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Sex differences in neural activation following different routes of oxytocin administration in awake adult rats

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Abstract

The neuropeptide oxytocin (OT) regulates social behavior in sex-specific ways across species. OT has promising effects on alleviating social deficits in sex-biased neuropsychiatric disorders. However little is known about potential sexually dimorphic effects of OT on brain function. Using the rat as a model organism, we determined whether OT administered centrally or peripherally induces sex differences in brain activation. Functional magnetic resonance imaging was used to examine blood oxygen level-dependent (BOLD) signal intensity changes in the brains of awake rats during the 20 min following intracerebroventricular (ICV; 1µg/5µl) or intraperitoneal (IP; 0.1mg/kg) OT administration as compared to baseline. ICV OT induced sex differences in BOLD activation in 26 out of 172 brain regions analyzed, with 20 regions showing a greater volume of activation in males (most notably the nucleus accumbens and insular cortex), and 6 regions showing a greater volume of activation in females (including the lateral and central amygdala). IP OT also elicited sex differences in BOLD activation with a greater volume of activation in males, but this activation was found in different and fewer (10) brain regions compared to ICV OT. In conclusion, exogenous OT modulates neural activation differently in male versus female rats with the pattern and magnitude, but not the direction, of sex differences depending on the route of administration. These findings highlight the need to include both sexes in basic and clinical studies to fully understand the role of OT on brain function.

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Contributions

Authors Kelly M. Dumais, Alexa H. Veenema, and Craig F. Ferris designed the study. Author Kelly M. Dumais performed all experiments and analyzed the data. Author Praveen P. Kulkarni assisted with data analysis. Authors Kelly M. Dumais and Alexa H. Veenema wrote the manuscript, and Craig F. Ferris and Praveen P. Kulkarni edited the manuscript. All authors have approved the final manuscript.

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Keywords

oxytocin; sex differences; amygdala; nucleus accumbens; fMRI; rat

1. Introduction

Oxytocin (OT) is a neuropeptide synthesized mainly in the paraventricular and supraoptic nuclei of the hypothalamus (Buijs and Swaab, 1979). OT acts through the OT receptor (OTR) centrally as a neuromodulator and peripherally as a hormone via release from the posterior pituitary (Buijs and Swaab, 1979; Knobloch et al., 2012). Across species, OT regulates various social behaviors (Guastella and MacLeod, 2012; Veenema and Neumann, 2008) and often does so in sex-specific ways (Dumais and Veenema, 2016a, 2016b). For example, in humans, intranasal OT administration impaired social recognition memory and emotion perception in men, but not women (Herzmann et al., 2013; Lynn et al., 2014). In rats, intracerebroventricular (ICV) OT administration enhanced social recognition and reversed social defeat-induced social avoidance in males (Benelli et al., 1995; Lukas et al., 2011) but not females (Engelmann et al., 1998; Lukas and Neumann, 2014).

These sex-specific effects of OT on behavior suggest sex differences in neural activation in response to OT. Indeed, functional magnetic resonance imaging (fMRI) studies in humans have begun to uncover sex differences in neural activation following intranasal OT (Domes et al., 2007, 2010; Rilling et al., 2014). Surprisingly, fMRI studies examining neural activation in response to OT in awake rats have used males only (Ferris et al., 2015) or lactating females only (Febo et al., 2005). In male rats, ICV OT induced BOLD activation in brain regions such as the bed nucleus of the stria terminalis and lateral septum (Ferris et al., 2015). Interestingly, these brain regions show higher OTR binding density in male compared to female rats (Dumais et al., 2013) and are involved in sex-specific regulation of social behavior by OT (Bredewold et al., 2014; Dumais et al., 2016b). This suggests the potential for OT to modulate brain activation differently in male versus female rats.

Because OT does not readily pass the blood brain barrier, with only up to 1.3% of peripheral OT entering the brain (Mens et al., 1983; Ermisch et al., 1985) it is still debated whether OT administered via peripheral routes, including intranasal administration, directly affects brain activity (Leng and Ludwig, 2016). Therefore, it is important to understand how central versus peripheral routes of OT administration modulate neural activity, and whether these activation patterns are different in males compared to females. Rats can be a useful model to investigate potential sex differences in neural activation in response to OT because of the ability to compare different routes of administration including a direct route into the brain. To this end, we examined blood oxygen level dependent (BOLD) activation in awake male and female rats following central (ICV) or peripheral (intraperitoneal; IP) OT administration. The sex-specific effects of OT on social behavior (Dumais et al., 2016a, 2016b) as well as the sex differences in OTR binding density (Dumais et al., 2013) in rats led us to hypothesize that ICV OT administration would elicit sex differences in neural activation. Because peripheral OT has hampered access to the brain (Mens et al., 1983; Ermisch et al., 1985), and knowledge of sex differences in peripheral OTR is limited (except

for on reproductive systems; Gimpl and Fahrenholz, 2001), we hypothesized that there would be fewer sex differences in neural activation following IP OT administration compared to ICV administration.

2. Methods

2.1 Animals

Adult male (300–325g) and female (280–300g) Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA), and were given at least one week to acclimate to the facilities at Northeastern University. Rats were maintained on a 12 h light/dark cycle (lights on at 0800 h) and food and water were available *ad libitum*. Rats were housed in standard Plexiglas cages (13 × 8.25 × 7 in) in same-sex pairs until 3–5 days before the first day of scanning at which they were housed individually. All experiments were conducted according to the guidelines of the NIH and approved by the Northeastern University and Boston College Institutional Animal Care and Use Committees.

2.2 Acclimation for awake imaging

To reduce the stress associated with awake imaging, rats were acclimated to the imaging procedure (according to King et al., 2005) once a day for four days prior to the first imaging session. Rats were briefly anesthetized for securement into a padded head holder and a body tube to restrict overall movement. When fully conscious, restrained rats were placed into a black opaque box (“mock scanner”) for 30 min with a tape-recording of the MRI pulse sequence. This procedure was previously found to reduce respiration, heart rate, motor movements, and plasma corticosterone levels when comparing the first and last acclimation periods (King et al., 2005).

2.3 Stereotaxic surgery

For ICV injections, rats were cannulated with custom-designed cannulas created for compatibility with MRI (Plastics One, Roanoke, VA) 1–2 days after the last acclimation session. Using isoflurane as an anesthetic and a stereotaxic frame, guide cannulae were implanted unilaterally 1.5 mm dorsal to the lateral ventricle (0.5 mm caudal to bregma, –1.5 mm lateral to midline, 3.0 mm ventral to the skull surface) according to Paxinos and Watson (2007). Guide cannulae were fixed to the skull with plastic screws and acrylic glue. Scanning was performed 3 and 5 days after surgery. Only scans with cannula placements correctly aimed at the lateral ventricle (Fig. 1) were used in analyses.

2.4 Oxytocin preparation and administration

Separate cohorts of rats were used for ICV and IP injections. Each rat was scanned during two imaging sessions, two days apart, receiving either vehicle (Ringer’s solution for ICV; 0.9% NaCl for IP) or OT (1 µg/5 µl ICV, 0.1 mg/kg IP; Sigma, St. Louis, MO) in counterbalanced order. Doses of OT were chosen because they were effective in eliciting BOLD responses in adult male (Ferris et al., 2015) and lactating female (Febo et al., 2005) rats. For ICV injections, polyethylene tubing was connected to an injector cannula extending 1.5 mm beyond the guide cannula and approximately 30 cm in length to attach to a 10 µl

Hamilton syringe. For IP injections, polyethylene tubing was positioned in the peritoneal cavity and extended approximately 30 cm to attach to a 10 ml syringe.

2.5 MRI procedures

Rats were briefly anesthetized and positioned into a quadrature transmit/receive volume coil built into a head holder and restraining system (Animal Imaging Research, Holden, MA). Experiments were conducted using a Bruker Biospec 7.0T/20-cm USR horizontal magnet (Bruker, Billerica, MA), and a 20-G/cm magnetic field gradient insert (inner diameter 12 cm; 120 μ s rise time). The scanner was controlled by Bruker Paravision 5.1, which automatically finds the basic frequency, shims, power requirements for 90° and 180° pulses, and sets the receiver gain. Functional images were collected using a multi-slice half-Fourier acquisition single-shot turbo spin echo (HASTE) pulse sequence (22 slices; 1.1 mm thick; FOV 3.0 cm; matrix size 96 X 96; TR 6.0 s, TE 48 ms; NEX 1), providing an in-plane voxel resolution of 312 μ m². A total of 250 functional acquisitions were collected, for a total functional scan time of 25 min. Out of these 250 acquisitions, the first 50 acquisitions (acquisitions 1–50; min 1–5) were baseline acquisitions. Acute vehicle or OT injections were given immediately after the 5 min baseline, and was followed by a 20 min post-treatment period (acquisitions 51–250; min 5–25; see Fig. 1 for functional imaging paradigm). This continuous functional scan was chosen to be consistent with previous rodent fMRI studies (Ferris et al., 2015) and has the advantage of comparing BOLD signal intensity during the 20 min treatment period with the baseline scan within each subject. Moreover, behavioral changes are observed in rats within 20 min after OT injections (Benelli et al., 1995; Lukas et al., 2011).

2.6 Data analysis

Full details of the MRI data analysis have been previously reported (Ferris et al., 2008). Briefly, pre-processing included brain extraction, slice time correction, motion correction, drift correction, and smoothing. Motion correction and slice time correction was applied using SPM8's co-registrational code (quality: 0.97; spatial separation: 0.45 mm, interpolation: 2nd degree B-spline; resliced interpolation: 4th degree B-spline). In addition, an SPM smoothing algorithm was used with smoothing FWHM set at 0.8 mm (~2×2 voxel) in all axis. These pre-processing steps generated mean images of functional MRI data for each subject. Using Medical Image Analysis and Visualization software (MIVA), pre-processed mean functional images were then aligned and registered (by translating, rotating, and scaling each subject's anatomy) to a segmented and annotated 3D MRI rat brain atlas (Ekam Solutions, Boston, MA), which is delineated into 172 distinct regions of interest (ROIs). All transformed pixel locations of the mean images were tagged with the segmented atlas ROIs, creating a fully segmented representation of all ROIs for each subject in its own space.

Statistical t-tests were then performed on each voxel (~ 36,000) to determine differences between baseline BOLD signal intensity and post-injection BOLD signal intensity, using a baseline threshold of 2% BOLD change to account for normal fluctuations of BOLD signal in the rat brain under the awake condition (Brevard et al., 2003). The t-tests used a 95% confidence level ($p < 0.05$), two-tailed distributions, and heteroscedastic variance

assumptions. As a result of the multiple t tests performed, a false-positive detection controlling mechanism was introduced (Genovese et al., 2002). This subsequent filter guaranteed that, on average, the false positive detection rate was below our cutoff of 0.05.

The formulation of the filter satisfied the following expression: $P_{(i)} \leq \frac{i}{V} \frac{q}{c(V)}$, where $P_{(i)}$ is the p value based on the t test analysis. Each voxel (i) within the region of interest (ROI) containing (V) voxels was ranked based on its probability value. The false-positive filter value q was set to be 0.2, and the predetermined constant $c(V)$ was set to unity (Benjamini and Hochberg 1995). These analysis settings provided conservative estimates for significance.

These statistical t tests determined the number of voxels that were significantly different during post-injection compared to baseline (i.e., number of activated voxels). The number of activated voxels within each ROI (volume of activation) was then compared between treatment conditions (i.e., vehicle-treated males vs vehicle-treated females, vehicle-treated males vs OT-treated males, vehicle-treated females vs OT-treated females, OT-treated males vs OT-treated females) using the non-parametric Kruskal-Wallis test and a between-subject analysis. We compared the volume of activation between treatment conditions during the first 10 min post injection (Min 1–10) and the second 10 min post injection (Min 10–20). The breakdown into Min 1–10 and 10–20 was based on, and was used to be consistent with, a previous rodent fMRI study showing different patterns of activation between these two periods in response to ICV and IP OT in male rats (Ferris et al., 2015). To rule out differences in treatment conditions due to differences in motion, motion artifact was determined by analysis of voxel displacement and spikes of raw data activity over time. Motion (calculated as the average voxel displacement in the x- y- and z- plane of each subject over the 25 min imaging period) was compared between each treatment condition using unpaired, two-tailed t tests (reported as average $\pm$ SEM). Volume of activation and motion was considered to be different between experimental conditions when comparisons yielded p values less than 0.05.

Final group sizes were as follows: ICV vehicle treatment (males= 9, females=14), ICV OT treatment (males=10, females=11); IP vehicle treatment (males=12, females=11), IP OT treatment (males=9, females= 9). Estrus phase was determined via vaginal smears (according to Dumais et al., 2013) taken immediately following scanning. All female rats were observed to be cycling normally, and all stages of estrous were represented at scanning. Although not directly analyzed due to too low sample size for the different stages, results suggest that estrous cycle stage did not affect OT-induced activation because BOLD signal variability (as interpreted by the average standard deviation of BOLD signal change of all ROIs was no greater in females than in males.

3. Results

3.1 BOLD activation in response to ICV OT administration

3.1.1 ICV OT induced robust activation throughout the brain, including many regions dense in OTR—ICV OT induced positive BOLD activation in more brain regions in males than in females during the 20 min imaging period (Fig. 2). Specifically,

compared to vehicle-treated rats, ICV OT-treated males showed a greater volume of positive BOLD activation in 41 regions, and ICV OT-treated females showed a greater volume of positive BOLD activation in 26 brain regions. Most notable were numerous cortical and subcortical regions that showed BOLD activation in ICV OT-treated males but not females.

Eleven brain regions dense in OTR (Dumais et al., 2013; Smith et al., 2016; Tribollet et al., 1988) showed a greater volume of positive BOLD activation in OT-treated rats compared to vehicle-treated rats (Fig. 2, highlighted in gray). These regions were the insular cortex and nucleus accumbens shell (Min 1–10 and Min 10–20), ventral subiculum and lateral hypothalamus (Min 1–10), and anterior olfactory nucleus, paraventricular hypothalamus, and ventromedial hypothalamus (Min 10–20) in males, and the perirhinal cortex and basal amygdala (Min 1–10 and Min 10–20), and nucleus accumbens core and medial preoptic area (Min 1–10) in females.

Furthermore, compared to vehicle, the volume of negative BOLD activation was greater in OT-treated males in 7 brain regions and in OT-treated females in 21 brain regions (see Table S1 for details).

3.1.2 ICV OT induced robust sex differences in neural activation—A direct comparison between ICV OT-treated males and ICV OT-treated females revealed that ICV OT induced sex differences in the volume of positive BOLD activation in 26 brain regions during the 20 min imaging period (Fig. 3). Compared to ICV OT-treated females, ICV OT-treated males showed a greater volume of activation in 20 regions, including the ventral pallidum (Min 1–10), glomerular and external plexiform layers of the olfactory bulb (Min 10–20), and nucleus accumbens shell (Min 1–10 and Min 10–20; Fig. 3, 4). Compared to ICV OT-treated males, ICV OT-treated females showed a greater volume of activation in six regions, including the central amygdala (Min 1–10) and the lateral amygdala (Min 1–10 and Min 10–20; Fig. 3, 4).

Importantly, brain regions that showed ICV OT-induced sex differences in positive BOLD activation did not show sex differences in ICV vehicle-treated rats, with two notable exceptions. First, the insular cortex is less activated in males compared to females under vehicle treatment, but is more activated in males compared to females after ICV OT treatment (Fig. 3, 4; Table S2). Second, the vestibular nucleus is more activated in males compared to females under vehicle treatment, but is less activated in males compared to females after ICV OT treatment (Fig. 3, 4; Table S2).

Sex differences in negative BOLD volume of activation following ICV OT were found in 7 brain regions (see Table S3 for details).

3.2 BOLD activation in response to IP OT administration

3.2.1 IP OT induced robust activation throughout the brain, but in few regions dense in OTR—IP OT induced positive BOLD activation in a similar number of brain regions in males and females during the 20 min imaging period (Fig. 5). Specifically, compared to vehicle, IP OT-treated males showed a greater volume of positive BOLD activation in 31 regions, and IP OT-treated females showed a greater volume of positive

BOLD activation in 35 brain regions. In both sexes, several of these activated brain regions were located within the olfactory system, cerebellum, and brainstem. Notably, compared to vehicle, IP OT-treated females showed activation in more cortical and brainstem regions during Min 10–20 compared to males.

Five brain regions dense in OTR (Dumais et al., 2013; Smith et al., 2016; Tribollet et al., 1988) showed a greater volume of positive BOLD activation in OT-treated rats compared to vehicle-treated rats (Fig. 5, highlighted in gray). These were the nucleus accumbens shell (Min 1–10 and Min 10–20) and medial amygdala and perirhinal cortex (Min 10–20) in males, and the anterior olfactory nucleus (Min 1–10 and Min 10–20) and dorsal subiculum (Min 10–20) in females.

Finally, compared to vehicle, the volume of negative BOLD activation was greater in OT-treated males in 18 brain regions and in OT-treated females in 23 brain regions (see Table S4 for details).

3.2.2 IP OT induced few sex differences in neural activation—A direct comparison between IP OT-treated males and IP OT-treated females revealed that IP OT induced sex differences in positive BOLD activation in 10 brain regions, with all regions showing a greater volume of activation in OT-treated males (Fig. 6). These brain regions included the arcuate nucleus, premammillary nucleus, and root of trigeminal nerve (Min 1–10), and cortical amygdala, medial amygdala, and paraflocculus cerebellum (Min 10–20). Brain regions that showed sex differences in positive BOLD activation in response to IP OT did not show sex differences in IP vehicle-treated rats (Table S5), except for the medial geniculate and the paraflocculus cerebellum which also showed a greater volume of activation in vehicle-treated males compared to vehicle-treated females.

Sex differences in negative BOLD activation following IP OT were found in 4 brain regions (all showing a greater volume of activation in OT-treated females; Table S3).

3.3 Effects of sex and treatment on motion

Voxel displacement did not differ between sexes or between treatment groups (see Table 1 for details).

4. Discussion

This is the first rodent study to compare BOLD activation patterns between males and females in response to different routes of OT administration. ICV OT induced sex differences in more (26 versus 10) and in distinct (overlap in only 1) brain regions compared to IP OT. Interestingly, of the sex differences observed, males showed a greater volume of positive BOLD activation than females in nearly all brain regions following ICV OT (20 out of 26 regions) and in all brain regions following IP OT (10 out of 10 regions). We also show that motion parameters were not different between any groups, indicating that motion did not contribute to these treatment and sex effects. Overall, these results show that exogenous OT induces robust sex differences in neural activation, and that the pattern and the

magnitude, but not the direction, of these sex differences strongly depend on the route of administration.

4.1 Males show more BOLD activation in olfactory and reward processing regions following ICV OT

Following ICV OT, males showed a greater volume of positive BOLD activation compared to females in brain regions involved in social olfactory processing (olfactory bulb layers, olfactory tubercle, caudal piriform cortex) and in motivated/rewarding behaviors (nucleus accumbens shell, ventral pallidum). Interestingly, ICV OT facilitates social recognition and increases social approach (toward conspecifics that previously defeated them) in male but not female rats (Benelli et al., 1995; Engelmann et al., 1998; Lukas et al., 2011; Lukas and Neumann, 2014). It would therefore be interesting to determine whether the observed sex differences in olfactory and reward processing brain regions following ICV OT may contribute to the sex-specific regulation of these social behaviors by OT. Importantly, OT administration in humans also induced sex-specific activation of the nucleus accumbens. In detail, intranasal OT increased nucleus accumbens activation in response to reciprocated cooperation in men, but not women (Rilling et al., 2014). Because humans rely heavily on visual processing of social cues, as opposed to olfactory processing used by rodents (Baum and Cherry, 2015), it may be relevant to examine sex differences in activation of the visual system following exogenous OT in humans. Interestingly, high levels of OTR binding density are found in the olfactory system in rats and in the visual system in humans (Freeman and Young, 2016). This seems to support the idea that OT may serve as a social salience modulator by acting on the regions that are important for species-specific social information processing (Shamay-Tsoory and Abu-Akel, 2015).

4.2 Females show more BOLD activation in the amygdala following ICV OT

We recently showed that adult male and female rats have similar levels of endogenous OT release (Dumais et al., 2016a) and similar OTR binding density (Dumais et al., 2013; Smith et al., 2016) in the central amygdala. However, we now show that the response to exogenous OT elicits sex-specific activation of the amygdala. In detail, following ICV OT, females showed a greater volume of positive BOLD activation in the lateral and central amygdala compared to males. This is of particular interest because the amygdala is involved in social and emotional processing, and is a core region of sex-specific activation by OT in humans. For example, intranasal OT increased fear-induced amygdala activation in women, but decreased fear-induced amygdala activation in men (Domes et al., 2007, 2010). The opposite effect was found in response to positive social interactions, such that intranasal OT decreased amygdala activation in women, but increased amygdala activation in men, during cooperative social exchanges (Rilling et al., 2012, 2014). Given these findings in humans, it would be interesting to examine whether the direction of sex-specific activation in the amygdala following ICV OT in rats depends on exposure to negative or positive social stimuli. Furthermore, these data suggests that, across species, the amygdala is a site of sex-specific activation by exogenous OT.

4.3 Sex-specific activation of the insular cortex following ICV OT

Vehicle-treated females showed a greater volume of positive BOLD activation in the insular cortex compared to vehicle-treated males. The insular cortex links interoceptive information processing with emotional/social salience by integrating homeostatic, visceral, and nociceptive inputs with emotional/social cues (Craig, 2002). Sex differences have been found in the structure and function of the insular cortex, with female rats showing greater dendritic branching than male rats (Kolb and Stewart, 1991) and women showing higher insular cortex activity in response to negative images than men (Andreano et al., 2014). If the latter finding extrapolates to rats, then the sex difference in BOLD activation in the insular cortex in rats could reflect a sex difference in response to a negative or stressful stimulus, i.e., the imaging procedure. Indeed, as male and female rats show different stress responses following restraint stress (Gagliano et al., 2014), it is possible that males and females may respond to the acclimation procedure and/or imaging procedure differently, and therefore experience different levels of arousal and/or stress during imaging. Interestingly, OT reversed this pattern of insula activation, such that OT-treated males showed a greater volume of positive BOLD activation than OT-treated females. Strikingly, in humans, intranasal OT induced higher insular cortex activation in men compared to women during reciprocated cooperation (Rilling et al., 2014). Moreover, it has been suggested that a dysregulation of insular cortex-OT may contribute to social “self” deficits characteristic of autism, a male-biased social disorder (Quattrocki and Friston, 2014). Together, this suggests that rats could serve as a model system to gain insights into the mechanisms by which OT modulates insula-mediated emotional and social functioning in sex-specific ways, which, in turn, may be used to guide further studies in typically developing and autistic men and women.

4.4 Activation patterns following ICV OT administration: Potential mechanisms of action

As expected, several brain regions dense in OTR binding showed greater BOLD activation following ICV OT compared to ICV vehicle in both sexes. However, many regions not dense in OTR binding were also activated. This may be explained by indirect activation (i.e., activation of downstream targets of OTR-dense brain regions) reflecting the highly interconnected neuronal network of the rat brain (Gozzi and Schwarz, 2016). This could also be due to activation of the vasopressin 1a receptor (V1aR) by OT (Manning et al., 2012). Indeed, a number of brain regions not dense in OTR but highly dense in V1aR (i.e., suprachiasmatic nucleus, ventral anterior thalamus, somatosensory cortex; Dumais et al., 2013; Smith et al., 2016) were activated by OT. Likewise, sex differences in BOLD activation were found in regions that show sex differences in OTR binding density (i.e., insular cortex, paraventricular nucleus of the hypothalamus, ventromedial hypothalamus, perirhinal cortex; Dumais et al., 2013; Smith et al., 2016), in regions that show similar OTR binding density between the sexes (i.e., ventral subiculum, lateral hypothalamus, basal amygdala; Smith et al., 2016), and in regions that do not show dense OTR binding at all (glomerular layer; Ferris et al., 2015). Therefore, sex differences in OTR binding densities may explain several, but not all, of the sex-specific activation patterns following ICV OT. Other possibilities, such as sex differences in affinity of OT to OTR or in modes of action of the OTR (i.e., direct post-synaptic excitation or indirect modulation of synaptic inputs upon OT binding; Joëls, 2000; Raggenbass, 2001) may account for some sex-specific activation

patterns. While these hypotheses need further investigation, our findings demonstrate that BOLD activation, as well as sex-specific BOLD activation, occurs in more brain regions than can be predicted solely by OTR distribution in the male and female brain, and suggests that additional mechanisms play a role in the activation patterns elicited by ICV OT.

4.5 Sex-specific BOLD activation patterns following IP OT

Contrary to our hypothesis, there were also sex differences in BOLD activation following IP OT, albeit in fewer brain regions compared to ICV OT. These sex differences could be mediated by peripheral OT gaining access to the brain, although only up to 1.3% of peripheral OT is estimated to reach the brain (Mens et al., 1983; Ermisch et al., 1985). Therefore, it is likely that these sex differences are primarily mediated through OTR-expressing peripheral systems (Gimpl and Fahrenholz, 2001) that project via the vagus nerve to the brain or have spinal afferents to the brain (Menetrey and De Pommery, 1991). Interestingly, connections from the testis and ovaries, which both express OTR (Gimpl and Fahrenholz, 2001), to the brain (Lee et al., 2002; Ortega-Villalobos et al., 1990) are sexually dimorphic (Gerendai et al., 2002). This sex-specific innervation may provide one possible mechanism by which IP OT induced sex differences in BOLD activation. Our findings further suggest that IP OT may have sexually dimorphic effects on behavior. Though IP OT injections have been found to modulate various social behaviors in male and female rats (Holley et al., 2015; Ramos et al., 2015), direct comparisons between the sexes are lacking. These comparisons could provide insight into the behavioral consequences of the observed sex-specific BOLD activation patterns following IP OT.

4.6 BOLD activation following ICV OT and IP OT: Males show higher activation compared to females

Surprisingly, the total number of brain regions that showed BOLD activation following IP OT (males: 31 regions; females: 35 regions) was roughly at the same magnitude to those following ICV OT (males: 41; females 26 regions). This robust modulation of neural activity by IP OT may explain the behavioral changes seen after IP OT (Holley et al., 2015; Ramos et al., 2015). However, compared to ICV OT, sex differences in BOLD activation following IP OT were in different and far fewer brain regions. These distinct activation patterns likely reflect different modes of action of ICV OT (direct central mechanism) versus IP OT (indirect central mechanism) as discussed above. Interestingly, irrespective of route of administration, males showed a greater volume of BOLD activation compared to females in response to OT. This is an important observation because it suggests an overall higher sensitivity to exogenous OT in males versus females. However, this could be a dose dependent effect. It seems common practice in both animal and human studies to only use a single dose of OT to determine sex differences (Domes et al., 2007, 2010; Lukas et al., 2011; Lukas and Neumann, 2014; Rilling et al., 2012, 2014). Therefore, testing multiple doses of OT in both human and animal studies will be an essential next step to gain a better understanding of true sex differences in response to OT. Yet, the observed sex differences in response to a single dose of OT in the current study as well as in other studies may indicate that there are sex differences in the peripheral and central OT systems. Indeed, the central OT system is structurally and functionally sexually dimorphic in rats (reviewed in Dumais and Veenema, 2016a, 2016b), but whether this is also true for the peripheral OT system is

mostly unknown. Likewise, sex comparison studies on peripheral or central OT system parameters in humans are very limited (reviewed in Dumais and Veenema, 2016a, 2016b).

4.7 Considerations and future directions

The OT-induced BOLD activation patterns in male rats are generally consistent with our previous report (Ferris et al., 2015), with some differences. For example, we replicated the IP OT-induced activation in the olfactory system and brainstem (Ferris et al., 2015). We also replicated the ICV OT-induced activation in OTR-dense brain regions such as the nucleus accumbens shell, olfactory tubercles, and subiculum (Ferris et al., 2015), but found additional ICV OT-induced activation in the ventromedial hypothalamus, paraventricular nucleus of the hypothalamus, anterior olfactory nucleus, and insular cortex. These differences may be due to different ICV injection procedures (tubing placement immediately prior to scanning in Ferris et al. [2015] versus permanent cannula placement in the current study). However, the additional ICV-OT induced activation in the anterior olfactory nucleus and paraventricular hypothalamus has also been reported by others using Fos as a marker of neuronal activation (Ludwig et al., 2013).

When imaging awake animals, there is concern for the stress associated with head and body restraint. Therefore, protocols have been made to acclimate rats to the imaging procedure (see Methods). However, while signs of autonomic arousal and stress are reduced with acclimation (King et al., 2005), they are not eliminated entirely. Thus, the OT-induced changes in BOLD activation may have occurred against a backdrop of heightened arousal and stress. In addition, because OT injections were given in the absence of social stimuli, our paradigm represents a marked difference when compared to the task-based paradigms that are often used in human research. Therefore, our results may serve as a model for sex-specific activation profiles in response to OT under baseline conditions, which then can be compared with OT-induced activation following stimulus presentation.

Finally, while ICV and IP routes of administration in rats give valuable insight into how central versus peripheral OT modulate brain activation, it is unclear how this translates to intranasal OT administration, which is the preferred route in humans. Despite numerous human studies, it is still unknown whether intranasal OT administration modulates brain activation through central (via the nasal epithelium) or peripheral routes, or both (Churchland and Winkelman, 2012; Evans et al., 2014; Leng and Ludwig, 2016). Importantly, a recent study using fMRI in mice shows that activation patterns elicited by intranasal OT administration is distinct from activation elicited by peripheral OT administration (Galbusera et al., 2017), suggesting, at least partly, a central mechanism of action of intranasal OT. Future studies using intranasal OT in rats and humans and blocking peripheral effects of OT will be valuable in gaining a better understanding of how intranasal OT modulates brain activation and whether this occurs in sex-specific ways. This is also important given the sex-specific behavioral effects of intranasal OT administration in both rodents (Bales et al., 2013, 2014; Steinman et al., 2016) and humans (Herzmann et al., 2013; Lynn et al., 2014; Rilling et al., 2014; Patel et al., 2015).

5. Conclusions

The present work demonstrates that exogenous OT administration activated the rat brain in sex-specific ways, albeit more so after central than after peripheral OT administration. Importantly, irrespective of route, males showed a greater volume of BOLD activation in response to OT than females. Moreover, the notable similarities between rats and humans in the OT-induced sex-specific activation of the amygdala and insular cortex may provide an opportunity to use rats as a model system to further investigate the underlying mechanisms of these cross-species sex differences. Overall, these findings in rats provide incentive for more comprehensive investigation into OT-induced sex differences in neural activation and its behavioral consequences. In particular, it may be interesting to determine whether intranasal OT, the typical route of OT administration in humans, induces a BOLD activation pattern that resembles the pattern seen after ICV OT or IP OT, or perhaps induces a unique pattern. Given the potential of OT to alleviate social dysfunction in a range of sex-biased neuropsychiatric disorders (Hofmann et al, 2015), a better understanding of the sex-specific modulation of OT on brain function will be essential in order to determine whether OT can be used to treat social dysfunction in men and women alike.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- BOLD activation was analyzed in male and female rat brains after oxytocin injection
- Males showed a greater volume of activation following oxytocin compared to females
- Central oxytocin induced sex differences in more regions than peripheral oxytocin
- Males showed more activation in the nucleus accumbens following central oxytocin
- Females showed more activation in the amygdala following central oxytocin

Figure 1. Intracerebroventricular cannula placement and functional imaging paradigm

Anatomical image depicting a coronal section of the brain of an adult male rat showing the location of the guide cannula with injector directed at the lateral ventricle with a black arrow (left). Diagram showing the paradigm used for functional imaging (right). Functional imaging sessions consisted of a 25 min scan, in which an acute injection of OT or vehicle was administered after a 5 min baseline.

ICV Injections, Positive BOLD Volume of Activation														
Males					Females									
Min 1-10														
Region of Interest	Vehicle	OT	p value	Vehicle	OT	Region of Interest	Vehicle	OT	p value	Vehicle	OT			
	med	med					med	med						
cortical	insular ctx	0	3.5	0.001	cortical	perirhinal ctx	0	5	0.005	cortical				
	prelimbic ctx	0	5.5	0.003		lateral amygdala	0	14	0.006					
	cortical amygdala	0	5	0.004		ventral lateral striatum	0	3	0.010					
	secondary motor ctx	0	7.5	0.004		basal amygdala	0	3	0.017					
	caudal piriform ctx	0	3.5	0.012		lateral preoptic area	0	0.5	0.024					
	ventral subiculum	0	13	0.018		zona incerta	0	0.5	0.026					
	primary somatosensory ctx jaw	0	4.5	0.018		nucleus accumbens core	0	1	0.033					
	lateral orbital ctx	0	3	0.019		medial preoptic area	0	9	0.034					
	frontal association ctx	2	40.5	0.02		anterior hypothalamic area	0	12	0.034					
	primary motor ctx	0	11.5	0.021		white matter	11	21	0.04					
subcortical	temporal ctx	0	0.5	0.039	subcortical	ventral pallidum	0	0.5	0.042	subcortical				
	visual 2 ctx	0	1.5	0.047		central gray	0	0.5	0.042					
	substantia nigra reticularis	0	15	0.002		root of trigeminal nerve	1	8	0.038					
	interpeduncular nucleus	0	34	0.003		facial nucleus	0	3	0.04					
	nucleus accumbens shell	0	3.5	0.007										
	reuniens nucleus	0	8	0.023										
	ventral anterior thalamic nucleus	0	17	0.023										
	lateral hypothalamus	0	14.5	0.025										
	lateral posterior thalamic nucleus	0	1.5	0.027										
	olfactory tubercles	4	27.5	0.027										
brainstem/cerebellum	white matter	5	12.5	0.033	brainstem/cerebellum				brainstem/cerebellum					
	suprachiasmatic nucleus	0	0.5	0.039										
	ventrolateral thalamic nucleus	0	11	0.047										
	ventral tegmental area	0	5	0.048										
	lateral dorsal thalamic nucleus	0	33.5	0.049										
	perilovary nucleus	0	32	0.001										
	pontine nuclei	0	28	0.001										
	inferior olivary complex	0	74	0.006										
	trapezoid body	0	5	0.008										
	paraflocculus cerebellum	0	11	0.011										
pontine reticular nucleus oral	0	0.5	0.018											
flocculus cerebellum	0	0.5	0.039											
Min 10-20														
Region of Interest	Vehicle	OT	p value	Vehicle	OT	Region of Interest	Vehicle	OT	p value	Vehicle	OT			
	med	med				med	med							
cortical	prelimbic ctx	1	14.5	0.006	cortical	perirhinal ctx	1	4	0.008	cortical				
	insular ctx	0	9.5	0.026		insular ctx	9.5	1	0.030					
	primary somatosensory ctx upper	0	1	0.038		primary somatosensory ctx jaw	19	1	0.039					
	reuniens nucleus	0	17	0.003		caudal piriform ctx	2.5	0	0.043					
	anterior olfactory nucleus	3	16	0.006		central gray	0	0.5	0.006					
	ventral anterior thalamic nucleus	0	15	0.015		lateral preoptic area	0	4	0.008					
	nucleus accumbens shell	1	8	0.015		basal amygdala	0	9	0.011					
	white matter	8	13	0.017		posterior hypothalamic area	0	6	0.036					
	diagonal band of Broca	7	50	0.026		claustrum	0	0.5	0.042					
	paraventricular hypothalamic nucleus	0	22	0.037		paraflocculus cerebellum	4	21	0.005					
subcortical	posterior hypothalamic area	0	20.5	0.04	subcortical	interposed nucleus	0	0.5	0.006	subcortical				
	ventromedial hypothalamus	6	52.5	0.041		dorsal paraflocculus	0	5	0.012					
	substantia nigra compacta	0	11	0.042		nucleus	5	17	0.015					
	pontine reticular nucleus oral	0	7	0.003		vestibular nucleus	1	12	0.016					
	pontine nuclei	19	59	0.004		medial cerebellar nucleus	0	0.5	0.016					
	dorsal paraflocculus	5	0	0.008		fastigial	0	0.5	0.016					
	nucleus	5	0	0.008		first cerebellar lobule	0	0.5	0.042					
	trapezoid body	0	7.5	0.017		facial nucleus	0	8	0.045					

Gray shading = Regions with dense OTR binding (Tribollet et al., 1988; Dumais et al., 2013)

Black text = Regions showing sex differences in OTR binding density (Dumais et al., 2013; Smith et al., 2016)

Gray shading = Regions with dense OTR binding (Tribollet et al., 1988; Dumais et al., 2013)

Bold text = Regions showing sex differences in OTR binding density (Dumais et al., 2013; Smith et al., 2016)

Figure 2. ICV OT activated many brain regions in both sexes, but more so in male than in female rats

Listed are brain regions that showed differences in the volume of positive BOLD activation following ICV OT versus ICV vehicle within each sex ($p < 0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1–10) or second 10 minutes (Min 10–20) post vehicle or OT injection. Brain regions are clustered into cortical, subcortical, and brainstem/cerebellum regions. The 3D color model depicts the location of the listed brain regions in yellow. Positive BOLD responses for each voxel were averaged across subjects within each experimental condition, and in red is the composite average of voxels that showed a significant increase in positive BOLD compared to baseline. Brain regions highlighted in gray in the table show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013), while all other brain regions either do not show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013; Smith et al., 2016), or have not been examined for OTR binding density. Brain regions in bold show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016). ctx, cortex.

Listed are brain regions that showed sex differences in the volume of positive BOLD activation following ICV OT ($p < 0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1–10) or second 10 minutes (Min 10–20). In the left column are brain regions that showed a greater volume of activation in OT-treated males compared to OT-treated females, and in the right column are brain regions that showed a greater volume of activation in OT-treated females compared to OT-treated males. Brain regions are clustered into olfactory bulb, cortical, subcortical, pituitary, and brainstem regions. The 3D color model depicts the location of the listed brain regions within each time point in yellow. Positive BOLD responses for each voxel were averaged across subjects within each experimental condition, and in red is the composite average of voxels that showed a significant increase in positive BOLD compared to baseline in regions that show a greater volume of activation in males (OT Males) and in regions that show a greater volume of activation in females (OT Females). Brain regions highlighted in gray in the table show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013), while all other brain regions either do not show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013; Smith et al., 2016), or have not been

examined for OTR binding density. Brain regions in bold show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016). ctx, cortex.

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Figure 4. Selection of brain regions that showed sex or sex x treatment effects in positive BOLD activation following ICV OT

(A) Volume of positive BOLD activation was greater in OT-treated males compared to OT-treated females and vehicle-treated males in the nucleus accumbens (NAc) shell, and greater in OT-treated males compared to OT-treated females in the ventral pallidum (VP). (B) Volume of positive BOLD activation was greater in OT-treated females compared to OT-treated males and vehicle-treated females in the lateral amygdala (LA), and greater in OT-treated females compared to OT-treated males in the central amygdala (CeA). (C) Volume of positive BOLD activation in the insular cortex was greater in vehicle-treated females compared to vehicle-treated males and OT-treated females and greater in OT-treated males compared to vehicle-treated males and OT-treated females. Volume of positive BOLD activation in the vestibular nucleus was greater in vehicle-treated males compared to vehicle-treated females and greater in OT-treated females compared to vehicle-treated females and OT-treated males. Bar graphs represent median number of voxels showing positive BOLD activation in each condition during Min 1–10 (LA, CeA, insular cortex) or Min 10–20 (NAc, VP, vestibular nucleus). Numbers above each bar show the minimum and maximum number of voxels showing positive BOLD activation in each condition. Activation maps of positive BOLD activation are shown on coronal images of the rat brain atlas. Time-course plots show the percent change in positive BOLD signal over time. * $p < 0.05$, versus other sex in same treatment condition, Kruskal-Wallis test; # $p < 0.05$, versus same-sex vehicle condition, Kruskal-Wallis test.

IP Injections, Positive BOLD Volume of Activation												
Males						Females						
Min 1-10												
Region of Interest		Vehicle	OT		Vehicle	OT	Region of Interest		Vehicle	OT		
cortical	olfactory bulb	external plexiform layer	0	10	0.001		external plexiform layer	0	3.5	0.001
		granular cell layer	0	18	0.005			granular cell layer	0	24.5		0.001
		glomerular layer	0.5	29	0.007			granular cell layer	0	2		0.001
		rostral piriform ctb	5.5	17	0.041			frontal association ctb	0	9		0.001
		arcuate nucleus						lateral orbital ctb	0	4		0.001
	subcortical	hypothalamus	0	2	0.023	prelimbic ctb	0	0.5	0.019			
		medial prefrontal area	0.5	0	0.03	secondary motor ctb	0	5	0.049			
		premamillary nucleus	0	3	0.031	anterior olfactory nucleus	0	5	0.004			
		magnocellular preoptic nucleus	0	0.5	0.035	parafascicular thalamic nucleus	0	0.5	0.04			
		nucleus accumbens shell	0	0.5	0.036	nucleus accumbens shell	0	0.5	0.04			
subcortical	ventral pallidum	lateral preoptic area	0	0.5	0.036	ventral pallidum	0	0.5	0.04			
		ventral pallidum	0	0.5	0.036	reticular nucleus midbrain	0	2.5	0.01			
		neural lobe pituitary	0	5	0.037	lemniscal nucleus	0	0.5	0.019			
		root of trigeminal nerve	0	20	0.009	simple lobule cerebellum	0	1	0.031			
		principal sensory nucleus				flocculus cerebellum	0	0.5	0.04			
	transverse/cerebellum	trigeminal	0	24	0.018							
		vestibular nucleus	0	5	0.034							
		10th cerebellar lobule	0	0.5	0.041							
		lemniscal nucleus	0	7	0.047							
Min 10-20												
Region of Interest		Vehicle	OT		Vehicle	OT	Region of Interest		Vehicle	OT		
cortical	olfactory bulb	external plexiform layer	1	19	0.001		external plexiform layer	0	52	0.001
		granular cell layer	9.5	63	0.013			granular cell layer	0	14.5		0.001
		perirhinal ctb	2.5	16	0.008			granular cell layer	0	16.5		0.001
		cortical amygdala	1.5	15	0.016			frontal association ctb	0	10.5		0.001
		CA1 ventral hippocampus	0	6	0.035			lateral orbital ctb	0	16.5		0.001
	subcortical	posterior thalamic nucleus	0	4	0.003	medial orbital ctb	0	8	0.001			
		nucleus accumbens shell	0	6	0.007	prelimbic ctb	0	10	0.001			
		parafascicular thalamic nucleus	0	4	0.009	CA1 dorsal hippocampus	0	20.5	0.006			
		olfactory tubercles	1	53	0.015	ventral orbital ctb	0	0.5	0.018			
		ventral pallidum	0	1	0.026	secondary somatosensory ctb	0	4	0.031			
subcortical	medial amygdala	medial amygdala	0.5	16	0.027	subiculum dorsal	0	18	0.028			
		medial prefrontal area	0	0.5	0.032	dentate gyrus dorsal	2	22	0.031			
		zona incerta	0	7	0.032	dentate gyrus ventral	3	15	0.042			
		extended amygdala	0	0.5	0.035	anterior cingulate area	4	16.5	0.048			
		prerubral field	0	0.5	0.036	anterior olfactory nucleus	0	10	0.001			
	transverse/cerebellum	substantia nigra compacta	0	1	0.038	habenula nucleus	2	8	0.016			
		medial geniculate	2	19	0.04	reticular nucleus midbrain	2	16	0.026			
		reticular nucleus midbrain	10.5	33	0.027	medial geniculate	0	5.5	0.039			
		root of trigeminal nerve	9.5	33	0.036	lateral posterior thalamic nucleus	0	8	0.04			
						substantia nigra reticularis	0	10.5	0.049			
subcortical	ventral pallidum	simple lobule cerebellum	0	24.5	0.002		simple lobule cerebellum	0	24.5	0.002
		4th cerebellar lobule	0	7.5	0.003			4th cerebellar lobule	0	7.5		0.003
		flocculus cerebellum	0	1.5	0.004			flocculus cerebellum	0	1.5		0.004
		superior colliculus	2	57	0.004			superior colliculus	2	57		0.004
		lemniscal nucleus	0	10	0.007			lemniscal nucleus	0	10		0.007
	transverse/cerebellum	inferior colliculus	14	47.5	0.008		inferior colliculus	14	47.5	0.008
		5th cerebellar lobule	7	33.5	0.032			5th cerebellar lobule	7	33.5		0.032
		pontine reticular nucleus caudal	0	0.5	0.019			pontine reticular nucleus caudal	0	0.5		0.019
		cochlear nucleus	0	0.5	0.028			cochlear nucleus	0	0.5		0.028
		interposed nucleus	0	0.5	0.04			interposed nucleus	0	0.5		0.04
subcortical	trapezoid body	trapezoid body	0	0.5	0.04		trapezoid body	0	0.5	0.04
		sub coeruleus nucleus	0	0.5	0.04			sub coeruleus nucleus	0	0.5		0.04
	transverse/cerebellum											

Gray shading = Regions with dense OTR binding (Tribollet et al., 1988; Dumais et al., 2013)

Bold text = Regions showing sex differences in OTR binding density (Dumais et al., 2013; Smith et al., 2016)

Figure 5. IP OT activated many brain regions in both sexes

Listed are brain regions that showed differences in the volume of positive BOLD activation following ICV OT versus ICV vehicle within each sex ($p < 0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1–10) or second 10 minutes (Min 10–20) post vehicle or OT injection. Brain regions are clustered into olfactory bulb, cortical, subcortical, pituitary, and brainstem/cerebellum regions. The 3D color model depicts the location of the listed brain regions in yellow. Positive BOLD responses for each voxel were averaged across subjects within each experimental condition, and in red is the composite average of voxels that showed a significant increase in positive BOLD compared to baseline. Brain regions highlighted in gray in the table show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013), while all other brain regions either do not show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013; Smith et al., 2016), or have not been examined

for OTR binding density. Brain regions in bold show sex differences in OTR binding densities (Dumais et al., 2013; Smith et al., 2016). ctx, cortex.

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Figure 6. IP OT induced few sex differences in BOLD activation

Listed are brain regions that showed sex differences in the volume of positive BOLD activation following IP OT ($p < 0.05$), with the corresponding median (med) number of voxels showing positive BOLD activation during either the first 10 minutes (Min 1–10) or second 10 minutes (Min 10–20). All brain regions that showed sex differences had a greater volume of activation in males compared to females, and brain regions are clustered into cortical, subcortical, brainstem, and cerebellum regions. The 3D color model depicts the location of the listed brain regions within each time point in yellow. Positive BOLD responses for each voxel were averaged across subjects within each experimental condition, and in red is the composite average of voxels that showed a significant increase in positive BOLD compared to baseline in regions that showed a greater volume of activation in males (OT Males) and in regions that showed a greater volume of activation in females (OT Females). The medial amygdala is highlighted in gray in the table and is in bold text because this region shows a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013), and shows a sex difference in OTR binding density (Dumais et al., 2013; Smith et al., 2016). All other brain regions do not show a high density of OTR binding (Tribollet et al., 1988; Dumais et al., 2013; Smith et al., 2016), or have not been examined for OTR binding density.

Table 1
Motion characteristics of male and female rats during scanning

Data represents mean $\pm$ SEM voxel displacements (across x- y- and z- planes) in millimeters (mm). Each group is represented in each table to show both sex effects and treatment effects on motion.

Route	Treatment	Males	Females	Sex effect
ICV	Vehicle	0.013 $\pm$ 0.004	0.002 $\pm$ 0.007	t(21)=-1.66, p=0.11
	OT	0.016 $\pm$ 0.007	0.003 $\pm$ 0.015	t(19)=-1.16, p=0.26
IP	Vehicle	0.005 $\pm$ 0.004	0.008 $\pm$ 0.007	t(21)=0.33, p=0.75
	OT	0.006 $\pm$ 0.005	0.002 $\pm$ 0.014	t(16)=-0.55, p=0.59

Route	Sex	Vehicle	OT	Treatment effect
ICV	Males	0.013 $\pm$ 0.004	0.016 $\pm$ 0.007	t(17)=-0.33, p=0.74
	Females	0.002 $\pm$ 0.007	0.003 $\pm$ 0.015	t(23)=0.06, p=0.95
IP	Males	0.005 $\pm$ 0.004	0.006 $\pm$ 0.005	t(19)=-0.17, p=0.87
	Females	0.008 $\pm$ 0.007	0.002 $\pm$ 0.014	t(18)=0.65, p=0.52