Published in final edited form as:

Physiol Behav. 2012 January 18; 105(2): 529–535. doi:10.1016/j.physbeh.2011.09.015.

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

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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, nearly 30% of autistic patients show elevated blood levels of serotonin (5-HT) and, therefore, various genetic and environmental factors that are known to elevate 5-HT levels may play a role in the development of ASDs. In the present study, we used the socially monogamous male prairie vole (*Microtus ochrogaster*) as an animal model to examine the effects of perinatal exposure to 5-methoxytryptamine (5-MT), a non-selective serotonin agonist, on subsequent behavioural and neurochemical changes in the brain. 5-MT treated males showed a decrease in affiliation and an increase in anxiety-related behavior, as well as a decrease in the density of 5-HT immunoreactive (ir) fibers in the amygdala and oxytocin-ir and vasopressin-ir cells in the paraventricular nucleus of the hypothalamus, compared to saline treated controls. These data indicate that exposure to 5-HT during early development can induce abnormalities in various neurochemical systems which, in turn, may underlie deficits in social and anxiety-related behaviors. In addition, these data will help to establish the prairie vole model to study the neurobiological underpinnings of complex neuropsychiatric disorders such as ASDs.

Keywords

Autism; Social Affiliation; Anxiety; Serotonin; Oxytocin; Vasopressin

1. Introduction

Autistic spectrum disorders (ASDs), or more commonly referred to as autism, are considered to be developmental disorders characterized by abnormalities in various cognitive and behavioral functions, including increased anxiety, stereotyped and repetitive behaviors, hypersensitivity to sensory stimuli, and deficits in social interactions and communication [1]. Although the etiologies are still largely unknown, genetic mutations in

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serotonin transporter (SERT) gene [2, 3] and maternal use of monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and cocaine (for review, see [4]) - all of which elevate serotonin (5-HT) levels - are thought to increase the risk of autism. Interestingly, nearly 30% of patients with autism demonstrate elevated blood levels of 5-HT (for review, see [5]). 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.

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, neurite outgrowth, synaptogenesis, dendritic branching, and neurogenesis in target brain regions and also serves to autoregulate its own neuronal outgrowth via a negative feedback mechanism (for review, see [6]). Thus, the 5-HT concentration in the developing brain is crucial for proper development. For instance, it has been shown that early exposure to 5-methoxytryptamine (5-MT), a non-selective 5-HT agonist, decreases serotonergic outgrowth [7] whereas prenatal depletion of 5-HT delays the onset of neurogenesis in 5-HT target brain regions [8]. Additionally, such neuroanatomical changes that result from 5-HT depletion correlate with behavioral changes including altered exploratory, social, and fear-related behaviors as well as increased resistance to change [9, 10]. Developmental exposure to elevated levels of 5-HT also has been shown to disrupt proper development of other neurotransmitter systems such that 5-MT treated subjects had fewer oxytocin (OT) cells in the paraventricular nucleus of the hypothalamus (PVN) in the rat brain compared to saline controls [11]. In this same study, 5-MT treated rats were also found to have decreased litter bonding behavior and reduced olfactory-based social interactions, indicating that these neuroanatomical changes may underlie the behavioral deficits seen in 5-MT treated subjects [11].

Although various neuroanatomical, neurochemical, and behavioral abnormalities have been associated with ASDs, the specific neuromechanisms that underlie the deficits in social behaviors are still unknown, 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 (for review, see [12]). Studies have shown that male and female prairie voles form long lasting pair bonds following mating [13–15] and that these behaviors are regulated by a variety of neurochemical systems including OT [15, 16], arginine vasopressin (AVP) [13, 17, 18], dopamine (DA) [19] and 5-HT [20]. Therefore, the prairie vole model may provide an excellent opportunity to study the neurochemical mechanisms underlying complex psychiatric disorders such as ASDs. In the present study we examined the effects of 5-MT perinatal exposure on social affiliation and anxiety-related behavior in adult male prairie voles. We also examined 5-MT's influence on the expression of 5-HT, OT, AVP, and DA - neurochemicals which have been previously implicated in social and anxiety-related behaviors. As the 4:1 male:female gender ratio was found in ASDs [21], the present study was focused on male prairie voles.

2. Materials and Methods

2.1 Subjects

Capture-bred male and female prairie voles (*Microtus ochrogaster*) were housed in large polycarbonate cages (25×45×20 cm) with cedar chip bedding and hay covering, and allowed to produce multiple litters to ensure breeding reliability. Upon weaning, male subjects were housed in same-sex sibling pairs in polycarbonate cages (18×29×13 cm). All cages were maintained under a 14L:10D photoperiod. The room temperature was kept at 21 ± 1°C. Food and water were provided *ad libitum*. Behavioral testing began once subjects reached adulthood at approximately postnatal day 80 (PND80). All animals received three

behavioral tests which were conducted one per day for three consecutive days. The open field and the elevated plus maze tests were conducted between 0900 and 1100 on days 1 and 3, respectively, whereas the social affiliation test was conducted between 1300 and 1500 on day 2.

2.2 Drug Treatment

Prairie vole dams are immediately impregnated following a gestation period (GD) and birth of a new litter [22]. Thus, pregnant-timed prairie vole dams received prenatal daily injections of either saline (control, $n=5$) or saline containing 5-MT (1mg/kg, $n=8$) from GD12 (when 5-HT neurons first become evident [23]) to GD21. 5-MT is a non-selective 5-HT agonist with relatively high potency at all tested 5-HT receptors [24]. Beginning at parturition (PND0), male pups continued to receive daily injections of either saline or 5-MT (1mg/kg) until PND20 (when peak 5-HT synaptogenesis typically ends [25]). All injections were given between 1100 and 1200. It is important to note that this is an established paradigm used in rat studies [11, 26] to manipulate 5-HT system during a time when 5-HT has its peak developmental influence [23].

2.3 Social Affiliation Test

The apparatus consists of two polycarbonate cages (28×16×12cm) connected via a hollow tube (7.5×16 cm) and has been previously established to test social affiliation in prairie voles [27–29]. An unfamiliar, same age male prairie vole was loosely tethered in the stimulus cage whereas the subject was placed into the empty, non-stimulus cage but allowed to move freely throughout the apparatus. The 30-min test was video-recorded. The duration and frequency of cage entries and contacts with the stimulus animal were later scored and analyzed.

2.4 Open Field Test

The open field test has been validated to measure locomotor activity and anxiety-related behavior in voles [19, 29, 30]. The apparatus consists of an open-field box (56×56×20 cm) in which the floor is equally divided into 16 squares (14×14 cm). To begin, subjects were placed in the center of the apparatus and the 10-min test was video-recorded. Frequency of square crossings and the duration of time spent in the center, corner, and peripheral squares were later scored and analyzed.

2.5 Elevated Plus Maze Test

The elevated plus maze (EPM) has been validated to test anxiety-related behavior in voles [31, 32]. The EPM apparatus 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). To begin, subjects were placed in the center of the EPM apparatus and were observed by the experimenter throughout the entire 5-min test to ensure that the subject remained on the apparatus. This 5-min test was also video-recorded and the duration and frequency of open and closed arm entries were later scored and analyzed.

2.6 Immunohistochemistry

Sixty minutes following the EPM test, subjects received 0.1 ml of ketamine and were perfused with 4% paraformaldehyde and their brains were stored in a solution containing 30% sucrose and 0.1M phosphate buffer (PB). Brains were sliced into 40 μ m sections using a microtome. Four sets of floating sections at 240 μ m intervals were processed for 5-HT, OT, AVP, and tyrosine hydroxylase (TH) immunostaining using previously established protocols [10, 33, 34]. TH is a rate limiting enzyme for DA synthesis and is therefore a marker of DA containing cells. Briefly, sections were incubated in 1% NaBH₄, rinsed in 0.1M PB,

incubated in 0.5% H₂O₂ in order to block endogenous peroxidase activity and then in a blocking solution containing 10% normal rabbit serum (NRS), 0.3% triton, and 0.1M PB. Each set of sections was incubated with either 5-HT polyclonal goat IgG antibody (1:5k, Thermo Scientific, Inc., Rockford, IL), OT polyclonal rabbit IgG antibody (1:10k, gift from Dr. Morris, Wright State University), AVP polyclonal rabbit IgG antibody (1:8k, Millipore Corporation, Temecula, CA), or TH polyclonal rabbit IgG antibody (1:5k, Millipore Corporation, Temecula, CA) in 2% NRS, 0.3% triton, and 0.1M PB for either 40 hrs (5-HT and OT) or 16 hrs (AVP and TH) at 4°C. Thereafter, sections were rinsed with 0.3% triton and incubated in a solution containing either biotinylated rabbit anti-goat secondary antibody (for 5-HT) or biotinylated goat anti-rabbit secondary antibody (for OT, AVP, or TH) (1:300 Vector Laboratories, Inc., Burlingame, CA) for two hrs and then in ABC complex (Vector Laboratories, Inc.) for 90 mins. Lastly, sections were stained with Nickel-DAB (Vector Laboratories, Inc.) and mounted on slides and cover-slipped. In order to control for variability, all sections for each staining were processed simultaneously.

2.7 Data Quantification and Analysis

Using a Zeiss Axioskop II microscope with StereoInvestigator software, images were taken from the dorsal raphe (DR), amygdala (AMY), PVN, and ventral tegmental area (VTA). For each neurochemical marker staining in the brain areas, images were taken from either 4 (AVP, TH, 5-HT) or 6 (OT) anatomically matched brain sections across the experimental groups. The PVN and VTA are brain regions with distinct borders and so the OT, AVP, and TH cells were counted within a given area that best represented these regions to give a cell density measurement. However, due to the undefined boundary of the DR, the total number of 5-HT cells was quantified. Further, 5-HT optic fiber densities in the central (CeA), basolateral (BLA), medial (MeA), and cortical (CoA) amygdalar nuclei were quantified in 3 consecutive brain sections using ImageJ software. Group differences in the number of 5-HT, OT, AVP and TH immunoreactive (ir) stained cells, 5-HT fiber densities, and social affiliation, anxiety-related, and open field behaviors were all analyzed by independent samples t-tests.

3. Results

3.1 Effects of 5-MT treatment on behaviors

Treatment with 5-MT during early development significantly altered behaviors of male prairie voles when later tested during adulthood. In the social affiliation test, 5-MT treated males spent less time in the stimulus animal cage (Figure 1A) ($t = 2.64$, $p < 0.05$) and had less side-by-side contact with the stimulus animal (Figure 1C) ($t = 2.45$, $p < 0.05$), compared to saline treated controls. No group differences were found in the number of cage entries into either the empty cage ($t = 0.88$, $p = 0.40$), the stimulus cage ($t = 0.86$, $p = 0.41$) (Figure 1B) or in the total number of cage crossings ($t = 1.03$, $p = 0.33$), suggesting that subjects' locomotor activity was not affected by perinatal 5-MT treatment. Additionally, the frequency of contacts with the stimulus animal did not differ (Figure 1D) ($t = 1.07$, $p = 0.31$). In the EPM test, 5-MT treated males entered the open arm less (Figure 2B) ($t = 1.84$, $p < 0.05$) than saline treated controls whereas no differences were found in the total number of open and closed arm entries ($t = 0.87$, $p = 0.40$). In the open field test, 5-MT treated males spent significantly more time in the corners of the arena compared to saline treated controls (Figure 2C) ($t = 3.09$, $p < 0.05$), whereas the two groups did not differ in locomotor activity (indicated by the number of square crossings) ($t = 1.60$, $p = 0.14$).

3.2 Effects of 5-MT treatment on neurochemical marker staining

5-HT labeled cells were found in the DR in the prairie vole brain (Figure 3). 5-MT treated males seemed to have fewer 5-HT cells in the DR compared to saline treated controls,

however, this difference did not reach statistical significance (Figure 3A–C). 5-HT stained fibers were found in many areas in the prairie vole brain. We focused on the AMY due to its involvement in regulating social and anxiety-related behaviors [35, 36]. 5-MT treated males had decreased optic densities of 5-HT fibers in the CeA ($t = 5.34$, $p < 0.05$), B1A ($t = 2.67$, $p < 0.05$), MeA ($t = 3.37$, $p < 0.05$), and CoA ($t = 2.59$, $p < 0.05$) nuclei when compared to saline treated controls (Figure 3D & E).

The PVN has been shown to contain the cell bodies of various neurotransmitter systems including OT, AVP, and DA and is a brain region that plays an important role in regulating both social and anxiety-related behaviors [13, 17–19, 37]. Similar to the AMY, the PVN also receives dense serotonergic innervations [38], and therefore 5-HT can potentially influence the development of this brain region and these neurotransmitter systems. We focused on the PVN to examine the effects of perinatal 5-MT exposure on OT, AVP, and TH expression. 5-MT treated males showed decreased OT ($t = 3.03$, $p < 0.05$) and AVP ($t = 3.73$, $p < 0.05$) cell densities within the PVN, whereas no differences were found in TH cell densities, compared to saline treated controls (Figure 4A–E). As the VTA provides a major source of DA-ergic projects in the mesolimbic pathway, we also counted TH stained cells in the VTA, however, no significant group differences were found (Figure 4E).

4. 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 increased anxiety-related behavior [1]. It has been shown 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 [4, 5]. In the present study, we found that perinatal exposure to 5-MT, a 5-HT agonist, impaired social affiliation, increased anxiety-related behavior, and decreased 5-HT expression in 4 distinct subnuclei of the AMY as well as OT and AVP expression in the PVN in the socially monogamous male prairie vole. We used a paradigm that has been previously established in rat studies [11] to manipulate the 5-HT system during a time when it has its peak developmental influence [23]. The dose of 5-MT used in these experiments, 1mg/kg, has been shown to be sufficient in inducing neurochemical and behavioral changes in rats [7, 11, 39]. It is important to note that post-partum fluoxetine treatment in prairie vole dams does not affect maternal behavior [20] and, therefore, pre-partum 5-MT effects on post-partum maternal behavior was not expected.

Although various animal models have been developed to study the neurochemical regulation of the behavioral phenotypes associated with ASDs, the investigation of social behaviors has been limited to pup play behavior and olfactory social investigation [11, 40]. In the present study using the highly social male prairie voles, we found that the perinatal exposure to 5-MT significantly impaired social affiliation. In similar studies in rats, perinatal exposure to 5-MT also impaired social interactions in that 5-MT treated subjects participated in less pup play behavior and as adults spent less time engaged in olfactory-based social interactions [11]. In addition, genetic studies investigating SERT null mutant mice behavior have revealed decreased sociability such that these mutant mice failed to show a preference for the compartment containing the novel conspecific [41]. Here, our data provide additional evidence to support the notion that excess 5-HT exposure during early development induces deficits in social behaviors.

In the present study, 5-MT treated male voles also displayed increased anxiety-related behavior, indicated by fewer entries into the open arm in an EMP test and increased time spent in the corner squares in an open field test, compared to saline treated controls.

Although a similar 5-MT treatment was ineffective to significantly alter anxiety-related behavior in rats [11], genetic animal models of autism have successfully been able to show increased anxiety-related behavior in mice [42, 43]. Our data are consistent with these genetic animal models and the tendency for patients with autism to display increased anxiety. Together, our behavioral data show that, in male prairie voles perinatal manipulation of 5-HT system can alter social affiliation and anxiety-related behaviors – two characteristic behavioral phenotypes associated with ASDs [1].

It has been demonstrated that 5-HT can act as a developmental signal and that elevated levels of 5-HT during early brain development induces abnormalities among various brain regions and neurotransmitter systems [6, 23, 44]. As 5-HT neurons contain autoreceptors that help to regulate proper serotonergic outgrowth [45], high levels of 5-HT during early development may feedback to inhibit its own development, thereby reducing the number of 5-HT neurons in the DR and causing a subsequent decrease in 5-HT projections into various brain regions [7, 46]. Our data are consistent with this observation. The fact that perinatal 5-MT treatment significantly decreased 5-HT projections in the AMY is particularly interesting. The CoA and MeA have been implicated in social behavior [35] whereas the CeA is important in fear and anxiety-related behavior [47, 48]. The AMY receives dense 5-HT innervations from the DR [38, 49] and released 5-HT within the AMY provides an inhibitory response [49, 50]. Thus, a decrease in 5-HT afferent fibers, as our data indicate, would lead to a decrease in inhibition on the amygdalar neurons which would, in turn, result in an increase in amygdalar output activity. Interestingly, fMRI studies have shown that increased amygdalar activity can be associated with anxiety-related and fear behaviors [51, 52] as well as social phobia [53].

The neuropeptides OT and AVP are involved in the regulation of social and anxiety-related behaviors [54, 55]. Our findings that perinatal exposure to 5-MT leads to a significant decrease in OT neurons in the PVN is consistent with previous findings in rats [11]. Interestingly in our study, OT neurons in the PVN also seemed to show much less projections in the 5-MT treated male voles (Figure 4). Besides the posterior pituitary, OT neurons in the PVN also project into some forebrain regions important for behavioral and cognitive functions, [56, 57]. Given the role of OT in social and anxiety-related behaviors in prairie voles [58, 59], fewer OT neurons in the PVN and less projections may likely contribute to the altered social affiliation and anxiety-related behaviors in this species. In addition, it may also indicate a decreased OT release in the blood stream. Interestingly, patients with autism typically have an associated decrease in blood OT levels [60, 61], and OT infusion in ASD patients reduces repetitive behaviors [62] and increases social cognition [63] thereby rescuing some of the behavioral deficits typically seen in ASDs. Furthermore, as PVN AVP inhibits the hypothalamic-pituitary-adrenal axis and thus the stress response [64], decreased AVP by 5-MT treatment may imply a reduction in AVP's inhibition on the stress response. Such alteration in central AVP may also affect vole social behaviors [13, 65]. It is worth mentioning that the AVP receptor gene, AVPR1a, is considered to be an autism susceptibility gene, and clinical studies have demonstrated that variation in the 5' flanking microsatellite region of the AVPR1a gene can contribute to the onset and severity of autism [66–68]. Finally, the lack of 5-MT effects on DA indicate a neuropeptide specific effect that 5-HT has on the OT and AVP systems.

5. Conclusion

Prairie voles are a unique animal model in that they display highly affiliative behaviors towards conspecifics and form long-lasting pair bonds after mating [13–15]. Given that patients with autism typically demonstrate decreased social bonding, the prairie vole may provide an excellent opportunity to investigate the underlying neuromechanisms that are

associated with the deficits in social behaviors typically seen in ASDs [69, 70]. Data from our present study show that a disruption in the 5-HT system during early development leads to altered social affiliation and anxiety-related behaviors associated with abnormalities in 5-HT, OT, and AVP systems. These data not only provide further evidence to support the notion that the 5-HT system may play a large role in the pathogenesis of autism but also help to establish the prairie vole model to study both the behavioral phenotype and the underlying neuromechanisms in such a complex psychiatric disorder. Future studies will need to investigate any neurochemical and behavioral changes in female prairie voles; other types of behavioral characteristics typically associated with autism including stereotyped and repetitive behaviors, social communication, and learning and memory; and causal relationships between the altered neurochemical systems and behaviors.

Acknowledgments

We thank Dr. Kimberly Young, Claudia Lieberwirth, Kelly Lei, Adam Smith, and Matt D'Alessandro for their critical reading of this manuscript. This work was supported by the National Institutes of Health grant MHR01-058616 to ZXW.

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Highlights

1. 5-HT acts as a developmental signal during early brain development.
2. Early exposure to a 5-HT agonist, 5-MT, altered social and anxiety behaviors.
3. Early exposure to 5-MT also decreased OT, AVP, and 5-HT staining in the brain.
4. The prairie vole model may provide an excellent opportunity to study ASDs.

Figure 1.

Perinatal 5-MT treatment decreases social behavior in adult male prairie voles (~PND80). During a social affiliation test, 5-MT treated males ($n=8$) preferred to spend less time in the cage with the stimulus animal compared to saline controls ($n=5$) (**A**). No group differences were found in the number of cage entries, indicating that decreased time spent in the stimulus cage was not due to altered exploratory behavior (**B**). 5-MT treated males also spent less time in contact with the stimulus animal compared to saline treated controls (**C**). The number of total contacts did not differ between treatment groups indicating that decreased contact duration was not due to altered investigative behavior (**D**).

Figure 2.

Perinatal 5-MT treatment increases anxiety-related behavior in adult male prairie voles (~PND80). In an elevated plus maze test, no differences were found between 5-MT treated males (n=8) and saline controls (n=5) in the amount of time spent in the open arm (**A**), however, 5-MT treated males entered the open arm less frequently compared to saline controls (**B**). In the open field test, 5-MT treated males (n=8) spent more time in the corner squares compared to saline controls (n=5) (**C**). No differences were found in the number of entries into either the center or corner squares suggesting that locomotor activity was not affected.

Figure 3.

Perinatal 5-MT treatment affects 5-HT expression in the brain of adult male prairie voles (~PND80). 5-MT treated males (n=4) seemed to have fewer 5-HT stained cells in the dorsal raphe nucleus compared to saline controls (n=3) (A–C; scale bar = 100µm). 5-MT treated males also showed a decreased optic density of 5-HT fibers in the central (CeA), basolateral (BIA), medial (MeA), and cortical (CoA) subnuclei of the amygdala compared to saline controls (D–E) (scale bar = 1000µm for panels A, B & D, and 10µm for the insert in D).

Figure 4. Perinatal 5-MT treatment decreases OT and AVP, but not TH, expression in the brain of adult male prairie voles (~PND80). 5-MT treated males (n=4) had decreased densities of OT (A, B, & E) and AVP (C, D, & E) cells in the PVN compared to saline controls (n=3). In contrast, no group differences were found in the density of TH stained cells in either the PVN or VTA (E). Scale bar = 100µm.