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Effects of neonatal oxytocin manipulations on male reproductive potential in prairie voles

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Abstract

Oxytocin (OT) modulates adult mammalian sexual behavior, sperm production and transport, and steroidogenesis; however, the consequences of developmental manipulations of oxytocin have received little attention. The purpose of this experiment was to determine whether neonatal exposure to OT, an oxytocin antagonist (OTA), saline (SAL), or handling (HAN)-only would have long-term effects on reproductive potential in male prairie voles (*Microtus ochrogaster*). Adult males were observed for 24 h with a sexually receptive female and sexual behavior was recorded. Females were subsequently lavaged and smears were examined for sperm. Reproductive parameters including motility of epididymal sperm, testis weight, and plasma androgen levels were in the normal range. OT-treated males that did not mate within the first 30 min did not mate at all, and in comparison to controls, a higher proportion of those OT-treated and OTA-treated males that did mate did not transfer sperm to the females. OTA-treated males also had significantly higher testicular sperm concentrations than HAN-only males, and significantly lower epididymal sperm concentrations. These differences suggest that in males, developmental manipulations of OT may have the potential to influence the subsequent expression of sexual behavior and sperm transport.
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1. Introduction

Neuropeptides, including oxytocin (OT) modulate mammalian male sexual behavior (reviews in Refs. [1–4]). In male rats, plasma OT levels become elevated following exposure to a female, and correlate with the intensity of copulation [5]. In rats, OT injections can facilitate penile erection [4], whereas treatment with oxytocin receptor antagonists (OTAs) reduced male sexual behavior [6,7]. OT also reinstated the ejaculatory response portion of sexual behavior after its loss due to treatment with fluoxetine [8]. Lesioning the paraventricular nucleus of the hypothalamus (PVN), which contains OT perikarya, results in a decrease in emission of seminal fluid [9]. In contrast to rats, central (intracerebroventricular) injections of OT inhibited sexual behavior in male prairie voles (*Microtus ochrogaster*) [10].

In addition to affecting sexual behavior, OT has been implicated in local effects in the testicular/epididymal system. OT knockout mice matured later than wild-type mice and had lower epididymal sperm count [11]. Intratesticular injection of OT resulted in elevations of basal testosterone levels in rats [12]. Both OT and the related peptide arginine vasopressin (AVP) increased contractility of the rat epididymis in vitro [13]. In sheep, OT, but not AVP, increased fluid output and the number of spermatozoa [14]. OT receptors have been identified in the epididymis in mammalian species, including pigs [15] and primates [16,17], and are believed to regulate contractility in vivo.

OT has been implicated in the development of the reproductive system and behavior. OT receptors are present in the central nervous system on postnatal day 1 in the prairie vole brain [18], as well as in the neonatal mouse kidney [19]. OT itself is first measurable by immunohistochemistry during the first few days of life in rats [20] and voles [21]. A previous study of female sexual development implicated OT in abnormal ovarian development [22]. However, the consequences of exposure to exogenous OT on male sexual behavior and reproductive potential are not known.

The purpose of the present experiment was to examine the effects of neonatal exposure to OT on male sexual

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behavior and reproductive potential. Prairie voles were studied because this rodent is sensitive to the behavioral effects of neuropeptides, and because other behavioral investigations included tests of partner preference behavior and male alloparenting; a monogamous species was therefore necessary [23–25]. In addition, adult social behavior, including the formation of a pair bond, is impacted by neonatal manipulations of OT in this species [26]. We hypothesized that manipulations of the OT system would have long-term consequences for reproductive potential and/or sexual behavior (as in Refs. [4,6,7,11,13,14]).

2. Materials and methods

2.1. Subjects, treatments, and behavioral testing

Subjects were laboratory-bred male prairie voles, F_{3-4} descendants of a wild stock originally caught near Champaign, Illinois. Stock has been systematically outbred. Animals were maintained on a 14:10-h light/dark cycle and were provided food (Purina high fiber rabbit chow) and water ad libitum. Breeding pairs were maintained in large polycarbonate cages (44 × 22 × 16 cm) and provided with cotton nesting material. This study was carried out in the facilities of the Department of Biology at the University of Maryland, and approved by the IACUC committee of the University of Maryland.

Within 24 h postpartum, pups were removed from their parents and were weighed, sexed, treated, and toe-clipped for identification. Pups received either of the following: (1) a 3-μg injection of OT (Peninsula Laboratories, San Carlos, CA); (2) a 0.3-μg injection of an OTA (see below); (3) an injection of isotonic saline (SAL); or (4) were handled without injection (HAN). Dosages were based on the literature on rats [27] and our experience with voles [25,26]. All injections were 50 μl in volume and were administered intraperitoneally using gas-tight syringes. Within a litter, at least one male was assigned randomly to a control group and one male to a treatment group, with each treatment group represented only once within a litter. The OT receptor antagonist ($[d(CH_2)_5$, $Tyr(Me)^2$, $Orn^8]$-Vasotocin), was designed by Bankowski et al. [28], and is commercially available from Peninsula Laboratories. This OTA has been tested extensively in behavioral studies, including sexual behavior [29], and is known to affect male copulatory behavior in rats [6].

Following injection, the pups were returned to their parents and left undisturbed until weaning, except for normal colony management. At 21 days of age, offspring were removed and housed in same-sexed sibling pairs in smaller (27 × 16 × 13 cm) cages. Each animal received several other noninvasive behavioral tests, including two parental care (10-min exposure to an unrelated infant; [30]), plus-maze (elevated arena with two closed and two open arms, [31]) and same-sex aggression tests (5-min exposure to a male; [32]), and a partner preference test (1-h cohabitation with a nonestrus partner female and 3-h choice test between the partner and a nonestrus stranger female; [33]), prior to their participation in the mating test described here. In all cases, males were still virgin at the beginning of the mating test.

At 75–90 days of age, males were tested for sexual behavior, by being placed in a 27 × 16 × 13 cm polycarbonate cage with an ovariectomized, sexually receptive stimulus female. Stimulus females were primed to be sexually receptive using a subcutaneous injection of estradiol benzoate (10 μg suspended in 50 μl of sesame oil) once a day for 2 days, starting 48 h before testing. Prior to testing, primed females were tested for receptivity by placing them with a sexually experienced stud male. Only stimulus females that displayed lordosis in response to being mounted by the stud male were used in tests. The experimental male remained with the female for 24 h. Behavior was recorded in real time for 30 min, then by time-lapse video (recording one frame every 12 s) for the next 23.5 h. After lights out, recording was carried out under red light using an infrared-sensitive video camera. At the end of 24 h, the male was returned to his home cage. The female was vaginally lavaged immediately afterwards with distilled water, and the smear was examined for the presence or absence of sperm. Group sizes consisted of 10–13 males per treatment.

Behavior was scored using Observer 3.0 software (Noldus) by experimentally blind observers. Behaviors scored during the first 30 min included mounts, intromissions, and ejaculations. Aggression and side-by-side contact are not considered here because of their relatively rare occurrence during the first 30 min. If no sperm was detected in the vaginal lavage, and the male was not observed to ejaculate within 30 min, the rest of the time-lapsed video was examined to determine whether or not each male showed a behavioral ejaculation in the subsequent 24 h of cohabitation.

At 6–8 months of age, a subset of males from each group were sacrificed. One week before sacrifice, each male was sedated under ketamine/xylazine and a blood sample was collected from the supraorbital sinus and was assayed for androgens. At the time of sacrifice, organs were excised and weighed, including the testes, epididymides, prostates, and seminal vesicles.

2.2. Sperm motility and concentration

Immediately after the dissection of the right epididymis (2–3 min), a drop of epididymal fluid containing spermatozoa was collected. The sample was taken from the end of the caudal portion of the epididymis. The sample was placed on a prewarmed slide with two drops of phosphate-buffered saline, PBS (37 °C), and was coverslipped. Slides were analyzed under ×400 by two independent, experimentally blind observers, who examined five separate fields for each

sample/observer. The percentage of sperm that was motile was estimated. A spermatozoon was considered motile if it showed forward movement in a progressive consistent path. This progressive motility was estimated to the nearest 10% for each sample and the two ratings were averaged per sample.

The left epididymis and testis were minced and homogenized in 0.5 and 1.0 ml of SAL solution (0.9% NaCl with two drops of 2% eosin to stain the sperm head), respectively. Samples were homogenized manually in an eppendorf tube using a pellet pestle. A standard hemocytometer was used to count spermatozoa (dilution rate was factored into the final calculation of sperm count). Each sample was counted 10 times and the counts were averaged. Final concentration was expressed as sperm count/mg tissue [34–36].

2.3. Histological sections

The right testis from each animal was removed and fixed in Bouin's solution. Samples were washed in 50% ethanol (three changes, 6 h each), dehydrated through a series of ascending concentrations of alcohol, followed by clearing (Hemo-De; Fisher Scientific, Fairlawn, NJ), then infiltrated (melted paraffin wax at 60 C) in a vacuum oven, and embedded in paraffin blocks. Tissue blocks from three males per treatment were sectioned (5 μm thickness) on a rotary microtome and mounted on Mayer's egg-albumin-coated microscope slides. Sections were deparaffinized, rehydrated, and stained according to Berg [37] (also validated in Ref. [38]). In this method, spermatozoa appear as brilliant red, while the other tissue components stain blue to purple. Histological sections were evaluated by an experimentally blind observer by measuring the relative area occupied by spermatazoa within a seminiferous tubule. Furthermore, spermatogenic stages were checked to verify normal progression in spermatozoan development and maturation. The area within the testis occupied by sperm cells (red staining) was determined using image analyses (Biovision System with Nikkon microscope; Biovision Technologies, Exton, PA) and termed sperm cell area. Tubules with visible lumens were measured to have the proper (and similar) orientation of the tubular measurements across all animals. The area occupied by cells within a seminiferous tubule was also determined, which would include both spermatozoa and Sertoli cells. Fifteen randomly selected seminiferous tubules were measured in each animal by an experimentally blind observer. In previous studies, investigators have used this approach to ascertain morphological impact and to determine if further analyses are warranted (see Ref. [11]).

2.4. Androgen assay

Plasma androgen was measured using a radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA) validated for the prairie vole. Sensitivity was 2 pg/tube and inter- and intra-assay coefficients of variance were less than 10%.

2.5. Statistical analysis

Because of the nonparametric distribution of much of the behavioral and hormonal data, these variables were analyzed by one-way nonparametric analyses of the median using the Wilcoxon Two-sample test [39]. Where nonparametric statistics were used, results are presented as medians rather than means. Proportions were analyzed by Fisher's Exact Tests.

Body weights, organ weights, and sperm motility and concentration were analyzed by mixed-model ANOVAs [40] in SAS 8.0 (SAS Institute, Cary, NC). The animal's birth litter and cage-mate identification were included as random factors. Data were transformed where necessary by square- or quad-root transformations [39]. Finally, logistic regression was used to statistically predict which males did or did not mate, and which males did or did not leave sperm, based on androgen levels.

3. Results

3.1. Behavior

Neonatal treatment did not affect sexual behavior in a variety of measures (Table 1). The total proportion of males in each group that successfully mated in the first 30 min and over the 24-h test did not differ (Fisher's Exact Test; first 30 min: P=.457; over 24 h: P=.397; Fig. 1). However, there was a difference in the proportion of males that were able to successfully mate only after the first 30 min of the testing period (Fisher's Exact Test; P=.03; Fig. 1). Specifically, OT-treated males that did not mate in the first 30 min showed no mating behavior (0 out of 4 males), while 57% (4/7) of the HAN-only, 75% (6/8) of the SAL-treated, and 88% (7/8) of the OTA-treated males that did not mate within the first 30 min, did mate at some point in the subsequent 23.5-h observation period.

3.2. Sperm in female tract

There was a significant effect of treatment on the probability of sperm deposition (Fisher's Exact Test, P=.012; Fig. 2). While 90% (9/10) of HAN-only and 100% (10/10) of SAL-treated males that were behaviorally observed to mate left sperm in the female tract, only 50% (3/6) of OT-treated males that mated and 50% (5/10) of OTA-treated males that mated deposited sperm.

3.3. Sperm motility and concentration, and organ weights

There were no overall differences in the motility of epididymal sperm, body weight, testes weights, prostate,

Table 1
Behavioral variables by neonatal treatment (median ± interquartile range)

Behavior	Oxytocin ($n=10$)	Oxytocin antagonist ($n=12$)	Saline ($n=13$)	Handled only ($n=13$)	Test statistic (χ^2)	*P* value
Number of mounts	5.0 ± 13	0 ± 17.5	5.0 ± 9	6.0 ± 12	2.06	.560
Latency to mount (s)	497.0 ± 1682	1800.0 ± 1560	702.0 ± 1567	511.0 ± 1707	2.06	.560
Total duration of mounting (s) including nonmounters	76.5 ± 138	0 ± 85.5	45.0 ± 93	26.0 ± 122	1.85	.605
Total duration of mounting (s) not including nonmounters	117.5 ± 77 ($n=6$)	137.5 ± 97.5 ($n=4$)	93.0 ± 103 ($n=9$)	113.0 ± 137 ($n=8$)	0.55	.907
Latency to intromit (s) only animals that mounted	551.0 ± 1680	1800.0 ± 1002	1692.0 ± 1196	915.0 ± 1589	1.85	.605
Number of ejaculations including nonmounters	1.0 ± 2.0	0 ± 0.5	0 ± 1.0	0 ± 1.0	3.39	.335
Number of ejaculations not including nonmounters	1.5 ± 1.0 ($n=6$)	1.5 ± 1.5 ($n=4$)	0.5 ± 1.5 ($n=9$)	1.0 ± 1.0 ($n=8$)	3.24	.356
Latency to ejaculate (s) including nonejaculators	897.5 ± 1525	1800.0 ± 643.5	1800.0 ± 101	1800.0 ± 975	3.39	.335
Latency to ejaculate (s) not including nonejaculators	275.5 ± 154 ($n=6$)	351.0 ± 169 ($n=3$)	573.5 ± 985 ($n=4$)	579.5 ± 641 ($n=6$)	0.90	.825

Group sizes are as indicated at the top of the table unless otherwise stated.

epididymides, and seminal vesicles or in the relative weight of these organs expressed as a percent of body weight (data not shown). However, both testicular ($F=2.83$, $P=.069$; Fig. 3a) and epididymal ($F=2.61$, $P=.08$; Fig. 3b) sperm concentrations tended to vary by treatment. The direction of variation differed from controls as tested in planned comparisons, which showed that testicular sperm concentrations of the OTA-treated group were significantly higher than the HAN-only males ($t=-2.86$, $P=.01$), whereas OTA-treated males had significantly lower concentrations of epididymal sperm than HAN-only males ($t=-2.74$, $P=.013$). However, because SAL-treated animals showed an intermediate number of epididymal sperm when compared to HAN-only controls, we cannot exclude the possibility that some of the effects on this measure were due to stress associated with the neonatal injection procedures.

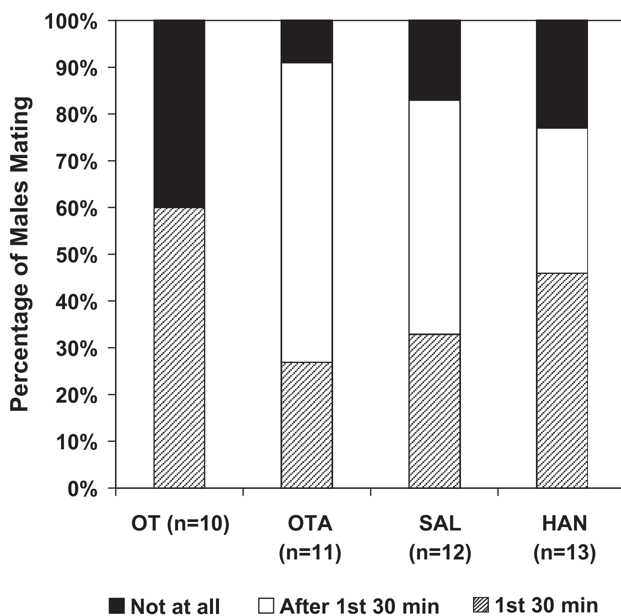

Fig. 1. Proportion of males from each treatment that mated in the first 30 min of cohabitation with a female, after the first 30 min of cohabitation, and not at all. The proportion of males mating after the first 30 min differed significantly by treatment (Fisher's Exact Test; $P=.03$).

3.4. Androgen levels

Group differences in baseline androgens were not significantly different, and androgen levels were not statistically predictive of whether or not a male would mate or whether or not he would deposit sperm. Group androgen levels (median ± interquartile range) were as follows: OT-treated, 162.36 ± 215.28 pg/ml; OTA-treated, 60.40 ± 54.03 pg/ml; SAL-treated, 106.67 ± 114.75; and HAN-only, 93.23 ± 190.97 (Wilcoxon Two-sample Test, $\chi^2=3.35$, $P=.340$).

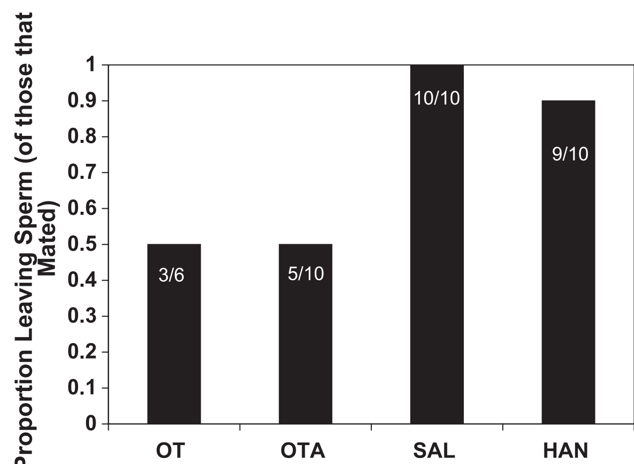

Fig. 2. Proportion of males leaving sperm in the female (of those that mated; Fisher's Exact Test, $P=.012$).

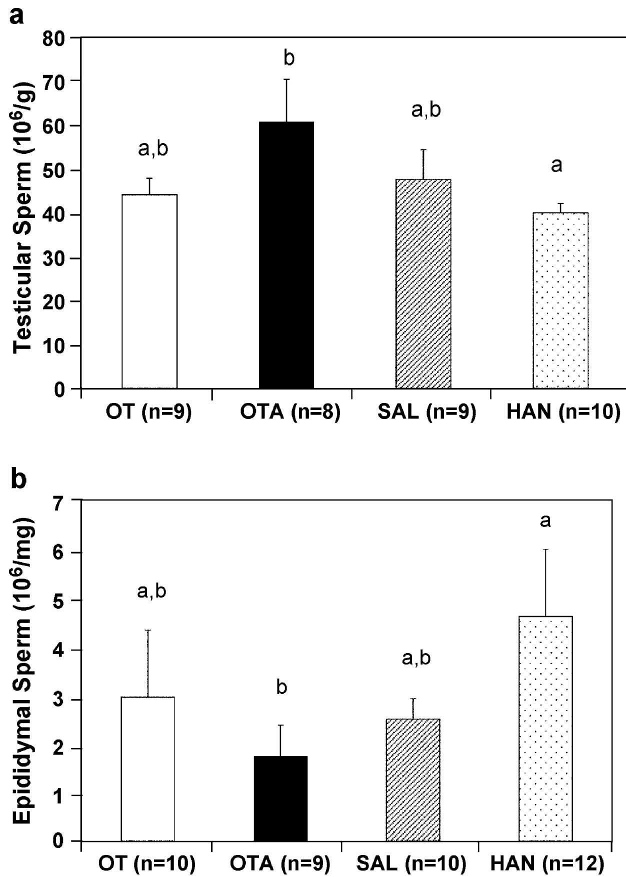

Fig. 3. (a) Testicular sperm concentration (10^6/g). Bars with differing letters are different at $P<.05$. (b) Epididymal sperm concentration (10^6/mg). Bars with differing letters are different at $P<.05$.

3.5. Testicular histology

Histological examination showed no differences in the testicular spermatogenic cycle or in the seminiferous tubules in the testes. Although areas occupied by spermatozoa in the testes were slightly higher in treatment males (OT-treated and OTA-treated) compared to the SAL-treated control, this difference did not prove to be significant (OT-treated: 6.3 ± 1.8 mm$^2 \times 10^{-4}$; OTA-treated: 5.3 ± 2.1 mm$^2 \times 10^{-4}$; HAN-only: 4.7 ± 2.6 mm$^2 \times 10^{-4}$; and SAL-treated, 1.1 ± 4.3 mm$^2 \times 10^{-4}$; other data not shown). Similarly, there were no significant differences in the total area or diameter of the seminiferous tubules, or in area within the tubule occupied by sperm cells. Based on these results, further histological study was not concluded.

4. Discussion

Treatment with OT or OTA did not result in large differences in gonadal weight, sperm motility, androgen levels, or cumulative expression of sexual behavior. However, there were more subtle differences in behavior, sperm deposition, and sperm transport. There was a difference in the OT-treated and OTA-treated groups in terms of the timing of initiation of sexual behavior; while 60% of the OT-treated males initiated sexual behavior within 30 min, fewer than 30% of OTA-treated males showed sexual behavior in the first 30 min with the female. While over 90% of the OTA-treated males eventually mated, the OT-treated males that did not mate in the first 30 min did not show mating behavior at all. Moreover, approximately half of the OT- and OTA-treated males failed to deliver sperm to the female's tract. The SAL-treated and HAN-only males showed mating patterns that were intermediate to the treatment groups, with 30–40% of the males mating in the first 30 min and roughly 80% of the males showing mating during the entire trial. Practically, this means that only 3 (30%) of the OT-treated males and 5 (45%) of the OTA-treated males would have the potential to fertilize the female as compared to a total of 19 of the 25 SAL-treated and HAN-only males (76%). OTA-treated males also had a higher sperm concentration in the testes and lower sperm concentration in the epididymis compared to the HAN-only treatment. This suggests less stored and maturing sperm in the epididymis. This observation is more difficult to interpret because the OT-treated and SAL-treated males did not differ significantly from the OTA-treated males. Possible effects of stress from the SAL injection as well as separate effects of OT may underlie the partial response observed in these parameters. Based on these findings, it appears that manipulations of the OT system, through either addition or removal during development could have consequences for male reproductive behavior and sperm transfer, with potential impact on the male's ability to successfully reproduce. Given the similarity of outcome for both OT and OTA treatments, it is also possible that the developmental processes underlying sperm deposition are nonspecifically vulnerable to manipulations of this system.

4.1. Developmental effects of OT

OT-treated males tended to mate within the first 30 min of exposure to a female or not at all. Of those that mated, only half left detectable sperm in the female reproductive tract. The lack of sperm in OT-treated males is unlikely to be due to the fact that these males mated earlier than those in other groups, as we were able to recover sperm from the partners of all control males that mated during that time period. The reduction in sexual behavior observed in the neonatally OT-treated males is consistent with results from an earlier study in adult prairie voles in which OT inhibited sexual behavior [10]. These data offer indirect support for a role for OT in sexual satiety (for review, see Ref. [1]).

Developmental exposure to OT is known to affect several other physiological variables in rats. In particular, postnatal OT treatment (using a repeated injection procedure) in female rats resulted in increased adiposity, placental, and fetal growth in later life [41]. OT administered to male rats in early life also ameliorated the detrimental effects of

prenatal food restriction on body weight, glucose, and corticosterone levels [27]. Furthermore, early OT administered to both male and female rats led to higher adult body weights and more resistance to pain [42]. In male voles tested under the paradigm described here, a single exposure to postnatal OT facilitated the onset of a preference for a familiar partner, as well as increasing overall social contact, when compared to SAL controls [26].

4.2. Developmental effects of OTA

There are indications from the present study that both administering OT and blocking OT receptors with OTA can disrupt certain aspects of male reproduction. While males treated with OTA displayed a more normal temporal patterning of mating behavior, especially when compared to OT-treated males, the OTA-treated males also were somewhat less likely than controls to leave sperm in the female reproductive tract. We have also observed that a single postnatal treatment with OTA was associated later in life with reductions in parental behavior and an unexpectedly high incidence of pup-directed aggression in male prairie voles [43].

A lower dose of OTA than OT was used because in studies regarding rats, OTA has been shown to have a much higher affinity for OT receptors, which is approximately 10–100 times higher than the natural ligand [44,45]. It is highly probable that these peripheral injections are active centrally. Numerous studies have shown that OT and OTA cross the blood–brain barrier in small amounts, even in adults whose blood–brain barrier would be more fully developed than that of the pups injected in this study (1.3% in Ref. [46]; also Refs. [47,48]). Other studies have shown that neonatal OT and OTA as administered in this study can produce a rapid change in the expression of c-Fos, an immediate early gene indicating activation of a certain brain area [49] and long-lasting changes in social behavior [26,43].

The absence of significant differences in organ weights, sperm motility, and sperm production suggests that the observed reductions in sperm transfer in both the OT- and OTA-treated groups may be due to differential sperm transport in a subset of males. Additional evidence for this hypothesis comes from the observation that OTA-treated males, while having a higher concentration of testicular sperm than HAN-only males, had lower concentrations of epididymal sperm. It is possible that either treatment was capable of influencing the production of OT and/or OT receptors in the seminiferous tubules or epididymis. These results also bear some resemblance to studies of developmental effects of environmental toxicants, which can affect fluid balance in the male reproductive system and alter time needed for sperm transport [50].

4.2.1. Clinical applications of drugs that manipulate OT: relevance for human health

Pitocin, a synthetic form of OT, is widely administered to induce or hasten labor, while the OTA (Atosiban) is approved for use in 43 countries for the prevention of prematurity [51]. There is considerable variation in the amount and duration in the clinical applications of pitocin and Atosiban. Although we administered our treatment to pups rather than mothers, it is possible that clinically used OT and especially OTAs may reach and potentially affect human fetuses. The extent to which pitocin or endogenous maternal OT actually crosses the placental barrier is not easily determined and one recent study did not find support for transfer [52]. However, a study in baboons found that Atosiban crossed the placental barrier relatively freely [53]. In addition, as stated above, research in animals suggests that peripherally injected OT can have physiological consequences [27,41,42] and even in adulthood, OT may cross the blood–brain barrier, albeit in small amounts [46–48,54]. In infants, and especially during stressful experiences, perhaps including birth, both the blood–brain and fetal–placental barriers may have reduced effectiveness in blocking drugs. It is also likely that there are individual differences in the amount of endogenous OT produced by each neonate, because the release of OT is affected by various stimuli and experiences, including suckling, physical contact, and stress [55]. Each of these could contribute to individual differences in later reproductive physiology and behavior; by studying the effects of neuropeptides on development in voles, we are able to look at a range of social behaviors larger than that of a rat or a mouse.

The OTA Atosiban has significantly fewer maternal side effects than other available agents used to prevent premature labor, and for this reason has received an enthusiastic response in obstetrics [51,56,57]. Atosiban is most effective when administered in bolus form [51], similar to the treatments used here. The results of the present study, and others using an OTA with features similar to those of Atosiban [26,43], suggest caution in the manipulation of OT in early life, because animal research reveals that such treatments can have long-term consequences for social behavior, brain development, and reproductive processes [58].

4.3. Summary

Manipulations of OT, using exogenous OT or an OTA, can have long-term effects on male reproductive behavior and physiology [58]. In the present study, both treatments had deleterious effects on a subset of males, suggesting that appropriate development of the male reproductive system and subsequent physiology and behavior rely on exposure to an optimal level of activity in the OT system in early life.

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