ABSTRACT

Title of Thesis: THE EFFECTS OF NEONATAL OXYTOCIN ON SEXUAL MATURATION AND THE EXPRESSION OF SOCIOSEXUAL REPRODUCTIVE BEHAVIOR IN FEMALE RATS

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While the role of oxytocin (OT) in regulating adult behavior data is thoroughly documented, increasing evidence suggests that neonatal exposure to OT can have long-term effects on behavior and physiology. Based upon the role of OT in regulating adult behavior, I predicted that neonatal OT would affect the expression of adult female sociosexual behavior and sexual maturation. Female Sprague-Dawley rats were treated for the first 7 days of life with intraperitoneal injections of either OT (1 μg/g), an OT antagonist (0.1 μg/g), isotonic saline, or handled only. Parameters measured included age of vaginal opening, age of first estrus, a 10-hr paced sex test during first estrus, and body weight on postnatal days 70, 91 and 136. Treatment with OT significantly delayed the age of vaginal opening and first estrus. OTA significantly reduced mating frequency from an expected rate of 33%. There was no effect on weight at any age.

THE EFFECTS OF NEONATAL OXYTOCIN ON SEXUAL MATURATION AND THE EXPRESSION OF SOCIOSEXUAL REPRODUCTIVE BEHAVIOR IN FEMALE RATS

by

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INTRODUCTION

While physiology and behavior are often studied in terms of individual systems, i.e. digestive, cardiovascular, nervous, endocrine etc, the body is a complex unit designed to do two things, survive and reproduce. If not from a strictly biological standpoint, then at least from an evolutionary perspective an argument can be made that sexual development and mating behavior, including the proper expression of the prosocial behaviors that facilitate mating, are critical to the continuation of a species. Therefore, it is not surprising that reproduction, especially in higher vertebrates, involves the complex interaction of all the systems of the body and that development of the underlying structures of reproduction begins early.

In asexual species reproduction is an individual event, however, in many species reproduction involves the intimate association of a male and female and the transfer of gametes. This means that sexual reproduction is a social event, requiring significant changes from typical day-to-day expression of social behavior to those that facilitate mating, and successful reproduction. For example, in many species of mammals adult females generally avoid adult males. However, during mating females must be willing to socially engage males. Perhaps because of the complexity of sexual reproduction, it is not surprising that reproduction can be affected by not only the physiological condition of the individual but also by external factors, such as parental and peer interactions.

The goal of this study is to try to gain insight into understanding the early mechanisms that may play a role in the development and ultimate expression of sociosexual behaviors that are essential for successful reproduction, especially in females.

Hormones play a critical role in regulating and facilitating all aspects of reproduction, including sexual differentiation, maturation, sociosexual behavior, mating, and parental care. We examine here the effects of neonatal manipulation of the hormone oxytocin on the expression of female reproduction.

While roles of steroids are the most well-known in reproduction, many hormones are involved in successful reproduction. Oxytocin is known to play an important role in almost all aspects of reproduction, including social recognition (23,27,44), the formation of pair bonds (15,17), the physical act of mating (2,8,36), parturition (32), maternal behavior (39,44,58), and nursing (55). One can appreciate how numerous and diverse these roles are, suggesting a trophic role. Added to this are recent findings that neonatal manipulation of oxytocin can alter adult physiology (including weight gain, stress, and some receptor populations), suggesting the hypothesis that during the neonatal period oxytocin could play a developmental role in the expression of sexually related functions. If true this would also suggest a mechanism through which environmental conditions, such as early social experience, could be reflected in sexual development and reproduction as oxytocin is release in response to social contact (47,71,78) as well as various types of stressors (41,52,60,75). Therefore the purpose of the research presented in this thesis was to determine if neonatal exposure to oxytocin can influence reproductive parameters in adult females.

REVIEW OF THE LITERATURE

Oxytocin Structure

Oxytocin (OT) is a nine amino acid neuropeptide hormone that can be released from nerve terminals ending in the posterior pituitary gland into the general circulation, acting as a hormone or released within the brain acting as a neurotransmitter. While it is produced in other regions the two primary nuclei within the brain responsible for the production of OT are the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nuclei (SON) situated on either side of the third ventricle and above the optic chiasm of the brain. These nuclei contain one of two types of oxytocinergic neurons, magnocellular and parvocellular neurons, which either extend their axons down through the infundibular stalk to terminate in the posterior pituitary gland (magnocellular), or diverge from their hypothalamic nuclei to directly innervate nearby nuclei (parvocellular). Although the majority of oxytocinergic magnocellular neurons terminate in the neurohypophesis, magnocellular neurons may also extend to distant regions of the brain such as the thalamus, olfactory bulb, and limbic system.

The nine amino acid sequence of OT forms a six-residue cyclic structure with a COOH terminated three amino acid tail (Figure 1). It shares structural similarity with vasopressin (AVP), a sister neuorpeptide/hormone which is also produced in the PVN and SON and when released as a hormone from the posterior pituitary plays a major role in the regulation of water balance. The presence of a neutral amino acid at position 8 classifies a cyclic nonapeptide into the OT family. Variation of residues at positions 2,3,4, and 8 make up the variations within the family (1). Cross-stimulation of receptors by various OT-like peptides does occur, however an isoleucine residue at position 3 is

necessary for properly stimulating OT receptors in placental mammals. This structural feature is considered essential for OT to interact appropriately within the binding cleft of the receptor (4).

OT and AVP are old hormones – similarly structured hormones are found in almost all vertebrates, and it is estimated that the ancestral gene is more than 500 million years old (34). The most primitive species to have an OT-like hormone is the earthworm *Eisenia foetidia* with its OT analog annetocin (exogenous annetocin injected into this earthworm induces egg-laying behavior) (56). OT retains its reproductive role even into advanced vertebrates where it is classically associated with uterine contraction and milk letdown in mammals. The preservation of OT-like peptide hormones in so many species suggests a strong selective pressure and essential role of the hormone.

Oxytocin Release

OT is released in response to a number of stimuli. Breast and genital stimulation are the most potent releasers of OT, but it can also be released by licking, grooming, and light touch (41,47,71,78). Tactile stimulation itself is not required for some OT release - urogenital olfactory cues and even conditioning such as hearing a baby's cry can release OT in a lactating female (12). OT can induce its own release – positive feedback followed by a negative inhibition (possibly due to receptor down-regulation, a threshold level of PKC products in the cytosol, or depletion of pre-formed neurosecretory vesicles) is a probable explanation for OT's characteristic pulsatile release. Once released, OT is enzymatically degraded in the blood, with a half-life of 19 minutes in rats (25).

Oxytocin Receptor

The Oxytocin receptor is a 7 trans-membrane domain G-protein coupled receptor. Interestingly, all OT receptors sequenced share the same homology, and unlike many receptors to this date there is still only one described form. The OT receptor does not seem to vary between locations (34). Binding activates phospholipase C leading to the generation of inositol triphosphate and diacylglycerol. Inositol triphosphate triggers Ca^{++} influx from intracellular stores as well as inhibiting Ca/Mg ATPase activity. Diacylglycerol activates protein kinase C. These second messengers can lead to subsequent changes in protein phosphorylation, membrane excitability, and/or gene transcription (34).

OT receptors are under complex physiologic regulation. They have both a low- and a high-affinity binding state of approximately K_d 50 nM and K_d 1 nM, respectively (35). For high affinity binding, divalent cations and cholesterol must be present. Divalent metals such as Mg increase both binding capacity and affinity, and are thought to influence positive cooperativity (63). Cholesterol similarly enhances binding capacity and affinity, stabilizing the receptor into its high-affinity state (45).

Gonadal steroids have a dramatic effect on OT receptor expression. Ovarian steroids alter the number of OT receptors in the uterus (48). In the brain Estrogen (E) increases both receptors and OT neuropeptide levels (42). Progesterone (P) effects are less clear; some studies have shown P to decrease binding and some show increases in binding (42). Some studies suggest membrane cholesterol modulation as a possible mechanism for progesterone's complex effects on OT receptor populations (46).

Testosterone in males has similar actions to E on OT receptors – most likely due to its active metabolites E and DHT in the brain (42).

Persistent stimulation of OT receptors results in a rapid desensitization and receptor internalization (29). This could be a mechanism for the pulsatile pattern of OT release, however OT receptor downregulation is long-lasting making a role in the pulsatile pattern unlikely. This down-regulation is rapid: approximately a 60% decrease within 10 minutes and a 10-fold decrease in 20 hours. Furthermore, these receptors are not recycled back to the cell surface (34). These decreases were also accompanied by a decrease in OT receptor mRNA (62), prolonging synthesis of replacement receptors and potentially making desensitization long lasting. Despite the demonstration of feedback loops and receptor down-regulation under normal physiological conditions (31), OT production does not appear necessary for the normal receptor distribution, at least in mice, as OT knock-out mice display normal receptor binding and distribution (76). Besides OT receptor down-regulation, high OT levels can alter other receptor populations, demonstrated in an increase in sensitivity in noradrenergic and estrogen receptor populations (17,21,22). Detailed information on the mechanism of these and other changes is still lacking**.**

Oxytocin Targets

OT targets in the periphery include the uterus, ovary, mammary glands, testis, prostate, heart, kidney, pancreas, adrenal gland, and adipose tissue (34). OT binding also occurs in the thymus, presumably to induce tolerance of the immune system to OT (33). OT receptors in the brain are predominantly located in the hypothalamus, although

receptors have also been localized in the thalamus, olfactory system, limbic system, and brainstem (34). Interestingly, OT receptor localization changes dramatically with age (70). In the pre-pubescent rat, the extrahypothalamic areas of greatest binding include the areas associated with stress and social contact such as the cingulate cortex, restrosplenial cortex, mammilary nuclei, globus pallidus, dorsal subiculum, and trigeminal nucleus. In the adult, the areas of greatest extrahypothalamic binding shifts to the peduncular cortex, ventral pallidum cell groups, ventral subiculum, amygdala, islands of calleja, and bed nucleus of the stria terminalis (BNST)(34). The roles of OT in each of these areas are still being elucidated.

Peripheral Action

The 'classic' roles of OT include uterine contraction and milk letdown. Uterine contraction is indeed a potent action of OT-like agents, however in-vivo uterine contraction occurs without an increase in serum OT levels. Rather, it occurs by a rapid up-regulation and expression of OT receptors in the myometrium which increases binding and then later leads to increases in prostaglandins and prostaglandin receptors (32). Although this is a 'well known' function of OT, in OT knockout mice labor was still successful (55), suggesting that OT plays a role in the birthing process may not be necessary and essential for labor induction.

Milk-letdown is a less ambiguous peripheral function of OT. Tactile stimulation of the nipple almost immediately causes synchronized high frequency bursts of activity from OT releasing neurons in the hypothalamus (34). Oxytocin is dumped into the peripheral bloodstream and contraction of breast myoepithelial cells occurs. This is a

critical role of OT, as knockout mice were incapable of nursing offspring. Peripheral OT injections restored the ability to lactate (55).

OT can affect the gonads, where it speeds the development of mouse blastocycts in the ovary. Although OT receptors have yet to be demonstrated in rat ovaries, they are present in male rat testis, where they are involved in regulation of the seminiferous tubules and modulation of steroidogenesis (53, 54). OT receptors are also present on the prostate, where they increase smooth muscle tone and possibly facilitate ejaculation (6).

OT is also involved in blood homeostasis. Rising osmolarity of the blood releases both OT and its sister hormone AVP exponentially with sodium concentration (74). While OT does not have the substantial osmotic effect AVP does, it still plays a role in renal function, with acute administration to rats causing a mild increase in glomerular filtration rate due to a decrease in tubular sodium reabsorption (74). In the heart, OT synthesis and receptors have been demonstrated, where it appears that OT works in concert with atrial naturetic peptide in a paracrine manner to decrease blood pressure and pulse rate (40). Glucose levels in the blood can be affected by pancreatic binding of OT in the islets, with an infusion of OT causing increases in glucagon and insulin blood levels (24). Studies in humans further illustrated a rapid increase in glucagon, followed later by long-lasting increases in both insulin and epinephrine (57).

Oxytocin is involved in the hypothalamic-pituitary-adrenal axis relating to stress response and corticosterone release. OT immunoreactivity appears in both the cortex and the medulla of rat adrenal glands (37,) but the function is not clear. A bolus of OT perfused into the rat at physiological levels stimulates a release of ACTH, corticosterone, and aldosterone (60,64). In contrast**,** long-term perfusion or repeated injections of high

doses, in the micromolar range, caused a sustained decrease in these hormones, as well as a decreased response to a stressful event. Although several research groups claim OT has a definite role as a suppressor of the stress axis, these results are always obtained after large repeated (5 day) regimens of OT prior to testing. Single treatments tend to show increases in stress hormones, or no significant interaction (52,60,75). The evidence shown for down-regulation of OT receptors in response to high levels of stimulation (29,34 62) makes the effect of OT on the stress axis unclear, as receptor desensitization is a plausible explanation for the decreased response resulting from 5-day treatment regimens. Testing of isolated in vitro cultures of various adrenal cells has thus far failed to provide a clear physiologic role (69, 38).

When considering the roles of OT in the periphery – uterine contraction, lactation, gonadal steroid modulation, regulation of blood osmolarity, pressure, and glucose mobilization and storage, as well as involvement in the stress axis, a global role presents itself. Although specific actions and mechanisms are still being elucidated, the global function of OT as a reproductive regulator with smooth muscle and homeostatic action can be appreciated. This 'background' regulation (and a primary role in lactation) for reproductive functions is further illustrated in OT action upon the central nervous system.

Central Action

Oxytocin receptors are scattered across the limbic cortex and the brainstem. It comes as no surprise that OT infused into brainstem nuclei can affect homeostatic autonomics relating to its peripheral actions (34), but the most profound effects of oxytocin are behavioral. Nulliparous rats, which do not form social groups and who

normally display avoidance behavior when presented with pups, can be induced to display full maternal behavior with intracranial injections of OT following standard estrogen/progesterone priming. This effect has been repeatedly confirmed (39,44,58). Endogenous OT released from genital stimulation is sufficient to stimulate this response in female rats. Maternal effects vary among species; mice show more subtle maternal effects from OT treatment (51), and the OT knock-out mouse displayed normal maternal behavior (55). Additionally, research suggests that OT's role is only in stimulating maternal behavior, but not maintaining it: oxytocin antagonists (OTA) did not diminish the expression of maternal behavior after it had been initiated (77).

Intracerebral infusions of OT have been shown to facilitate sexual behavior in rats as well as other species. In ovariectomized female rats primed with estrogen and progesterone, oxytocin infused into the PVN of the hypothalamus was shown to facilitate lordosis as well as solicitation behaviors (2,8,36). Estrogen/progesterone priming was necessary to elicit this effect. OTA infused into the medial preoptic area of the hypothalamus inhibited lordosis and increased rejection of males (10). Similar results were seen in intact female rats in spontaneous behavioral estrus in response to intracerebroventricular (ICV) injections of either OT or an OT antagonist (5). Notably, the inhibiting effects of OTA on sexual behavior were not seen with ovariectomized rats primed with estradiol alone – suggesting that progesterone is essential for OT's effects upon reproductive behavior (77) although the exact mechanism of this remains unknown.

Besides sexual and maternal activity, a number of behaviors exist in mammals that increase the likelihood of mating and increase survivability of social groups and progeny. Examples of such behavior include territorial aggression, mate guarding, and

affiliative behavior (non-sexual social proximity/contact) with other members of the species. Several studies have shown a modulatory effect of OT on a number of these social behaviors. Witt et al. (1992) showed dramatic (double) increases in the duration of physical contact over 6 hours in male/female rat pairs where the male was given a chronic central OT infusion via osmotic minipump (78). These increases in physical contact were observed even in the absence of sexual behavior. It has also been demonstrated that central OT infusion, especially into the olfactory bulb, prolongs social and maternal memory through an adrenergic modulatory mechanism (23). OTA, when infused into the central amygdala of female rats, increases aggression toward intruders, and abolished a dam's ability to discriminate between a novel and a familiar pup (27,49). OT knock-out mice fail to recognize familiar conspecifics, even after repeated exposures (76). Exogenous OT (in OT knock-out mice) or withdrawal of the OT antagonist (in intact rats) restores all of these behaviors. Interestingly, a social defeat experience selectively stimulates the release of OT in rats (26), thus providing a plausible mechanism for the maintenance of a social hierarchy and suppression of aggression within a social structure or confined space (like a laboratory cage).

The most profound data on social effects of OT come not from rats, but from prairie voles (*Microtus ochrogaster*). These North American voles have a complex biparental and monogamous social structure. Exposure to a novel male induces estrus in the female, which will subsequently become receptive to mating in about 24 hours (11,13). Prairie voles form a long-term social bond after mating and display a number of monogamous behaviors including biparental care, increased physical contact, and mate guarding. Pair-bonded voles typically remain together for life and further exposures to

novel males results in aggressive behavior. Cho et al. (1999) showed that under control conditions, 1 hour of cohabitation does not result in a partner preference when later tested in a Y-shaped maze with the familiar and a stranger conspecific (15). However a highly significant partner preference was displayed in female voles given an intraventricular administration of 100 ng of OT just prior to the initial 1-hour cohabitation. This result was highly reproducible. AVP was also able to elicit the resulting partner preference, but co-administration of OTA prevented the formation of pair bonds in both OT and AVP treated animals, suggesting OT involvement in a common pathway (15).

OT effects in voles were both social and sexual. In addition to facilitating the formation of pair-bonds, OT increased total social contact in female voles during partner preference tests (15). Cushing and Carter (1999) showed that pre-treatment with OT increases the likelihood that a sexually naive female vole will mate in a 48-hour sex test with an experienced male. A follow up experiment in ovariectomized female voles showed that OT pre-treatment increased sensitivity to very low doses of estradiol (17).

Of particular importance is that the effect on sexual receptivity observed by Cushing and Carter was seen with high-dose (20 μg) subcutaneous injections of OT (17). This illustrates that peripheral OT can reach the central nervous system. The normal adult rat blood-brain barrier (BBB) displays a 1-2% non-saturable permeability to OT across most areas, with BBB leaky areas such as the BNST displaying permeabilities up to 30 times higher (28). Thus the limited permeability to OT is still sufficient for large peripheral exposures to attain high enough levels within the brain to affect behavior.

Persistent Effects

The broad range of effects OT has upon the periphery and the CNS begs the question of how long these effects persist. The demonstrated interplay of OT with gonadal steroids alone provides rationale to expect that long-term changes can occur, especially in sexual development and behavior. The facilitation of pair-bonds seen in prairie voles provide direct evidence that OT can initiate changes that persist throughout life. Exposures during developmental periods could result in even higher CNS levels, due to the leaky nature of the infant BBB. These high central levels of hormone could cause organizational changes later manifesting as altered behavior or growth patterns that persist well into adulthood.

Despite their importance, long-term effects of OT have only recently been investigated and described. Uvnas-Moberg et al. (March 1998) showed a modest increase in weight in male and female rats given daily subcutaneous OT injections on day 10-14 of life. The increased body weight was more apparent in females and was first seen at day 40, but the increase persisted until the rats were terminated at 60 days of age (73). In the same study they also reported a slight increase in circulating levels of cholecystokinin in the OT-treated animals as well as increased threshold of temperature-nocioception, measured by a tail-flick test (73). A recent paper from the same laboratory reported that subcutaneous OT treatment in female rats on day 1-14 of life increased adiposity as well as increased placental weight and insulin-like growth factor during late pregnancy (68). None of these results have yet been replicated in other labs, but these

findings clearly support the hypothesis that neonatal treatment with OT can cause long-term changes.

Centrally, OT has been shown to cause long-term changes in the expression and distribution of receptors. Diaz-Cabiale et al. (2000) has shown that chronic OT treatment causes a long-term increase in receptor sensitivity among noradrenergic and estrogen receptor populations in the brain (21,22). Treatment with OT and/or OTA on the day of birth produces changes in neuronal activity, as indicated by c-fos expression (18). Yamamato et al. (2004, in press) demonstrated that a single intraperitoneal injection of OT in female prairie voles caused an increase in hypothalamic OT immunoreactivity on day 21. Interestingly, both OT and OTA injections on day 1 caused an increase in OT immunoreactivity on day 21. Male neonates also showed day 21 changes from a day 1 injection of OTA, but these changes were in AVP immunoreactivity not OT (79).

It is not known how these changes manifest in adulthood with regard to physiology and behavior, other than the increased weight gain and placental weight described earlier (68,73). Research has only just begun to uncover the mechanism of OT's trophic actions, and every behavior and peripheral function of OT is a potential site of long-term change. The fact that OT analogues have been used clinically in human health care for many years necessitates continued investigation and understanding of the basic science of oxytocin action and the persistence of these actions. Needless to say, continued research on the long-term physiological and behavioral significance of OT-exposure related changes is of principal interest.

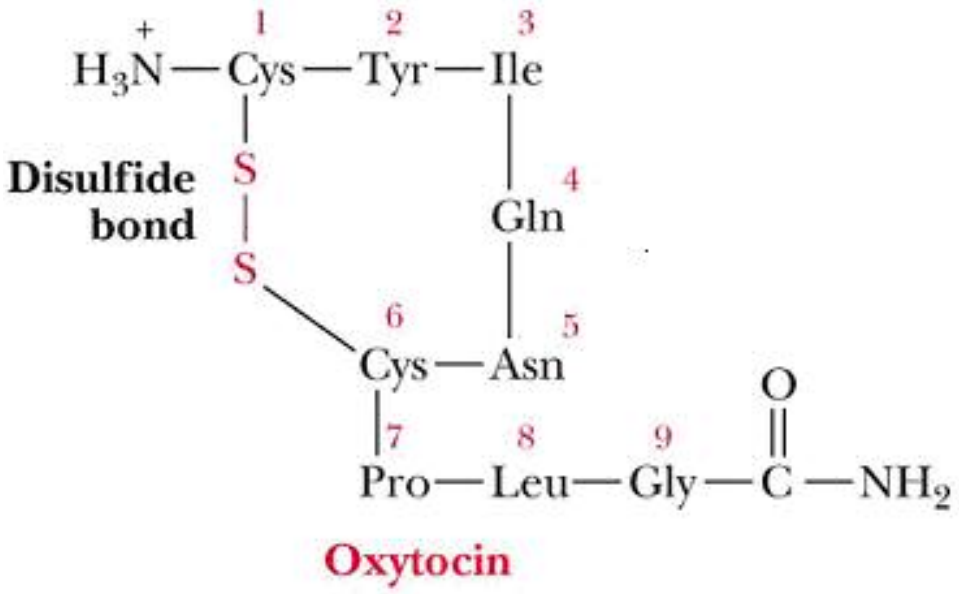

Figure 1: Oxytocin structure. The nine amino acid sequence of OT forms a 6 residue cyclic structure with an amide terminated three amino acid tail. The presence of a neutral amino acid at position 8 classifies a cyclic nonapeptide into the OT family. (Figure from Campbell Biochemistry 3e: Fig 3.12 p.312)

HYPOTHESES

Based on OT's capacity to facilitate sexual behavior in the acute setting, the capacity of OT to increase estrogen sensitivity in some animal models, and demonstrated long-lasting effects of neonatal OT in rats, I predict that neonatal manipulation of OT will affect the subsequent expression of sexual maturation and adult reproductive behavior in female Sprague-Dawley rats. There are several possible outcomes. If the neonatal system is responsive to exogenous OT then treatment with OT will affect the development and expression of female reproduction. Latency to vaginal opening, latency to 1st estrus, mating frequency, and prosocial behavior represent physiological and behavioral parameters that could be affected by neonatal manipulation of OT. Alternatively, endogenous OT may already produce a maximal effect and additional exogenous OT may not affect the development of expression of sociosexual behaviors or sexual maturation. In this case OTA, which inhibits the effects of endogenous OT, would be predicted to elicit an observable change in sexual maturation and the expression of female reproductive behavior. Finally, it is possible that treatment with both OT and OTA will affect the expression of female reproductive characteristics. In this case the two treatments would be predicted to produce opposite outcomes.

METHODS

The purpose of the study was to determine if neonatal manipulation of OT affects the subsequent expression of reproduction in adult females. More specifically, sexual maturation and reproductive activity were investigated, including prosocial behavior at first estrus. Sprague-Dawley rats were used as the model system, and a series of measures were taken and tests conducted to determine if neonatal manipulation of OT, either through treatment with exogenous OT or inhibition of endogenous OT using an OT antagonist (OTA), affects the subsequent expression of female reproduction. Vaginal opening and onset of first estrus were used as indicators of sexual maturation. Females were weighed at several ages, as previous studies have indicated a potential effect of neonatal OT on body mass and as body fat deposition is associated with female sexual maturation. Finally, estrous females participated in a sex test, where the effects of neonatal treatment on sexual receptivity, prosocial behavior and mating success were determined.

Animal Husbandry

Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) and used to establish a breeding colony from which experimental animals were produced. Breeding adults were ear-clipped for identification, while experimental pups were toe-clipped at the time of initial treatment for identification. Toe clipping was used because the ears of neonates are too small. All rats were maintained on a 14:10 light:dark cycle (lights on at 0500h), 22° C, 60% humidity, with *ad libidum* access to water and food

(Purina Rat Chow). Rats were housed in same sex cages with 2 to 3 rats per cage in standard rat cages.

To generate timed pregnancies after 60 days of age females were placed in a cage with an adult male. After placing the female with the male a daily vaginal lavage was performed, within two hours of lights on, until sperm was detected in the vaginal lavage, at which time the pair was separated. Rats have a 22 to 23 day gestation period and in this way it was possible to predict the day of birth for the purposes of treating the newborns. On the day of birth litters were culled to a maximum of 10 pups, which included a minimum of two males. At the same time experimental pups were treated and identified (see below). Litters were weaned at 22 days of age and housed in same sex sibling groups of 2-3 per cage.

Treatment Groups

Because of factors unique to first litters, such as physiological changes in nulliparous females, inexperience of the parents, etc., all animals used in this study were from 2nd or subsequent litters. Additionally, only mixed-sex litters were used because of potentially confounding prenatal and postnatal effects unique to single-sex litters. Within 24 hours of birth, female pups were weighed, toe-clipped for identification, and randomly assigned to one of four treatment groups, with each treatment group being represented at least once per litter to yield a randomized incomplete block design (RIBD) design. Female pups received an intraperitoneal injection of one of the following : OT (1μg/g body weight), OTA (([d(CH_2)$_5$, Tyr(Me)^2, Orn^8]-Vasotocin - Peninsula Laboratories, Belmont, California)) (0.1 μg/g body weight), isotonic saline (SAL)

volume: 6.25 µl/g body weight, or were handled only (HAN). Doses were chosen based upon doses described in the literature that yielded significant effects when used peripherally (73). All injections were in an isotonic saline vehicle (volume: 6.25 µl/g body weight). Weighing and injection occurred between 12:00 and 14:00 every day for 7 days.

Latency to Vaginal Opening and First Vaginal Estrus

Latency to vaginal opening and first vaginal estrus were used as physical indicators of physical sexual maturation. Vaginal opening, vaginal cytology, and the estrous cycle are regulated by circulating levels of estrogen. Therefore, vaginal opening and vaginal cytology provide an indirect measure of the effects of estrogen in response to neonatal manipulation of OT. OT has been shown to increase sensitivity to low doses of estrogen in prairie voles (17).

Beginning on postpartum day 27, females were examined daily at 14:30 to determine age of vaginal opening. Handling involved gently picking up the female and examining the genitalia beneath the tail. As the female matures and estrogen levels rise, the vestibular skin covering the vaginal opening thins, creases, and finally opens, exposing the vagina. For this experiment vaginal opening was defined as an opening large enough to allow entrance of PE50 tubing without disturbing the vestibular skin.

Once vaginal opening was confirmed, first estrus was determined by performing a vaginal lavage (see below for details) once a day starting on the day of vaginal opening. In total, 66 animals (18 OT, 16 OTA, 16 SAL, 16 HAN) were treated with this protocol and measured for analysis of latency to vaginal opening and first vaginal estrus. 26 of

these animals were subsequently used for another protocol while 40 of these animals went on to be sex tested in an overnight paced sex test, and subsequently measured for long-term effects on weight.

Vaginal Lavage and Estrous Cycle Determination

Vaginal lavage was performed with a 1 ml syringe fitted with a 26-gauge 5/8" hypodermic needle. To prevent any possible damage to the female the tip of the needle was covered with PE50 tubing and only the tubing was placed into the vagina. A small amount of distilled water (0.05 ml) was then used to flush the vagina and the fluid collected in the syringe. A small drop of water from the lavage was then placed on a microscope slide and allowed to dry. Once dried the cellular composition of the lavage was examined at low magnification (4x) to determine the phase of the estrous cycle: diestrus, proestrus, estrus, or metestrus (Figure 2). Vaginal diestrus, which is associated with low circulating levels of estrogen consists primarily of leukocytes and a small number of squamous epithelial cells. Proestrus, associated with increasing levels of estrogen, consists of a combination of squamous epithelial cells, with some cornified epithelial cells and a few leukocytes. During vaginal estrus cornified epithelial cells dominate the smear. Metaestrus is similar to proestrus, as hormone levels are decreasing and the tissue is transitioning back from estrous to diestrous cytology. During a typical 4 day estrous cycle a female would be in diestrus for two days, proestrus for 4 to 8 hrs preceding estrus, a day in estrus and then metaestrus which would lead back into diestrus, with the cycle repeating. Animals not found to be in estrus were lavaged each day at 14:30 until a smear indicating late proestrus or estrus was obtained.

Sex and Affiliative Behavior

Sexual receptivity and prosocial behavior were used as measures of the effects of neonatal manipulation of OT on reproduction and reproductive behavior.

To examine prosocial behavior and sexual receptivity, females were filmed overnight on time-lapse recording during a 10 hr paced sex test. In a paced sex test a female controls access to the male (16). This is accomplished by using a two chambered testing apparatus made of Plexiglas with a divider in the middle (Figure 3). The divider has a small hole (4.5 cm diameter) which allows the smaller female to freely pass into both chambers, while restricting the male to his portion of the cage. Testing began 2 hr into the dark phase (2100 hours) with the male being placed on one side of the chamber 15 to 30 min prior to the female. This is done to allow the male to acclimate to the test chamber. The female was then placed on the opposite side. Females were removed at the end of the 10 hr test (2 hr into the light cycle). Interactions between the female and the male were observed via time-lapse video (see following section for details). At the end of the test, females were returned to their cages and monitored for the next 23 days to determine if they were pregnant. Females that produced a litter were removed from the data set analyzing long-term weight gain.

Behavioral interactions were recorded using a time-lapse VCR (with a compression ratio of 12:1 reducing the total tape time to 1 hour 50 min). A low-light sensitive video camera was used and during the dark phase a 25-watt red light was used to provide illumination. An experimentally blind scorer later scored data. Parameters scored included mounts, lordosis, positional data, and social interaction. Data analyzed

included the expression of prosocial behavior, time spent in home and male cage, latency to lordosis, lordosis quotient and mating frequency.

Lordosis quotient (LQ) is a standard measure of willingness or interest of a female in mating (77). Lordosis quotient is measured by scoring the times a test female displays lordosis for the first 10 mounts by a male, with LQ ranging from 0 – 1. Latency to lordosis is the time a test animal spends in a sex test before displaying lordosis for the first time. Mating frequency is the percentage of test animals that display lordosis at least once during a sex test by treatment.

Prosocial behavior was measured by determining the effect of treatment on the willingness of the female to be in the vicinity of the male, i.e. time spent in the male side of the cage and time spent in side-by-side contact with a male. Positional data was designated as home cage or male cage, with cage location signifying the willingness of the female to associate with the male: in the home cage the stud had no access to the female, and in the male cage the stud had full access to the female.

Social interactions, with the female as the focal subject, were divided into three categories: exploratory, side-by-side, or uninterested. The exploratory state included active movement in either the home or male cage and sniffing at objects, including sniffing at the stud male from a short distance. Side-by side contact was defined as physical contact with the male, including grooming of the male or allowing the male to groom the test female, prolonged sniffing longer than 6 seconds (sometimes lasting several minutes), and inactivity while in physical contact such as laying together. Females were classified as uninterested if they were inactive, while not in contact with the male, or while feeding, drinking, or performing self-grooming. The inherent error in

classifying some exploratory and side-by side behavior necessitates a single treatment-blind scorer to score all recorded tapes to prevent bias.

Long-term weight change

Measures of weight were taken for two reasons. First, the onset of puberty and sexual maturation is determined by a number of developmental events, and are influenced by genetics, age and weight. Age and weight are two measures that can be easily obtained from the rats. Second it has been previously reported that neonatal peripheral treatment with OT affected the weight of female Sprague-Dawley rats, with OT treated females showing a significant increase in weight (73).

In addition to being weighed on postpartum days 1-7 treated females that did not produce a litter as a result of pairing with a male during the sex test were weighed weekly beginning on day 70 (10 weeks) until day 154 (22 weeks). Preliminary growth curves blind to treatment showed a sigmoid curve with greatest variation in weight at day 91 (13 weeks) and the peak of the plateau being reached by day 133 (19 weeks). Hypotheses of weight differences were made for these periods to maximize statistical power while minimizing type 1 error.

Statistical Analysis

SAS 8.0 was used for all statistical calculations. Results were considered significant at $P < 0.05$. In ANOVA tests and *post-hoc* LSD contrasts degrees of freedom were determined by the Satterthwaite method.

Latency to Vaginal Opening and First Estrus

Latency to vaginal opening and latency to first estrus were tested by mixed model ANOVA, with treatment a fixed variable and litter variation blocked out as a random variable. Where overall treatment effects were significant, pair-wise *post-hoc* comparisons were made using LSD t contrasts.

Sexual Receptivity during paced sex test

Animals were classified as either mating or not mating. The control females are assumed to represent the normal probability that a female in first estrus will mate, as indicated by lordosis. Therefore the number of control females that mated was used to generate a predicted probability of mating. This produced a binomial distribution (mated or did not mate) with a probability of 33%. Differences were considered significant at $P < 0.05$.

Positional Data during paced sex test

Positional scoring during the overnight sex test was totaled into percentages of the total 10-hour testing time. These percentages were then arcsin transformed before ANOVA analysis. Treatment was a fixed variable and litter variation was blocked as a random variable. The parameters analyzed were time spent in the Home cage and time spent in the Male cage.

Affiliative behavior during paced sex test

Data scored for side-by-side, exploratory, and uninterested behavior were totaled into percentages of the 10-hour testing time. These percentages were arcsin transformed before ANOVA analysis of side-by side and exploratory behavior. Treatment was a fixed variable and litter variation was blocked as a random variable.

Long-term weight change

Long-term weight change was analyzed by examining weight at day 70 (to compare to published literature), 91 (maximum variation due to differences in the rate of growth) and day 133 (plateau weight of the adult rats). Weights were analyzed via ANOVA with treatment fixed and variation between litters blocked as a random variable. To assure that the initial treatment randomization did not produce an artificial weight difference (as big rat pups tend to grow into big rats), day 1 weight was also treated as a covariate.

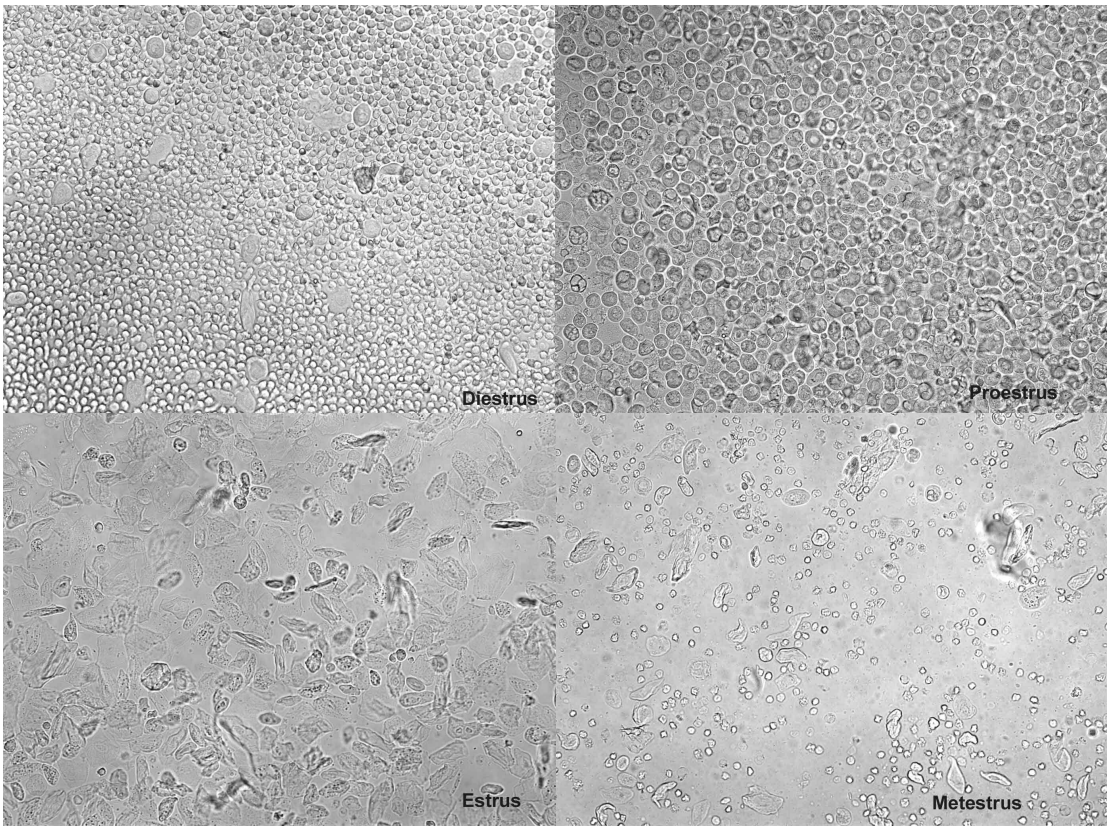

Figure 2: Examples of unstained vaginal smears by light microscopy. Test animals were lavaged every day at 1430 hours beginning on day 27 to catch the onset of first vaginal estrus. During a typical 4 day estrous cycle a female would be in diestrus for two days, proestrus for 4 to 8 hrs preceding estrus, a day in estrus and then metestrus which would lead back into diestrus, with the cycle repeating. Vaginal opening does not necessarily begin with the female rat in a particular stage of the estrus cycle, however smears taken at vaginal opening overwhelmingly demonstrated proestrus and estrus smears. Animals not found to be in proestrus or estrus were lavaged each day at 14:30 until a smear indicating late proestrus or estrus was obtained. (Images from Altmann Laboratory, Department of Ecology and Evolutionary Biology, Princeton University.)

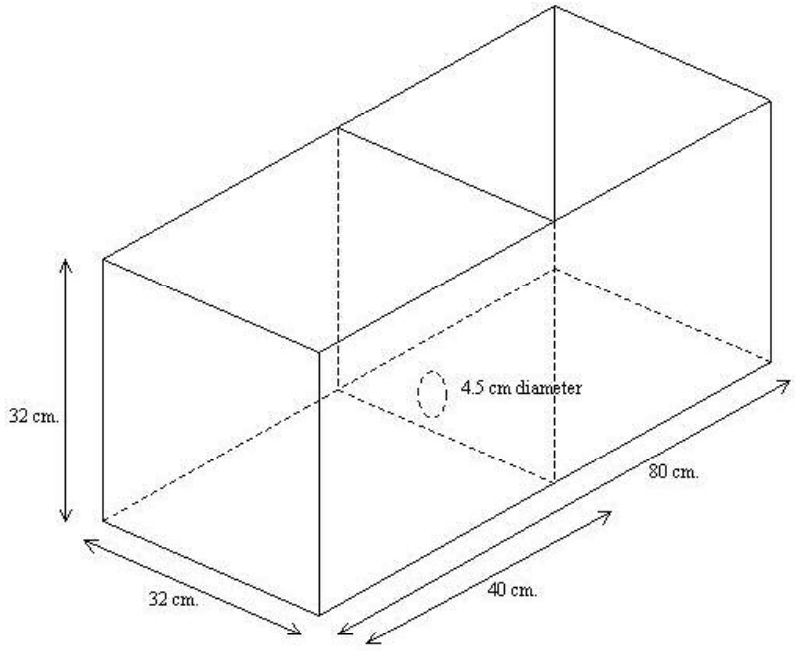

Figure 3: The two-chambered apparatus for paced sex-tests. The hole in the central divider allows the smaller female to freely pass into both chambers, while restricting the male to one side of the cage. The apparatus is constructed of 3mm Plexiglas.

RESULTS

Effects of Oxytocin treatment on latency to vaginal opening and menarche

There was a significant treatment effect on the age of vaginal opening (mixed model ANOVA $F(3,51.2)=5.08, p<0.01$). The treatment effect was due to a significant delay in vaginal opening in females treated with OT compared to both control groups HAN (OT vs. HAN: 1.57 day delay, SE 0.49, $p<0.01$) and SAL female (OT vs. SAL: 1.37 day delay, SE 0.50, $p<0.01$)(Figure 4). The latency of OT treated rats averaged 33.9 days. HAN, OTA, and Saline treated rats were insignificantly different from one another and clustered around 32 days.

The occurrence of first observable estrus is highly correlated with vaginal opening, as increasing levels of estrogen are involved in both processes. While most rats displayed estrous vaginal smears on the same day of vaginal opening, approximately 25% of them had smears consistent with diestrus or proestrus upon vaginal opening. These animals produced smears consistent with vaginal estrus one or two days later. These delays did not appear to be correlated with treatment and were scattered across all treatment groups. Therefore it is not surprising that the ANOVA analysis of latency to first estrus also revealed a significant treatment effect ($F(3,51.1)=3.46, p<0.05$). Pair wise comparisons (PLSD contrasts) again indicated that there was a significant difference between OT treated females and both HAN (OT vs. HAN: 1.47 day delay, SE 0.51, $p<0.01$) and SAL treated (OT vs. SAL: 0.95 day delay, SE 0.52, $p<0.05$) females, with OT delaying the age of first estrus (Figure 4). Treatment with OTA did not affect the age of first estrus compared to the control females.

Litter variation also had a large and highly significant effect on latency that was adjusted for by treating litter as a block variable (the latency of siblings clustered around different litter means among each litter).

Effects of Oxytocin on first estrus mating frequency

OTA caused a significant decrease in mating frequency from an expected rate of 33%. The animals in the OTA treatment group displayed a 0% mating frequency (95% confidence limit: 0%-26%, *n=10*). The OT treatment group mating frequency of 27% did not significantly differ from the expected rate of 33% (Figure 5**).** SAS 8.0 did not detect significant litter variation, therefore this analysis is equivalent to a completely randomized design (CRD).

Effect of Oxytocin on prosocial behavior during first estrus 10-hour paced sex tests

There was a significant treatment effect on time spent in the male cage (*F(3,36)=3.46, p<0.05).* Post-hoc pair-wise comparison (PLSD contrast) revealed a significant saline effect as SAL females spent significantly more time in the male cage than all other treatments (vs. HAN *p<0.05*), (vs. OT *p<0.01*), (vs. OTA*p<0.05*)(Figure 6). OT, OTA and HAN animals were all statistically insignificant from one another. Overall, saline treated animals spent 64% of their time (SE 5%) in the male cage, while all other treatment groups spent approximately 45% of their time on the male side. SAS 8.0 did not detect significant litter variation, therefore this analysis is equivalent to a completely randomized design (CRD).

Effect of Oxytocin on social interactions during first estrus paced sex test

ANOVA tests did not show significant treatment effects on side-by-side or exploratory behavior (*side by side: F(3,36)=2.38, ns)(exploratory: F(3,36)=0.57, ns).* Overall, the animals spent 11.6% (SE 1.7%, *n*=40) of their time displaying side-by-side behavior and 39% (SE 1.4%, *n*=40) of their time displaying exploratory behavior.

Effects of Oxytocin on adult weight

There was no apparent treatment effect on weight at any age, day 70 (ANOVA F(3,30.8) = 0.67, ns), day 91 (F(3,39.8) = 0.6, ns), or at day 133 (F(3,29.8) = 0.27, ns). The data is summarized in Figure 7.

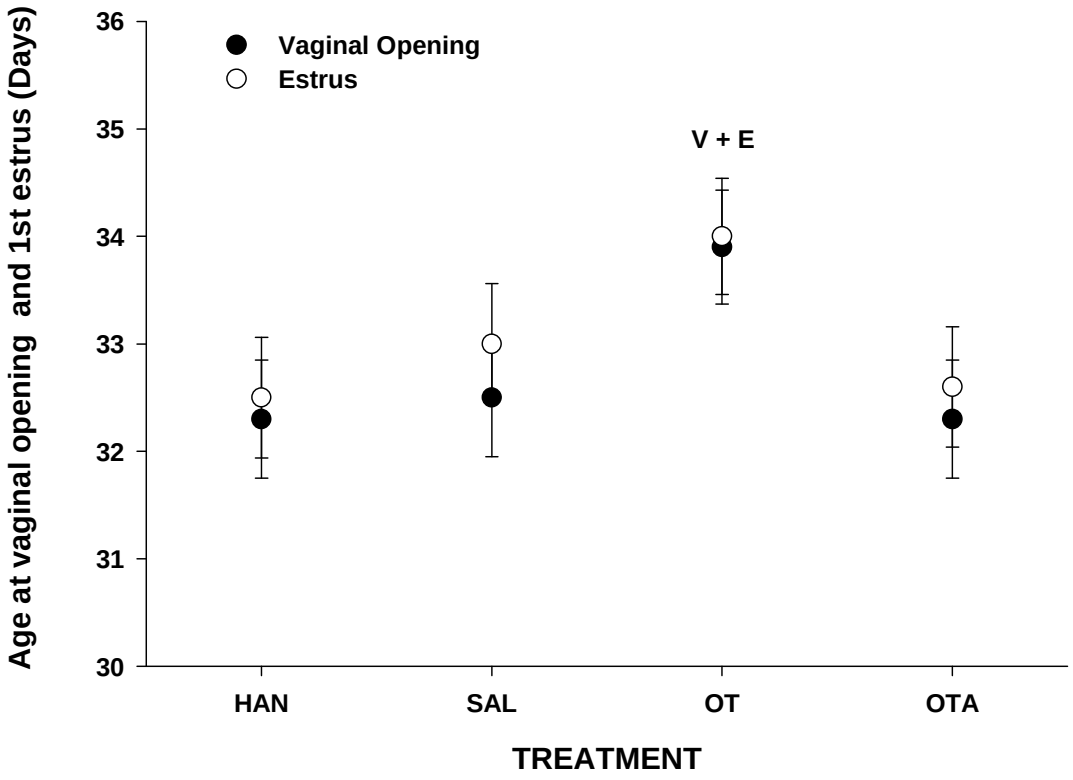

Figure 4: OT treatment significantly delayed latency to both vaginal opening and first estrus when compared to saline injected and handled-only controls. Treatment with OTA did not affect the age of first estrus compared to the control females. “V” and “E” on the figure represent significance from the unlabeled data points.

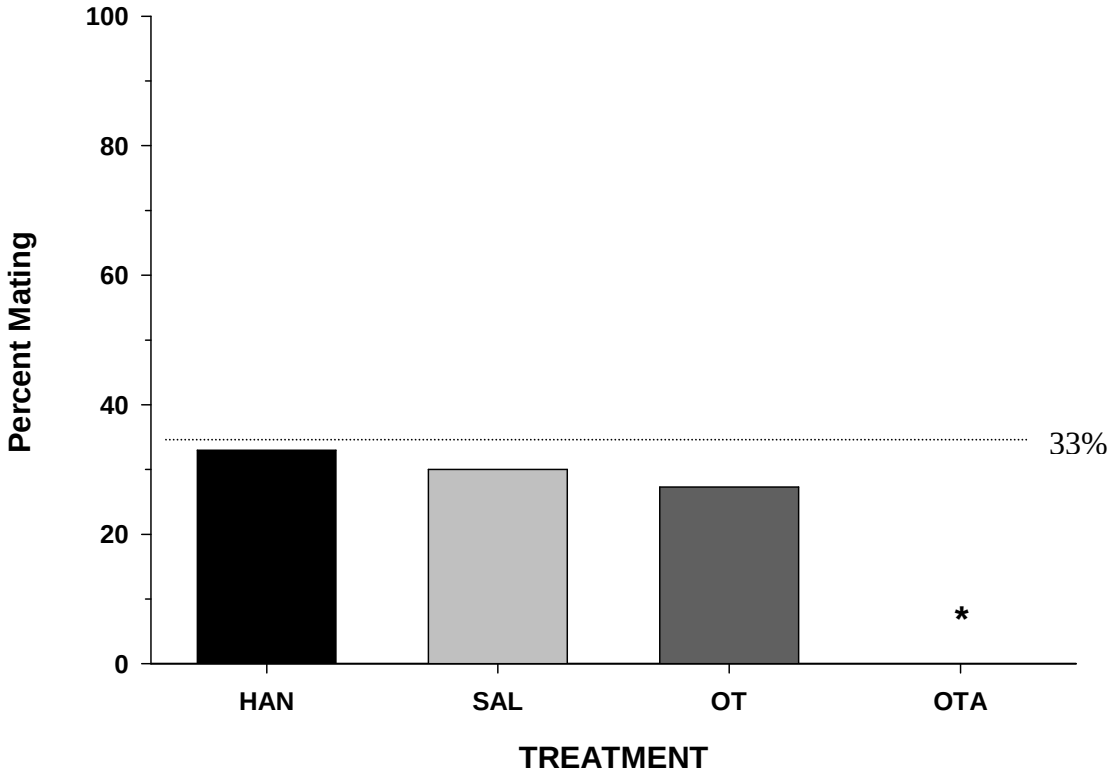

Figure 5: OTA caused a significant decrease in mating frequency from an expected rate of 33%. The animals in the OTA treatment group displayed a 0% mating frequency (95% confidence limit: 0%-26%, *n=10*).

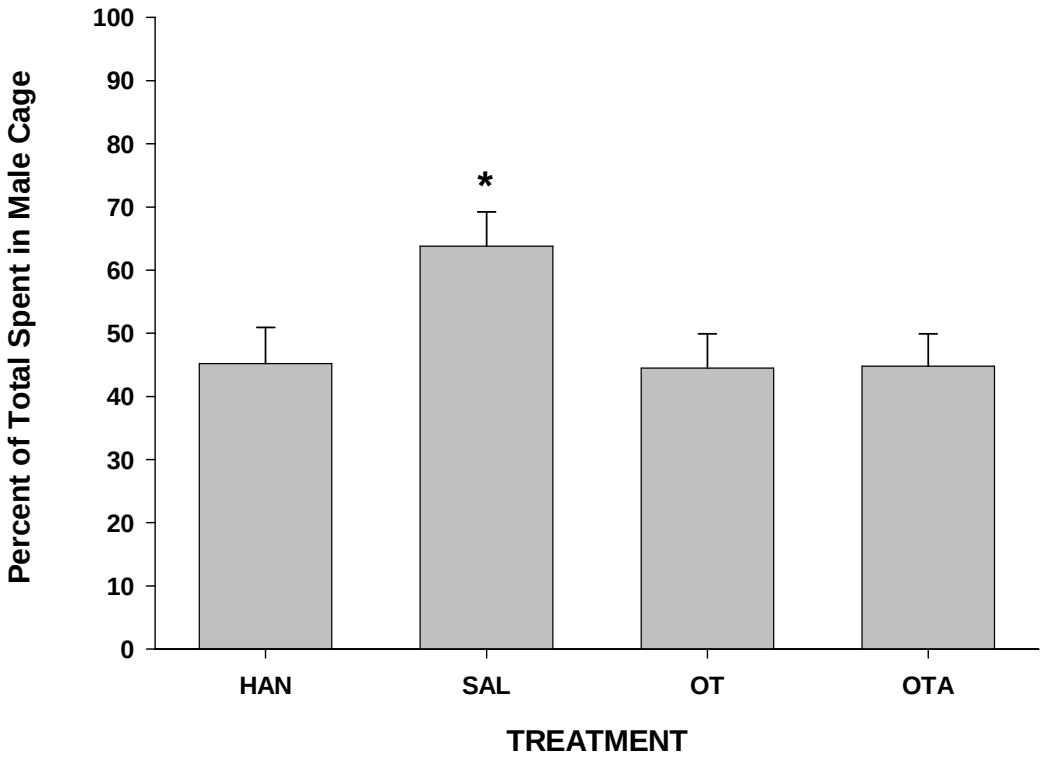

Figure 6: SAL treated females spent significantly more time in the male cage than all other treatments (vs. HAN *p<0.017*), (vs. OT *p<0.010*), (vs. OTA *p<0.012*).

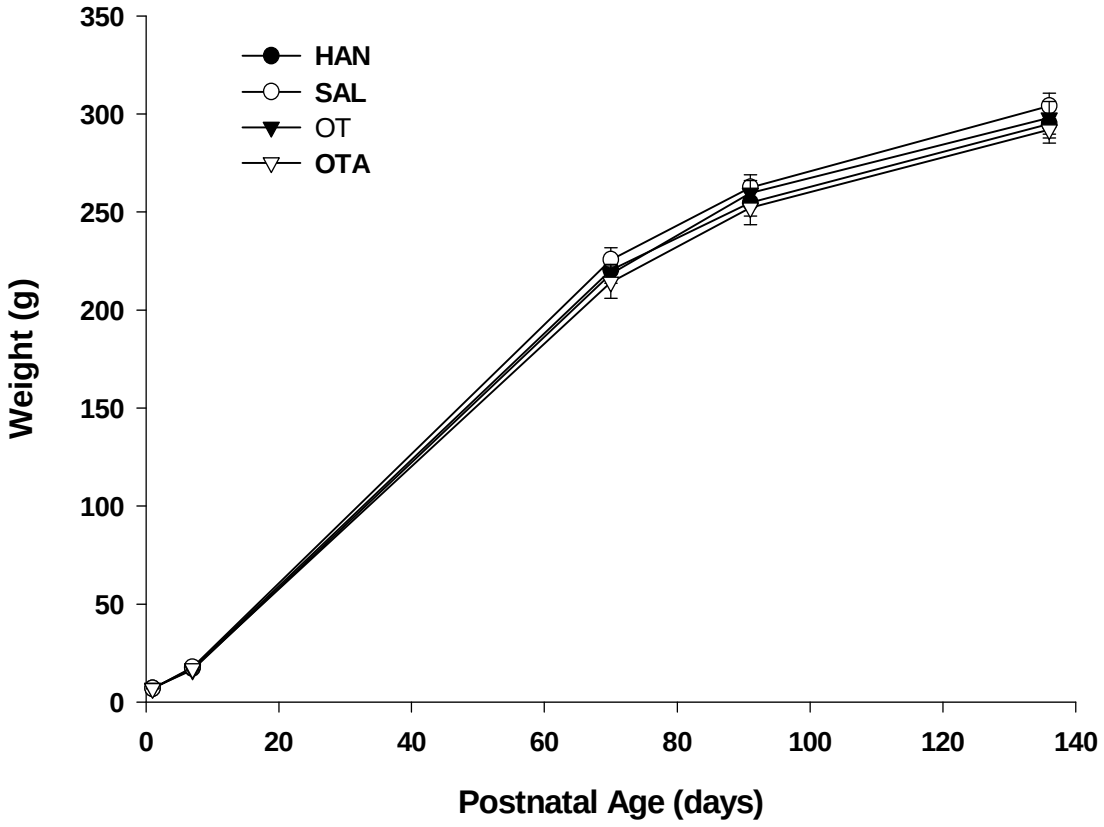

Figure 7: There was no apparent treatment effect on weight at any age, day 70 (ANOVA $F(3,30.8) = 0.67$, ns), day 91 ($F(3,39.8) = 0.6$, ns), or at day 133 ($F(3,29.8) = 0.27$, ns).

DISCUSSION

The results from this study supported the hypothesis that neonatal manipulation of OT can affect the development and expression of female reproductive activity. Neonatal treatment with OT resulted in a significant delay in the age of vaginal opening and the age of first observable estrus, while neonatal treatment with OTA decreased mating frequency (95% confidence limit for our sample size 0-26%) . Conversely neonatal manipulation did not affect weight or the expression of direct social interactions, i.e. side-by-side contact expressed by estrous females.

Neonatal manipulation of OT, sexual maturation and reproduction

In adults OT regulates a variety of social behaviors and physiological responses with most know effects being short-term and transient (34), with many of OT's documented roles appearing to play critical modulating functions in female reproduction (2,5,8,32,36,55). Treatment of estrogen-primed female rats with OT facilitates mating behavior, including lordosis and proceptive behaviors (2,8,36), while treatment with an OT antagonist (OTA) reduced or inhibited the expression of sexual behavior (7,9,10,59,77).

In contrast, in the neonate many of the behaviors regulated in the adult by OT do not occur or are irrelevant, such as uterine contractions and lordosis. This does not mean that neonatal OT has no effect; instead it appears that during the neonatal period OT acts to organize behavioral and physiological responses, producing long-term effects. Some already demonstrated long-term effects include weight, responses to pain, hormone levels (73) and the development and growth of the placenta in adult females that had been

treated with OT as pups (68). In prairie voles neonatal manipulation of OT affected the formation of partner preferences (3), aggression (61), basal corticosterone levels, and ultrasonic vocalizations in response to social stress (Kramer, KM et al; data from our lab, manuscript currently in review).

Because neonatal OT has been shown to have long-term effects on estrogen-related adult parameters as well as the HPA axis similar to effects seen in acute adult OT exposures (68,73), I predicted that neonatal manipulation of OT would affect sexual development and female reproductive activity. The results from this study showing changes in latency to vaginal opening, latency to first estrus, and the likelihood of mating supported this prediction and suggest that the long-term effects of neonatal manipulation of OT are in fact acting upon some of the same systems that are regulated by OT in adults.

In addition to exogenous OT affecting sexual development there are indications that endogenous OT may also affect sexual development. Although treatment with OTA, did not affect vaginal opening or the onset of estrus, females treated with OTA failed to mate during a paced sex test during first estrus. The lack of mating in OTA treated females suggests that endogenous OT may be a part of an important trophic mechanism in setting up the pattern that will lead to normal sexual function in adulthood. However, this finding and conclusion must be tempered by the fact that mating frequency was low in all groups. Testing females at an older age and after several estrous cycles might provide a better indication of the long-term effects of OTA on mating behavior and the expression of sexual receptivity.

Early social experience can affect the development of the oxytocinergic system. Female pups raised by high licking and grooming mothers showed higher level of social behavior than those raised by low licking and grooming females (30). This social contact stimulates the release of OT (41,47,71,78). In female prairie voles neonatal exposure to exogenous OT increased the number of cells in the PVN that produced OT (Yamamoto et al. 2004). These results combined with the findings from the current study suggest the intriguing possibility that social conditions during neonatal development can have a direct impact on the sexual development of females.

Neonatal manipulation of OT and the subsequent expression of prosocial behavior

As previously mentioned, OT facilitates the expression of a variety of prosocial behaviors in the adult, such as social memory (23,27,49), partner preference (15), and maternal behavior (39,44,58). Rat pups exposed to higher levels of early social contact (those raised by high licking and grooming females) also display higher levels of prosocial behavior as adults (30). High licking and grooming by the mothers had a direct effect on the oxytocinergic system**,** suggesting that changes in OT or sensitivity to OT may be responsible for the increase in the expression of prosocial behavior.

In prairie voles neonatal treatment with OT has been shown to affect the subsequent expression of alloparental behavior and female aggression (3,61). If the mechanisms behind these behaviors are not unique to voles, these studies suggest the possibility that in rats neonatal manipulation of OT could affect the subsequent expression of social behavior. However, the results of this experiment failed to show that

OT could affect the expression of prosocial behavior as an adult, as measured by time spent near the male or in physical contact with the male.

It is possible that direct manipulation of OT has no effect on the development and subsequent expression of prosocial behavior in a reproductive setting. However, this seems unlikely given the findings that early social experience alters the oxytocinergic system and the expression of maternal social behaviors (30). It is also possible that this research lacked sufficient power to detect differences in social behavior; the expression of prosocial behavior was extremely variable, and this experiment was limited to approximately 10 animals per treatment group.

Alternatively, early treatment with OT may selectively alter the expression of some social behaviors and not others. In adult rats social interactions between adult males and females are generally limited to mating. This means that social interaction between non-familial males and females occurs after females become sexual receptive, which may explain why in rats that estrogen regulates the effects of OT. Estrogen stimulates sexual activation, while also facilitating the females' willingness to interact with the male, a prerequisite for successful mating. Conversely, in prairie voles, sexual receptivity in females is induced by prolonged exposure to an adult male, thus social interaction precedes sexual activation in prairie voles. This may explain why, in prairie voles, OT enhances the effects of estrogen (17). If this is correct then in rats the only social interaction between males and females that may be regulated by OT is the physical act of mating (lordosis), which was not observed in our OTA treated females.

Neonatal manipulation of OT and weight

Previous research documented a subtle, but significant change in weight observable in adulthood after neonatal OT treatment (73). However, in the current study there was no treatment effect on weight at any age. This differs from the previous studies where females displayed a significant and long-lasting increase in weight, due to increased fat deposition starting around postnatal day 70 (73). These apparently contradictory results may have occurred for several reasons. First, the period of treatment differed between the two groups. In this study, neonates were treated on postnatal days 1-7, whereas in the other study females were treated with OT on days 10-14. The difference in the age of treatment could explain the differences between the two studies. OT receptor patterns change during the early neonatal period and therefore treatment at different ages could produce markedly different effects. Second, the published effects on weight are of a very small magnitude. Normal variation and the sample size may have accounted for the lack of a treatment effect in our study. Third, while out-bred strains of Sprague-Dawley rats were used in both studies, the source of the rats varied. This may be important as there can be significant physiological variation between different out-bred strains of Sprague-Dawley rats (7). This concept is also supported by comparing the average age of vaginal opening between Sprague-Dawley rats from various suppliers. The rats in this study were from Taconic Farms and the average age of vaginal opening was 32.9 +/- 2.0 days. In a different study we conducted in Sweden, Sprague-Dawley rats were obtained from B&K Universal and first vaginal opening occurred on average more than five days later in untreated females (38.1 $\pm$ 2.6 days, unpublished data). Given that the reproductive life span of a Sprague-Dawley rat is

approximately 1 year (+/- a few months), a difference of 3 days is relatively large and illustrates the potential difference between sub-populations.

Mechanisms of the effects of neonatal OT

Puberty and the process of sexual maturation is a complex process involving the interactions of genes, hormones, and the environment. Changes in any one of these could alter, delay, or perhaps advance the process. Females for this study were obtained from the same source and randomly assigned to treatments; reducing the probability that genetic difference would explain the observed results. Additionally, all rats were raised under the same conditions. Therefore, changes in hormones or their effect are most likely the underlying cause of changes in sexual maturation and reproduction.

To understand how and where neonatal treatment with OT might be acting to delay sexual maturation it is important to understand the hormonal process involved in puberty. Reviewing the hypothalamic-pituitary-gonadal axis, GnRH is released from the hypothalamus to stimulate the release of LH and FSH that in turn stimulate thecal cell growth and proliferation of the granulosa layer within ovarian follicles. Development of the granulosa layer leads to production of estrogen, which is involved in a positive feedback mechanism stimulating follicle cells to undergo further proliferation, increasing the production of estrogen. Circulating estrogen eventually feeds back upon the pituitary and hypothalamus to decrease GnRH, LH, and FSH. As the juvenile female matures the sensitivity of the hypothalamus decreases and GnRH is again released. This process of changing the sensitivity of the hypothalamus to the effects of estrogen continues and results in increasing levels of estrogen. Sexual maturation, ovulation, and sexual

receptivity occur when estrogen levels are high enough so that the ovarian follicles continue to develop in response to estrogen, while at the same time estrogen suppresses the release of gonadotropins through negative feedback at the hypothalamus. As the ovarian follicles mature, circulating estrogen reaches a high threshold level that stimulates a positive feedback loop in the hypothalamus resulting in the release of a large pulse of GnRH, which in turn triggers a surge in LH. The LH surge facilitates the rupturing of the mature follicles and ovulation. The period just prior to ovulation and following ovulation correlates with the expression of the first estrus and sexual maturation. Changes in the timing of the production, release, or the response to any of these hormones could effectively alter sexual maturation, either accelerating or delaying it.

While this study was not designed to specifically address the mechanisms of the action of OT on sexual maturation there are several mechanisms that could explain the effects of neonatal OT. In adults OT has been shown to influence the production or release of both of gonadotropins (43, 65, 66) and progesterone (67, 77, 10), both of which play a critical role in reproduction. Progesterone levels do increase slightly before ovulation but the primary increase in progesterone occurs following ovulation, when the remaining granulosa cells from the ruptured follicle are converted to luteal cells forming the corpus luteum. Additionally, vaginal opening and vaginal estrus are stimulated by estrogen alone. Therefore changes in the onset of vaginal opening and first estrus are unlikely to be associated with an affect of neonatal OT on either the production or sensitivity to progesterone. This suggests that neonatal manipulation of OT is most like acting by either altering sensitivity to or the production of gonadotropins and/or estrogen.

In adult females the relationship between OT and estrogen is variable. In rats estrogen stimulates increased OT sensitivity as it up-regulates OT receptors (42). In contrast in female prairie voles estrogen does not affect OTR, except in the accessory olfactory bulb (77). However, there is still an OT-estrogen relationship in prairie voles; treatment with OT increases sensitivity to estrogen (17). While the response differs between rats and voles both would seem to suggest that the interaction between OT and estrogen is positive, facilitating sexual activity. These findings would seem to suggest that neonatal OT would be predicted to enhance sexual maturity, an estrogen dependent process. However, neonatal OT exposure did not speed the development of sexual maturity, instead, neonatal OT caused a delay in the sexual maturation.

Two recently published studies suggest that during development the effect of OT on estrogen may be different from that seen in the adult. In a line of breast cancer cells OT down-regulated estrogen receptors and inhibited the mitotic effect of estrogen (14). While this is a cell line derived from adult females, cancer cells like developing cells are less differentiated than mature cells. A down-regulation of estrogen receptors would in effect reduce sensitivity to estrogen, which could delay the effect of estrogen as higher circulating levels would be required to stimulate the same effect. In addition to reducing sensitivity to estrogen neonatal OT may also have an effect on the production of estrogen. Early neonatal treatment with OT increased apoptosis in the neonatal ovary (50). This could delay maturation of the ovary, which would affect the production and release of circulating estrogen. Interestingly, this ovarian effect of neonatal OT is opposite that of estrogen treatment, which increases cell survival and ovarian volume (50). An assay of circulating levels of estrogen might shed light onto the effect of OT on the production of

estrogen, but on the other hand these difference might be hard to detect. Very subtle differences might account for the shift in the onset of vaginal opening, or the timing of the effect could have a developmental effect. In other words, by 32 days of age there may no longer be a difference in the level of production of estrogen, but there may have been differences at an earlier age that shifted the timing of maturation. Finally, long-lasting receptor internalization of OT receptors in response to potent stimulation may compound the mechanism of OT action; future studies must include a factorial design spanning high and low doses of OT.

While direct changes in the sensitivity or production of estrogen may account for the delay in sexual maturation it is also possible that the effect of OT on estrogen is directly or indirectly related the production of gonadotropins. There have been reported OT effects on gonadotropins published in the literature (43,65, 66); like the interaction between OT and estrogen, these studies suggest a positive correlation with OT facilitating the production/release of LH during proestrus (43,65). This literature may initially seem contradictory to the delay in first vaginal estrus seen in our results, but both effects could actually be the result of the same mechanism. If OT increases sensitivity to estrogen in the hypothalamus this could effectively reduce the circulating levels of estrogen required to trigger the release of GnRH and the resulting surge in LH. In the developing female rat that is not yet cycling, an increase in hypothalamic estrogen sensitivity would make the hypothalamus more sensitive to circulating estrogen, thereby creating a new 'set point' for hypothalamic negative feedback, with lower levels of estrogen inhibiting the release of GnRH. In this scenario the feedback loop within the HPG axis could result in an increase in the time required for maturation of the ovary and the onset of estrus.

Regardless of the mechanism of the effect of neonatal manipulation of OT on sexual maturation, OT did not disrupt maturation, but only altered the timing. The degree of handling and/or the amount of social contact that a pup receives can alter the release of endogenous OT. The response of the system to exogenous OT treatment, suggests the possibility that the effect observed in this study may not just be pharmacological, but instead may represent a system that is designed to respond to social experience.

Conclusions

In conclusion, peripheral OT exposure during the neonatal period was shown to have significant effects on sexual development in female Sprague-Dawley rats by delaying vaginal opening and first estrus. At first estrus, OTA reduced the likelihood of mating, a finding consistent with the effects of adult exposure to OTA. Affiliative behavior at that time was not affected. Furthermore, the delay in physical pubertal development was not accompanied by a difference in adult body weight. The results from this study suggest that effects of OT may be long lasting, and may have a time component, with different observable results depending on the age of exposure.

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