Oxytocin has sex-specific effects on social behaviour and hypothalamic oxytocin immunoreactive cells but not hippocampal neurogenesis in adult rats.

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Abstract

Oxytocin regulates social behaviours, pair bonding and hippocampal neurogenesis but most studies have used adult males. Our study investigated the effects of oxytocin on social investigation and adult hippocampal neurogenesis in male and female rats. Oxytocin has poor penetration of the blood-brain barrier, therefore we tested a nanoparticle drug, TRIOZANTM (Ovensa Inc.), which permits greater blood-brain-barrier penetration. Adult male and female rats were injected daily (i.p.) for 10 days with either: oxytocin in PBS (0.5 or 1.0 mg/kg), oxytocin in TRIOZANTM (0.5 or 1.0 mg/kg), or vehicle (PBS) and tested for social investigation. Oxytocin decreased body mass and increased social investigation and number of oxytocin-immunoreactive cells in the supraoptic nucleus (SON) of the hypothalamus in male rats only. In both sexes, oxytocin decreased the number of immature neurons (doublecortin+ cells) in the ventral hippocampus and reduced plasma 17 β -estradiol levels in a dose- and delivery-dependent way. Oxytocin in TRIOZANTM reduced sedation observed post-injection and increased some central effects (oxytocin levels in the hypothalamus and ventral hippocampus neurogenesis) relative to oxytocin in PBS indicating that the nanoparticle may be used as an alternative brain delivery system. We showed that oxytocin has sex-specific effects on social investigation, body mass, sedation, and the oxytocin system. In contrast, similar effects were observed in both sexes in neurogenesis and plasma 17 β -estradiol. Our work suggests that sex differences in oxytocin regulation of brain endpoints is region-specific (hypothalamus versus hippocampus) and that oxytocin does not promote social investigation in females.

Introduction

Oxytocin is well known for regulating social behaviours and pair bonding (Lim and Young, 2006) and is mainly synthesized and secreted in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON). Surprisingly, few studies have examined roles for oxytocin in both sexes even though there are sex differences in the effects of oxytocin on social behaviour and brain activation in both humans and animal models (DiBenedictis et al., 2017; Dumais et al., 2017, 2016b; Lukas and Neumann, 2014; Rilling et al., 2014; Smith et al., 2017; Steinman et al., 2016; and reviewed by Bredewold and Veenema, 2018; Caldwell, 2018). Social behaviour regulation is associated with a network that includes the prefrontal cortex, medial amygdala, bed nucleus of the stria terminalis and hypothalamus (Ko, 2017) and many of these areas are also connected to the hippocampus (reviewed in Jurek and Neumann, 2018). Thus, perhaps it is not surprising that the integrity of the hippocampus has been implicated in social cognition (Alexander et al., 2016; Danjo et al., 2018; Kumaran et al., 2016; Matta et al., 2017; Okuyama et al., 2016; Omer et al., 2018; Raam et al., 2017). In mammals, the hippocampus retains the ability to produce new neurons throughout the lifespan (Kempermann et al., 2018). New neurons in the hippocampus have been implicated in pattern separation, stress resilience, and anxiety-related behaviours (Anacker et al., 2018; Clelland et al., 2009; Snyder et al., 2011). Furthermore, social isolation (Leasure and Decker, 2009; Stranahan et al., 2006), social stress, (McCormick et al., 2012, 2010) and social interactions (Peragine et al., 2016, 2014; Tzeng et al., 2016) alter neurogenesis in the hippocampus. Hormones, such as sex steroids and prolactin, regulate different aspects of neurogenesis, proliferation, differentiation and survival of new neurons, in a sex-dependent way (reviewed in Mahmoud et al., 2016). In male rodents, oxytocin promotes social behaviour (Lukas et al., 2011; Ramos et al., 2013) and increases adult

hippocampal neurogenesis (Leuner et al., 2012; Opendak et al., 2016; Sánchez-Vidaña et al., 2016). However, it is unknown whether oxytocin regulates hippocampal neurogenesis in females. Oxytocin receptors (OTR) are expressed in the hippocampus of both sexes in rodents (Dumais et al., 2013; Lin et al., 2017; Yoshida et al., 2009). However, there are sex differences with female rats having lower OTR binding densities in many regions including the CA1 of the hippocampus compared to males (Dumais et al., 2013). Furthermore, oxytocin activates brain regions differently in males and females (Dumais et al., 2017) and it is possible that the regulation of hippocampal neurogenesis and social investigation by oxytocin is different between the sexes.

Oxytocin is a large hydrophilic peptide, which has poor penetration of the blood-brain barrier (BBB) and a very short half-life in the blood (~3-9 min) (Churchland and Winkielman, 2012; Leng and Ludwig, 2016; Modi and Young, 2012). Following systemic oxytocin, only up to 1.3% of peripheral oxytocin enters the brain (Ermisch et al., 1985; Mens et al., 1983). The use of a nanotechnology drug delivery system that both permits BBB penetration and lengthens the half-life may offer greater delivery of oxytocin to the brain. TRIOZAN™ nanomedicine delivery platform (Ovensa Inc) is based on a high value-added chitosan derivative called trimethyl chitosan (TMC), which enables efficient encapsulation resulting in protection of drug molecules against degradation, effectively maintaining therapeutic integrity (Kulkarni et al., 2017; Mourya and Inamdar, 2008; Ovensa Inc., 2018). TRIOZAN™ is a safe, biodegradable and biocompatible biopolymer and has the ability to accumulate in the targeted tissues with minimal loss, enabling controlled release of therapeutics via effective penetration of the BBB (Ovensa Inc., 2018). TMC nanoparticles have been previously used for drug delivery to different tissues, including the brain (Cardia et al., 2019; Kumar et al., 2013; Wang et al., 2010), because TMC enhances transport of

large molecules through the adsorptive-mediated transcytosis mechanism (Lu, 2012; Ovensa Inc., 2018). Therefore, in the present study we compared the effects of systemic oxytocin with and without the nanoparticle TRIOZANTM.

The objective of this study was to investigate whether systemic oxytocin delivered in vehicle or with a nanoparticle drug delivery platform (TRIOZANTM) regulates social investigation, oxytocin levels in the brain and periphery, oxytocin-immunoreactive cells in the hypothalamus (SON and PVN) and adult hippocampal neurogenesis in male and female rats. We hypothesized that oxytocin would impact the sexes differentially and that TRIOZANTM would increase delivery of oxytocin to the brain and result in enhanced effects on social investigation and hippocampal neurogenesis.

Materials and Methods

Animals and treatments

Adult male and female Sprague–Dawley rats (n=5-8/treatment/sex; 60 days old and tested around 80 days old) were obtained from Charles River (Quebec, Canada) and double-housed with food and water available ad libitum. All protocols were approved by the Institutional Animal Care Committee at the University of British Columbia and conformed to the guidelines set out by the Canadian Council for Animal Care. Ten days after arrival, males and females were randomly divided into one of the following treatment groups: oxytocin in 0.1 M PBS (0.5 or 1.0 mg/kg), oxytocin delivered in TRIOZANTM (0.5 or 1.0 mg/kg, prepared by PO-Laboratories, Dorval, QC, Canada), and vehicle control (equivalent volume of 0.1 M PBS, pH=7.4). The volume of injection was 1.0-1.2 ml for females and 1.5-2.0 ml for males. Oxytocin encapsulated in TRIOZANTM was prepared using PBS and the resulting solution contained both

free (dissolved) and encapsulated oxytocin. Concentrations of free and encapsulated oxytocin were determined by high performance liquid chromatography (HPLC) and resulted in 42.2% of oxytocin encapsulated in TRIOZANTM (PO-Laboratories). Animals received daily i.p. injections for 10 days between 9:00 and 11:00. As oxytocin injections can cause sedation (e.g., Uvnäs-Moberg et al., 1994; the current study), we monitored sedation 5-10 mins post-injection over the course of the 10 days using a binary score (0 = no sedation, 1 = sedation indicated by a clear lack of locomotor activity and rearing, eyes closed and rats appearing asleep). In addition, animals were behaviourally tested two hours after injection on day 9 to avoid confounding sedative and/or muscular effects of oxytocin that would affect locomotion (Elabd et al., 2014; Hess et al., 2016; Leong et al., 2016; Uvnäs-Moberg et al., 1994). On day 10, brains were collected 30 mins after oxytocin injection. This timeline coincides with peak levels in microdialysates sampled within amygdala and hippocampus (after an i.p. injection of oxytocin in male mice; Neumann et al., 2013). Similar nanoparticles to TRIOZANTM, such as TMC, increase delivery of drugs within 30 mins (after inhalation of progesterone bound to TMC in male mice; Cardia et al., 2019) and between 60-240 mins (after injection with anti-neuroexcitation peptide bound to TMC in male mice; Wang et al., 2010). Therefore, we chose 30 mins as it was within the range of previous studies using a nanoparticle to deliver a drug to the brain and the temporal profile of oxytocin in saline.

Social Investigation

The three-chambered social paradigm allows for the measurement of social investigation in rodents (Crawley, 2004; Ku et al., 2016; Nadler et al., 2004). On day 9, two hours after injection, animals were allowed to explore a novel arena (100 cm x 40 cm x 40 cm) composed of

three connecting chambers separated by open sliding doors (Figure 3) for 10 min. Locomotion, grooming, rearing, behaviours were assessed during this time to evaluate general activity. Time spent in each of the chambers was also measured to ensure animals did not have a preference for a particular side of the chamber. Following this habituation period, animals were briefly kept (for ~2-3 min) in the central chamber with sliding doors closed while an unfamiliar and novel same sex conspecific rat was placed in one of the outer chambers under a small wire cage. An identical empty wire cage was secured in the other outer chamber. The unfamiliar rat was randomly assigned to either the right or left side of the chamber. The test animal was then allowed to explore the three chambers for a further 10 min. All sessions were recorded and scored by an observer unaware of treatment conditions. Videos were scored for (1) time spent in the chamber that housed the novel conspecific rat (i.e., social stimulus rat) and (2) time spent engaging in investigatory behaviour with the novel rat (sniffing and touching the basket where the novel rat was confined; i.e. social investigation).

Tissue and blood collection

Vaginal lavages were conducted on days 9 (before behaviour) and 10 (at euthanasia) to account for estrous cycle phase effects on our measures. On day 10, 30 mins after receiving the treatment, all rats received a lethal overdose of sodium pentobarbital. Blood was collected via cardiac puncture into cold EDTA coated tubes and centrifuged 4 h later for 10 min at 4°C. Plasma was stored at -80°C until ELISA and radioimmunoassay (RIA). Adrenals were extracted and weighed. Hypothalamus was quickly dissected from the left hemisphere, flash frozen on dry ice, and stored at -80°C. The right hemisphere was fixed for 24 h in 4% paraformaldehyde in 0.1M PBS (4°C) and then transferred to a 30% sucrose/0.1 M phosphate buffer solution for

cryoprotection until brains sunk. Serial coronal sections (30 μ m) were cut with a freezing microtome (SM2000R; Leica, Richmond Hill, ON) across the extent of the hippocampus (collected in 10 series) and stored in an antifreeze solution (containing 0.1M PBS, 30% ethylene glycol, and 20% glycerol) and stored at -20°C.

Oxytocin and 17 β -estradiol concentrations

Oxytocin concentrations were analyzed using an enzyme-linked immunosorbent assay kit (Enzo Life Sciences, ADI-900-153A) on plasma and hypothalamus homogenate samples. Plasma samples were run in duplicate according to the manufacturer's instructions. Hypothalamus (and other brain regions, frontal region and hippocampus) samples were homogenized in 200 μ l of cold Tris lysis buffer (150 mM NaCl, 20 mM Tris, pH=7.5, 1 mM EDTA, 1 mM EGTA , 1% Triton X) containing a cocktail of protease and phosphatase inhibitors using a bead disruptor (Omni, VWR). Homogenates were centrifuged at 2100g for 10 min at 4°C and supernatants stored at -80°C. Hypothalamus homogenates were run in duplicate on a modified ELISA using the lysis buffer to prepare the standard curve (Enzo Life Sciences). Oxytocin levels were normalized to protein content using a BCA protein assay (ThermoFisher) and expressed as pg/mg protein. The oxytocin antibody is highly specific with 7.5% cross-reactivity with Arginine-Vasotocin. The sensitivity of the assay is 15 pg/ml and average intra- and inter-assay coefficients of variation were <10%. 17 β -estradiol levels were determined in plasma using a commercially available radioimmunoassay kit (ultrasensitive estradiol RIA, Beckman Coulter, DSL4800). All samples were run in duplicate. The assay sensitivity is 2.2 pg/ml and average intra-assay coefficient of variation was <20%. Samples below the zero standard curve point were replaced with 0 pg/ml.

Doublecortin Immunohistochemistry

Free floating sections were rinsed three times for ten minutes in 0.1M phosphate buffered saline (PBS; pH=7.4) in between each step listed below, unless otherwise stated. Endogenous peroxidase activity was quenched with a 30 min incubation in 0.6% H₂O₂. Tissue was incubated in a goat anti-DCX polyclonal antibody (1:1000 in PBS containing 3% normal rabbit serum and 0.4% Triton X-100; Santa Cruz Biotechnology, Cat# sc-8066, RRID: AB_2088494) for 24 h at 4°C. Tissue was rinsed five times for ten minutes in PBS and then incubated overnight at 4°C in a rabbit anti-goat secondary (1:500; Vector Laboratories) diluted in PBS. Sections were then washed five times for ten minutes in PBS and then incubated in an avidin-biotin horseradish peroxidase complex (1:1000; ABC Vector Elite kit; Vector Laboratories) for 4 h at room temperature. Tissue was then washed in sodium acetate buffer (0.175 M; pH= 7.3) two times for 2 min each before reacting with diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate Kit, Vector Laboratories) according to the manufacturer instructions. Sections were mounted onto Superfrost/Plus slides (Fisher Scientific, Ontario, Canada), dried several days, dehydrated in ethanol and cleared with xylene, and then cover slipped with Permount (Fisher Scientific). The primary antibody was omitted in a control sample and did not contain any immunoreactivity.

Oxytocin immunofluorescence

Free floating sections were rinsed three times for ten minutes in 0.1M phosphate buffered saline (PBS; pH 7.4) in between each step listed below, unless otherwise stated. Sections were incubated for 10 min in 0.1% sodium borohydride in PBS on ice and then blocked in PBS

containing 10% normal goat serum (NGS) for 30 min. Sections were then incubated in mouse anti-oxytocin monoclonal antibody (1:2000, Millipore, Cat# MAB5296, RRID: AB_2157626) in 3% NGS and 0.5% Triton-X for 44 h at 4°C and then in goat anti-mouse secondary antibody Alexa 488 (1:500; Invitrogen) in 3% NGS and 0.5% Triton-X for 2 h. After four rinses in PBS, sections were mounted onto Superfrost/Plus slides (Fisher Scientific) and coverslipped using polyvinyl alcohol with DABCO (Sigma-Aldrich).

Microscopy

A researcher blinded to experimental conditions counted doublecortin (DCX)+ cells using a standard light microscope. DCX+ cells were counted in the entire granule cell layer of the right hippocampus at 400x magnification. Sections were considered ventral when the dentate gyrus at the bottom of the section was obviously present, that is between -5.2 mm to -6.7 mm Bregma (Paxinos and Watson, 2005). DCX is a microtubule-associated protein expressed in proliferating progenitor cells and immature neurons, from 2 h to 3 weeks after birth in rats (Brown et al., 2003). DCX+ cells can be classified into developmental stages: proliferative (type 1, no or short processes present), intermediate (type 2, medium process with no branching), or post-mitotic (type 3, branching dendrites; Figure 4A). For DCX+ cell phenotyping, a total of 120 cells per rat were randomly selected from three different sections from dorsal (60) and ventral (60) regions of the hippocampus (Gobinath et al., 2016; Workman et al., 2016). Total GCL volumes (mm³) from dorsal and ventral sections were quantified from digitized images using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>). OT-immunoreactive (ir) cells were counted at 400x magnification in one section of the supraoptic nucleus (SON) and one section of the paraventricular nucleus (PVN) of the

hypothalamus for each brain using an epifluorescence microscope. Sections of the SON (around Bregma -1.44 and -1.56 mm) and PVN (around Bregma -1.80 and -1.92 mm; Paxinos and Watson, 2005) were anatomically matched across all animals.

Statistical Analysis

All analyses were performed using Statistica v.8.0 (StatSoft Inc, Tulsa, OK). Time spent in the social stimulus chamber (time spent on side of the chamber containing novel rat divided by total time in the arena), time spent investigating the stimulus rat (time spent sniffing and touching the basket containing novel rat divided by total time in the arena), and maturity of DCX+ cells were transformed before analysis using the arcsine transformation. The oxytocin in plasma and hypothalamus data were log transformed and the 17 β -estradiol data were square root transformed to meet normality and heteroscedasticity requirements. All data (unless specified) were analyzed using ANOVA with sex (male, female) and treatment (PBS control, oxytocin in PBS (0.5 or 1.0 mg/kg), oxytocin in TRIOZANTM (0.5 or 1.0 mg/kg)) as between-subject factors. Number (number of cells divided by number of sections counted) and maturity of DCX+ cells, and GCL volume were each analyzed using repeated measures ANOVA with hippocampal region (dorsal, ventral) and an additional within-subjects factor for maturity of DCX+ cells (proliferative, intermediate, post-mitotic). The data for OT-ir cells in the SON were not normal or homoscedastic with female counts being more variable than male counts, even after transformation. We therefore analyzed males and females separately to meet ANOVA assumptions. 17 β -estradiol data were analyzed using ANOVA separately for both sexes and using estrous cycle as between-subject factors in females. We identified estrous cycle phase but due to low sample sizes (N=1-2 for some treatment groups in each phase) we used estrous cycle

stage or 17 β -estradiol as a covariate on our measures and the covariate effect is only presented when significant. When appropriate, post-hoc analysis used the Neuman-Keul's procedure. Test statistics were considered significant if $p \leq 0.05$. A priori comparisons were subject to Bonferroni corrections. Pearson's correlations were performed between variables of interest.

Results

Body and adrenal mass

Body mass immediately prior to the start of the injections did not differ between treatment groups (all p 's>0.3) however males ($405.6\text{g} \pm 4.8\text{g}$ (SEM)) were larger than females (mean $\pm$ SEM= $252.5 \pm 1.6\text{g}$; Cohen's $d = 7.31$; main effect of sex $F(1,58)=829.93$; $p<0.0001$; partial $\eta^2 = 0.93$; Table 1). Net body mass gain (last day minus first day of injections) was reduced in rats treated with oxytocin in PBS relative to the PBS controls (0.5 and 1.0 mg/kg doses; $p=0.04$ and 0.003 , respectively; main effect of treatment $F(4,58)=6.16$; $p<0.001$; partial $\eta^2 = 0.30$) but this effect was driven by the males. In males, oxytocin in PBS or in TRIOZANTM (0.5 and 1.0 mg/kg) reduced body mass gain relative to the PBS male controls (a priori comparisons, oxytocin in PBS by dose: $p=0.01$ and $p=0.0003$; Cohen's $d = 1.15$ and 1.46 ; oxytocin in TRIOZANTM by dose: p 's<0.0001; Cohen's $d = 2.04$ and 1.88 , respectively), while oxytocin treated females were not affected relative to PBS female controls (p 's>0.14, respectively). No significant differences were observed between oxytocin in PBS and oxytocin delivered in TRIOZANTM (p 's>0.2). There was also a sex difference with males having a larger body mass gain than females (main effect of sex $F(1,58)=32.14$; $p<0.0001$; partial $\eta^2 = 0.36$; Cohen's $d = 1.30$). Treatment with oxytocin did not affect adrenal mass (adrenal mass/body mass; all p 's>0.15) although we observed an effect of sex ($F(1,58)=120.59$; $p<0.0001$; partial η^2

= 0.68; Cohen's $d = 2.83$) with females having significantly larger relative adrenal mass compared to males.

Oxytocin caused sedation

 Injections of oxytocin caused sedation observed 5-10 mins post-injection (Uvnäs-Moberg et al., 1994). We calculated a sedation score using a binary score (0 = no sedation, 1 = sedation indicated by a clear lack of locomotor activity and rearing, eyes closed and rats appearing asleep) over the course of 10 days for a maximum possible score of 10. All the PBS control animals had a sedation score of 0 (i.e., no sedation). In contrast, all doses and delivery methods of oxytocin resulted in levels of sedation higher than 0 (Figure 1A). We therefore compared sedation scores for the oxytocin groups only. Male rats had higher levels of sedation than female rats for the low doses of oxytocin (0.5 mg/kg), regardless of delivery method (interaction between treatment and sex $F(3,44)=4.08$; $p<0.05$; partial $\eta^2 = 0.22$; main effect of sex: $F(1,44)= 50.04$; $p<0.0001$; partial $\eta^2 = 0.53$). Furthermore, for female rats oxytocin in TRIOZANTM (0.5 mg/kg) resulted in lower sedation relative to the same dose of oxytocin delivered in PBS ($p=0.05$; Cohen's $d = 0.88$) and relative to the high dose of oxytocin in TRIOZANTM (1.0 mg/kg; $p=0.0001$; Cohen's $d = 2.78$; Figure 1A).

Oxytocin injections increased oxytocin levels and decreased 17 β -estradiol levels in both sexes

 Daily oxytocin (both doses) injections increased circulating levels of plasma oxytocin in males and females regardless of delivery method (all p 's <0.001 ; main effect of treatment $F(4,48)=358.3$; $p<0.0001$; partial $\eta^2 = 0.97$; Figure 1B). In males and females, both doses of oxytocin delivered in TRIOZANTM (0.5 and 1.0 mg/kg) resulted in lower plasma levels relative to oxytocin in PBS ($p<0.01$ and 0.0001 ; Cohen's $d = 1.49$ and 2.23 , respectively). Oxytocin

treatment (0.5 and 1.0 mg/kg in PBS and TRIOZANTM) reduced plasma levels of 17 β -estradiol in females relative to PBS controls, regardless of delivery method and estrous cycle phase (main effect of treatment $F(4,22)=3.53$, $p<0.05$; partial $\eta^2 = 0.39$; Figure 1C). In males, oxytocin delivered in TRIOZANTM (0.5 and 1.0 mg/kg) reduced circulating levels of 17 β -estradiol relative to PBS controls ($p<0.05$ and $p<0.001$, respectively; main effect of treatment $F(4,29)=11.18$, $p<0.001$; partial $\eta^2 = 0.61$). The high dose of oxytocin delivered in TRIOZANTM (1.0 mg/kg) also reduced circulating levels of 17 β -estradiol relative to oxytocin delivered in PBS (1.0 mg/kg; $p<0.001$).

Oxytocin treatment increased brain levels of oxytocin when delivered in TRIOZANTM only

Oxytocin delivered in TRIOZANTM (1.0 mg/kg) increased oxytocin concentrations in the hypothalamus in males and females relative to oxytocin delivered in PBS (1.0 mg/kg; main effect of treatment $F(4,49)=3.16$, $p=0.02$, partial $\eta^2 = 0.21$; a significant covariate effect of 17 β -estradiol ($F(1,49)=5.84$, $p=0.02$; partial $\eta^2 = 0.10$) to decrease oxytocin levels; Figure 2A). Oxytocin delivered in PBS did not affect the levels of oxytocin in the hypothalamus relative to the PBS controls in both sexes (p 's >0.3). To investigate how peripheral and hypothalamic levels of oxytocin compare between the treatment groups, we calculated the ratio of hypothalamus to plasma oxytocin content. Compared to the PBS controls, all oxytocin treatments significantly decreased the hypothalamus to plasma ratio in both males and females (all p 's <0.001 ; main effect of treatment $F(4,36)=28.71$, $p<0.0001$; partial $\eta^2 = 0.76$; a significant covariate effect of 17 β -estradiol ($F(1,36)=4.11$, $p=0.05$; partial $\eta^2 = 0.10$) to increase the hypothalamus to plasma ratio; Figure 2B). In addition, oxytocin in PBS (1.0 mg/kg) resulted in a lower hypothalamus to plasma ratio compared to the oxytocin delivered in TRIOZANTM (1mg/kg; $p<0.01$; Cohen's $d =$

2.15). Using ELISA, we were not able to detect oxytocin levels in other homogenized brain regions (frontal region, hippocampus). To complement the total levels of oxytocin in the homogenized hypothalamus, we used immunofluorescence to investigate the effect of oxytocin treatments on the number of OT-ir cells in two hypothalamic nuclei, SON and PVN (Figure 2C and D). There was a weak correlation ($r = -0.248$; $p = 0.08$) between plasma 17β -estradiol and the number of OT-ir cells in the PVN, therefore we used 17β -estradiol levels as a covariate in the analysis of OT-ir cells. In females, oxytocin in PBS and oxytocin delivered in TRIOZANTM did not affect the number of OT-ir cells in the SON and PVN (all p 's > 0.3 ; Figure 2C and D). In males, oxytocin in PBS (1.0 mg/kg; $p = 0.03$; Cohen's $d = 2.00$) and oxytocin delivered in TRIOZANTM (0.5 and 1.0 mg/kg; $p = 0.02$ and 0.03 ; Cohen's $d = 1.98$ and 2.33 , respectively) increased the number of OT-ir cells in the SON relative to PBS controls (main effect of treatment $F(4, 19) = 3.63$; $p < 0.05$; partial $\eta^2 = 0.43$; Figure 2D). Oxytocin delivered in TRIOZANTM (0.5 mg/kg) increased the number of OT-ir cells relative to oxytocin in PBS (0.5 mg/kg; $p = 0.04$; Cohen's $d = 1.56$). In the PVN, oxytocin did not significantly affect the number of OT-ir cells in males (p 's > 0.7).

Oxytocin treatment did not affect time spent in locomotion or grooming but decreased time spent rearing

Because we observed sedation shortly after oxytocin injection, we conducted social investigation tests 2 hours after oxytocin injection to ensure locomotion was not affected during the social investigation test. During the habituation period in the three-chambered arena, time spent in locomotion was not affected by oxytocin treatment (p 's > 0.26 ; Table 2), but there was a trend for a main effect of sex indicating that females were more active than males ($F(1, 58) = 3.53$ $p = 0.06$; partial $\eta^2 = 0.06$). Oxytocin treatment did not affect the amount of time spent grooming

(all p 's>0.41) but oxytocin in PBS (0.5 and 1.0 mg/kg) and in TRIOZANTM (1.0 mg/kg) decreased the time spent rearing during the habituation period relative to the PBS control (p 's=0.046, 0.018 and 0.014 respectively; Cohen's d = 0.76, 1.34 and 1.34, respectively) in males and females (main effect of treatment $F(4,58)=4.15$; $p<0.01$; partial η^2 = 0.22), and females reared more than males (main effect of sex $F(1,58)=11.37$, $p<0.01$; partial η^2 = 0.16; for females a significant covariate effect of estrous cycle ($F(1,27)=4.44$, $p=0.04$; partial η^2 =0.14)).

Oxytocin increased social investigation in males but not females

During habituation, the time spent in each of the three chambers was not significantly different between treatments indicating that animals did not have a preference for a particular side of the chamber (all p 's>0.4; data not shown). We quantified the time spent in the social stimulus third of the chamber and the time investigating the social stimulus rat (i.e. sniffing and touching the basket where the stimulus rat was confined) as two measures of social investigation. Regardless of treatment and sex, rats spent significantly more time in the chamber with the social stimulus rat compared to the empty or middle chambers (main effect of side $F(2,116)=191.3$, $p<0.0001$; partial η^2 = 0.77; Table 3). Surprisingly, none of the oxytocin treatments affected the time spent in chamber with the social stimulus (p 's>0.13; Figure 3B). However, we did find effects of oxytocin on time spent investigating the stimulus basket. Oxytocin in PBS (0.5 mg/kg) increased the time spent investigating the stimulus basket in males ($p=0.049$; Cohen's d = 1.83) but not female rats relative to PBS controls (p 's>0.6; main effect of treatment, $F(4,58)=3.67$, $p<0.01$, partial η^2 = 0.20; interaction between treatment and sex, $F(4,58)=2.50$, $p=0.05$, partial η^2 = 0.15; no significant main effect of sex, $p>0.12$; Figure 3C). Social investigation of the stimulus

rat was not significantly different between oxytocin in PBS and oxytocin in TRIOZANTM groups (p's>0.2).

Oxytocin decreased hippocampal neurogenesis in males and females

The volume of the GCL was larger in males than in females (main effect of sex F(1,58)=18.01, p<0.0001, partial η^2 =0.24; Table 4) and the volume of the ventral GCL was larger than the volume of the dorsal GCL (main effect of region; F(1,58)=212.5; p<0.0001, partial η^2 =0.79). Oxytocin treatments had no effect on GCL volumes (p's>0.11), therefore we present number of DCX+ cells. Oxytocin in PBS (1.0 mg/kg) decreased the number of DCX+ cells in the dorsal GCL relative to PBS control in males and females (p=0.015; Cohen's *d* = 0.86; interaction between region and treatment F(4,54)=4.17, p<0.01, partial η^2 = 0.24; Figure 4B). In the ventral region, oxytocin in PBS (0.5 and 1.0 mg/kg) and delivered in TRIOZANTM (1.0 mg/kg) decreased the number of DCX+ cells relative to PBS control in both sexes (p=0.0002, 0.0001, 0.002, respectively; Cohen's *d*=0.95, 1.26, 0.72, respectively; Figure 4C). The latter effect of oxytocin in TRIOZANTM (1.0 mg/kg) relative to PBS was driven by the females (a priori comparisons; p<0.001, Cohen's *d*= 1.59 in females; p=0.50 in males). Oxytocin in PBS (0.5 mg/kg) also decreased the number of DCX+ cells in the ventral region relative to oxytocin in TRIOZANTM (0.5 mg/kg) in males and females (p=0.002; Cohen's *d*=0.85). Males had more DCX+ cells in the hippocampus than females (main effect of sex F(1,54)=9.98, p<0.01, partial η^2 =0.16) and the ventral region had more DCX+ cells than the dorsal region in both sexes (main effect of region F(1,54)=385.9, p<0.0001, partial η^2 =0.88). We also examined the maturity of DCX+ cells in the dorsal and ventral hippocampus (Table 4). Oxytocin (in PBS or TRIOZANTM) had no effect on the percentage of DCX+ cells at each stage of maturity in males and females

(p 's>0.4). There were significantly more type 3 DCX+ cells relative to type 1 and 2 (main effect of type of DCX+ cell; $F(2,116)=23.09$, $p<0.0001$; partial $\eta^2=0.28$).

Hippocampal neurogenesis was associated with 17 β -estradiol in females and social investigation in males

We found that increased numbers of DCX+ cells were significantly associated with increased 17 β -estradiol in the dorsal (Pearson's correlation; $r(31)=0.44$; $p=0.01$) and ventral ($r(31)=0.48$; $p=0.005$) hippocampus in female but not male rats ($r(29)=-0.013$, $p=0.95$; $r(29)=-0.05$, $p=0.80$, respectively; Figure 5A and B). After removing one outlier in females with high 17 β -estradiol levels (~32 pg/ml; Figure 5A and B include this outlier), the only correlation that remains significant is in the ventral region ($r(30)=0.36$, $p=0.04$). In females, the correlation between DCX+ cells and 17 β -estradiol was observed in the PBS control (dorsal, $r(6)=0.84$, $p=0.009$; ventral, $r(6)=0.70$, $P=0.05$; although these were not significant with the female outlier removed: dorsal, $r(5)=0.69$, $p=0.09$; ventral, $r(5)=0.67$, $p=0.10$) but not in any of the oxytocin groups (p 's>0.4). We also found that increased DCX+ cells in the ventral hippocampus was associated with decreased social investigation in males ($r(32)=-0.40$, $p=0.03$) but not females ($r(32)=0.07$, $p=0.71$; Figure 5C). Finally, we found no significant correlations between neurogenesis (ventral or dorsal) and OT-ir cells in the PVN and SON.

Discussion

In the present study, we found that oxytocin treatment decreased body mass, increased social investigation and the number of OT-ir cells in the SON in male but not female rats. In contrast in both sexes, oxytocin reduced plasma 17 β -estradiol levels and neurogenesis in the

ventral hippocampus in a dose- and delivery-dependent way. Oxytocin treatment, regardless of delivery and sex, resulted in sedation effects immediately after injection. Oxytocin delivered in TRIOZANTM produced some effects that differed from oxytocin delivered in PBS. Principally, oxytocin delivered in TRIOZANTM reduced sedation and plasma oxytocin levels relative to oxytocin delivered in PBS. In addition, oxytocin in TRIOZANTM increased central measures such as hypothalamic oxytocin levels and ventral neurogenesis relative to oxytocin in PBS in males and females. Altogether, oxytocin resulted in sex-specific effects on social investigation and OT-ir cells in the SON but similar effects between the sexes on hippocampal neurogenesis, plasma 17 β -estradiol and hypothalamus oxytocin levels, although some of these effects were dependent on the dose and delivery system used.

Oxytocin increased social investigation in males but not females

In this study, both males and females displayed a preference for the social stimulus in line with previous research in rats (Lukas and Neumann, 2014) and mice (Crawley et al., 2007). We found that treatment with oxytocin for 10 days increased social investigation in male, but not in female, rats. The finding in males is consistent with previous research in male mice and rats (Lukas et al., 2011; Ramos et al., 2013). However, in our experiment, oxytocin did not increase social investigation in female rats, which is similar to the previous findings using central infusions of either oxytocin or an OTR antagonist (Lukas and Neumann, 2014). Thus, oxytocin appears to have no significant influence on social investigation in non-postpartum female rats, across a variety of paradigms and injection procedures. Consistent with our work, in rats, infusion of an OTR antagonist in the amygdala reduced social investigation in males only

(Dumais et al., 2016a). These findings suggest that oxytocin manipulations influence social investigation in males but not females.

There are very few studies doing direct comparisons between males and females, nevertheless sex differences in the effects of oxytocin on neural activation have been observed in humans and rodents. In humans, intranasal oxytocin resulted in sex-specific effects on brain activity during reciprocated cooperation (Rilling et al., 2014). Oxytocin increased brain activity in males in areas important for reward, social bonding, arousal, and memory (the striatum, basal forebrain, insula, amygdala and hippocampus), whereas, oxytocin had no effect or decreased this response in certain areas in females (Rilling et al., 2014). In rats, oxytocin treatment also resulted in sex differences in neural activation patterns as males had more activation than females in the nucleus accumbens, insular cortex, pituitary, and some regions of the hypothalamus, whereas, females had more activation in the amygdala than males (Dumais et al., 2017). In addition, males have more OTR binding sites in hypothalamic and amygdala areas compared to females (DiBenedictis et al., 2017; Smith et al., 2017). In the current study, we found that oxytocin increased the number of OT-ir cells in the SON in males only. Oxytocin can autoregulate its own secretion in the hypothalamus by binding to OTR in male mice (Lopatina et al., 2010) and during parturition in female rats (Neumann et al., 1996). Therefore, it is possible that in male rats oxytocin treatment resulted in further oxytocin release in the SON via autoregulation but this positive feedback is not present in females (outside of parturition). Altogether, the lack of an effect of oxytocin in females on social investigation could be related to sex differences in the effects of oxytocin on OT-ir cells, OTR binding, autoregulation of oxytocin release, and neural activation.

Oxytocin decreased ventral hippocampal neurogenesis in male and female rats

Contrary to previous studies in males, we found that oxytocin treatment decreased the number of immature neurons in the ventral hippocampus of both female and male rats. To our knowledge this is the first study investigating the effects of oxytocin on adult neurogenesis in female rodents. Previous studies in male rats have used similar doses and routes of exposure of oxytocin but different experimental designs (i.e., length of exposure, markers of neurogenesis, behavioural testing) making it difficult to directly compare our current results. In male rats, a single i.p. injection or central infusion of oxytocin increased cell proliferation in the ventral hippocampus (Leuner et al., 2012). Furthermore, oxytocin treatment for 7 days increased new neuron survival 1 or 3 weeks *after* the last injection (Leuner et al., 2012; Opendak et al., 2016). Similarly, oxytocin treatment for 14 days increased DCX+ cells in adult male rats that also underwent behaviour (Sánchez-Vidaña et al., 2016). Altogether, this suggests that in males, oxytocin increases neurogenesis after prolonged treatment (14 days) or 1 to 3 weeks after 7 days of treatment, however in the present study we found that oxytocin decreased neurogenesis (DCX+ cells) after 10 days of exposure. Thus, it is possible that in the current study we may have observed an increase after more prolonged exposure to oxytocin or a longer time prior to brain collection.

Interestingly, the effects of oxytocin on adult neurogenesis were mostly observed in the ventral hippocampus, similar to previous studies in rats (Leuner et al., 2012; Opendak et al., 2016). In contrast, other studies have seen effects in either both regions (in mice; Lin et al., 2017) or in the dorsal region only (in rats; Sánchez-Vidaña et al., 2016). The dorsal and ventral regions of the hippocampus have distinct functions (Bannerman et al., 2004; Fanselow and Dong, 2010; Hawley et al., 2012; Wu and Hen, 2014). Newly generated neurons in the dorsal

and ventral hippocampus also project to different areas (reviewed in Strange et al., 2014). We observed a significant correlation between ventral neurogenesis and social investigation in males. It is possible that increased levels of ventral neurogenesis are associated with lower levels of social investigation in males. In addition, it is also possible that the regulation of ventral neurogenesis by oxytocin is related to other social-, stress-, or anxiety-related behaviours (Anacker et al., 2018; Kheirbek et al., 2013; Opendak et al., 2016; Snyder et al., 2011; Wu and Hen, 2014). As oxytocin also reduced 17 β -estradiol levels (discussed below), and estradiol can regulate neurogenesis in both males and females (Barker and Galea, 2008; Eid et al., 2019; McClure et al., 2013; Ormerod et al., 2004), it is possible the effects we saw were related to oxytocin's effects on circulating 17 β -estradiol. However, in males this is unlikely as oxytocin reduced neurogenesis and 17 β -estradiol either has no significant effect on neurogenesis in rats (after 15 days: Barker and Galea, 2008) or increases neurogenesis (after 5 days) in voles (Ormerod et al., 2004). Furthermore, no significant correlation was found between 17 β -estradiol levels and DCX+ cell counts in males. In female rats, 17 β -estradiol can suppress (after 15 days Barker and Galea, 2008) or increase neurogenesis (after 20 days, McClure et al., 2013; after 47 days, Eid et al., 2019). In the present study, we found a significant correlation between 17 β -estradiol and DCX+ cell counts but this was mostly observed in the PBS controls. Although it is possible that in females the effects of oxytocin were mediated by effects on circulating 17 β -estradiol, the lack of a correlation in the oxytocin groups suggest this was not a large contribution.

In the current study, we found no significant effects of oxytocin on the proportion of cells at different stages of maturation (proliferative, intermediate and mature) immediately after 10 days. In contrast, oxytocin treatment (1.0 mg/kg) for 14 days increased cell proliferation, number

of mature DCX+ cells (i.e., cells with tertiary dendrites), and dendritic complexity in the dorsal hippocampus in male rats (Sánchez-Vidaña et al., 2016). In the latter study, animals were treated with oxytocin for a longer period (14 days vs 10 days present study) and were subjected to a battery of behavioural tests (including, forced swim test (FST)) which may have influenced the results. For example, behavioural despair tests (FST) can increase oxytocin levels in the brain of male rats (Yan et al., 2014). Altogether, our work suggests that oxytocin similarly regulates adult hippocampal neurogenesis in males and females but based on previous research, the effects may be affected by length of exposure, neurogenesis endpoints (proliferation, maturation or survival), and interaction with experiences.

Nanoparticle increased oxytocin levels in the brain and reduced oxytocin sedation side effect

We found that delivery of oxytocin with the nanoparticle TRIOZAN™ increased the levels of oxytocin in the hypothalamus and reduced sedation and plasma oxytocin levels relative to delivery with PBS. Thus, we saw oxytocin delivered in TRIOZAN™ reduced peripheral effects by decreasing sedation and increased some central effects by increasing hypothalamic oxytocin levels relative to oxytocin delivered in PBS. Penetration of peripheral oxytocin into the brain is observed in small amounts (Neumann et al., 2013) calculated at ~0.002-0.003% (Mens et al., 1983), 0.1% (Jones and Robinson, 1982), and 1.3% (Ermisch et al., 1985) but sufficient to induce behavioural and brain changes in hippocampal neurogenesis and brain activation (present study; Dumais et al., 2017; Leuner et al., 2012; Ring et al., 2006; Sánchez-Vidaña et al., 2016; Uvnäs-Moberg et al., 1992). Indeed, for a number of our measures we did not observe large differences between oxytocin delivered in PBS or in TRIOZAN™.

In the current study, we observed sedation effects with both doses of oxytocin tested (0.5 and 1.0 mg/kg) similar to previous research showing that oxytocin causes sedation at high levels (0.25 and 1.0 mg/kg) and anxiolytic effects at lower levels (<0.004 mg/kg; Uvnäs-Moberg et al., 1994). However, female rats receiving oxytocin delivered in TRIOZANTM had less sedation compared to oxytocin in PBS which would suggest either a slow action or release of oxytocin in TRIOZANTM or a peripheral effect of oxytocin to cause sedation. There is evidence that oxytocin regulates skeletal muscle regeneration (Elabd et al., 2014) and that sleep is regulated by peripheral tissues such as skeletal muscle in mice (Ehlen et al., 2017). The nanoparticle TRIOZANTM can protect molecules from degradation (Ovensa Inc., 2018) and plasma levels of oxytocin were lower in the oxytocin in TRIOZANTM groups compared to oxytocin in PBS, which may suggest that oxytocin is more actively transported to tissues including the brain where it can either be stored or slowly released.

In our study, one limitation is that we cannot distinguish between endogenous and exogenous oxytocin. One possibility is that oxytocin delivered in TRIOZANTM increased endogenous production in the hypothalamus via indirect mechanisms (e.g., through projections from the periphery to the brain). Altogether, our work suggests that oxytocin in TRIOZANTM penetrated the brain but resulted in different behavioural and brain outcomes compared to oxytocin in PBS.

Interactions between oxytocin and 17 β -estradiol

In the present study, oxytocin treatment decreased plasma levels of 17 β -estradiol in both sexes. There is evidence that oxytocin and estrogens interact (reviewed in Engel et al., 2019) but to date, research has focused on the effect of estrogens on the oxytocin system (Lim and Young,

2006; McCarthy et al., 1996; Tribollet et al., 1990). In human females, oxytocin levels decreased during times of lower estradiol phases during the menstrual cycle (meta-analysis by Engel et al., 2019) and with menopause (Maestrini et al., 2018). To our knowledge, our study is the first demonstration that oxytocin regulates circulating 17β -estradiol levels indicating that the interaction between these hormones is bidirectional. It is possible that the oxytocin regulation of neurogenesis is affected by endogenous levels of estradiol and potentially other sex steroids, which should be investigated.

Summary

We found sex differences in the oxytocin regulation of social investigation and OT-ir cells in the SON of the hypothalamus. Oxytocin increased social investigation and the number of OT-ir cells in the SON in males but not females. In contrast, oxytocin decreased plasma 17β -estradiol and neurogenesis in the hippocampus of both males and females. We also show that the nanoparticle TRIOZANTM increased oxytocin levels in the hypothalamus in males and females and reduced sedation in females indicating that it can be a viable delivery system for oxytocin. Our study contributes to the evidence that oxytocin does not promote social investigation in both sexes.

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References

- Alexander, G.M., Farris, S., Pirone, J.R., Zheng, C., Colgin, L.L., Dudek, S.M., 2016. Social and novel contexts modify hippocampal CA2 representations of space. *Nat Commun* 7, 10300. <https://doi.org/10.1038/ncomms10300>
- Anacker, C., Luna, V.M., Stevens, G., Millette, A., Shores, R., Jimenez, J.C., Chen, B., Hen, R., 2018. Hippocampal neurogenesis confers stress resilience by inhibiting the ventral dentate gyrus. *Nature* 559, 98–102. <https://doi.org/10.1038/s41586-018-0262-4>
- Bannerman, D.M., Rawlins, J.N.P., McHugh, S.B., Deacon, R.M.J., Yee, B.K., Bast, T., Zhang, W.-N., Pothuizen, H.H.J., Feldon, J., 2004. Regional dissociations within the hippocampus—memory and anxiety. *Neuroscience & Biobehavioral Reviews, Festschrift in Honour of Jeffrey Gray - Issue 1: Anxiety and Neuroticism* 28, 273–283. <https://doi.org/10.1016/j.neubiorev.2004.03.004>
- Barker, J.M., Galea, L.A.M., 2008. Repeated estradiol administration alters different aspects of neurogenesis and cell death in the hippocampus of female, but not male, rats. *Neuroscience* 152, 888–902. <https://doi.org/10.1016/j.neuroscience.2007.10.071>
- Bredewold, R., Veenema, A.H., 2018. Sex differences in the regulation of social and anxiety-related behaviors: insights from vasopressin and oxytocin brain systems. *Current Opinion in Neurobiology, Neurobiology of Behavior* 49, 132–140. <https://doi.org/10.1016/j.conb.2018.02.011>
- Brown, J.P., Couillard-Després, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L., Kuhn, H.G., 2003. Transient expression of doublecortin during adult neurogenesis: Doublecortin Expression in Adult Neurogenesis. *Journal of Comparative Neurology* 467, 1–10. <https://doi.org/10.1002/cne.10874>
- Caldwell, H.K., 2018. Oxytocin and sex differences in behavior. *Current Opinion in Behavioral Sciences, Sex and Gender* 23, 13–20. <https://doi.org/10.1016/j.cobeha.2018.02.002>
- Cardia, M.C., Carta, A.R., Caboni, P., Maccioni, A.M., Erbi, S., Boi, L., Meloni, M.C., Lai, F., Sinico, C., 2019. Trimethyl Chitosan Hydrogel Nanoparticles for Progesterone Delivery in Neurodegenerative Disorders. *Pharmaceutics* 11. <https://doi.org/10.3390/pharmaceutics11120657>
- Churchland, P.S., Winkielman, P., 2012. Modulating social behavior with oxytocin: How does it work? What does it mean? *Hormones and Behavior* 61, 392–399. <https://doi.org/10.1016/j.yhbeh.2011.12.003>
- Clelland, C.D., Choi, M., Romberg, C., Clemenson, G.D., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., Bussey, T.J., 2009. A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science* 325, 210–213. <https://doi.org/10.1126/science.1173215>
- Crawley, J.N., 2004. Designing mouse behavioral tasks relevant to autistic-like behaviors. *Ment Retard Dev Disabil Res Rev* 10, 248–258. <https://doi.org/10.1002/mrdd.20039>
- Crawley, J.N., Chen, T., Puri, A., Washburn, R., Sullivan, T.L., Hill, J.M., Young, N.B., Nadler, J.J., Moy, S.S., Young, L.J., Caldwell, H.K., Young, W.S., 2007. Social approach behaviors in oxytocin knockout mice: Comparison of two independent lines tested in different laboratory environments. *Neuropeptides* 41, 145–163. <https://doi.org/10.1016/j.npep.2007.02.002>
- Danjo, T., Toyozumi, T., Fujisawa, S., 2018. Spatial representations of self and other in the hippocampus. *Science* 359, 213–218. <https://doi.org/10.1126/science.aao3898>

- DiBenedictis, B.T., Nussbaum, E.R., Cheung, H.K., Veenema, A.H., 2017. Quantitative mapping reveals age and sex differences in vasopressin, but not oxytocin, immunoreactivity in the rat social behavior neural network. *Journal of Comparative Neurology* 525, 2549–2570. <https://doi.org/10.1002/cne.24216>
- Dumais, K.M., Alonso, A.G., Bredewold, R., Veenema, A.H., 2016a. Role of the oxytocin system in amygdala subregions in the regulation of social interest in male and female rats. *Neuroscience* 330, 138–149. <https://doi.org/10.1016/j.neuroscience.2016.05.036>
- Dumais, K.M., Alonso, A.G., Immormino, M.A., Bredewold, R., Veenema, A.H., 2016b. Involvement of the oxytocin system in the bed nucleus of the stria terminalis in the sex-specific regulation of social recognition. *Psychoneuroendocrinology* 64, 79–88. <https://doi.org/10.1016/j.psyneuen.2015.11.007>
- Dumais, K.M., Bredewold, R., Mayer, T.E., Veenema, A.H., 2013. Sex differences in oxytocin receptor binding in forebrain regions: Correlations with social interest in brain region- and sex- specific ways. *Hormones and Behavior* 64, 693–701. <https://doi.org/10.1016/j.yhbeh.2013.08.012>
- Dumais, K.M., Kulkarni, P.P., Ferris, C.F., Veenema, A.H., 2017. Sex differences in neural activation following different routes of oxytocin administration in awake adult rats. *Psychoneuroendocrinology* 81, 52–62. <https://doi.org/10.1016/j.psyneuen.2017.04.003>
- Ehlen, J.C., Brager, A.J., Baggs, J., Pinckney, L., Gray, C.L., DeBruyne, J.P., Esser, K.A., Takahashi, J.S., Paul, K.N., 2017. Bmal1 function in skeletal muscle regulates sleep. *eLife* 6, e26557. <https://doi.org/10.7554/eLife.26557>
- Eid, R.S., Lieblich, S.E., Duarte-Guterman, P., Chaiton, J.A., Mah, A.G., Wong, S.J., Wen, Y., Galea, L.A.M., 2019. Selective activation of estrogen receptors α and β : Implications for depressive-like phenotypes in female mice exposed to chronic unpredictable stress. *Hormones and Behavior* 119, 104651. <https://doi.org/10.1016/j.yhbeh.2019.104651>
- Elabd, C., Cousin, W., Upadhyayula, P., Chen, R.Y., Chooljian, M.S., Li, J., Kung, S., Jiang, K.P., Conboy, I.M., 2014. Oxytocin is an age-specific circulating hormone that is necessary for muscle maintenance and regeneration. *Nat Commun* 5, 4082. <https://doi.org/10.1038/ncomms5082>
- Engel, S., Klusmann, H., Ditzen, B., Knaevelsrud, C., Schumacher, S., 2019. Menstrual cycle-related fluctuations in oxytocin concentrations: A systematic review and meta-analysis. *Frontiers in Neuroendocrinology* 52, 144–155. <https://doi.org/10.1016/j.yfrne.2018.11.002>
- Ermisch, A., Rühle, H.-J., Landgraf, R., Hess, J., 1985. Blood—Brain Barrier and Peptides. *Journal of Cerebral Blood Flow & Metabolism* 5, 350–357. <https://doi.org/10.1038/jcbfm.1985.49>
- Fanselow, M.S., Dong, H.-W., 2010. Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? *Neuron* 65, 7–19. <https://doi.org/10.1016/j.neuron.2009.11.031>
- Gobinath, A.R., Workman, J.L., Chow, C., Lieblich, S.E., Galea, L.A.M., 2016. Maternal postpartum corticosterone and fluoxetine differentially affect adult male and female offspring on anxiety-like behavior, stress reactivity, and hippocampal neurogenesis. *Neuropharmacology* 101, 165–178. <https://doi.org/10.1016/j.neuropharm.2015.09.001>
- Hawley, D.F., Morch, K., Christie, B.R., Leasure, J.L., 2012. Differential Response of Hippocampal Subregions to Stress and Learning. *PLOS ONE* 7, e53126. <https://doi.org/10.1371/journal.pone.0053126>

- Hess, L., Votava, M., Málek, J., Kurzová, A., Slíva, J., 2016. Sedative effects of intranasal oxytocin in rabbits and rhesus monkeys. *Physiol Res* 65, S473–S480.
- Jones, P.M., Robinson, I.C., 1982. Differential Clearance of Neurophysin and Neurohypophysial Peptides from the Cerebrospinal Fluid in Conscious Guinea Pigs. *NEN* 34, 297–302. <https://doi.org/10.1159/000123316>
- Jurek, B., Neumann, I.D., 2018. The Oxytocin Receptor: From Intracellular Signaling to Behavior. *Physiological Reviews* 98, 1805–1908. <https://doi.org/10.1152/physrev.00031.2017>
- Kempermann, G., Gage, F.H., Aigner, L., Song, H., Curtis, M.A., Thuret, S., Kuhn, H.G., Jessberger, S., Frankland, P.W., Cameron, H.A., Gould, E., Hen, R., Abrous, D.N., Toni, N., Schinder, A.F., Zhao, X., Lucassen, P.J., Frisén, J., 2018. Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell* 23, 25–30. <https://doi.org/10.1016/j.stem.2018.04.004>
- Kheirbek, M.A., Drew, L.J., Burghardt, N.S., Costantini, D.O., Tannenholz, L., Ahmari, S.E., Zeng, H., Fenton, A.A., Hen, R., 2013. Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron* 77, 955–968. <https://doi.org/10.1016/j.neuron.2012.12.038>
- Ko, J., 2017. Neuroanatomical Substrates of Rodent Social Behavior: The Medial Prefrontal Cortex and Its Projection Patterns. *Front Neural Circuits* 11. <https://doi.org/10.3389/fncir.2017.00041>
- Ku, K.M., Weir, R.K., Silverman, J.L., Berman, R.F., Bauman, M.D., 2016. Behavioral Phenotyping of Juvenile Long-Evans and Sprague-Dawley Rats: Implications for Preclinical Models of Autism Spectrum Disorders. *PLOS ONE* 11, e0158150. <https://doi.org/10.1371/journal.pone.0158150>
- Kulkarni, A.D., Patel, H.M., Surana, S.J., Vanjari, Y.H., Belgamwar, V.S., Pardeshi, C.V., 2017. N,N,N-Trimethyl chitosan: An advanced polymer with myriad of opportunities in nanomedicine. *Carbohydrate Polymers* 157, 875–902. <https://doi.org/10.1016/j.carbpol.2016.10.041>
- Kumar, M., Pandey, R.S., Patra, K.C., Jain, S.K., Soni, M.L., Dangi, J.S., Madan, J., 2013. Evaluation of neuropeptide loaded trimethyl chitosan nanoparticles for nose to brain delivery. *International Journal of Biological Macromolecules* 61, 189–195. <https://doi.org/10.1016/j.ijbiomac.2013.06.041>
- Kumaran, D., Banino, A., Blundell, C., Hassabis, D., Dayan, P., 2016. Computations Underlying Social Hierarchy Learning: Distinct Neural Mechanisms for Updating and Representing Self-Relevant Information. *Neuron* 92, 1135–1147. <https://doi.org/10.1016/j.neuron.2016.10.052>
- Leasure, J.L., Decker, L., 2009. Social isolation prevents exercise-induced proliferation of hippocampal progenitor cells in female rats. *Hippocampus* 19, 907–912. <https://doi.org/10.1002/hipo.20563>
- Leng, G., Ludwig, M., 2016. Intranasal Oxytocin: Myths and Delusions. *Biological Psychiatry* 79, 243–250. <https://doi.org/10.1016/j.biopsych.2015.05.003>
- Leong, K.-C., Zhou, L., Ghee, S.M., See, R.E., Reichel, C.M., 2016. Oxytocin decreases cocaine taking, cocaine seeking, and locomotor activity in female rats. *Exp Clin Psychopharmacol* 24, 55–64. <https://doi.org/10.1037/pha0000058>

- Leuner, B., Caponiti, J.M., Gould, E., 2012. Oxytocin stimulates adult neurogenesis even under conditions of stress and elevated glucocorticoids. *Hippocampus* 22, 861–868. <https://doi.org/10.1002/hipo.20947>
- Lim, M.M., Young, L.J., 2006. Neuropeptidergic regulation of affiliative behavior and social bonding in animals. *Hormones and Behavior, Translational Topics in Behavioral Neuroendocrinology* 50, 506–517. <https://doi.org/10.1016/j.yhbeh.2006.06.028>
- Lin, Y.-T., Chen, C.-C., Huang, C.-C., Nishimori, K., Hsu, K.-S., 2017. Oxytocin stimulates hippocampal neurogenesis via oxytocin receptor expressed in CA3 pyramidal neurons. *Nature Communications* 8. <https://doi.org/10.1038/s41467-017-00675-5>
- Lopatina, O., Liu, H.-X., Amina, S., Hashii, M., Higashida, H., 2010. Oxytocin-induced elevation of ADP-ribosyl cyclase activity, cyclic ADP-ribose or Ca²⁺ concentrations is involved in autoregulation of oxytocin secretion in the hypothalamus and posterior pituitary in male mice. *Neuropharmacology* 58, 50–55. <https://doi.org/10.1016/j.neuropharm.2009.06.012>
- Lu, W., 2012. Adsorptive-mediated brain delivery systems. *Curr Pharm Biotechnol* 13, 2340–2348. <https://doi.org/10.2174/138920112803341851>
- Lukas, M., Neumann, I.D., 2014. Social preference and maternal defeat-induced social avoidance in virgin female rats: Sex differences in involvement of brain oxytocin and vasopressin. *Journal of Neuroscience Methods, Measuring Behavior* 234, 101–107. <https://doi.org/10.1016/j.jneumeth.2014.03.013>
- Lukas, M., Toth, I., Reber, S.O., Slattery, D.A., Veenema, A.H., Neumann, I.D., 2011. The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. *Neuropsychopharmacology* 36, 2159.
- Maestrini, S., Mele, C., Mai, S., Vietti, R., Blasio, A.D., Castello, L., Surico, D., Aimaretti, G., Scacchi, M., Marzullo, P., 2018. Plasma Oxytocin Concentration in Pre- and Postmenopausal Women: Its Relationship with Obesity, Body Composition and Metabolic Variables. *OFA* 11, 429–439. <https://doi.org/10.1159/000492001>
- Mahmoud, R., Wainwright, S.R., Galea, L.A.M., 2016. Sex hormones and adult hippocampal neurogenesis: Regulation, implications, and potential mechanisms. *Frontiers in Neuroendocrinology* 41, 129–152. <https://doi.org/10.1016/j.yfrne.2016.03.002>
- Matta, R., Tiessen, A.N., Choleris, E., 2017. The Role of Dorsal Hippocampal Dopamine D1-Type Receptors in Social Learning, Social Interactions, and Food Intake in Male and Female Mice. *Neuropsychopharmacology* 42, 2344–2353. <https://doi.org/10.1038/npp.2017.43>
- McCarthy, M.M., McDonald, C.H., Brooks, P.J., Goldman, D., 1996. An anxiolytic action of oxytocin is enhanced by estrogen in the mouse. *Physiology & behavior* 60, 1209–1215.
- McClure, R.E.S., Barha, C.K., Galea, L.A.M., 2013. 17 β -Estradiol, but not estrone, increases the survival and activation of new neurons in the hippocampus in response to spatial memory in adult female rats. *Horm Behav* 63, 144–157. <https://doi.org/10.1016/j.yhbeh.2012.09.011>
- McCormick, C.M., Nixon, F., Thomas, C., Lowie, B., Dyck, J., 2010. Hippocampal cell proliferation and spatial memory performance after social instability stress in adolescence in female rats. *Behav. Brain Res.* 208, 23–29. <https://doi.org/10.1016/j.bbr.2009.11.003>
- McCormick, C.M., Thomas, C.M., Sheridan, C.S., Nixon, F., Flynn, J.A., Mathews, I.Z., 2012. Social instability stress in adolescent male rats alters hippocampal neurogenesis and

produces deficits in spatial location memory in adulthood. *Hippocampus* 22, 1300–1312.
<https://doi.org/10.1002/hipo.20966>

Mens, W.B.J., Witter, A., Van Wimersma Greidanus, T.B., 1983. Penetration of neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): Half-times of disappearance of these neuropeptides from CSF. *Brain Research* 262, 143–149.
[https://doi.org/10.1016/0006-8993\(83\)90478-X](https://doi.org/10.1016/0006-8993(83)90478-X)

Modi, M.E., Young, L.J., 2012. The oxytocin system in drug discovery for autism: Animal models and novel therapeutic strategies. *Hormones and Behavior* 61, 340–350.
<https://doi.org/10.1016/j.yhbeh.2011.12.010>

Mourya, V.K., Inamdar, N.N., 2008. Trimethyl chitosan and its applications in drug delivery. *J Mater Sci: Mater Med* 20, 1057. <https://doi.org/10.1007/s10856-008-3659-z>

Nadler, J.J., Moy, S.S., Dold, G., Simmons, N., Perez, A., Young, N.B., Barbaro, R.P., Piven, J., Magnuson, T.R., Crawley, J.N., 2004. Automated apparatus for quantitation of social approach behaviors in mice. *Genes, Brain and Behavior* 3, 303–314.
<https://doi.org/10.1111/j.1601-183X.2004.00071.x>

Neumann, I., Douglas, A.J., Pittman, Q.J., Russell, J.A., Landgraf, R., 1996. Oxytocin Released within the Supraoptic Nucleus of the Rat Brain by Positive Feedback Action is Involved in Parturition-Related Events. *Journal of Neuroendocrinology* 8, 227–233.
<https://doi.org/10.1046/j.1365-2826.1996.04557.x>

Neumann, I.D., Maloumby, R., Beiderbeck, D.I., Lukas, M., Landgraf, R., 2013. Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. *Psychoneuroendocrinology* 38, 1985–1993.
<https://doi.org/10.1016/j.psyneuen.2013.03.003>

Okuyama, T., Kitamura, T., Roy, D.S., Itohara, S., Tonegawa, S., 2016. Ventral CA1 neurons store social memory. *Science* 353, 1536–1541. <https://doi.org/10.1126/science.aaf7003>

Omer, D.B., Maimon, S.R., Las, L., Ulanovsky, N., 2018. Social place-cells in the bat hippocampus. *Science* 359, 218–224. <https://doi.org/10.1126/science.aao3474>

Opendak, M., Offit, L., Monari, P., Schoenfeld, T.J., Sonti, A.N., Cameron, H.A., Gould, E., 2016. Lasting Adaptations in Social Behavior Produced by Social Disruption and Inhibition of Adult Neurogenesis. *J. Neurosci.* 36, 7027–7038.
<https://doi.org/10.1523/JNEUROSCI.4435-15.2016>

Ormerod, B.K., Lee, T.T.-Y., Galea, L.A.M., 2004. Estradiol enhances neurogenesis in the dentate gyri of adult male meadow voles by increasing the survival of young granule neurons. *Neuroscience* 128, 645–654. <https://doi.org/10.1016/j.neuroscience.2004.06.039>

Ovensa Inc., 2018. TRIOZAN™ Nanomedicine Delivery Platform. <http://ovensa.com/triozan-nanomedicine-delivery-platform/> (accessed October 28, 2019).

Paxinos, G., Watson, C., 2005. The rat brain in stereotaxic coordinates. 5th ed. San Diego: Elsevier Academic Press; 2005.

Peragine, D.E., Simpson, J.A., Mooney, S.J., Lovern, M.B., Holmes, M.M., 2014. Social regulation of adult neurogenesis in a eusocial mammal. *Neuroscience* 268, 10–20.
<https://doi.org/10.1016/j.neuroscience.2014.02.044>

Peragine, D.E., Yousuf, Y., Fu, Y., Swift-Gallant, A., Ginzberg, K., Holmes, M.M., 2016. Contrasting effects of opposite- versus same-sex housing on hormones, behavior and neurogenesis in a eusocial mammal. *Hormones and Behavior* 81, 28–37.
<https://doi.org/10.1016/j.yhbeh.2016.03.005>

- 796 Raam, T., McAvoy, K.M., Besnard, A., Veenema, A.H., Sahay, A., 2017. Hippocampal oxytocin
797 receptors are necessary for discrimination of social stimuli. *Nat Commun* 8, 1–14.
798 <https://doi.org/10.1038/s41467-017-02173-0>
- 799 Ramos, L., Hicks, C., Kevin, R., Caminer, A., Narlawar, R., Kassiou, M., McGregor, I.S., 2013.
800 Acute prosocial effects of oxytocin and vasopressin when given alone or in combination
801 with 3,4-methylenedioxymethamphetamine in rats: involvement of the V1A receptor.
802 *Neuropsychopharmacology* 38, 2249–2259. <https://doi.org/10.1038/npp.2013.125>
- 803 Rilling, J.K., DeMarco, A.C., Hackett, P.D., Chen, X., Gautam, P., Stair, S., Haroon, E.,
804 Thompson, R., Ditzen, B., Patel, R., Pagnoni, G., 2014. Sex differences in the neural and
805 behavioral response to intranasal oxytocin and vasopressin during human social
806 interaction. *Psychoneuroendocrinology* 39, 237–248.
807 <https://doi.org/10.1016/j.psyneuen.2013.09.022>
- 808 Ring, R.H., Malberg, J.E., Potestio, L., Ping, J., Boikess, S., Luo, B., Schechter, L.E., Rizzo, S.,
809 Rahman, Z., Rosenzweig-Lipson, S., 2006. Anxiolytic-like activity of oxytocin in male
810 mice: behavioral and autonomic evidence, therapeutic implications. *Psychopharmacology*
811 185, 218–225. <https://doi.org/10.1007/s00213-005-0293-z>
- 812 Sánchez-Vidaña, D.I., Chan, N.-M.J., Chan, A.H.L., Hui, K.K.Y., Lee, S., Chan, H.-Y., Law,
813 Y.S., Sze, M.Y., Tsui, W.-C.S., Fung, T.K.H., Lau, B.W.-M., Lai, C.Y.Y., 2016.
814 Repeated treatment with oxytocin promotes hippocampal cell proliferation, dendritic
815 maturation and affects socio-emotional behavior. *Neuroscience* 333, 65–77.
816 <https://doi.org/10.1016/j.neuroscience.2016.07.005>
- 817 Smith, C.J.W., Poehlmann, M.L., Li, S., Ratnaseelan, A.M., Bredewold, R., Veenema, A.H.,
818 2017. Age and sex differences in oxytocin and vasopressin V1a receptor binding
819 densities in the rat brain: focus on the social decision-making network. *Brain Structure*
820 and Function 222, 981–1006. <https://doi.org/10.1007/s00429-016-1260-7>
- 821 Snyder, J.S., Soumier, A., Brewer, M., Pickel, J., Cameron, H.A., 2011. Adult hippocampal
822 neurogenesis buffers stress responses and depressive behaviour. *Nature* 476, 458–461.
823 <https://doi.org/10.1038/nature10287>
- 824 Steinman, M.Q., Duque-Wilckens, N., Greenberg, G.D., Hao, R., Campi, K.L., Laredo, S.A.,
825 Laman-Maharg, A., Manning, C.E., Doig, I.E., Lopez, E.M., Walch, K., Bales, K.L.,
826 Trainor, B.C., 2016. Sex-Specific Effects of Stress on Oxytocin Neurons Correspond
827 With Responses to Intranasal Oxytocin. *Biological Psychiatry* 80, 406–414.
828 <https://doi.org/10.1016/j.biopsych.2015.10.007>
- 829 Stranahan, A.M., Khalil, D., Gould, E., 2006. Social isolation delays the positive effects of
830 running on adult neurogenesis. *Nat Neurosci* 9, 526–533. <https://doi.org/10.1038/nn1668>
- 831 Strange, B.A., Witter, M.P., Lein, E.S., Moser, E.I., 2014. Functional organization of the
832 hippocampal longitudinal axis. *Nature Reviews Neuroscience* 15, 655–669.
833 <https://doi.org/10.1038/nrn3785>
- 834 Tribollet, E., Audigier, S., Dubois-Dauphin, M., Dreifuss, J.J., 1990. Gonadal steroids regulate
835 oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An
836 autoradiographical study. *Brain Research* 511, 129–140. [https://doi.org/10.1016/0006-8993\(90\)90232-Z](https://doi.org/10.1016/0006-8993(90)90232-Z)
- 837
838 Tzeng, W.-Y., Wu, H.-H., Wang, C.-Y., Chen, J.-C., Yu, L., Cherng, C.G., 2016. Sex
839 Differences in Stress and Group Housing Effects on the Number of Newly Proliferated
840 Cells and Neuroblasts in Middle-Aged Dentate Gyrus. *Front Behav Neurosci* 10, 249.
841 <https://doi.org/10.3389/fnbeh.2016.00249>

- Uvnäs-Moberg, K., Ahlenius, S., Hillegaart, V., Alster, P., 1994. High doses of oxytocin cause sedation and low doses cause an anxiolytic-like effect in male rats. *Pharmacology Biochemistry and Behavior* 49, 101–106. [https://doi.org/10.1016/0091-3057\(94\)90462-6](https://doi.org/10.1016/0091-3057(94)90462-6)
- Uvnäs-Moberg, K., Alster, P., Hillegaart, V., Ahlenius, S., 1992. Oxytocin reduces exploratory motor behaviour and shifts the activity towards the centre of the arena in male rats. *Acta Physiologica Scandinavica* 145, 429–430. <https://doi.org/10.1111/j.1748-1716.1992.tb09385.x>
- Wang, S., Jiang, T., Ma, M., Hu, Y., Zhang, J., 2010. Preparation and evaluation of anti-neuroexcitation peptide (ANEP) loaded N-trimethyl chitosan chloride nanoparticles for brain-targeting. *International Journal of Pharmaceutics* 386, 249–255. <https://doi.org/10.1016/j.ijpharm.2009.11.002>
- Workman, J.L., Gobinath, A.R., Kitay, N.F., Chow, C., Brummelte, S., Galea, L.A.M., 2016. Parity modifies the effects of fluoxetine and corticosterone on behavior, stress reactivity, and hippocampal neurogenesis. *Neuropharmacology* 105, 443–453. <https://doi.org/10.1016/j.neuropharm.2015.11.027>
- Wu, M.V., Hen, R., 2014. Functional dissociation of adult-born neurons along the dorsoventral axis of the dentate gyrus. *Hippocampus* 24, 751–761. <https://doi.org/10.1002/hipo.22265>
- Yan, Y., Wang, Y.-L., Su, Z., Zhang, Y., Guo, S.-X., Liu, A.-J., Wang, C.-H., Sun, F.-J., Yang, J., 2014. Effect of oxytocin on the behavioral activity in the behavioral despair depression rat model. *Neuropeptides* 48, 83–89. <https://doi.org/10.1016/j.npep.2014.01.001>
- Yoshida, M., Takayanagi, Y., Inoue, K., Kimura, T., Young, L.J., Onaka, T., Nishimori, K., 2009. Evidence That Oxytocin Exerts Anxiolytic Effects via Oxytocin Receptor Expressed in Serotonergic Neurons in Mice. *J. Neurosci.* 29, 2259–2271. <https://doi.org/10.1523/JNEUROSCI.5593-08.2009>

FIGURES

Figure 1. Oxytocin treatment caused sedation, increased plasma oxytocin and reduced

plasma 17 β -estradiol. **A.** Sedation scores in male and female rats immediately after injection

with either PBS, oxytocin (OT, 0.5 or 1.0 mg/kg) or oxytocin in TRIOZANTM (OTT, 0.5 or

1.0mg/kg). Sedation scores higher than 0 (i.e. 0 = no sedation) were observed after oxytocin

injection and not in the PBS controls. Males had higher sedation scores than females in the low

doses of oxytocin, regardless of delivery method (*** indicates significantly different from

males treated with oxytocin, OT and OTT 0.5 mg/kg; p<0.001). In females, oxytocin in

TRIOZANTM (OTT 0.5 mg/kg) resulted in lower sedation scores relative to oxytocin in PBS (OT

0.5 mg/kg; δ indicates significant difference from OT 0.5; p=0.05) and relative to oxytocin in

TRIOZANTM (1.0 mg/kg; β indicates significant difference between OTT 0.5 and OTT 1.0; p=

0.0001). **B.** Oxytocin treatment increased plasma levels of oxytocin in males and females relative

to controls (*** p<0.001) and oxytocin in TRIOZANTM (OTT 1.0 mg/kg) resulted in lower

plasma oxytocin compared to oxytocin in PBS (OT 1.0 mg/kg; $\delta\delta\delta$ indicates significant

difference from OT 1.0; p<0.001). **C.** Oxytocin treatment reduced plasma 17 β -estradiol levels in

females and males relative to PBS controls (*** p<0.001). In females, all doses and delivery

methods reduced 17 β -estradiol levels. In males both doses of oxytocin in TRIOZANTM reduced

17 β -estradiol in plasma. In addition, the highest dose of oxytocin in TRIOZANTM (OTT 1.0

mg/kg) resulted in lower plasma 17 β -estradiol relative to oxytocin in PBS (OT 1.0 mg/kg; $\delta\delta\delta$

indicates significant difference from OT 1.0; p<0.001).

Figure 2. Oxytocin delivered in TRIOZANTM increased oxytocin levels in the

hypothalamus. **A.** Oxytocin levels measured by ELISA in the hypothalamus were significantly

higher in oxytocin in TRIOZANTM (OTT, 1.0mg/kg) compared to oxytocin in PBS in both sexes (OT, 1.0 mg/kg; indicated by δ , $p < 0.05$). **B.** Ratio of hypothalamus to plasma levels of oxytocin were significantly lower in all oxytocin treatment groups (** $p < 0.001$) but oxytocin delivered in TRIOZANTM (OTT, 1.0 mg/kg) resulted in a higher ratio compared to oxytocin in PBS (OT, 1.0 mg/kg; indicated by $\delta\delta$, $p < 0.01$). **C.** In the PVN, the number of OT-ir cells was not affected by oxytocin treatment. **D.** There were more OT-ir cells in the SON of male rats treated with oxytocin in PBS (OT, 1.0 mg/kg) and in TRIOZANTM (OTT, 0.5 and 1.0 mg/kg) relative to PBS controls ($p < 0.05$). The number of OT-ir cells in the SON was higher in oxytocin delivered in TRIOZANTM (OTT, 0.5 mg/kg) compared to oxytocin in PBS (OT, 0.5 mg/kg; indicated by δ , $p < 0.05$). Representative images showing OT-ir cells in the PVN (**C**) and SON (**D**).

Figure 3. Oxytocin increased social investigation in male but not female rats in the three chambered social approach test. **A.** Three-chambered apparatus for quantifying social investigation. **B.** Oxytocin did not affect the percent time spent on social stimulus side. **C.** In males, oxytocin in PBS (OT 0.5 mg/kg) increased time spent investigating social stimulus relative to control ($p < 0.05$).

Figure 4. Oxytocin affected adult neurogenesis in a region- and delivery-dependent way. **A.** Representative images of DCX+ cells in the granule cell layer of the hippocampus and the three types of DCX+ cells based on morphology (scale bar is 10 μ m). **B.** In the dorsal hippocampus, oxytocin in PBS (OT 1.0 mg/kg) decreased the number of DCX+ cells in males and females relative to PBS controls ($p < 0.05$). **C.** In the ventral hippocampus, oxytocin in PBS (OT 0.5 and 1.0 mg/kg) decreased the number of DCX+ cells in males and females ($p < 0.001$). In females,

oxytocin delivered in TRIOZANTM (OTT 1.0 mg/kg) decreased the number of DCX+ cells relative to PBS controls ($p < 0.001$). In both sexes, oxytocin delivered in TRIOZANTM (OTT 0.5 mg/kg) increased the number of DCX+ cells relative to oxytocin in PBS (OT 0.5 mg/kg; $\delta\delta$ indicates significantly different from OT 0.5, $p < 0.01$).

Figure 5. Number of DCX+ cells was significantly associated with plasma 17 β -estradiol in females and social investigation in males. Increased numbers of DCX+ cells were significantly associated with increased 17 β -estradiol in the dorsal (**A**, $r(31) = 0.44$; $p = 0.01$) and ventral (**B**, $r(31) = 0.48$; $p = 0.005$) in female but not male rats ($p > 0.80$). In females, the correlation between DCX+ cells and 17 β -estradiol was observed in the PBS control (dorsal, $r(6) = 0.84$, $p = 0.009$; ventral, $r(6) = 0.70$, $P = 0.05$) but not in any of the oxytocin groups (p 's > 0.4). Increased DCX+ cell counts in the ventral hippocampus were associated with decreased social investigation in males (**C**, $r(32) = -0.40$, $p = 0.03$) but not females ($r(32) = 0.07$, $p = 0.71$).

Table 1. Mean (SEM) body mass at the start of injections (g), net body mass gain (g), and adrenal mass/body mass in male and female rats treated with oxytocin in PBS (OT 0.5 and 1.0 mg/kg) and in TRIOZANTM (OTT 0.5 and 1.0 mg/kg). Asterisks denote significant differences from control PBS males (p<0.05). ^a Denotes main effect of sex (p<0.001).

Treatment	Sex	Sample Size	Initial body mass ^a (g)	Net body mass gain ^a (g)	Adrenal mass/body mass ^a
PBS	F	8	252.13 (3.90)	21.38 (3.01)	0.025 (0.001)
	M	8	416.38 (13.49)	55.00 (5.70)	0.015 (0.001)
OT 0.5	F	8	248.38 (2.34)	16.50 (2.01)	0.028 (0.001)
	M	8	411.63 (11.25)	37.88 (4.79)*	0.013 (0.001)
OT 1	F	8	255.50 (2.06)	11.50 (2.78)	0.026 (0.002)
	M	8	406.00 (8.87)	29.50 (6.64)*	0.013 (0.001)
OTT 0.5	F	5	253.40 (5.85)	10.80 (5.80)	0.023 (0.003)
	M	5	390.20 (6.26)	25.60 (5.60)*	0.016 (0.001)
OTT 1	F	5	254.20 (6.03)	14.80 (2.60)	0.024 (0.003)
	M	5	393.80 (4.42)	19.60 (9.50)*	0.014 (0.001)

Table 2. Total (SEM) amount of time (in seconds) spent in locomotion, grooming, and rearing during the habituation of the three-chamber approach test in male and female rats treated with oxytocin in PBS (OT 0.5 and 1.0 mg/kg) and in TRIOZANTM (OTT 0.5 and 1.0 mg/kg). Asterisks denote significantly different from PBS (p<0.05). ^a Denotes main sex effect (p<0.01).

Treatment	Sex	Sample Size	Locomotion (s)	Grooming (s)	Rearing ^a (s)
PBS	F	8	202.24 (18.45)	13.75 (2.66)	169.80 (9.73)
	M	8	168.94 (13.41)	13.97 (4.09)	137.43 (16.70)
OT 0.5	F	8	185.90 (14.44)	12.17 (3.13)	141.74 (20.30)*
	M	8	171.14 (33.88)	11.20 (3.76)	85.18 (20.26)*
OT 1	F	8	184.48 (19.38)	9.24 (2.67)	119.18 (9.21)*
	M	8	129.49 (11.92)	8.20 (2.11)	81.97 (12.28)*
OTT 0.5	F	5	166.29 (21.60)	14.95 (5.68)	144.29 (15.33)
	M	5	157.94 (17.04)	11.66 (2.73)	116.69 (13.33)
OTT 1	F	5	149.51 (15.41)	9.84 (7.28)	112.86 (15.76)
	M	5	136.56 (22.45)	22.41 (6.67)	90.50 (16.86)

Table 3. Percent time spent (SEM) in each of the chambers during the social behaviour test in male and female rats treated with oxytocin in PBS (OT 0.5 and 1.0 mg/kg) and in TRIOZANTM (OTT 0.5 and 1.0 mg/kg). Rats spent more time in the social stimulus side compared to the empty and middle chambers, regardless of treatment and sex (^a denotes a significant main effect of side; $p < 0.0001$).

Treatment	Sex	Sample Size	Time in Social Stimulus Chamber ^a	Time in Empty Chamber	Time in Middle Chamber
PBS	F	8	60.13 (3.52)	28.35 (3.03)	11.52 (1.18)
	M	8	53.34 (4.50)	23.79 (4.35)	22.84 (5.82)
OT 0.5	F	8	58.83 (1.41)	29.26 (1.91)	11.9 (2.23)
	M	8	66.92 (3.51)	22.17 (3.45)	10.91 (1.98)
OT 1	F	8	54.00 (4.51)	26.15 (3.01)	19.84 (5.58)
	M	8	64.59 (4.13)	19.21 (3.47)	16.23 (3.41)
OTT 0.5	F	5	59.73 (3.70)	27.92 (3.75)	12.35 (2.47)
	M	5	61.12 (2.65)	25.96 (2.10)	12.92 (2.15)
OTT 1	F	5	53.30 (7.10)	24.60 (5.20)	22.09 (10.64)
	M	5	60.07 (4.72)	27.36 (4.80)	12.57 (2.79)

Table 4. Mean (SEM) dorsal and ventral volumes (mm³) of the granule cell layer (GCL) of the right hippocampus and percent of DCX+ cells in different stages of maturity (proliferative, type 1; intermediate, type 2; post-mitotic, type 3) in male and female rats treated with oxytocin in PBS (OT 0.5 and 1.0 mg/kg) and in TRIOZANTM (OTT 0.5 and 1.0 mg/kg). Males had significantly larger GCL volume compared to females (main effect of sex; $p < 0.05$). Ventral volumes were larger than dorsal volumes (main effect of region; $p < 0.0001$). Oxytocin treatments did not affect the volume of the GCL (all p 's > 0.11). There were significantly more type 3 DCX+ cells relative to type 1 and 2 (main effect of type of DCX+ cell; $p < 0.0001$). Oxytocin treatments did not affect the proportion of DCX+ cells in each stage of maturation (all p 's > 0.4).

Treatment	Sex	Sample Size	Dorsal GCL Volume	Ventral GCL Volume	Dorsal			Ventral		
					Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
PBS	F	8	0.58 (0.04)	1.09 (0.07)	22.50 (3.55)	31.67 (1.92)	45.83 (2.76)	31.04 (2.78)	35.00 (1.70)	33.96 (2.11)
	M	8	0.71 (0.05)	1.16 (0.11)	24.22 (3.00)	28.21 (2.71)	47.57 (2.94)	32.92 (3.18)	33.33 (3.42)	33.75 (2.69)
OT 0.5	F	8	0.63 (0.06)	1.02 (0.08)	24.72 (3.64)	28.90 (2.75)	46.38 (3.31)	34.66 (2.98)	32.56 (2.71)	32.78 (2.70)
	M	8	0.65 (0.06)	1.15 (0.08)	26.08 (3.67)	27.57 (1.75)	46.36 (4.67)	34.09 (3.59)	33.47 (3.63)	32.44 (2.17)
OT 1	F	8	0.52 (0.03)	1.02 (0.06)	30.07 (2.90)	27.97 (2.55)	41.96 (2.18)	35.20 (2.70)	34.17 (2.83)	30.63 (2.58)
	M	8	0.68 (0.03)	1.19 (0.10)	26.88 (4.48)	27.08 (2.15)	46.04 (4.76)	33.83 (3.22)	34.65 (1.16)	31.52 (3.16)
OTT 0.5	F	5	0.65 (0.10)	1.09 (0.04)	23.21 (6.57)	29.06 (2.71)	47.73 (5.48)	30.43 (4.89)	35.10 (3.12)	34.47 (3.66)
	M	5	0.71 (0.06)	1.23 (0.07)	27.44 (6.53)	24.74 (1.90)	47.82 (5.03)	32.33 (3.82)	31.67 (2.47)	36.00 (3.44)
OTT 1	F	5	0.61 (0.02)	0.86 (0.05)	27.08 (7.13)	28.76 (3.26)	44.16 (4.26)	30.00 (5.53)	35.00 (1.90)	35.00 (4.94)
	M	5	0.64 (0.05)	1.28 (0.08)	27.33 (3.82)	30.33 (1.43)	42.33 (3.23)	27.67 (4.67)	32.67 (3.44)	39.67 (2.32)

Figure 1

Figure 2

Figure 3

A Three-chambered social paradigm

B Male Female

Figure 4

Figure 5