



Effects of developmental hyperserotonemia on juvenile play behavior, oxytocin and serotonin receptor expression in the hypothalamus are age and sex dependent



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HIGHLIGHTS

- Excess developmental 5HT alters 5HT receptors differently in juvenile males & females.
- Juvenile play is disrupted & OXT cell counts are altered after serotonin treatment.
- The 5HT_{2A} receptor expression pattern changes over the second week of life.
- These findings may shed light on the sex difference in ASD etiology.
- Further comparison between males and females in studies of this kind is warranted.

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ABSTRACT

There is a striking sex difference in the diagnosis of Autism Spectrum Disorder (ASD), such that males are diagnosed more often than females, usually in early childhood. Given that recent research has implicated elevated blood serotonin (hyperserotonemia) in perinatal development as a potential factor in the pathogenesis of ASD, we sought to evaluate the effects of developmental hyperserotonemia on social behavior and relevant brain morphology in juvenile males and females. Administration of 5-methoxytryptamine (5-MT) both pre- and postnatally was found to disrupt normal social play behavior in juveniles. In addition, alterations in the number of oxytocinergic cells in the lateral and medial paraventricular nucleus (PVN) were evident on postnatal day 18 (PND18) in 5-MT treated females, but not treated males. 5-MT treatment also changed the relative expression of 5-HT_{1A} and 5-HT_{2A} receptors in the PVN, in males at PND10 and in females at PND18. These data suggest that serotonin plays an organizing role in the development of the PVN in a sexually dimorphic fashion, and that elevated serotonin levels during perinatal development may disrupt normal organization, leading to neurochemical and behavioral changes. Importantly, these data also suggest that the inclusion of both juvenile males and females in studies will be necessary to fully understand the role of serotonin in development, especially in relation to ASD.

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1. Introduction

Although perhaps best known as a neurotransmitter in the adult brain, serotonin (5-HT) also acts as a foundational signal during development, critically involved in the maturation of its target tissues and its own future cellular network [1–4]—by regulating processes such as neurite outgrowth [1–3,5], neuronal differentiation/organization [5,6],

synaptogenesis [7] and even neurogenesis [8,9]. These fundamental developmental mechanisms differ between males and females, mainly due to the actions of gonadal hormones during prenatal and early postnatal development [10–12]. Importantly, serotonin release and metabolism also exhibit a sexually dimorphic profile, with females maintaining a relatively static level of 5-HT in the brain across early postnatal development, while males exhibit a transient and significant decrease [13–15]. This transient change in 5-HT is necessary for full masculinization of the rodent brain, since prevention of this 5-HT drop results in female-typical brain morphology and function. For example, administration of an exogenous 5-HT agonist demasculinizes the size of the sexually dimorphic nucleus (SDN) and the anteroventral periventricular nucleus (AVPV) and also reduces male sex behavior,

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but supports the appearance and maintenance of the female-typical LH surge, in males and testosterone treated females [15–17]. Thus, proper regulation of the serotonin system during early life appears to be a key regulator of sexual differentiation for brain structures, physiology, and behaviors.

One of the most robust sexually dimorphic patterns of behavior in humans is the incidence/diagnosis of neurological disease and disorder, with developmental disorders such as Autism Spectrum Disorder (ASD) observed much more often in males than in females. In fact, even though ASD diagnoses have increased approximately 78% between 2002 and 2008 alone, the strikingly male-dominated sex difference persists, such that boys are diagnosed at least 4.6:1 over girls [18]. ASD is characterized by a number of different behaviors and given this diverse symptomology, identifying a consistent underlying pathophysiology has been challenging. One physiological condition seen in an unusually high percentage of ASD individuals (about 35%), is hyperserotonemia, or high serum levels of 5-HT [19–21]. The origin of this increased blood serotonin is currently unknown, but may be due to differences in serotonin production and/or metabolism in the individual during fetal development or be of maternal origin [22,23]. Indeed, at least one study has pointed to a potential link between *in utero* exposure to selective serotonin reuptake inhibitors (SSRIs) and a modestly increased risk for ASD in later childhood [24]. Additionally, serotonin synthesis in the brains of children with ASD may be dysregulated [25]. In rats, treatment of pregnant dams with the non-selective 5-HT receptor agonist, 5-methoxytryptamine (5-MT) results in developmental blood hyperserotonemia in the offspring similar to that observed in ASD individuals, as well as similarly aberrant social interactions, including reduced vocalizations, reduced preference for a conspecific over a novel object in sociability testing, and reduced rough and tumble play behavior [3,26,27]. In addition, developmental hyperserotonemia results in a reduced number of oxytocinergic cells in the paraventricular nucleus of the hypothalamus (PVN; [3,26]) seen in the offspring as adults. It is unclear, however, if changes in a peptide known to support social behaviors might also underlie the changes noted in juvenile social play behavior, which only occurs during adolescence in rodents—far earlier than the observation of reduced oxytocin (OXT) reported in adult tissue. It is equally unclear how an increase in serotonin activity during the perinatal period might alter the organization of the oxytocin-producing PVN, though the serotonin receptors present throughout the PVN presumably play a role. The two most likely candidates among those receptors are the 5-HT_{1A} and 5-HT_{2A} receptors, both of which 1) colocalize together in the same cells with OXT, 2) are known to influence OXT release in the PVN, and 3) are also implicated in sexual differentiation of other brain areas [17,28–30].

Given the higher number of males diagnosed with ASD, most animal studies have used males exclusively. A few recent studies have included females, and have shown that the cellular and behavioral changes resulting from SSRI-induced increases in 5-HT signaling are more apparent in males, including an increase in abnormally formed cells in the corpus callosum, as well as decreased novel object exploration, increased neophobia, and reduced juvenile play and male sex behavior [31,32]. These findings add to the growing evidence that the developing male brain is somehow more sensitive to perturbation than the developing female brain, thereby making experiments directly comparing males and females crucial to fully understanding the etiology of any developmental disorder, especially one as male-dominant as ASD. Further, observations of cellular and, to a lesser extent, behavioral responses to developmental hyperserotonemia in juveniles are lacking, as most experiments have reported effects in treated animals only as adults. Therefore, our investigation of the effects of hyperserotonemia included both male and female animals, with immunocytochemical detection of 5-HT_{1A}, 5-HT_{2A}, and OXT in the PVN at PND10 and PND18, and basal locomotor activity and observation of juvenile social play behavior between PND30 and 42.

2. Materials and methods

2.1. Subjects

Timed pregnant Sprague-Dawley rats were purchased (Charles River) and maintained on a reversed 12:12 light cycle (lights on 2200 h), housed in clear high-temp polycarbonate cages (48 cm × 25 cm × 22 cm), and provided tap water and standard rat chow (Prolab RMH 3000) *ad libitum*. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at the University of Massachusetts Boston in accordance with the national guidelines for research animal care and use.

Starting on gestational day (GD) 12, when the serotonin system is first functional in rats [33], dams were injected with 1 mg/kg 5-methoxytryptamine (5-MT; from Sigma-Aldrich for experiment 1 and MP Biomedicals for experiment 2) or vehicle (0.85% saline with no more than 0.4% DMSO), s.c. each morning between 0900 and 1000 until birth. Following parturition and observation of milk bands, pups were removed from the home cage, injected with 5-MT or vehicle s.c. and returned within 1 h. Litters were culled to a maximum of 12 pups. A total of 18 litters, with no more than 2 pups per litter contributing to each experimental group, were used. $N = 4–8$ animals per experimental group (vehicle male, vehicle female, 5-MT male, 5-MT female) at each age.

2.1.1. Experiment 1—effect of 5-MT treatment on PVN at PND10

Pups were injected with 5-MT or vehicle in accordance with their prenatal drug exposure, from day of birth (DOB) to postnatal day PND10, when they were sacrificed and tissue was processed for immunocytochemistry.

2.1.2. Experiment 2—effect of 5-MT treatment on PVN at PND18, and juvenile play behavior

On DOB, pups were cross-fostered within condition and injected with 5-MT or vehicle daily until PND16, in accordance with their prenatal drug exposure. Half of each litter was perfused on PND18 for immunocytochemistry. The second half of each litter was left undisturbed with the dam until weaning at PND21. Pups were weaned (6 per cage) quasi-randomly into mixed sex and treatment housing for behavioral testing such that no more than 2 animals from the same experimental group (vehicle males, vehicle females, 5-MT males, 5-MT females) were present in the cage. Every 2–3 days, the pups were handled and tail-marked with permanent non-toxic marker for maintenance of group identification. The tail marks and flank marks used during behavioral testing were coded such that the mark did not identify the animal's treatment status until decoded at the end of all experimentation.

2.2. Behavioral testing

All behavioral testing took place during the dark phase of the light cycle during the animals' normal active period, under dim red light. On postnatal days 30, 31, 36, 37, and 42—ages corresponding to the peak period of play in Sprague-Dawley rats (PND30–40; [34]) and prior to the onset of sexual maturity (PND50+; [35])—home cage behavior was videorecorded for 15 min. Due to space and filming constraints, the 15 min recorded session started either just after lights out for half of the cages or 30 min after lights out for the other half, with observation cage order counterbalanced across days. Observations were limited to this time as pilot work in our lab found that pups were generally sleeping three hours after lights out, a second common observation time [36]. Rough-and-tumble play behavior was coded (Noldus The Observer, version 12.3) by an observer blind to the treatment group, using the following operational definitions: 1) Boxing/Wrestling: two animals engaged in rolling and tumbling over one another or making jabbing movements at one another with the forepaws; 2) Bite: one animal

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