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Chronic intranasal oxytocin has dose-dependent effects on central oxytocin and vasopressin systems in prairie voles (*Microtus ochrogaster*)

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Abstract

Oxytocin (Oxt) is a neuropeptide with many functions, including modulation of social behavior(s) and anxiety. Due to its notable pro-social effects, it has been proposed as a treatment in the management of neuropsychiatric disorders, such as autism spectrum disorder (ASD), schizophrenia, and social anxiety; however, effects of long-term daily treatment are still being explored. Previously, we have shown that in male prairie voles (*Microtus ochrogaster)* exposure to Oxt during the peri-adolescent period impaired adult pair bonding in a dose-dependent fashion. In females, the medium dose used (0.8 IU/kg) appeared to facilitate pair bonding, and the low and medium doses were associated with fewer lines crossed in the open field. In this study, we examined central receptor binding and immunoreactive (IR) protein for Oxt and vasopressin (Avp), a closely related peptide. Voles were treated with saline vehicle, or one of three doses of Oxt (0.08, 0.8, 8.0 IU/kg) for three weeks from postnatal days 21–42, and euthanized as adults. We used autoradiography to examine Oxt and Avp receptor binding and immunohistochemistry to examine Oxt and Avp –immunoreactive cells in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Females that received the medium dose of Oxt had higher Oxt receptor binding in the nucleus accumbens shell (NAS), while males that received the medium

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dose had lower Avp-immunoreactive cells in the PVN. In summary, we found sex-specific effects of long-term exposure to intranasal Oxt on the Oxt and Avp systems at the weight-adjusted dose currently being used in clinical trials in humans.

Introduction

Oxytocin (Oxt) is a hypothalamic neuropeptide shown to be widely involved in mammalian social behavior and reproduction (Carter, 2014, Veening et al., 2015). In the last decade, intranasal delivery of compounds has opened up possibilities for use in human research on topics such as trust and empathy (Kosfeld et al., 2005, Barraza and Zak, 2009, van Ijzendoorn and Bakersmans-Kranenburg, 2012). Recent advances using this method include confirmation that intranasal administration of Oxt does indeed access the central nervous system (Lee et al., 2017), validation of techniques for measurement of Oxt receptors in human brain tissue (Freeman et al., 2017), and further development in the assay of Oxt in peripheral fluids, which has been controversial in the past (Brandtzaeg et al., 2016). With this growing body of evidence in the literature, we can now consider translational use in humans with increasing confidence.

In addition to its role in emotion, intranasal Oxt has been proposed as a therapeutic for multiple neuropsychiatric disorders, especially those which include disordered social behavior, including schizophrenia (Bradley and Woolley, 2017, Williams and Burkner, 2017), frontotemporal dementia (Tampi et al., 2017) and addictive behavior (Zanos et al., 2017). The most compelling idea has been to use OT to treat autism spectrum disorder (ASD), due mostly to the fact that social deficits are considered to be the central feature of ASD, as well as some evidence that OT might be deficient in ASD (Modahl et al., 1992, Insel et al., 1999, Jacob et al., 2007, Gregory et al., 2009, Campbell et al., 2011, Teng et al., 2013, Alvares et al., 2017). However, the enthusiasm for intranasal Oxt treatment quickly outstripped the available knowledge of both short-term and long-term effects (Miller, 2013). Clinical trials in humans have now determined that there is a very low incidence of acute side effects of Oxt when given intranasally to children and adolescents with ASD (Anagnostou et al., 2014). Meta-analyses of the available clinical trials have generally concluded that there are still reasons to believe that intranasal Oxt produces modest improvements in social behavior with few or no acute side effects (Bakermans-Kranenburg and Van Ijzendoorn, 2013, Alvares et al., 2017). However, these clinical trials tend to be relatively short, with the longest published trials lasting around six months (Tachibana et al., 2013) and having relatively small sample sizes. With this gap in literature on the long term effects of chronic administration of Oxt, concerns remain that it may result in reduction of receptor binding or other changes to the Oxt system. These changes could result in long-term behavioral effects differing from its prosocial effects found in studies on acute OT administration (Bales and Perkeybile, 2012).

Several animal studies have attempted to determine the long-term behavioral and neural effects of repeated developmental exposure to Oxt. In 2013, we published a paper on the behavioral effects of daily peri-adolescent exposure to intranasal Oxt in prairie voles (Bales et al., 2013). The peri-adolescent period was chosen because at that time, active clinical

trials were being conducted in this age group (ClinicalTrials.gov identifiers: NCT01908205 and NCT01308749). In this study, we found that low dose (0.08 IU/kg) and medium dose (0.8 IU/kg) of Oxt caused later deficits in pair-bonding in male prairie voles (*Microtus ochrogaster*). Females did not show a change in pair-bonding when all doses were considered; however, if comparing only the medium dose to saline, Oxt actually facilitated the pair bond in females (data shown in Bales et al., 2013; analysis of saline vs. medium dose Oxt unpublished). In addition, Oxt decreased line-crossing during open field testing in females treated with a low or medium dose. The effects of the medium dose were of particular interest because it was the weight-adjusted dose closest to what has been used in humans in clinical trials (Macdonald and Macdonald, 2010, Bales et al., 2013, Anagnostou et al., 2014).

Using a dose of Oxt slightly higher than our highest dose in voles (app. 11.0 IU/kg), Huang and colleagues (Huang et al., 2014) found both a reduction in social interaction and a widespread down-regulation of central OT receptors in C57Bl/6J mice. In our own study on both C57Bl/6J and BTBR T+ ltpr3tf/J mice (an inbred mouse with low sociability), at the 0.8 IU/kg Oxt dose, we found no behavioral changes at all (Bales et al., 2014), and therefore did not examine the neural changes. However, we continued to question whether the neural changes subserving the behavioral changes in partner preference and emotionality in the prairie voles would be similar to those seen in mice by Huang and colleagues. Alternatively, they could be due to changes in the peptide rather than the receptor, or in the closely related arginine vasopressin (Avp) system, as has previously been seen with neonatal Oxt injections (Yamamoto et al., 2004, Bales et al., 2007a). The present study therefore used brains from the same subjects in Bales et al. (2013) to look for potential changes in Oxt receptors (Oxtr) and Avp receptors (Avpr1a). We also utilized a second cohort of animals which underwent identical treatment conditions, but no behavioral testing, to examine central peptide levels via immunohistochemistry. Based on previous studies of the neurobiology of pair bonding, we predicted that we would see decreased receptor binding of Avpr1a in the ventral pallidum (VP), a critical area for male pair bonding (Lim and Young, 2004), and/or fewer Avp cells in the paraventricular nucleus of the hypothalamus (PVN). Given the faciliatory effect that the medium dose had on female partner preference behavior, we further predicted increased Oxtr receptor binding in females in the nucleus accumbens, a critical area for female pair bonding (Ross et al., 2009b). We predicted these regional changes, rather than brain-wide changes, based on our previous findings of regional and sex-specific changes in receptors based on neonatal exposure to OXT (Bales et al., 2007a).

Experimental Procedures

Subjects

Animals were housed as offspring of breeding pairs in polycarbonate cages (44 × 22 × 16 cm) on a 14:10 light cycle maintained at approximately 70°F and were provided with water and food *ad libitum* (Purina High Fiber Rabbit Chow, PMI Nutrition International, Brentwood, Missouri). The 173 prairie vole subjects were pseudorandomly selected from the breeding colony located at the University of California, Davis. At postnatal day (P) 20, subjects were weaned, marked with nontoxic Nyanzol D dye for identification (American

Color and Chemical Corporation, Charlotte, North Carolina), and housed in same-sex pairs in smaller cages (27 × 16 × 13 cm) with a sibling when available or an age-matched non sibling. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis.

Intranasal OT Treatments

Subjects were randomly assigned to 1 of 4 treatment groups: low (0.08 IU/kg), medium (0.8 IU/kg), high (8.0 IU/kg), or a saline control. From P21 to P42 (juvenile to pubertal period), subjects received 25 uL of intranasal Oxt treatment between 7 a.m. and 12 p.m. Awake animals were held by their scruff, tilted slightly backward so that the treatment would not drip out. Treatments were administered into the nasal mucosa using a Hamilton syringe (Bachem, Torrance, California) attached to cannula tubing and blunt cannula needle (33 gauge, 2.8 mm length; Plastics One, Roanoke, Virginia). This fine gauge cannula allowed experimenters to administer minute amounts of solutions without coming into direct contact with the subject's nostrils, as natural breathing resulted in fluid uptake into the nasal cavity. Administration was thus very similar to that described previously in rats and mice (Lukas and Neumann, 2012, Neumann et al., 2013), and shown to lead to increased OXT levels in the amygdala and hippocampus (Neumann et al., 2013). Immediately after administration, the subject was returned to its home cage. Syringes and cannulae were cleaned with a 70% isopropyl alcohol solution and rinsed with deionized water between treatment sessions. Initial treatment order for cage mates was randomized, and then alternated each day throughout the 3-week treatment period. After treatment, the subjects were further broken down into two groups: the first 89 subjects underwent behavioral testing and were used to measure OxtR and AvpR receptor autoradiography, and the remaining 84 did not undergo behavioral testing and were used to measure Oxt-ir and Avp-ir.

Receptor Autoradiography

Receptor autoradiography was performed on 89 of 173 subjects. Sample sizes per group were: Saline (M = 14, F = 15); Low dose (M = 10, F = 11); Medium dose (M = 10, F = 10); and High dose (M = 10, F = 9). Following all behavioral testing (results reported in Bales et. al, 2013), test subjects were sacrificed (~P60), brains were removed and immediately flash-frozen on dry ice, and stored at −80°C. Brains were sectioned at 20-μm thickness using a cryostat, mounted onto Super-frost slides, and stored at −80°C. At the time of assay, tissue was thawed to room temperature and immersed in 0.1% paraformaldehyde for 2min. Slides were then rinsed 3 times in 50mM Tris–HCl buffer (pH 7.4) at room temperature for 5min and incubated for 60min at room temperature in a solution of 50mM Tris–HCl (pH 7.4) with 10mM $MgCl_2$, 0.1% bovine serum albumin, and 50pM of radiotracer.

For Oxtr binding, [^{125}I]-ornithine vasotocin analog [(^{125}I)OVTA] was used [vasotocin, d(CH2)5[Tyr(Me)2,Thr4,Orn8, (^{125}I)Tyr9–NH2]; 2200 Ci/mmol]; (NEN Nuclear, Boston, MA, USA). For Avpr1a binding, ^{125}I–lin–vasopressin [^{125}I–phenylacetyl–D–Tyr(ME)–Phe–Gln–Asn–Arg–Pro–Arg–Tyr–NH2]; (NEN Nuclear) was used. Slides were then washed 4 times at 5 min intervals in 50mm Tris–HCl buffer (pH 7.4) with 10mM $MgCl_2$ at 4°C, and followed by a final rinse in this same buffer for 30 min while stirred with a magnetic bar. To finish, the slides were quickly dipped in cold dH_2O and dried with a stream of cold air.

Slides were opposed to Kodak BioMaxMR film (Kodak, Rochester, NY, USA). The ^{125}I-receptor binding was then quantified from film using the National Institutes of Health Image J program to measure optical density (Figure 1). Background was quantified for each section of tissue from a cortical area lacking receptors. The number of sections scored for each area varied, but averaged 9 sections. Each hemisphere was quantified separately for each area, and compared for potential differences. Finding no differences between the two sides, we averaged them together and the resulting means were used in the analyses.

Immunohistochemistry

All remaining test subjects (84 of 173 subjects) were sacrificed at P50 to P55 without behavioral testing, and brains were removed for Oxt and Avp cell body density quantification. Sample sizes per group were: Saline (M = 17, F = 17); Low dose (M = 9, F = 8); Medium dose (M = 8, F = 8); and High dose (M = 7, F = 8). PVN measures were based on 9.14 ± 0.43 slices (mean ± standard error) and SON measures were based on 5.81 ± 0.38 slices (mean ± standard error). Following removal, the tissue was fixed using 4% PFA and acrolein for two hours, then switched to 30% sucrose for 24 hours and stored in 30% sucrose and sodium azide until sliced. The tissue was sectioned at 40 μm, immersed in cryoprotectant, and stored at −20°C until the time of assay. Tissue sections were rinsed in 0.05 M KPBS and incubated in 0.014% phenyl hydrazine for 15 min. After another KPBS rinse, sections were then incubated in rabbit Oxt antisera at 1:150,000 or rabbit anti-Avp (MP Biomedicals, Irvine, CA, USA) at 1:100,000 dilution in 0.05 M KPBS-0.4% Triton X-100 for 1 hour at room temperature. Sections were then incubated for 48 hours at 4°C, rinsed in KPBS, and incubated again for 1 hour at room temperature in biotinylated goat, anti-rabbit IgG (1:600 dilution in KPBS-0.4% Triton X-100; H + L, BA-1000; Vector Laboratories, Burlingame, CA, USA). Sections were rinsed in KPBS and incubated in an avidin-biotin peroxidase complex (4.5μl A and 4.5μl B per 1 mL KPBS-0.4% Triton X-100; Vectastain ABC kit-elite pi-6100 standard; Vector Laboratories) for 1 hour at room temperature. Sections were rinsed in KPBS and then rinsed in 0.175 M sodium acetate. Finally, Oxt-immunoreactivity (Oxt-IR) and Avp-IR by incubating the sections in a nickel sulfate-diaminobenzidine chromogen solution (250 mg Nickel II Sulfate, 2 mg DAB, 8.3 μL 3% H_2O_2 per 10 mL 0.175 M sodium acetate) for 15 min, then rinsed in sodium acetate followed by KPBS rinses. Following Oxt or Avp immuno-labeling, sections were mounted onto subbed glass slides and air-dried overnight. Slides were then dehydrated in ascending ethanol solutions, cleared in Histoclear (National Diagnostics, Atlanta, GA, USA), and cover-slipped with Histomount (National Diagnostics).

Images were captured using a Nikon Eclipse E 800 microscope, Sensi-cam camera, and IP Lab Software. Pictures for analysis were taken at 100x magnification (Figure 2). Analyses were performed using Image J software (National Institutes of Health, Bethesda, MD, USA). All cells for all treatments were counted and measured on ImageJ by the same blind observer. Cells were counted if they were dark brown/black color, rather than light brown. Cells were only counted if at least 75% of the cell boundary was visible.

Statistical Analyses

Prior to analyzing differences in the neural regions, we first tested to see if any of the dependent variables were subject to hemisphere effects using one-way ANOVAs. Having found no significant differences between the left and right hemispheres for any of the variables, we averaged the two hemispheres together for further analysis. Thereby, the density measures shown in the following analyses represent the average of the right and left hemispheres.

Males and females were analyzed separately, as behavioral effects were previously found to be sexually dimorphic (Bales et al., 2013). Thus, all regions were assessed using one-way ANOVAs with treatment group as the independent variable. To control for litter effects, we assigned each animal from a particular litter a unique identifier which was then used as a random effect in all models. Each ANOVA was analyzed using Wald Chi-square tests and alpha was set to $p = 0.05$. We hypothesized that the treatment could both increase and decrease receptor binding depending on the area, so all tests on receptor binding were two-tailed. However, because the low and medium doses specifically dysregulated pair bonding in males (Bales et al., 2013), and because pair bonding in males is an Avp-dependent behavior (Lim et al., 2004a), we used one-tailed analyses for this measure.

All models were created in R version 3.3.2 (The R Foundation; www.r-project.org) using the lme4, car, MASS, and lsmeans packages. After creating linear mixed models using lme4, we then utilized the car package to calculate p-values through Wald Chi-square tests. Upon finding a significant effect of treatment, we conducted Dunnett-adjusted post-hoc analyses using the lsmeans package. Any models that did not meet the assumptions for ANOVA testing were re-fit using the penalized quasilikelihood (PQL) method in the MASS package. These PQL models were then passed to the Anova function in the car package for Wald Chi-square tests to determine the significance of treatment effect.

In addition to exploring the potential effects of treatment on physiological measures, we also analyzed the relationships in (1) Oxtr binding across brain regions, (2) Avpr1a binding across brain regions, and (3) nonapeptide receptor binding with our previously published behavioral data (Bales et al., 2013). For the behavioral data, we included only the behaviors statistically analyzed in the original paper, which were: 1) for acute home cage observations, contact with the cagemate and autogrooming; 2) for juvenile affiliation testing, contact with the juvenile and autogrooming; 3) for alloparental care testing, contact with the pup and autogrooming; 4) for open field testing, line crosses and autogrooming; 5) for elevated plus-maze, the ratio of time spent in open arms over total time, and autogrooming; and 6) for partner preference, time spent in partner contact – time spent in stranger contact. We produced a correlation matrix that included all measured Oxt receptor and Avpr1a regions with corresponding behavioral data using the Hmisc package. The correlation matrix was partitioned into two groups: (1) receptor by receptor correlations and (2) receptor by behavior correlations. There were a total of 165 brain by behavior correlations per sex, and a total of 105 brain area by brain area correlations by sex. We used Benjamini Hochberg's adjustment in each group to control for multiple comparisons with alpha set to 0.05. For the brain by brain correlations, we report only the ones that survived adjustment; for the brain by behavior correlations, we report those that were significant before adjustment (none

survived adjustment). They are presented here as exploratory analyses to provide predictions for future research.

Results

Sex Differences in Control Animals

Receptor density comparisons by sex in control animals revealed differences in the NAS [χ^2(df = 1, N = 19) = 6.54, p < 0.05]; males had higher Oxtr in the NAS than females. Control comparisons for cell immunoreactivity also revealed differences in the PVN [χ^2(df = 1, N = 33) = 4.25, p < 0.05] and SON [χ^2(df = 1, N = 30) = 6.65, p < 0.01]; females had higher Avp immunoreactivity in both regions. No other sex differences were found for receptor binding or cell immunoreactivity in any measured brain region.

Females

While analyzing each region for changes in female Oxtr binding, we found a significant treatment effect in the NAS [χ^2(df = 3, N = 35) = 12.80, p = 0.01; Figure 3a]. Post-hoc analyses confirmed more Oxtr binding in the NAS of medium-dose females than control females [t(26.33) = 2.958, p = 0.02]. No other significant changes in Oxtr were found in females.

For Avpr1a binding in females, we found a significant treatment effect in the MPA [χ^2(df = 3, N = 35) = 8.92, p = 0.03] and CeA [χ^2(df = 3, N = 37) = 8.35, p = 0.04], but these effects were not robust to adjustments for multiple comparisons. No other significant effects were found for Avpr1a in females.

No significant effects were found for either Oxt or Avp peptide immunoreactivity in either of the tested region in females. A list of descriptive statistics for female receptor autoradiography and peptide immunoreactivity analyses can be found in tables 1 and 2 respectively.

Males

No significant effects were found for Oxtr or Avpr1a in any of the tested regions in males. A list of descriptive statistics for male receptor autoradiography analyses can be found in table 3.

We also did not detect significant treatment effects for male Oxt peptide immunoreactivity in either of the tested regions. However, treatment influenced male Avp peptide immunoreactivity in the PVN [χ^2(df = 3, N = 34) = 7.69, p = 0.03]. Further analysis showed less cells immunoreactive for Avp in the medium-dose group than controls [t(20.91) = −2.761, p = 0.03; Figure 3b]. The SON did not show a detectable treatment effect in males. A list of descriptive statistics for male immunoreactivity analyses can be found in table 2.

Brain-Brain Correlations

There were a number of significant correlations in receptor binding measures between different brain areas. Those that survived corrections for multiple comparisons are reported in Table 4.

Brain-Behavior Correlations

No correlations between receptor binding measures and behavioral measures survived corrections for multiple comparisons. We report the correlations that were significant *prior* to the corrections in Table 5. These should not be considered statistically significant, but as potential avenues for future *a priori* investigations.

Discussion

In this study, we examined neural changes associated with long-term, daily treatment with intranasal Oxt in prairie voles. We found that changes in central Oxt and Avp systems showed different patterns in male and female subjects, and depended on the dose received, which is consistent with our previous behavioral findings (Bales et al., 2013). In both sexes, we found significant changes in the subjects that had received the medium dose, which is the weight-adjusted dose closest to the daily dose currently being used in human studies (Anagnostou et al., 2014). In both cases, the changes were consistent with current views on the neurobiology underlying pair bonding in voles (Numan and Young, 2016); that is, that Oxtr in the NAS are primarily mediating pair bonding in females, whereas Avp (via V1a in the VP) is primarily mediating pair bonding in males. Thus, in the present study an increase in Oxtr in the NAS was associated with a group that showed increased pair bonding in females, while a decrease in Avp was associated with a group that showed decreased pair bonding in males.

What possible mechanisms could account for the alterations we saw? Recent evidence supports the view that intranasal Oxt does enter the central nervous system (Neumann et al., 2013, Lee et al., 2017). However, it would be most common to predict lowered Oxt receptor binding in response to exogenous Oxt, as we have in the past (Bales and Perkeybile, 2012), and as has been found previously in mice (Huang et al., 2014). Desensitization of the receptor following exposure would presumably be due to receptor phosphorylation and internalization following stimulation (Gimpl et al., 2008, Busnelli and Chini, 2017). However, binding in medium-dose females was increased. Increased receptor binding has been posited as a “rebound” reaction to low Oxt levels (Zanos et al., 2014, Zanos et al., 2015), and theoretically causing Oxtr to undergo sensitization (as opposed to desensitization), under other circumstances like labor (Blanks et al., 2007). The response of Oxtr can differ by brain area (Busnelli and Chini, 2017). Finally, receptor binding was measured in behaviorally tested animals, while immunoreactivity was measured in behaviorally naïve animals, so we do not have comparable values from the same subjects. It is possible that behavioral testing led to lowered Oxt levels in the PVN (possibly via release), resulting in increased binding of Oxtr in the nucleus accumbens.

Likewise, the mechanism for changes in Avp immunoreactivity is not entirely clear. Higher Avp-ir could reflect either higher production or lower release. However, since these animals were behaviorally naïve and not exposed to any stimulus prior to euthanasia, we would argue that this measure should reflect baseline peptide produced and stored. Avp-ir in medium dose males could reflect the fact that Oxt can bind to Avpr1a (Zingg, 2002) and perhaps stimulated a feed-forward mechanism similar to that posited above for medium dose Oxt.

It is notable throughout the study that exogenous Oxt did not produce a linear dose-response curve. This is a common finding in studies of Oxt, including our own (Bales et al., 2007b, Bales et al., 2013), and those of others (Quintana et al., 2017, Spengler et al., 2017). It is tempting to speculate that the non-linearity of response is due to coupling with different G proteins, as discussed above, or binding to the Avpr1a receptor when high doses flood available Oxt receptors. However, at this time, these exact mechanisms are unknown.

We found somewhat unexpected sex differences in control animals: males had higher Oxtr binding in the NAS than females, whereas females had higher Avp-ir in the PVN than males. Sex differences in Oxtr in the NAS vary between species; in rats and voles, previous reports have not observed sex differences in Oxtr in the NAS (Lim et al., 2004b, Smith et al., 2017), but these studies were performed in sexually naive animals not exposed to behavioral testing. In naked mole-rats, breeding males had higher Oxtr in the NA than breeding females (Mooney et al., 2015). It is possible that some aspect of the behavioral experience that subjects underwent, including cohabitation with a partner and preference testing, affected Oxtr in the males and females differently. We also did not expect to see sex differences in Avp-ir in the PVN and SON; most documented sex differences in Avp (in voles and other species) are in the extended amygdala, not in the PVN (Wang et al., 1996, De Vries and Miller, 1998, Albers, 2015). It is worth noting, however, that our controls were not completely naïve; they were vehicle controls and the saline could potentially have affected Avp differentially in males and females.

It is also notable that we saw sexually dimorphic responses in both Oxtr binding and in Avp-ir. While both Oxt and Avp are involved in the neurobiology of sociality in both sexes (Cho et al., 1999, Bales et al., 2004, Dumais and Veenema, 2016), there are a large number of sex differences in the neuroanatomy and responsiveness of these two systems (Dumais and Veenema, 2016), including androgen-dependent synthesis of Avp in some brain areas (De Vries and Miller, 1998) and a subset of Oxtr that are estrogen-inducible (Champagne et al., 2001). We are not able to pinpoint the exact mechanism for sex differences in the present study, although this remains a fruitful avenue for future investigation.

One important question is why we did not detect any changes in low-dose males, which in our previous study showed a behavioral profile similar to that of medium dose males; that is, a preference for the stranger rather than normal pair bonding (Bales et al., 2013). While not statistically significant for any one area, it is notable that low dose males have the lowest Oxtr binding in four out of eight of the areas quantified (BST, CeA, NAC, and PCC; Table 3). In three of these areas (BST, NAC, and PCC), the medium and high dose has higher binding than saline and the low dose had lower binding than saline. Perhaps rather than

changes in any one area, small changes in OTR across several areas might have combined to contribute to behavioral changes in low-dose males. This viewpoint is consistent with the results of Huang and colleagues (Huang et al., 2014) in mice.

The brain-brain and brain-behavior correlations, while exploratory, suggest some areas for future research. Some of the most interesting brain-brain correlations are between the LS and other dopaminergic areas such as the NAC and VP. While all of these areas have been identified as important to prairie vole pair bonding (Liu et al., 2001, Lim et al., 2004b, Ross et al., 2009b), the LS has been less emphasized. In contrast, it has come out as a very important area to pair bonding in a monogamous primate, the titi monkey (Bales et al., 2017). Also of interest are the positive correlations between Oxtr in the nucleus accumbens (shell and core) and contact with pups in the alloparental care test in male subjects. While Oxtr binding in the nucleus accumbens was positively correlated with pup contact in female prairie voles (Olazabal and Young, 2006), to my knowledge this has not been tested in males. The role of Oxt in male pair bonding is now being explored more fully (Johnson et al., 2015, Johnson et al., 2016), but male alloparenting and Oxt, and in particular the role of the NAS and NAC, remain underexplored (Gordon et al., 2013, Olazabal, 2014, Gordon et al., 2016).

One limitation of this study was that extensive behavioral testing of animals that were then used for autoradiography could have affected the receptor binding results. In that case, Oxtr in the nucleus accumbens of medium-dose females might have been higher because of more time spent with the partner, rather than the other way around. However, options for exploring this relationship are limited unless we become able to measure Oxtr receptor binding in vivo, which has been elusive (Smith et al., 2013, Smith et al., 2016), or if we test behavior and receptor binding in different animals. However, it is possible that despite each treatment group experiencing all of the same testing, their subjective response to the testing might differ and affect their receptor binding levels.

We also did not distinguish between hypothalamic cells which were potentially parvocellular and magnocellular. Previous work in the prairie vole found that all oxytocinergic neurons in the SON stained for FluoroGold (indicating magnocellular neurons with terminals outside the blood-brain barrier) (Ross et al., 2009a). The Oxt positive cells in the PVN consisted of magnocellular neurons in the anterior and middle, interspersed throughout with isolated parvocellular neurons, as well as a cluster of parvocellular neurons in the dorsal posterior (Ross et al., 2009a). In this study, we did not attempt to differentiate parvocellular and magnocellular neurons in the PVN, which could have been illuminating as to the correlations with behavior. To our knowledge, parvo- vs. magnocellular locations of vasopressinergic neurons have not been mapped in prairie voles.

However, as a whole this study differed from the only other long-term study of intranasal Oxt administration in rodents (Huang et al., 2014), in that the present study did not find widespread, significant down-regulation of OT receptor binding. As mentioned above, the doses in our study were lower (with our high dose approximately 8.0 IU/kg, as compared to 11.0 IU/kg in the Huang study). We also gave our doses in 25 ul of saline rather than 5 ul of saline, as in the Huang study, which theoretically could have affected uptake or osmotic

balance. Autoradiography for the mouse study was carried out in brains from animals that had not been behaviorally tested, whereas the animals used for our receptor binding assays were tested extensively. Any of these differences could potentially have resulted in the contrasting results from the two studies.

In summary, we found that long-term administration of intranasal Oxt in socially monogamous prairie voles altered Oxtr binding in the NAS in females, and Avp peptide levels in the PVN in males. These results suggest possible mechanisms by which intranasal Oxt may achieve long-term behavioral results when given during peri-adolescent development.

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Highlights

- Voles got treated daily during adolescence with intranasal oxytocin
- Oxytocin-treated males showed fewer vasopressin immunoreactive neurons in the paraventricular nucleus.
- Oxytocin-treated females showed higher oxytocin receptor binding in the nucleus accumbens shell.

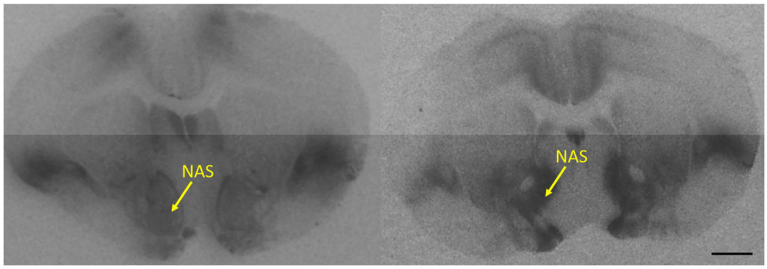

Figure 1.

Representative autoradiogram of OT receptor binding from saline (left) and medium dose OT (right) treated females at the level of the nucleus accumbens (scale bar = 1 mm)

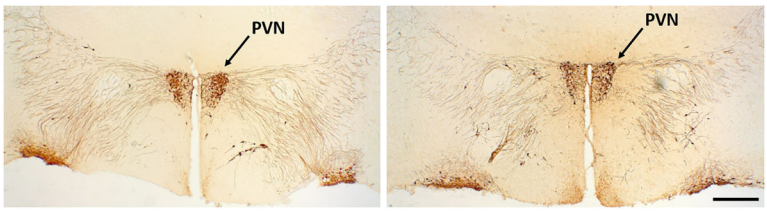

Figure 2.

Representative photos of Avp immunohistochemistry from saline (left) and medium dose OT (right) treated males (scale bar = 500 μm).

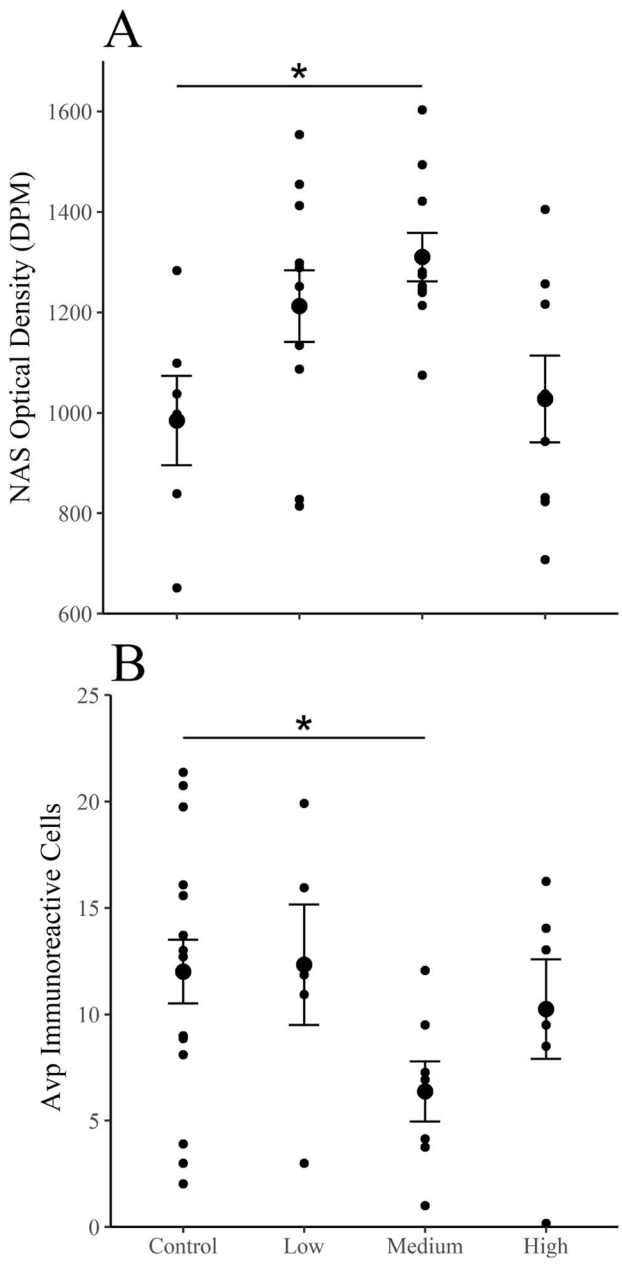

Figure 3.

A: Medium-dose females showed an increase in Oxtr binding in the nucleus accumbens shell [NAS: χ^2(df = 3, N = 35) = 12.80, p = 0.01].

B: Medium-dose males showed a decrease in Avp-immunoreactive cell counts in the paraventricular nucleus [PVN: χ^2(df = 3, N = 34) = 7.69, p = 0.03].

Table 1

Neuropeptide binding in females. Units are disintegrations per minute (DPM) with corresponding effect size measures for each comparison.

		Oxt Receptor Binding		Avp 1a Receptor Binding	
Area	**Predictor**	**Mean ± SEM**	**Cohen's d**	**Mean ± SEM**	**Cohen's d**
BST	Control	752.17 ± 59.59	-	951.62 ± 67.26	-
	Low	656.86 ± 52.58	−0.60	831.96 ± 69.46	−0.54
	Medium	779.03 ± 143.76	0.10	1063.67 ± 95.73	0.44
	High	559.95 ± 41.08	−1.45	856.52 ± 95.04	−0.39
CeA	Control	996.84 ± 76.75	-	489.63 ± 59.55	-
	Low	967.13 ± 61.42	−0.14	408.49 ± 42.54	−0.47
	Medium	1055.35 ± 55.51	0.29	747.78 ± 173.41	0.73
	High	1010.44 ± 37.4	0.08	458.67 ± 96.12	−0.13
LS	Control	765.56 ± 54.12	-	721.40 ± 51.33	-
	Low	837.44 ± 52.92	0.43	818.50 ± 53.95	0.56
	Medium	773.52 ± 63.52	0.04	757.70 ± 51.17	0.22
	High	676.88 ± 63.02	−0.54	718.16 ± 88.16	−0.02
MeA	Control	442.49 ± 73.41	-	896.88 ± 76.10	-
	Low	309.28 ± 37.01	−0.75	751.25 ± 75.94	−0.59
	Medium	331.40 ± 25.74	−0.67	1052.52 ± 158.28	0.43
	High	382.8 ± 46.88	−0.33	850.46 ± 89.02	−0.19
MPA	Control	386.66 ± 54.88	-	798.17 ± 70.35	-
	Low	283.70 ± 43.60	−0.69	783.95 ± 110.15	−0.05
	Medium	349.91 ± 43.38	−0.25	1031.96 ± 86.81	0.98
	High	374.17 ± 39.72	−0.09	930.90 ± 124.86	0.46
NAC	Control	1059.33 ± 90.99	-	-	-

		Oxt Receptor Binding		Avp 1a Receptor Binding	
Area	**Predictor**	**Mean ± SEM**	**Cohen's d**	**Mean ± SEM**	**Cohen's d**
	Low	1188.70 ± 67.67	0.56	-	-
	Medium	1187.58 ± 102.69	0.47	-	-
	High	1075.39 ± 91.82	0.06	-	-
NAS	Control	984.31 ± 88.99	-	-	-
	Low	1212.24 ± 71.27	1.00	-	-
	Medium*	1309.99 ± 48.43	1.73	-	-
	High	1027.49 ± 86.62	0.19	-	-
PCC	Control	575.66 ± 55.40	-	650.11 ± 46.47	-
	Low	493.47 ± 43.36	−0.53	710.76 ± 71.09	0.30
	Medium	573.63 ± 37.54	−0.01	808.00 ± 96.14	0.68
	High	520.66 ± 77.45	−0.28	690.75 ± 126.85	0.15
VP	Control	-	-	490.83 ± 48.35	-
	Low	-	-	416.14 ± 34.60	−0.54
	Medium	-	-	469.96 ± 37.15	−0.15
	High	-	-	478.63 ± 65.12	−0.07

Abbreviations: bed nucleus of the stria terminalis (BST), central amygdala (CeA), lateral septum (LS), medial amygdala (MeA), medial preoptic area (MPOA), nucleus accumbens core (NAC), nucleus accumbens shell (NAS), posterior cingulate cortex (PCC), ventral pallidum (VP);

*significantly different than control.

Table 2

Neuropeptide immunoreactivity in females and males. Units are in cell counts with corresponding effect size measures for each comparison.

		Oxt Immunoreactivity		Avp Immunoreactivity	
Area	**Predictor**	**Mean ± SEM**	**Cohen's d**	**Mean ± SEM**	**Cohen's d**
Females					
PVN	Control	49.08 ± 3.81	-	17.11 ± 1.98	-
	Low	44.43 ± 3.59	−0.35	22.12 ± 2.41	0.67
	Medium	50.23 ± 4.11	0.08	12.25 ± 2.73	−0.63
	High	44.39 ± 7.48	−0.25	18.82 ± 3.58	0.19
SON	Control	10.69 ± 1.05	-	19.53 ± 1.51	-
	Low	11.60 ± 1.82	0.19	20.12 ± 4.23	0.06
	Medium	15.11 ± 2.73	0.71	18.08 ± 3.05	−0.2
	High	12.38 ± 2.48	0.31	21.07 ± 5.46	0.14
Males					
PVN	Control	47.92 ± 1.96	-	12.01 ± 1.50	-
	Low	38.70 ± 3.56	−1.01	12.33 ± 2.83	0.05
	Medium	41.41 ± 5.14	−0.58	6.38 ± 1.41 *	1.13
	High	43.97 ± 3.33	−0.49	10.25 ± 2.33	−0.3
SON	Control	10.19 ± 1.43	-	13.65 ± 1.70	-
	Low	12.48 ± 1.58	0.48	16.99 ± 3.58	0.43
	Medium	7.92 ± 0.97	−0.55	8.55 ± 2.60	−0.79
	High	10.11 ± 3.21	−0.01	12.18 ± 3.35	−0.2

Abbreviations: paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON);

* significantly different than control.

Table 3

Neuropeptide receptor binding in males. Units are in disintegrations per minute (DPM) with corresponding effect size measures for each comparison.

		Oxt Receptor Binding		Avp 1a Receptor Binding	
Area	**Predictor**	**Mean ± SEM**	**Cohen's d**	**Mean ± SEM**	**Cohen's d**
BST	Control	765.89 ± 50.20	-	802.43 ± 74.63	-
	Low	706.97 ± 36.20	−0.42	984.98 ± 62.72	0.75
	Medium	736.96 ± 61.85	−0.17	732.81 ± 87.41	−0.25
	High	755.86 ± 43.79	−0.07	897.25 ± 102.62	0.31
CeA	Control	1040.15 ± 52.18	-	448.82 ± 49.78	-
	Low	928.85 ± 39.84	−0.68	439.04 ± 47.48	−0.06
	Medium	1055.23 ± 71.15	0.07	587.18 ± 126.53	0.47
	High	998.25 ± 57.05	−0.22	471.36 ± 33.11	0.16
LS	Control	763.36 ± 65.06	-	664.25 ± 41.54	-
	Low	822.47 ± 38.45	0.31	747.66 ± 51.64	0.52
	Medium	772.18 ± 68.59	0.04	654.56 ± 63.19	−0.05
	High	825.13 ± 49.19	0.30	707.13 ± 67.20	0.23
MeA	Control	354.01 ± 24.43	-	767.30 ± 66.25	-
	Low	373.78 ± 41.96	0.17	792.15 ± 179.92	0.06
	Medium	406.30 ± 42.68	0.45	709.65 ± 76.39	−0.25
	High	421.35 ± 49.06	0.53	724.61 ± 49.08	−0.21
MPA	Control	316.69 ± 38.84	-	602.88 ± 51.33	-
	Low	350.67 ± 37.29	0.26	633.08 ± 98.40	0.12
	Medium	318.81 ± 52.18	0.01	845.48 ± 102.16	1.01
	High	351.94 ± 47.39	0.25	516.81 ± 100.80	−0.35
NAC	Control	1259.86 ± 60.78	-	-	-

		Oxt Receptor Binding		Avp 1a Receptor Binding	
Area	**Predictor**	**Mean ± SEM**	**Cohen's d**	**Mean ± SEM**	**Cohen's d**
	Low	1226.30 ± 50.82	−0.18	-	-
	Medium	1272.64 ± 50.82	0.07	-	-
	High	1283.23 ± 64.79	0.11	-	-
NAS	Control	1240.88 ± 54.58	-	-	-
	Low	1259.31 ± 41.93	0.11	-	-
	Medium	1313.55 ± 45.16	0.42	-	-
	High	1275.65 ± 79.05	0.15	-	-
PCC	Control	491.69 ± 52.90	-	644.01 ± 34.71	-
	Low	425.17 ± 82.32	−0.29	740.27 ± 74.84	0.50
	Medium	646.81 ± 65.69	0.76	696.16 ± 94.67	0.24
	High	596.02 ± 37.19	0.64	649.26 ± 52.38	0.04
VP	Control	-	-	468.53 ± 52.71	-
	Low	-	-	523.58 ± 32.50	0.42
	Medium	-	-	445.93 ± 52.37	−0.14
	High	-	-	368.52 ± 48.37	−0.64

Abbreviations: bed nucleus of the stria terminalis (BST), central amygdala (CeA), lateral septum (LS), medial amygdala (MeA), medial preoptic area (MPOA), nucleus accumbens core (NAC), nucleus accumbens shell (NAS), posterior cingulate cortex (PCC), ventral pallidum (VP);

*significantly different than control.

Table 4

Receptor binding correlations. Only those areas with adjusted p-values under 0.05 are reported.

Oxtr x Oxtr Correlations			**Avpr1a x Avpr1a Correlations**			**Oxtr x Avpr1a Correlations**		
Area 1	**Area 2**	**r**	**Area 1**	**Area 2**	**r**	**OTR**	**AVPR1a**	**r**
Females								
LS	BNST	0.73 ***						
NAS	NAC	0.66 **						
Males								
LS	NAC	0.55 *	VP	BNST	0.57 *	MA	MPOA	−0.51 *
NAS	NAC	0.75 ***	VP	LS	0.57 *			
NAS	LS	0.51 *						

Significance is indicated with asterisks;

*** $p < 0.001$,

** $p < 0.01$,

* $p < 0.05$.

Table 5

Receptor binding correlations with behavior. Given the sheer number of brain regions, receptor types, and behaviors analyzed, none of these correlations are significant with adjustments for multiple comparisons. These correlations constitute exploratory analyses.

Receptor/Area	Behavior	Test	r
Females			
Oxtr/CA	Time ratio (open arms/closed arms)	Elevated plus maze	−0.50 **
Oxtr/CA	Autogrooming	Elevated plus maze	−0.39 *
Oxtr/NAS	Line crosses	Open field	−0.35 *
Avpr1a/LS	Line crosses	Open field	−0.39 *
Avpr1a/PC	Autogrooming	Juvenile affiliation	−0.33 *
Males			
Oxtr/NAC	Contact	Alloparental care	−0.44 **
Oxtr/NAS	Contact	Alloparental care	−0.38 *
Oxtr/PC	Contact	Juvenile affiliation	−0.31 *
Avpr1a/PC	Autogrooming	Alloparental care	−0.31 *
Avpr1a/PC	Time ratio (open arms/closed arms)	Elevated plus maze	−0.30 *

Unadjusted significance is indicated with asterisks;

** $p < 0.01$,

* $p < 0.05$.