibitory feedback mechanism [52, 54-56] and that the AMY receives dense 5-HT innervations from the DR [111, 113]. Therefore, a reduction in 5-HT fiber densities in the AMY was expected after perinatal 5-MT manipulation which is what we indeed found. The AMY,
however, not only receives 5-HT innervations from the DR, but also receives many diverse cortical, thalamic, hypothalamic, and brain stem afferent projections [66, 69, 111] thereby providing the AMY with an array converging information regarding current memory, autonomic, behavioral, and sensory information [69]. The AMY processes this converging information locally and then sends reciprocal efferent projections back to some of these regions including the hypothalamus and brain stem nuclei [69] so to coordinate autonomic and behavioral responses. Interestingly, it has been shown that 5-HT release within in AMY provides an inhibitory response [113, 114] and therefore a decrease in 5-HT afferent fibers, as our data indicate, would lead to a decrease in inhibition on the amygdalar neurons which typically receive 5-HT projections, which would result in an increase in amygdalar output activity. fMRI studies have then directly illustrated that this increased AMY activity can be associated with increased anxiety-related and fear behaviors [132, 136]. Therefore one can assume that a decrease in 5-HT afferent projections in the AMY would disrupt normal amygdalar function and subsequent behavioral outcomes. It should be noted, however, that the afferent and efferent connections in the AMY are diverse in that the AMY receives many different afferent projections from the OT, corticotrophin, dopaminergic, glutamatergic, and GABAergic neurotransmitters systems [69]. In addition, this neurophysiology is also further complicated by intra-AMY nuclei projections thereby making it difficult to determine the precise role that 5-HT plays on amygdalar function.

Neuroanatomically speaking, the AMY is a brain region composed of various sub-nuclei of which serve various functions. Specifically, the CoA and the MeA have been implicated in regulating social behavior. For instance, c-Fos activation in mice has been found in both of these AMY nuclei after a social encounter [120]. Studies have also established bidirectional connections between the CoA and the MeA [145, 146] suggesting a combined role in regulating social behavior. Taking this into consideration, the present behavioral data illustrates decreased social behavior and neurochemical data indicate fewer 5-HT projections in the CoA and MeA nuclei after perinatal 5-MT treatment. Collectively, these data suggest that the neuroanatomical changes in these particular amygdalar nuclei may underlie the deficits in social behavior seen in 5-MT subjects.

In contrast, fear and anxiety-related behaviors are thought to be partially regulated through the CeA and BlA nuclei. The BlA receives many afferent projections from the neocortex (e.g. prefrontal cortex), thalamus, and subicular region of the hippocampus before
processing this incoming information locally and sending heavy intra-AMY projections to the CeA [147] and therefore the BlA functions mainly to integrate afferent and efferent information. The CeA receives these BlA inputs as well as inputs from other AMY nuclei [148], the insular cortex, and the hypothalamus before processing this information locally and sending efferent projections to brain areas such as the hypothalamus and brain stem nuclei [69, 149] where it helps to regulate behavioral and autonomic responses. In fact, electrical stimulation of the AMY is sufficient to induce fear and anxiety-related behavior similar to that seen during a fearful event [67], whereas CeA lesions abolish normal fear responses in rodent species [150, 151]. Thus, evidence indicates that the BlA and CeA nuclei function to interpret and process incoming information thereby producing anxiety-related behavioral and autonomic responses. Taken together, these studies provide evidence that the BlA and CeA play an important role in the processing and expression of fear and anxiety-related behaviors and that abnormalities in the 5-HT system, as seen in 5-MT subjects, may underlie these changes in fear and anxiety-related behavior.

**Summary**

Our design represents the first approach to systematically examine the behavioral effects of developmental exposure to high levels of 5-HT in prairie voles. As previously mentioned, ASDs are characterized by a variety of behavioral abnormalities. Our selection of behavioral tests have therefore allowed us to evaluate the effects of perinatal 5-MT exposure on social and anxiety-related behaviors; although additional studies may want to investigate other types of behaviors typically associated with autism. In addition, because other neuropeptides, such as OT, are also implicated in autism, these neurotransmitter systems would also be worth investigating. Lastly, it is important to note that significance may not be found in all behavioral tests, which is not surprising given the complexity and variability of ASDs. In fact, most animal models of autism demonstrate only some of the behavioral deficits associated with ASDs. Nevertheless, the prairie vole model can be used to investigate important aspects of behavior, in particular social behavior, and a systematic array of behavioral tests will further establish a 5-MT behavioral phenotype in the male prairie vole, thus allowing us to further study the underlying neurobiology of autism.

These findings support the idea that elevated levels of 5-HT can induce abnormal serotonergic development and that neurochemical changes may correlate with subsequent
behavioral deficits. Specifically, it was found that 5-MT treated animals displayed decreased social affiliative behavior and increased anxiety-related behavior, and that these behavioral changes may be correlated with the decrease in the total number of 5-HT cells in the DR and the decrease in fiber optic densities in four separate nuclei of the AMY. Prairie voles are a unique animal model in that they display highly affiliative behaviors towards conspecifics and form long-lasting pair bonds after mating. Given that patients with autism typically demonstrate decreased social bonding and increased anxiety-related behaviors, the prairie vole animal model provides an excellent opportunity to investigate the underlying neuromechanisms that are associated with the behavioral deficits typically seen in ASDs. Taken together, these data help to establish the prairie vole animal model to study autism and also to provide evidence demonstrating that a dysregulation of the serotonergic system during early brain development can have subsequent long-lasting neurochemical and behavioral effects.
Animal Care and Use Committee (ACUC)
PROTOCOL REVIEW
COMMITTEE COMMENTS AND ACTION
Reviewed by the Animal Care and Use Committee on July, 29, 2010

Comments:

#1031, Dr. Zuoxin Wang, Triennial Resubmission, Confirm Approved, USDA covered species
Regarding new Protocol #1031, “Neurochemical Regulation of Social Attachment”, the primary objective of this research program is to investigate the neurochemical mechanism underlying social attachment between adult males and females and between parents and their offspring.

The protocol was submitted to the ACUC Committee members through the Designated Member Review process and was approved July 20, 2010. No questions, concerns or comments regarding the production or DMR approval were raised at the July meeting.

It was moved and seconded to confirm the Designated Member Review approval of the significant change to Protocol #1031. Approved unanimously.

OFFICIAL ACTION

APPROVED

Dr. Paul Q. Trombley, ACUC Chair
July 29, 2010
Date

Dr. Kathleen Harper, LAR Director and Attending Veterinarian
July 29, 2010
Date
REFERENCES


BIOGRAPHICAL SKETCH

EDUCATION:

Masters in Psychobiology  Psychology Department, Neuroscience Program  
Florida State University  Expected Graduation: April 2011

Bachelor of Science  Major: Biology  Minor: Chemistry  
The University of Tampa  Graduated: May 2005

RESEARCH EXPERIENCE:

- Graduate Research Assistant, Department of Psychology and Program in Neuroscience, Dr. Zuoxin Wang, Florida State University, 08/2008 – 04/2011
- Lab Technologist/Researcher, The Dow Chemical Company, Dr. Scott Gaynor, 01/2007 – 07/2008
- Lab Technologist, The Dow Chemical Company, Dr. Joyce Battjes, 02/2006 – 09/2006

TEACHING EXPERIENCE:

- Teaching Assistant, Sensation and Perception, Florida State University, Fall 2009 – Spring 2011
- Guest Lecturer, Sensation and Perception, Florida State University 2010
- Academic Center for Excellence Tutor, University of Tampa, 08/2004 – 05/2005
- Organic Chemistry Laboratory Assistant, University of Tampa, 08/2004 – 05/2005

AWARDS and HONORS:

- Achieved Top 90% List in which at least 90% of students rated me as excellent in response to the item, ‘overall assessment of instructor’.
- Graduated Cum Laude from the University of Tampa
- Graduated with Honors Program Distinction from the University of Tampa
- Dean’s List, University of Tampa, 2001-2005
- Elected Honor’s Program Officer 2004 - 2005
- Harvard Model United Nations Elected Representative for the University of Tampa, 2005
- Cross Country Team Captain, 2003 - 2004
- Track Team Captain, 2004-2005
- 8th Place Team Finish at the NCAA Division II National Championship, 2004
- Sunshine State Conference Student/Athlete Recognition, 2003-2004

SCHOLARSHIP/FELLOWSHIP SUPPORT:

- Congress of Graduate Students Grant, Florida State University, 2008-2010
- Neuroscience Fellowship, Florida State University, 2010
- Presidential Scholarship, University of Tamp 2001-2005
- Life Sciences Scholarship, University of Tamp 2001-2005
- Covenant Health Scholarship 2001
- Michigan Merit Scholarship 2001
- Athletic Scholarship 2002-2005

INVITED TALKS:

- Neuroscience Program Summer Seminar, Florida State University 2009
- Biology Senior Seminar, University of Tampa, 2005
- University of Tampa Research Presentation, 2004

CONFERENCE PROCEEDINGS:


MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

- Society for Neuroscience, 2008 - 2011
- Society for Behavioral Neuroendocrinology, 2009
- Golden Key Society Member
- Alpha Chi National Honors Society Member

SKILLS and TECHNIQUES:

- Behavioral Assay: Social affiliation, elevated plus maze, open field box
- Neuropharmacological Manipulations: Brain region-specific microinjections
• Pharmacological Manipulation: Subcutaneous injections
• Surgical Techniques: Stereotaxic cannulation, brain extraction
• Tissue Preparations: Cryostat and microtome tissue sectioning, brain region-specific microdissection, tissue mounting
• Histological Assays: Immunohistochemistry, bacterial isolation and transformation
• Molecular Assays: Western blotting, corticosterone radioimmunoassay, agarose gel electrophoresis, polymerase chain reaction, DNA, RNA, and protein isolation and purification, DNA digestion, Infrared and Mass Spectroscopy, nuclear magnetic resonance spectroscopy, Fulgent Method for DNA detection, thin layer chromatography
• Grant writing
• Manuscript review/editing

COMMUNITY OUTREACH:

• Brain Awareness Week, Spring 2010
• Brain Awareness Week, Fall 2010
• North Florida Brain Bee, February 2011