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Effects of oxytocin receptor antagonism on social function and corticosterone release after adolescent social instability in male rats

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ABSTRACT

Oxytocin influences social behaviour and hypothalamic-pituitary-adrenal (HPA) function. We previously found that social instability stress (SS) from postnatal day 30 to 45 increased oxytocin receptor (OTR) densities in the lateral septum and nucleus accumbens of adolescent male rats. Here, we investigated social behaviour and HPA function in adolescent male SS rats compared with age- and sex-matched controls after intraperitoneal treatment with an OTR antagonist L-368,899 (OTR-A). Regardless of OTR antagonism, adolescent SS rats spent more time in social approach (investigation through wire mesh) but less time in social interaction (physical interaction) with unfamiliar same-sex and same-age peers than did controls. However, OTR-A-treatment caused SS rats to be more socially avoidant than OTR-A-treated controls and saline-treated rats of the same condition. Additionally, the predicted rise in plasma corticosterone in response to OTR-A treatment was blunted in SS rats. Fos immunoreactivity (IR) was used as a marker of neural activation in social brain regions and oxytocin-IR was examined in the paraventricular nucleus of the hypothalamus (PVN) in response to interacting with unfamiliar peers in SS and control rats after OTR-A treatment. OTR-A treatment had little effect on Fos-IR and oxytocin-IR in the analyzed brain regions, but SS rats had lower Fos-IR and oxytocin-IR in the PVN and greater Fos-IR in subregions of the prefrontal cortex, and hippocampus, and lateral septum than did controls. Finally, binding density of OTR was measured in the PVN and hippocampus, and greater OTR binding density was found in the PVN of SS rats. Together, these data demonstrate a greater influence of OTR antagonism on social behaviour and a reduced influence of OTR antagonism on HPA responses after adolescent SS in male rats. The results also suggest that differences in neural functioning in the prefrontal cortex, hippocampus and lateral septum of adolescent SS rats may be involved in their altered social behaviour relative to that of controls.

1. Introduction

Social experiences in the adolescent period shape the development of future social behaviour and social brain regions (reviewed in Pellis et al., 2014; Pellis and Pellis, 2017). For example, social deprivation in adolescent male rats resulted in abnormal behaviour in situations of conflict (Einon and Potegal, 1991; Tulogdi et al., 2014; van den Berg et al., 1999), reduced social interactions with peers (van den Berg et al., 1999; Lukkes et al., 2009), and impaired social recognition of familiar and unfamiliar peers (Shahar-Gold et al., 2013). Further, more dendritic spines were found in the medial prefrontal cortex (involved in behavioural coordination with a peer, sexual interest in the opposite sex, and maternal behaviour; Bell et al., 2009; Nakajima et al., 2014; Sabihi et al., 2014) of female rats housed with an adult (low social play experience) compared with female rats housed with a same-age and same-sex peer (high social play experience) during adolescence (Bell et al., 2010). In addition, dendritic spine density and dendritic branching decreases in the medial prefrontal cortex and increases in the hippocampus (involved in displaying aggressive and submissive behaviours with peers, and displaying the correct sequence of behaviours in social interactions; Pagani et al., 2015; Maaswinkel et al., 1997) from adolescence to adulthood in male rodents (Gourley et al., 2012; Koss et al., 2014; Pokorný and Yamamoto, 1981). These changes may render social brain regions vulnerable to stressors during the adolescent

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period, with subsequent effects on social behaviours.

The hypothalamic-pituitary-adrenal (HPA) axis, activated in response to stressors, also undergoes development during adolescence (reviewed in Romeo et al., 2016). The HPA axis begins with the activation of the paraventricular nucleus (PVN) of the hypothalamus, and ultimately increases the release of glucocorticoids from the adrenal cortex. Glucocorticoids then bind to corticosteroid receptors to exert effects on the brain and the periphery (reviewed in Oyola and Handa, 2017). Adolescent rats have a greater and/or more prolonged release of corticosterone (the main glucocorticoid in rats) in response to certain stressors compared to adult rats (reviewed in McCormick et al., 2017; Romeo et al., 2016). The higher exposure to glucocorticoids in response to stressors in adolescence may underlie a greater vulnerability to stressors relative to adulthood (reviewed in Romeo, 2017). Moreover, previous studies demonstrate that neural development in rats is modified by stressor exposure and heightened glucocorticoid exposure during adolescence (Urban and Valentino, 2017; Jorgensen et al., 2017).

Exposure to stressors also results in the increased release of the neuropeptide oxytocin from the PVN and the supraoptic nucleus (Nishioka et al., 1998; reviewed in Stoop, 2012). Oxytocin and the oxytocin receptor (OTR) are involved in the release of glucocorticoids (reviewed in Neumann, 2008; Neumann and Landgraf, 2012). For example, oxytocin reduced plasma corticosterone whereas OTR antagonists increased plasma corticosterone when administered to the brains of adult male and female rodents (Neumann et al., 2000; Peters et al., 2014; Smith and Wang, 2014). Oxytocin and the OTR are also involved in the display of social behaviours (reviewed in Neumann, 2008; Neumann and Landgraf, 2012). Intraperitoneal injection of oxytocin increased the time adult male rats spent in side-by-side contact with a peer (Ramos et al., 2013), intranasal oxytocin reduced aggressive behaviour and increased social exploration in adult male rats (Calcagnoli et al., 2015), and intracerebroventricular administration of an OTR antagonist abolished the preference for a novel rat over a familiar rat in adult male rats (Lukas et al., 2013). In addition, OTR binding density in several brain regions, such as the PVN, hippocampus, and lateral septum, undergoes changes from adolescence to adulthood (Smith et al., 2017b), and stressor exposure in early life alters OTR binding density (Lukas et al., 2010) in male rats.

Social stressors that alter the quality of social experiences in adolescence also influence brain and behavioural development in rodents. Adolescent male rats that underwent social instability stress (SS; daily isolation for 1 h and return to an unfamiliar same-age peer from postnatal day [PND] 30–45) had increased OTR binding density in the dorsal lateral septum and nucleus accumbens shell compared with non-stressed controls (Hodges et al., 2017). OTR activation in these two brain regions has been shown to be involved in social novelty preference (Lukas et al., 2013; Veenema et al., 2012; Smith et al., 2017a). In addition, adolescent SS rats spent more time in social approach (investigating a peer behind wire mesh) and less time in social interaction (physical interaction with a peer) with an unfamiliar peer than did controls, and had impaired social recognition compared with controls (Hodges et al., 2017; Hodges et al., 2018). In response to social interaction, SS rats had reduced Fos (the protein product of an immediate-early-gene, used as a marker of neural activity; reviewed in Kovács, 2008) immunoreactive cells in the PVN and arcuate nuclei of the hypothalamus compared with control rats (Hodges et al., 2018). Moreover, adolescent SS rats had a pattern of reduced Fos immunoreactive cells in the medial amygdala and increased Fos immunoreactive cells in the nucleus accumbens compared with controls in response to social interaction with an unfamiliar peer (Hodges et al., 2018). In addition, although SS rats did not differ from controls in how rewarding they found social interactions with unfamiliar peers in a conditioned place preference task (Hodges et al., 2017), corticosterone remained elevated longer when returned to a new cage partner after 1 h isolation in SS rats compared with non-stressed controls (Hodges and McCormick, 2015). Thus, SS exposure during adolescence alters OTR in specific brain regions, various social behaviours, and stress-induced corticosterone responses, but the evidence for relationships among these endpoints is only correlational to date.

The current study is designed to address the causal relationship between SS-induced changes in OTR, social behaviour, and HPA axis responsiveness. We tested our hypothesis that adolescent SS disrupts the oxytocinergic regulation of social behaviour and HPA axis responsiveness in male rats. The OTR antagonist L-368,899 hydrochloride crosses the blood brain barrier (Boccia et al., 2007), and thus should influence social brain regions. In this study, adolescent SS and control male rats were treated intraperitoneally with 1 mg/kg L-368,899 hydrochloride, tested on social approach/avoidance and social interaction tasks with unfamiliar same-age peers, and plasma corticosterone was measured before and after the social tasks. If the higher OTR binding densities in social brain regions of SS rats represent changes in oxytocin signalling that in turn mediate the observed changes in social behaviours (Hodges et al., 2017), then we predict that OTR antagonism will reduce sociability to a greater extent in SS rats than in controls. Through measurement of plasma corticosterone, we also investigated whether there were differences in the effects of OTR antagonism on HPA responsiveness in SS and control rats. Based on the prolonged corticosterone response in SS rats when paired with a new cage partner (Hodges and McCormick, 2015), we predicted that SS rats would show a greater corticosterone response to OTR antagonism during social behaviour testing than would controls. We also speculated that SS rats may spend less time interacting with unfamiliar peers because of high corticosterone exposure increasing anxiety, and predicted that OTR antagonism might act to reduce social interactions in controls to match that of SS rats. We investigated whether SS and control rats differed in oxytocin immunoreactive cell numbers in the PVN and neural activity (as measured by Fos immunoreactive cell counts) in social brain regions in response to social approach/avoidance and social interaction. Finally, we determined whether SS treatment altered OTR binding in the PVN and hippocampus based on our finding of differences between SS and control rats in OTR binding density in other brain regions (Hodges et al., 2017).

2. Methods

2.1. Animals

Male Long-Evans rats ($N = 64$) were obtained from Charles River, Kingston, New York on postnatal day (PND) 24 and were housed in pairs. Rats were kept on a 12 h light/dark cycle (lights on at 8:00 a.m.) with food and water available ad libitum. The procedures involving the rats were approved by the Brock University Institutional Animal Care Committee and carried out in adherence to the Canadian Council on Animal Care guidelines.

2.2. Social instability stress procedure

Rats were randomly assigned to the non-stressed control (CTL, $n = 32$) or social instability stress (SS, $n = 32$) groups and housed in same condition pairs (CTL w/ CTL, SS w/ SS) on PND 30. From PND 30 to 45, SS rats were removed from their cage partners, isolated in 12 cm × 10 cm plastic containers for 1 h, and returned to a new cage and partner each day. Their new cage partner was also undergoing the isolation procedure. On PND 45, rats were returned to their original cage partner after isolation. To minimize habituation to the procedure, the SS procedure was conducted at various times during the lights on phase of the light/dark cycle. CTL rats remained in their home cages undisturbed until the testing procedures.

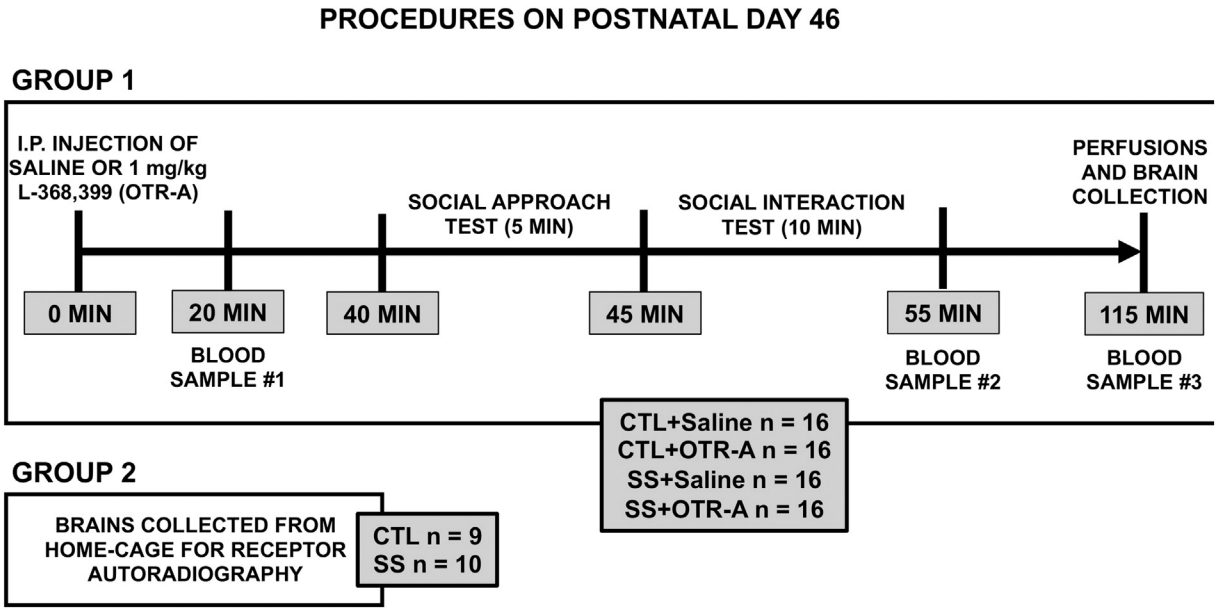

Fig. 1. Timeline of experimental procedures for rats that underwent social instability stress (SS) and non-stressed control (CTL) that were treated with either saline or the oxytocin receptor antagonist (OTR-A) (Group 1) or had their brains collected from the home-cage (Group 2) on postnatal day 46.

2.3. Oxytocin receptor antagonism

On PND 46, rats were randomly assigned to the saline or the oxytocin receptor antagonist (OTR-A), L-368,899 hydrochloride (Tocris Bioscience, Canada) injections groups. Saline (1 ml/kg) and L-368,899 (1 mg/kg) were administered intraperitoneally. Our dose of 1 mg/kg OTR-A was based on a study in rats that found reduced social approach toward females in adult males injected with 1 mg/kg of L-368,899 hydrochloride intraperitoneally compared with rats injected with sterile water (Blitzer et al., 2017). After the injection, rats were returned to their homecages and remained there for 40 min until social behaviour testing (L-369,899 is present in the brain 40 min after injection and reaches a peak in the brain 110 min after injection; Boccia et al., 2007). See Fig. 1 for the timeline of behavioural testing, blood collection, and brain collection after the saline or OTR-A injection.

2.4. Social behaviour testing

To assess social behaviour on PND 46, rats underwent a 5 min social approach test and then a 10 min social interaction test. Rats completed both social behaviour tests in a plastic arena (60 cm × 30 cm × 53 cm) with a removable metal mesh divider in the middle. A video-camera mounted on the ceiling above the arena was used to record the social behaviour tests. The arena was cleaned with 70% ethanol before each social behaviour test session. CTL and SS rats were placed into the arena with an unfamiliar peer undergoing the same stress (CTL or SS) and injection (saline or OTR-A) condition by experimenters who were blind to the condition of each rat. Rats were tested with unfamiliar partners from the same experimental group to maximize potential differences among groups (see Green et al., 2013). Social behaviour test sessions were conducted in red light during the light phase of the light/dark cycle when corticosterone concentrations are low to better capture any increase in concentrations from baseline that resulted from social interaction tests.

2.4.1. Social approach test

For the 5 min social approach test, the metal mesh divider remained in the middle of the arena. Each rat was placed on either side of the metal divider. Total time spent in social approach was defined as time spent sniffing the metal mesh. Social avoidance was also measured and was defined as time spent rearing and sniffing the two corners of the arena located the furthest away from the metal mesh. Social approach and social avoidance behaviours were measured by an experimenter blind to the condition of each rat.

2.4.2. Social interaction test

Immediately after the social approach test, the metal mesh between each pair of rats was removed and the 10 min social interaction test began. An experimenter blind to the condition of each rat measured the total time each rat spent in social interaction. Social interaction consisted of pouncing, crawling, anogenital sniffing, allogroom, and sniffing the face and/or body of the other rat. Pouncing was defined as a quick jump on any part of the other rat's body. Crawling was defined as at least half of the rat's body covering the other rat (excluding the tail). Anogenital sniffing was sniffing the genital or anal regions of the other rat. Allogroom was defined as grooming the neck and body of the other rat (see Green et al., 2013). All social behaviours were then totalled into one duration score (other physical interaction) other than anogenital sniffing, because no single behaviour other than anogenital sniffing was evident for more than a few seconds in the 10 min period of social interaction.

2.5. Blood sampling

Three blood samples were obtained from each rat via tail nick. Blood samples were taken (1) 20 min before the social behaviour test (i.e., 20 min post-injection), (2) immediately after the social interaction test (i.e., 55 min post-injection), and (3) 1 h after the social interaction test (i.e., 115 min post-injection). Blood from the tail (approximately 40 µl) was placed onto a Whatman™ blood stain card (approximately 1 cm × 2 cm; Sigma-Aldrich, Canada), left to dry overnight at room temperature, and stored at -80 °C until further processing (blood collection and assays were performed in keeping with the methods of Milot et al., 2012; Azogu et al., 2018).

Corticosterone concentrations from blood samples were determined using corticosterone enzyme-linked immunosorbent assay kits (Enzo life sciences, Farmingdale, NY). First, using a *gem* punch, a 3.0 mm circle was collected from the card and placed in 280 µl of assay buffer solution (1:10 dilution). Next, the tubes were covered with parafilm (VWR, Canada) and placed on a slow-moving rotating platform for 24 h.

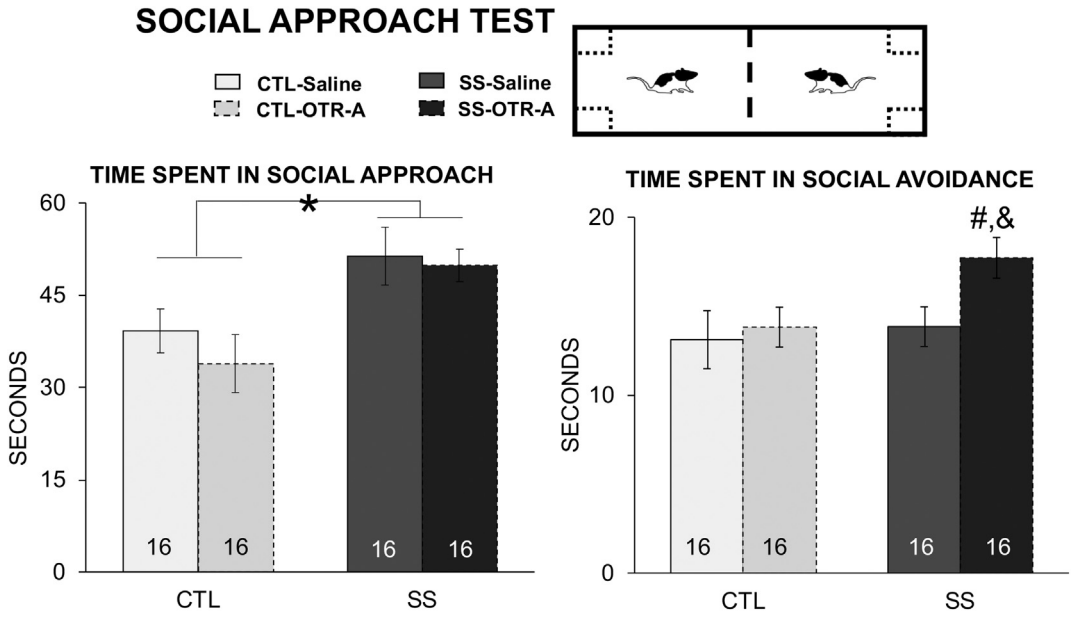

Fig. 2. Mean (± S.E.M.) time spent in social approach and social avoidance with an unfamiliar peer in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). Top right is a diagram showing the apparatus of the social approach test: the dashed line in the middle represents a metal mesh divider, dashed lines in each corner represents the areas of social avoidance. *main effect of Stress Group, $p < 0.01$. #higher than saline-injected SS rats, $p < 0.05$. &higher than OTR-A injected CTL rats, $p < 0.05$. Numbers in each bar indicate the *n* of each group.

The next day, 214.5 µl of each sample was mixed with 5.5 µl of steroid displacement reagent. Next, the standards and samples were prepared based on the manufacturer of the kit, added to the wells of the provided 96-well plates, and the plates were incubated on a slow-moving rotating platform for 2 h at room temperature. The contents of the wells were emptied, washed 3 times with 400 µl of wash buffer, and the wash buffer was then removed. Next, 200 µl of the pNpp substrate solution were added to every well and incubated at room temperature for 1 h without shaking. Lastly, 50 µl of the stop solution were added to every well and the plate was read using a Biotek Synergy plate reader. The cross reactivity of the assay with other steroids are: progesterone = 5.1%, cortisol = 1.1%, pregnenolone = 0.85%, testosterone = 0.12%. The sensitivity of the assay is 0.05 ng ml^{-1}. Intra-assay variance between duplicates was under 10% and inter-assay variance between the plate control means was under 15%. Assay sensitivity was 0.05 ng/ml^{-1}.

2.6. Immunohistochemistry

Immediately after the last blood sample was taken, rats were anaesthetized by an overdose of sodium pentobarbital (150 mg/kg). Rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde mixed in 0.1 M phosphate buffered saline (PBS; pH 7.4). Brains were removed from the skulls and post-fixed in a 4% paraformaldehyde and 30% sucrose solution at 4 °C for 48 h. Brains were sliced into 40 µm coronal sections using a cryostat and sections were collected throughout the prefrontal cortex, lateral septum, nucleus accumbens, paraventricular nucleus of the hypothalamus, arcuate nucleus, hippocampus, and medial amygdala. The sections were stored in cryoprotectant at −20 °C until subsequent immunohistochemical processing.

2.6.1. Single-labelling for Fos

Coronal sections were washed three times, 5 min per wash, in both 0.1 M PBS and PBS-X (PBS with 3% Triton-X). Sections were then incubated at room temperature in a 0.3% H_2O_2 (Fisher scientific, Inc., Hampton, NH) in PBS-X for 30 min. After another three, 5 min washes in PBS-X, sections were incubated at room temperature in a 10% normal goat serum solution (Sigma-Aldrich, Inc., St. Louis, MO) in PBS-X for 1 h. Sections were then incubated at 4 °C overnight in a 1:10,000 dilution of rabbit anti-Fos (sc-52; Santa Cruz Biotechnology, Inc., Dallas, TX) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 1 h in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA). Sections underwent another series of PBS-X washes and then they were incubated in an avidin-biotin horseradish peroxidase (1:500 avidin reagent, 1:500 biotin reagent; Vector Laboratories, Inc.) solution for 90 min at room temperature. Sections were then placed in a solution containing 1:50 3,3'diaminobenzidine (DAB), 1:90 3 M sodium acetate buffer, 1:62.5 H_2O_2, and 1:62.5 nickel (Vector Laboratories, Inc.) for 5 min. Another series of washes in PBS-X followed by 2 washes in distilled water were completed. Sections were then mounted onto Superfrost plus slides (Fisher Scientific, Inc.), dried and dehydrated in increasing increments of ethanol concentrations (70%, 95%, 100%), placed in xylene and coverslipped using permount mounting medium (Fisher Scientific, Inc.).

2.6.2. Double-labelling for Fos and oxytocin

Coronal sections from the paraventricular nucleus of the hypothalamus underwent a double-labelling procedure for Fos and oxytocin immunoreactivity. On the first day, sections underwent the same washes, 0.3% H_2O_2 incubation, and 10% normal goat serum incubation as single-labelled sections. However, sections were then incubated at 4 °C overnight in a 1:5000 dilution of rabbit anti-Fos (sc-52; Santa Cruz Biotechnology, Inc.) in PBS-X. The next day, sections underwent the same PBS-X washes, biotinylated goat anti-rabbit IgG incubation, avidin-biotin horseradish peroxidase incubation, and DAB incubation as single-labelled sections, but the sections were incubated in a 1:400 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc.) instead of a 1:200 dilution. After the DAB incubation, sections underwent three 5 min washes in PBS-X, an incubation for 10 min in 0.3% H_2O_2 in PBS-X, another three 5 min washes in PBS-X, and a 1 h incubation in 10% normal horse serum solution and 1% bovine serum albumin in PBS-X at room temperature. Sections were then incubated in a 1:20,000 dilution of mouse anti-oxytocin (MAB5296; EMD Millipore, Inc., Burlington, MA) in PBS-X at 4 °C overnight. On the third day,

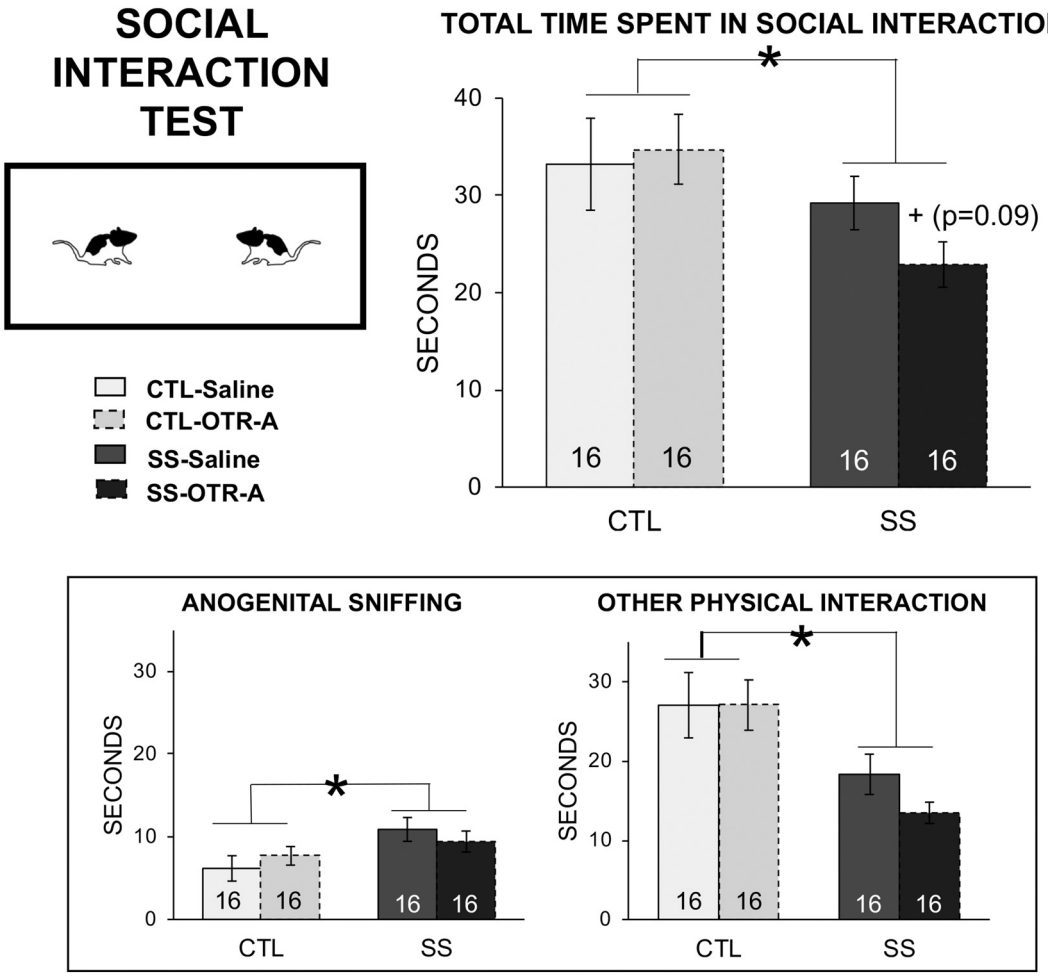

Fig. 3. Mean (± S.E.M.) time spent in social interaction with an unfamiliar peer (top right) and in social interaction divided into anogenital sniffing and other physical interaction (bottom) in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). Top left is a diagram showing the apparatus of the social interaction test. *main effect of Stress Group, $p < 0.05$. + lower than saline-injected SS rats, $p < 0.10$. Numbers in each bar indicate the *n* of each group.

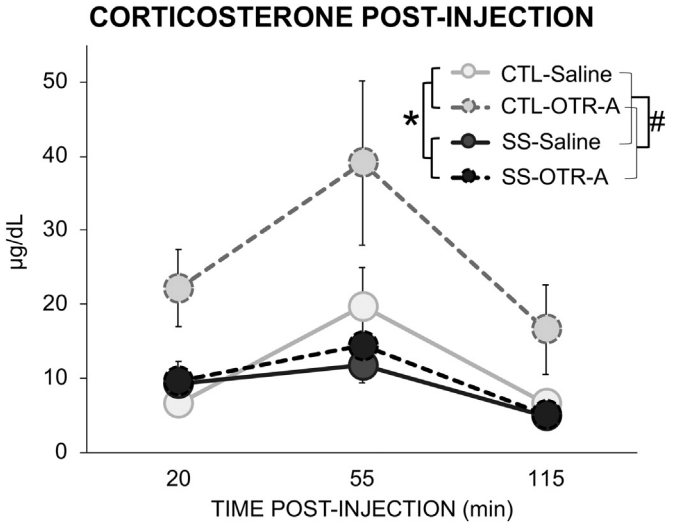

Fig. 4. Mean (± S.E.M.) concentrations of plasma corticosterone 20 min post-injection, 55 min post-injection (immediately after social behaviour tests), and 115 min post-injection in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A) (n = 10–16 per group). *main effect of Stress Group, $p < 0.05$. #main effect of Drug Treatment, $p < 0.05$.

sections underwent the same procedure as day 2 for single-labelled sections, but sections were incubated for 1 h in a 1:400 dilution of biotinylated horse anti-mouse IgG and 1% normal horse serum solution in PBS-X instead of 1:200 biotinylated goat anti-rabbit IgG, and DAB did not include nickel.

2.7. Microscopy and cell counts

Immunostained sections were analyzed using a Nikon Eclipse 80i microscope equipped with Nikon ACT-1 software and a digital camera (Nikon DXM1200F). Fos immunoreactive (IR) cells, oxytocin-IR cells, and cells expressing both Fos and oxytocin (double-labelled) were counted at a 40× objective (magnification of 400×) in a 250 µm^2 area of both hemispheres of each brain region in 2–4 sections for each brain region and then averaged. The percentage of double-labelled Fos-IR containing oxytocin-IR cells was calculated by dividing the number of cells expressing both Fos-IR and oxytocin-IR by the total number of oxytocin-IR cells. A limitation of the double labelling is that because it was not confirmed by confocal microscopy, it is possible that some counts represent overlapping single-labelled cells rather than double-labelled. Brain regions of interest were identified according to the atlas coordinates of Paxinos and Watson (2005); Cells were counted for the medial prefrontal cortex (mPFC) within bregma 3.72 mm and 2.76 mm, dorsal lateral septum (dLS) within bregma 1.44 mm and 0.36 mm, nucleus accumbens within bregma 1.92 mm and 0.84 mm, PVN within

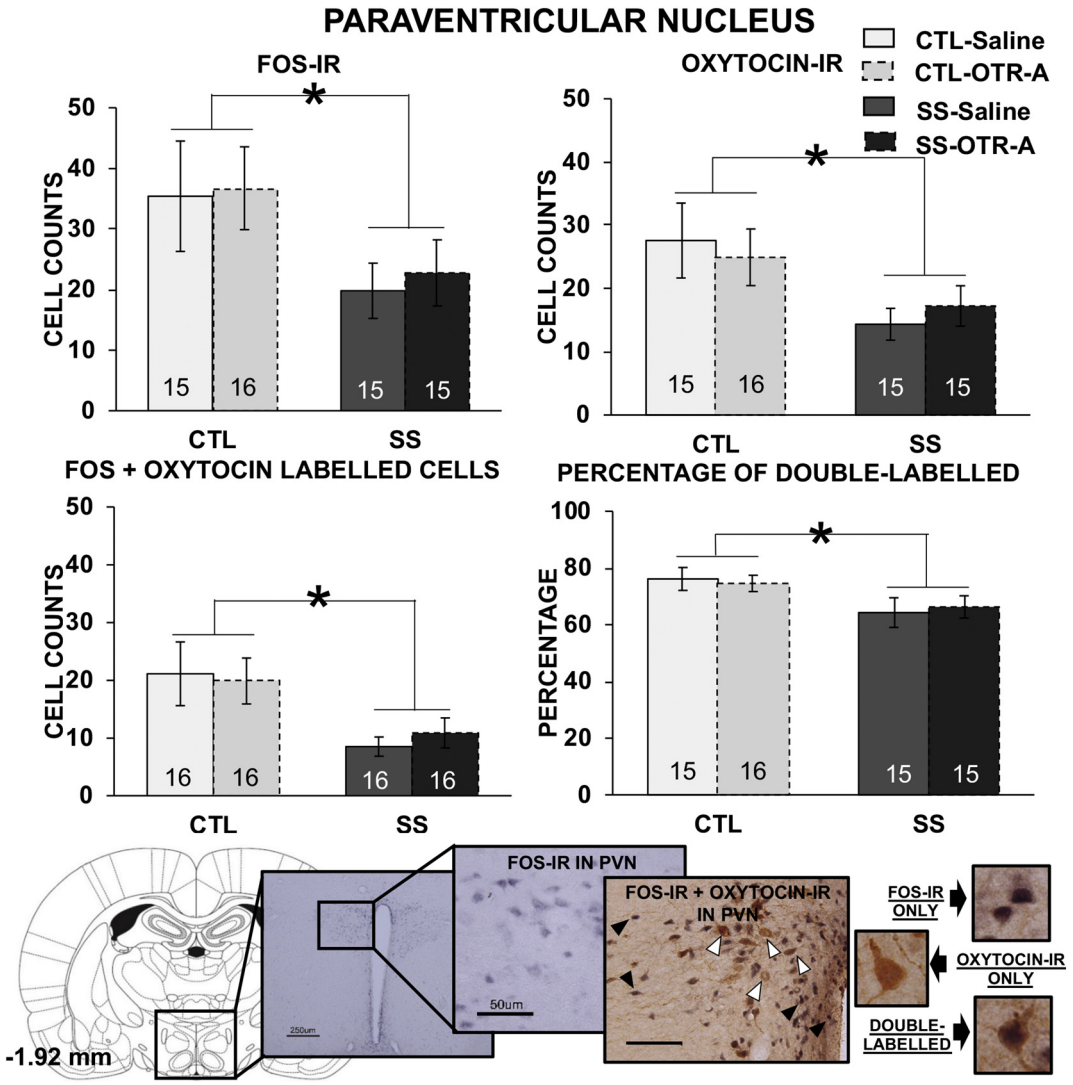

Fig. 5. Mean (± S.E.M.) Fos immunoreactive (Fos-IR), oxytocin immunoreactive (oxytocin-IR), and double-labelled (Fos + oxytocin) cells in the paraventricular nucleus (PVN) in response to social behaviour in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). An atlas image depicting the PVN (image used with permission from Paxinos and Watson, 2005) and images of Fos-IR and oxytocin-IR in the PVN at 40× magnification and 400× magnification are found at the bottom. Black triangles indicate Fos-IR cells, and white triangles with black outlines indicate oxytocin-IR cells. *main effect of Stress Group, $p < 0.05$. Numbers in each bar indicate the *n* of each group.

bregma −1.44 mm and −2.04 mm, arcuate nucleus (ARC) within bregma −2.04 mm and −3.36 mm, hippocampus within bregma −2.28 mm and −3.48 mm, and medial amygdala within bregma −1.80 mm and −3.24 mm. The medial prefrontal cortex was further divided into the cingulate gyrus (CG), infralimbic cortex (IL), and prelimbic cortex (PL). The nucleus accumbens was further divided into the nucleus accumbens core (NAcC) and shell (NAcS). The hippocampus was divided into the pyramidal layer of the hippocampus (CA1, CA2, CA3) and the suprapyramidal and infrapyramidal blades of the dentate gyrus (supraDG and infraDG respectively). The medial amygdala was further divided into the anterodorsal (MeAD), anteroventral (MeAV), and posterodorsal (MePD), and posteroventral (MePV) parts of the medial amygdala. Cell counts were performed by researchers blinded to the experimental condition of each rat.

2.8. Receptor autoradiography

Oxytocin receptor (OTR) binding density was determined in the PVN and hippocampus of adolescent SS ($n = 10$) and CTL ($n = 9$) male rats using autoradiography films that were used to determine OTR binding density in other brain regions in a previous study (Hodges et al., 2017). The current regions of interest were chosen because the PVN is a major region of oxytocin synthesis and a component of the HPA axis (reviewed in Stoop, 2012; Spencer and Deak, 2017), and the hippocampus is involved in regulating HPA axis activity and in social behaviours (Maaswinkel et al., 1997; reviewed in Herman et al., 2012).

Receptor autoradiography was conducted according to Smith et al. (2017b). In brief, 40 µm coronal sections sliced on a cryostat from approximately bregma −0.96 to −3.72 (Paxinos and Watson, 2005) were placed on glass slides and used for autoradiography. The tracer [125I]-Ornithine Vasotocin Analog d(CH2)5[-Tyr(Me)2,Thr4,Orn8, [125I]Tyr9-NH2]-OVTA (Perkin Elmer, Boston, MA, USA) was used for OTR. Sections containing the PVN and hippocampus of both CTL and SS rats were simultaneously processed, balanced across the two incubation chambers and exposure to Biomax MR films (VWR International, Pittsburgh, PA, USA). Films were exposed for 4 days and were then digitized using a Northern Light Illuminator (InterFocus Imaging, UK).

ImageJ (National Institute of Health; http://imagej.nih.gov/ij/) was used to measure optical densities of OTR and V1AR binding. The data were converted to dpm/mg (disintegrations per minute/mg of tissue)

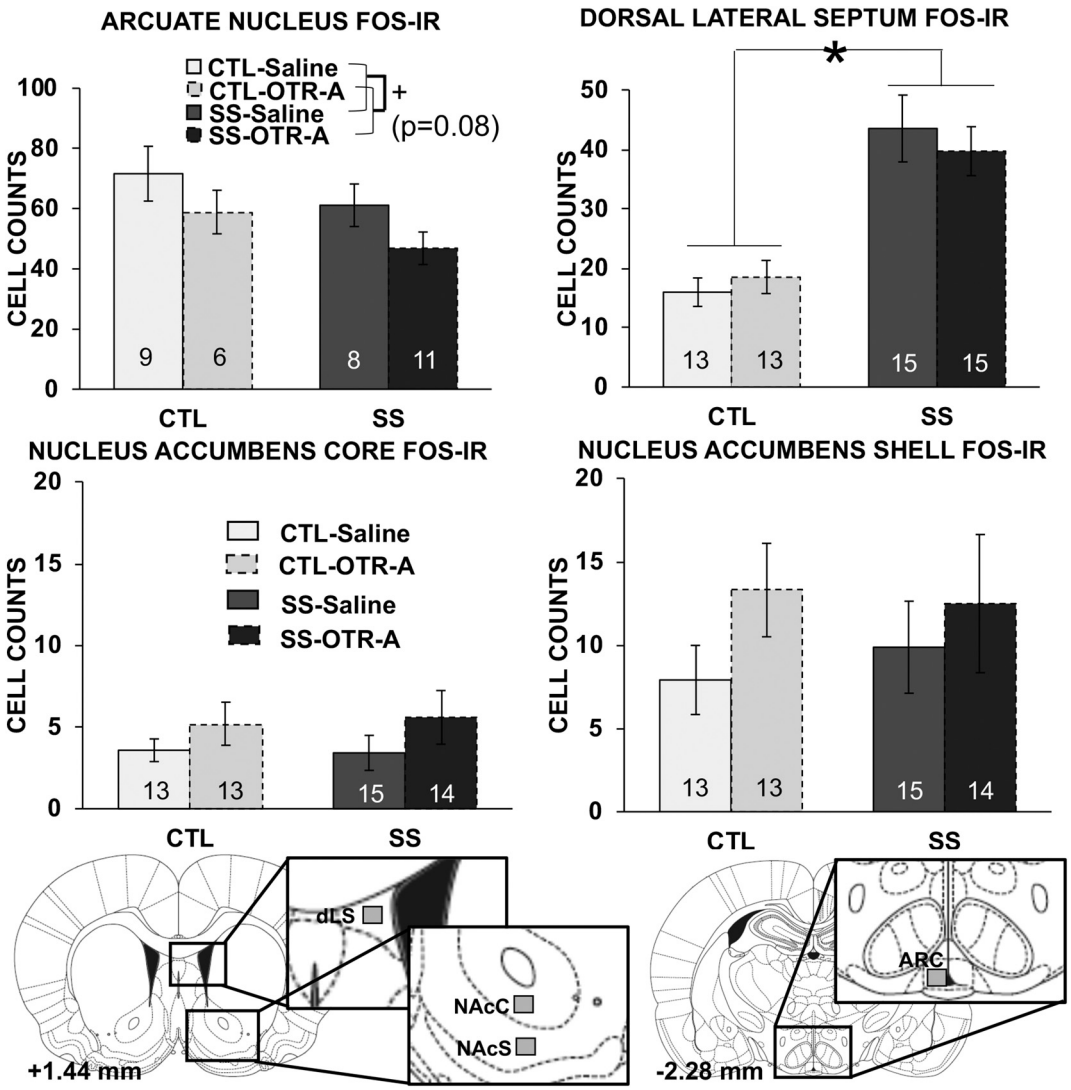

Fig. 6. Mean (± S.E.M.) Fos immunoreactive (Fos-IR) cells in the arcuate nucleus (ARC), dorsal lateral septum (dLS), and nucleus accumbens core (NAcC) and shell (NAcS) in response to social behaviour in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). Atlas images depicting brain regions of interest (images used with permission from Paxinos and Watson, 2005) are found at the bottom. Gray boxes indicate the areas measured for Fos-IR for each brain region of interest. *main effect of Stress Group, $p < 0.01$. +main effect of Drug Treatment in the ARC, $p < 0.10$.

using a [^{125}I] standard microscale (American Radiolabeled Chemicals Inc., St. Louis, MO, USA). OTR binding density was calculated by taking the mean of bilateral measurements from 3 sections per each brain region of interest and subtracting the film background for each section. OTR binding density was measured in the PVN (within bregma −1.56 mm and −2.40 mm) and pyramidal blade of the hippocampus (within bregma −2.76 mm and −4.44 mm).

2.9. Statistical analyses

Statistical analyses were performed using SPSS version 24 software. Analyses consisted of between-subjects (2 × 2) and mixed-factor (2 × 2 × 3) analysis of variances (ANOVAs), and independent *t*-tests. Follow-up analyses of social behaviour tests consisted of independent *t*-tests, and follow-up analyses of corticosterone concentrations consisted of independent and paired *t*-tests where appropriate. An alpha level of $p \leq 0.05$, two-tailed, was used to determine statistical significance. Between-subjects ANOVAs for the social behaviour tests involved the between-group factors of Stress Group (CTL, SS) and Drug Group (saline, OTR-A). The mixed-factor ANOVA for the corticosterone concentration data involved the between-group factors of Stress Group and Drug Group, and the within-group factor of Time Post-Treatment (20 min, 55 min, 115 min). For the Fos-IR and oxytocin-IR data, separate ANOVAs were performed for analyzing each brain region. Brain regions with multiple subregions were analyzed using mixed-factor ANOVAs with the between-group factors of Stress Group and Drug Treatment Group and the within-group factor of Subregion. Other brain regions were each analyzed using between-subjects ANOVAs with Stress Group and Drug Group as between-group factors. Partial correlation analyses controlling for drug treatment were used to determine correlations among time spent in social interaction, time spent in social avoidance, time at mesh, and Fos-IR cell counts in all brain regions. OTR binding density was analyzed using independent *t*-tests. Eta squares (η^2) are reported as a measure of effect size for main effects and interactions of ANOVAs. *t*-tests were used to probe interactions, and Cohen's *d* are reported as a measure of effect sizes for *t*-tests (calculated with online calculator at www.socscistatistics.com/effectsize/default3.aspx).

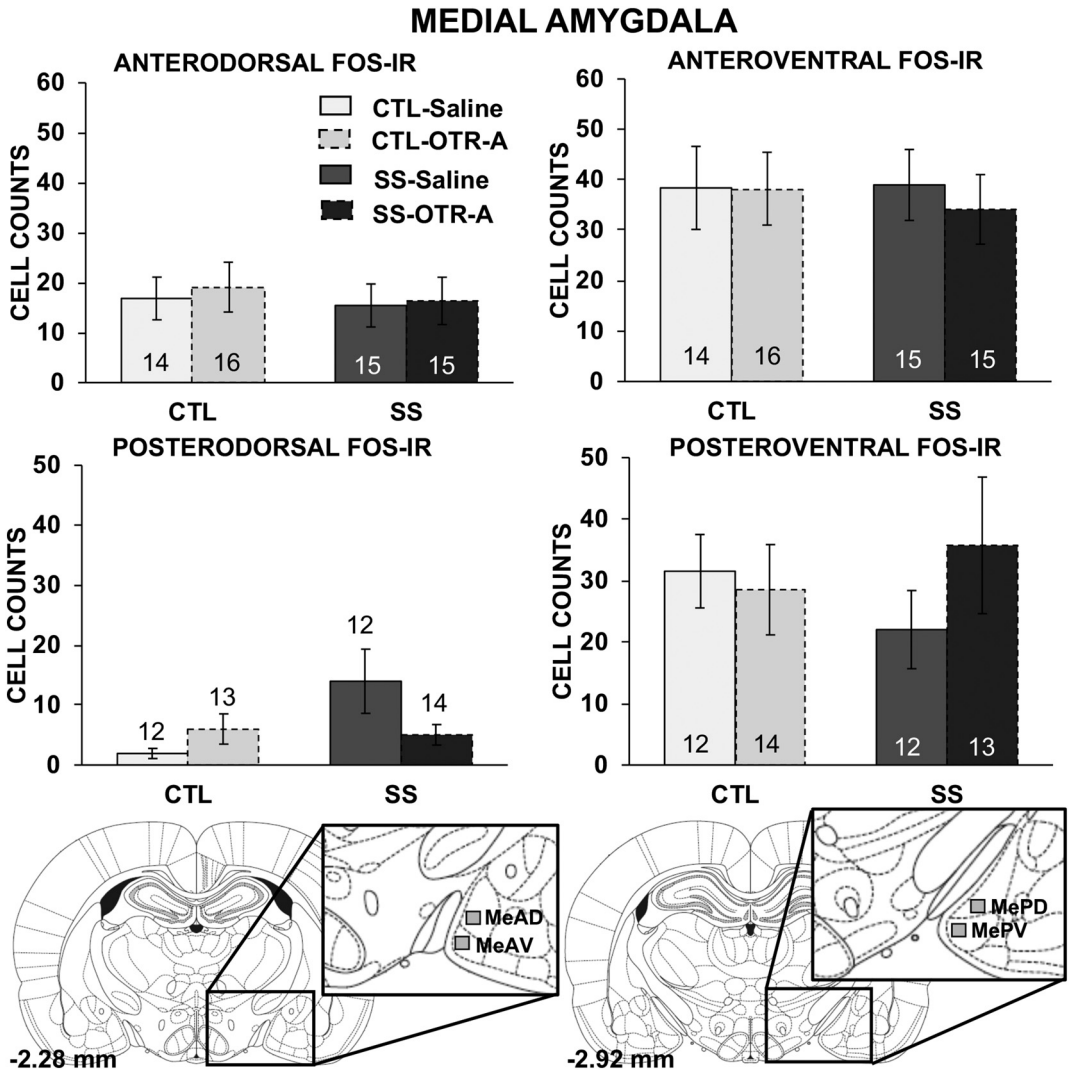

Fig. 7. Mean (± S.E.M.) Fos immunoreactive (Fos-IR) cells in the anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD) and posteroventral (MePV) parts of the medial amygdala in response to social behaviour in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). Atlas images depicting brain regions of interest (images used with permission from Paxinos and Watson, 2005) are found at the bottom. Gray boxes indicate the areas measured for Fos-IR for each brain region of interest.

3. Results

3.1. Social behaviour tests

3.1.1. Social approach

SS rats spent more time in social approach than did CTL rats (Stress effect: $F_{1,60} = 12.33$, $p = 0.001$, $\eta^2 = 0.02$). There was no significant main effect of Drug or Stress x Drug (all $p > 0.40$) (see Fig. 2).

3.1.2. Social avoidance

There was no significant main effect of Stress ($p = 0.07$), Drug ($p = 0.08$), or Stress x Drug ($p = 0.22$). Nevertheless, we probed our a priori hypothesis of an interaction with *t*-tests, and found that SS rats treated with OTR-A spent more time in social avoidance than did saline-treated SS rats ($p = 0.02$, $d = 0.86$) and OTR-A-treated CTL rats ($p = 0.02$, $d = 0.86$; Fig. 2).

3.1.3. Social interaction

SS rats spent less time in social interaction than did CTL rats (Stress effect: $F_{1,60} = 5.20$, $p = 0.03$, $\eta^2 = 0.01$). There was no significant main effect of Drug or Stress x Drug (both $p > 0.26$). *t*-tests indicated no significant effect of OTR-A on social interaction duration in SS rats ($p = 0.09$) or in CTL rats ($p = 0.80$) (see Fig. 3).

When examining anogenital sniffing behaviour and other physical interaction separately, we found that SS rats spent more time in anogenital sniffing (Stress effect: $F_{1,60} = 5.68$, $p = 0.02$, $\eta^2 = 0.03$) and less time in other physical contact (Stress effect: $F_{1,60} = 14.1$, $p < 0.001$, $\eta^2 = 0.05$) than did CTL rats. Neither the effect of Drug nor the Stress x Drug interaction were significant in either of these analyses (both $p > 0.27$) (see Fig. 3).

3.2. Corticosterone

SS rats had lower corticosterone than did CTL rats (Stress effect: $F_{1,46} = 4.26$, $p = 0.045$, $\eta^2 = 0.04$) and OTR-A-treated rats had higher corticosterone than did saline-treated rats (Drug effect: $F_{1,46} = 4.73$, $p = 0.04$, $\eta^2 = 0.05$), and the interaction of Stress x Drug was not significant ($p = 0.11$). Nevertheless, we probed our a priori hypothesis of an interaction. Among CTL rats, there was higher corticosterone in OTR-A-treated compared with saline-treated CTL rats (Drug effect: $F_{1,26} = 5.02$, $p = 0.03$, $\eta^2 = 0.09$) and no effect of Drug in SS rats ($p = 0.36$). SS and CTL rats did not differ significantly when the OTR-A ($p = 0.07$) and saline ($p = 0.58$) treatment groups were analyzed separately. The main effect of Time Post-injection was significant

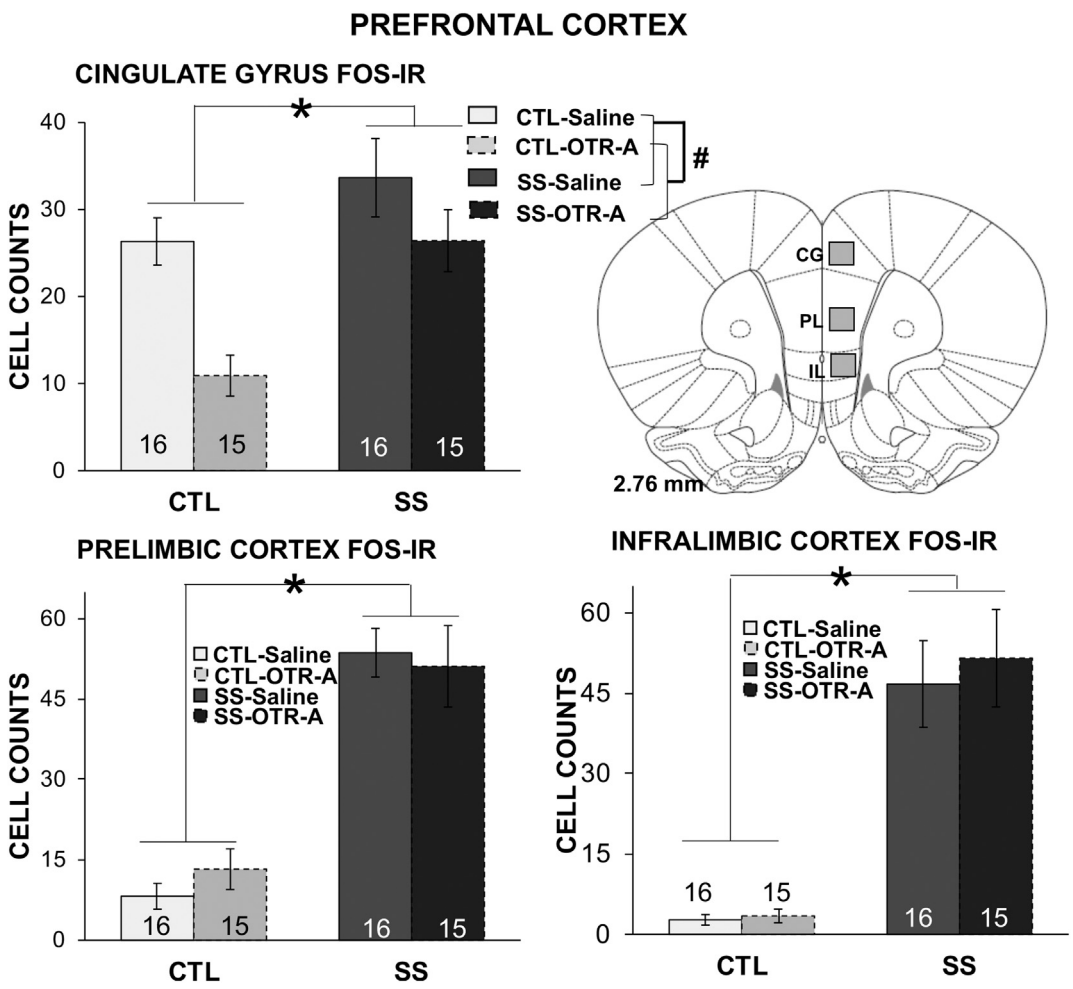

Fig. 8. Mean (± S.E.M.) Fos immunoreactive (Fos-IR) cells in the anterior cingulate (ACC), prelimbic (PL), and infralimbic (IL) cortices in response to social behaviour in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). An atlas image depicting the brain regions of interest (image used with permission from Paxinos and Watson, 2005) is found on the top right. Gray boxes indicate the areas measured for Fos-IR for each brain region of interest. *main effect of Stress Group, $p < 0.01$. #main effect of Drug Treatment in the ACC, $p < 0.01$.

($F_{1,92} = 13.4$, $p < 0.001$, $\eta^2 = 0.23$): corticosterone concentrations increased and peaked after social behaviour testing ($p < 0.001$, $d = 0.47$) and decreased 60 min thereafter ($p < 0.001$, $d = 0.71$) (see Fig. 4).

3.3. Immunohistochemistry

3.3.1. Fos and oxytocin immunoreactive cell counts in the paraventricular nucleus

SS rats had fewer Fos-IR cells (Stress effect: $F_{1,57} = 4.84$, $p = 0.04$, $\eta^2 = 0.04$), fewer oxytocin-IR cells (Stress effect: $F_{1,57} = 6.09$, $p = 0.02$, $\eta^2 = 0.04$), fewer double-labelled Fos-IR and oxytocin-IR cells (Stress effect: $F_{1,60} = 8.39$, $p = 0.005$, $\eta^2 = 0.06$), and a lower percentage of oxytocin cells expressing Fos-IR (Stress effect: $F_{1,57} = 6.12$, $p = 0.02$, $\eta_p^2 = 0.005$) in the PVN than did CTL rats. Neither the effect of Drug nor the interaction effect was significant in any of these analyses (all $p > 0.52$) (see Fig. 5).

3.3.2. Fos immunoreactive cell counts in social brain regions

SS rats had more Fos-IR cells than did CTL rats in the dorsal lateral septum (Stress effect: $F_{1,52} = 34.9$, $p < 0.001$, $\eta^2 = 0.12$) (see Fig. 6), in the PL (Stress effect: $F_{1,58} = 72.5$, $p < 0.001$, $\eta^2 = 0.24$), and in the IL (Stress effect: $F_{1,58} = 72.7$, $p < 0.001$, $\eta^2 = 0.33$) (see Fig. 8). In the CG, SS rats had more Fos-IR cells than did CTL rats (Stress effect: $F_{1,58} = 11.2$, $p = 0.001$, $\eta_p^2 = 0.04$) and OTR-A-treated rats had fewer Fos-IR cell counts than did saline-treated rats (Drug effect: $F_{1,58} = 11.03$, $p = 0.002$, $\eta_p^2 = 0.04$). In the hippocampus, SS rats had more Fos-IR cell counts than did CTL rats in the CA1 (Stress effect: $F_{1,47} = 9.93$, $p = 0.003$, $\eta^2 = 0.11$), CA3 (Stress effect: $F_{1,46} = 19.53$, $p < 0.001$, $\eta^2 = 0.22$), IPDG (Stress effect: $F_{1,43} = 31.7$, $p < 0.001$, $\eta^2 = 0.27$), SPDG (Stress effect: $F_{1,47} = 18.8$, $p < 0.001$, $\eta^2 = 0.19$), but not in the CA2 ($p = 0.53$) (see Fig. 9). Neither the effect of Drug nor the interaction effect was significant in brain regions other than the CG (all $p > 0.44$) (see Fig. 6).

There was no effect of Stress or Drug on Fos-IR in the arcuate nucleus, the core and shell of the nucleus accumbens or in the four parts of the medial amygdala (see Figs. 6 and 7) (all $p > 0.08$).

3.3.3. Correlations between social behaviour and Fos immunoreactive cell counts in social brain regions

In CTL rats, time spent in social approach was positively correlated with Fos-IR in the nucleus accumbens core ($r_{23} = 0.506$, $p = 0.02$) and shell ($r_{23} = 0.413$, $p = 0.04$) and negatively correlated with Fos-IR in the anterodorsal medial amygdala ($r_{27} = -0.385$, $p = 0.04$). In SS rats, time spent in social avoidance was negatively correlated with Fos-IR in the anteroventral medial amygdala ($r_{27} = -0.432$, $p = 0.02$), and time spent in social interaction was positively correlated with Fos-IR in the posterodorsal medial amygdala ($r_{23} = 0.391$, $p = 0.05$) (see Figs. 10 and 11A).

Correlations may be significant in only one group and yet not

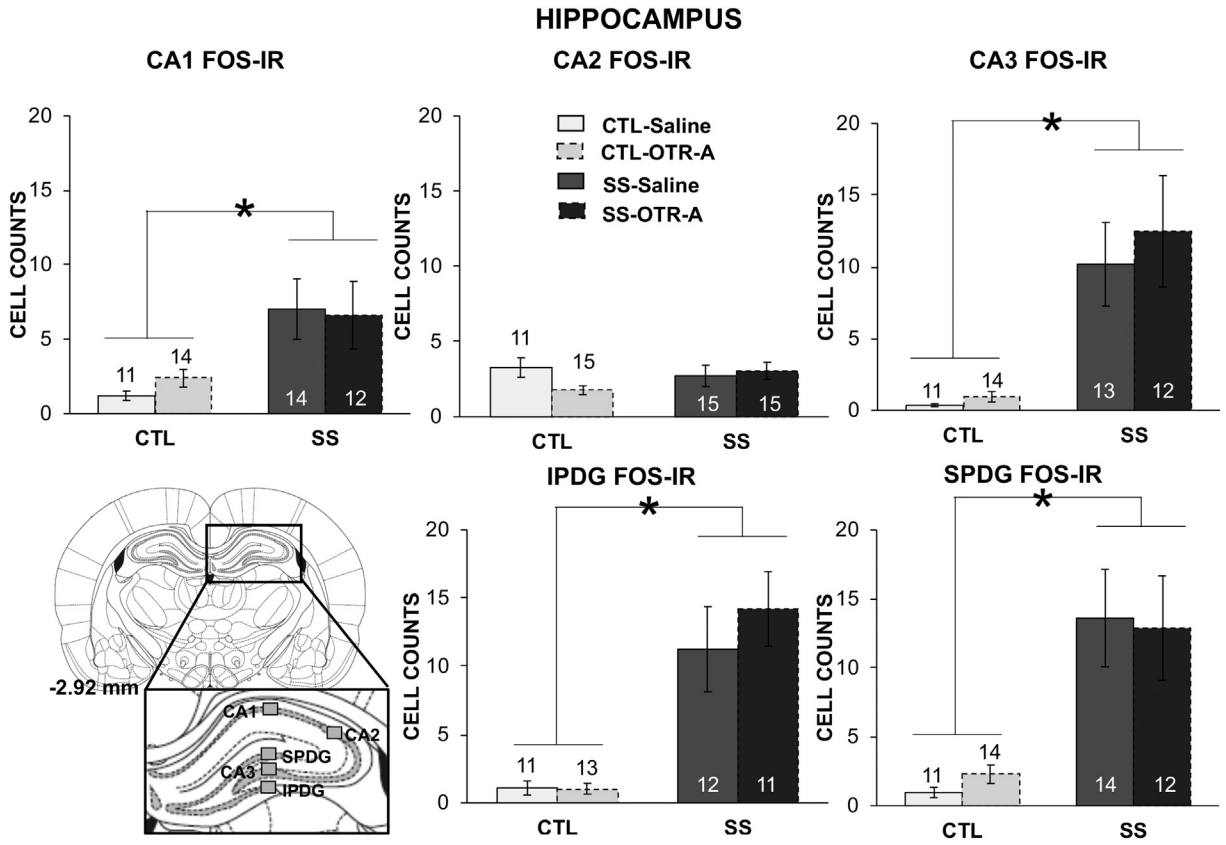

Fig. 9. Mean (± S.E.M.) Fos immunoreactive (Fos-IR) cells in the CA1, CA2, CA3, infrapyramidal blade of the dentate gyrus (IPDG), and suprapyramidal blade of the dentate gyrus (SPDG) of the hippocampus in response to social behaviour in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). An atlas image depicting the brain regions of interest (image used with permission from Paxinos and Watson, 2005) is found on the bottom left. Gray boxes indicate the areas measured for Fos-IR for each brain region of interest. *main effect of Stress Group, $p < 0.01$.

significantly different from the correlation in the other group (e.g., the similar strengths of association evident for Fos-IR in the anterodorsal medial amygdala and social approach in the two Stress Groups: $r = -0.385$ $p = 0.04$ in CTL, and $r = -0.285$, n.s. in SS rats). Thus, as a descriptive approach of the extent to which CTL and SS rats differed in neural activity during the social behaviour tests, Fig. 11B illustrates the partial correlations that differed by 0.5 or more between the two groups. The pattern that is evident includes: correlations between the hippocampal regions and prelimbic cortex that are positive in CTL rats and negative in SS rats; positive correlations in SS rats and negative correlations in CTL rats between hippocampal regions and the nucleus accumbens, medial amygdala, lateral septum, and hypothalamic regions; and no association in SS rats and a positive correlation in CTL rats between the nucleus accumbens and social approach.

3.4. OTR binding density

SS rats had higher OTR binding density in the PVN than did CTL rats ($t_9 = 2.49$, $p = 0.03$, $d = 1.50$). CTL and SS rats did not differ in OTR binding density in the pyramidal part of the hippocampus ($p = 0.267$) (see Fig. 12).

3.5. Summary of results

Table 1 provides a summary of the results.

4. Discussion

In brief, adolescent SS male rats spent more time in social approach (investigation through wire mesh) but less time in social interaction (physical interaction) with unfamiliar same-sex and same-age peers than did controls. SS rats treated intraperitoneally with the OTR-A L-368,899, and tested 40 min later, spent more time in social avoidance during the social approach test than did saline-treated SS rats and OTR-A-treated controls. OTR-A-treated rats had higher plasma corticosterone than did saline-treated rats, and control rats had higher plasma corticosterone than did SS rats. The effect of OTR-A on corticosterone, however, was driven by effects in control rats. Adolescent SS altered neural function in several brain regions, such that SS rats had fewer Fos-IR and oxytocin-IR cells in the PVN, and increased Fos-IR in the lateral septum, prefrontal cortex, and hippocampus compared with controls in response to social behaviour with an unfamiliar peer. OTR-A-treated rats had reduced Fos-IR in the CG compared with saline-injected rats in response to social behaviour. Additionally, SS rats had higher OTR binding density in the hypothalamic PVN than did controls.

4.1. Oxytocin receptor antagonism increased social avoidance in male rats exposed to adolescent social instability

In line with our previous findings, adolescent SS male rats spent more time interacting with unfamiliar peers behind a wire mesh (social approach) compared with adolescent male control rats (Hodges et al., 2017). The extent to which stressors influence social approach is mixed in the literature. For example, repeated restraint stress increased social approach in adult rats (Li et al., 2016), which is consistent with reduced social approach in adolescent male rats after SS. Social defeat in adolescent male rats, however, reduced (Vidal et al., 2007, 2011) or did not affect (Weathington et al., 2012) social approach compared with non-stressed rats when rats were tested as either adolescents or adults. We previously found that social approach did not differ between SS and

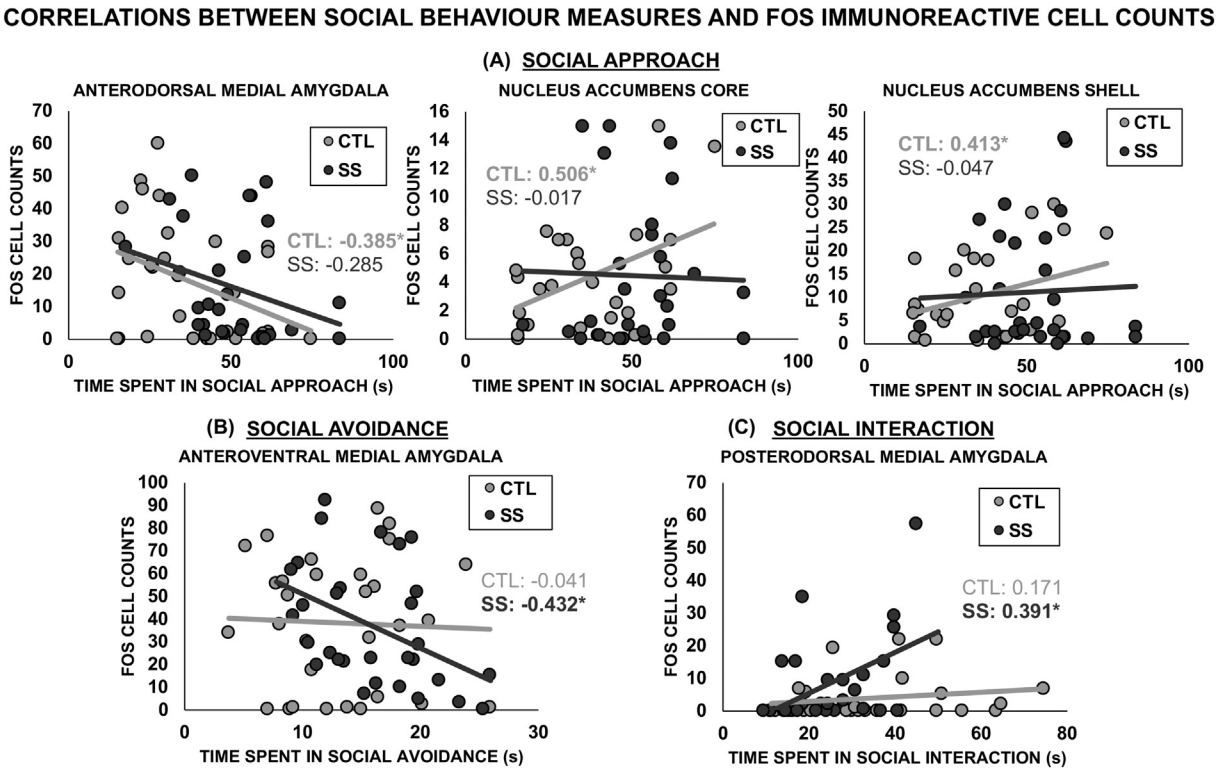

Fig. 10. Scatterplots, correlations, and regression lines demonstrating relationships between Fos immunoreactive (IR) cell counts in brain regions of interest and (A) social approach, (B) social avoidance, and (C) social interaction in CTL (control) and SS (social instability stress) rats. * and bold text indicates a significant correlation, $p \leq 0.05$.

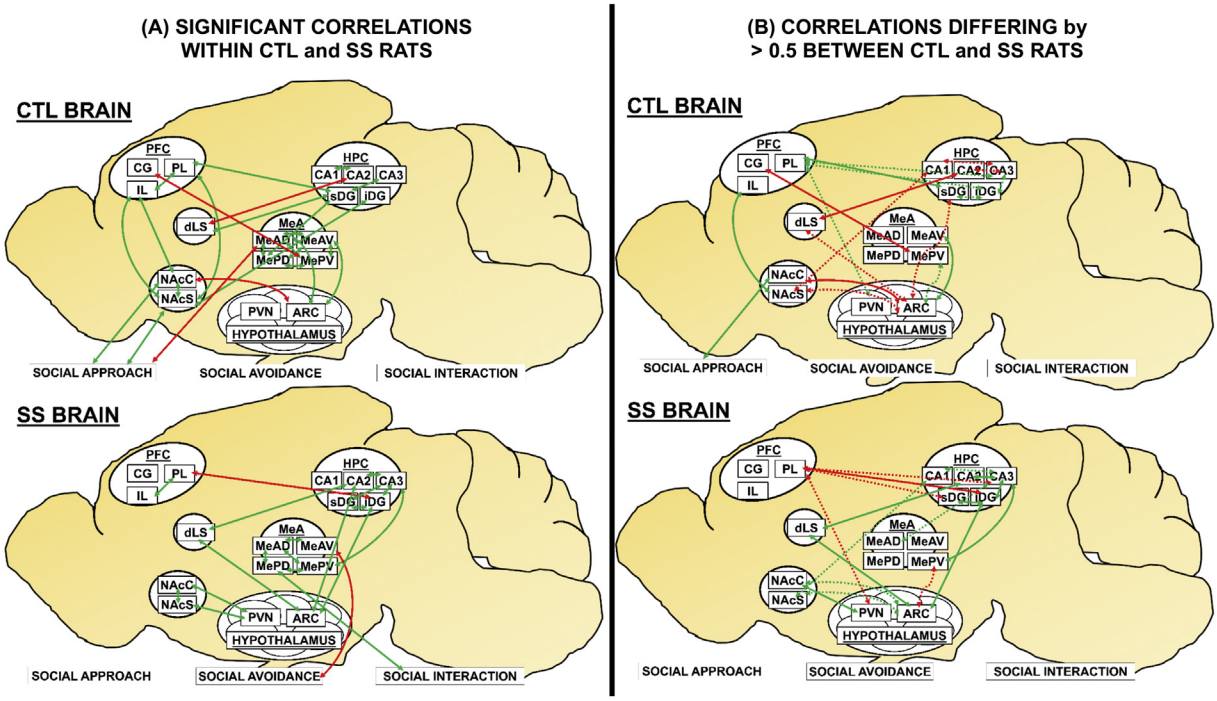

Fig. 11. (A) Illustration of the significant partial correlations (controlling for drug treatment) among brain regions and social behaviour measures within CTL and SS rats. Green arrows indicate positive correlations and red arrows indicate negative correlations. (B) Illustration of correlations that differed by $r > 0.5$ between SS and CTL rats. Dotted green and red lines are correlations that are not significant within either SS or CTL rats that are $r > 0.2$ or $r < -0.2$. Correlations that differed by $r > 0.5$ between SS and CTL rats that were within $r > -0.2$ and $r < 0.2$ are not shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

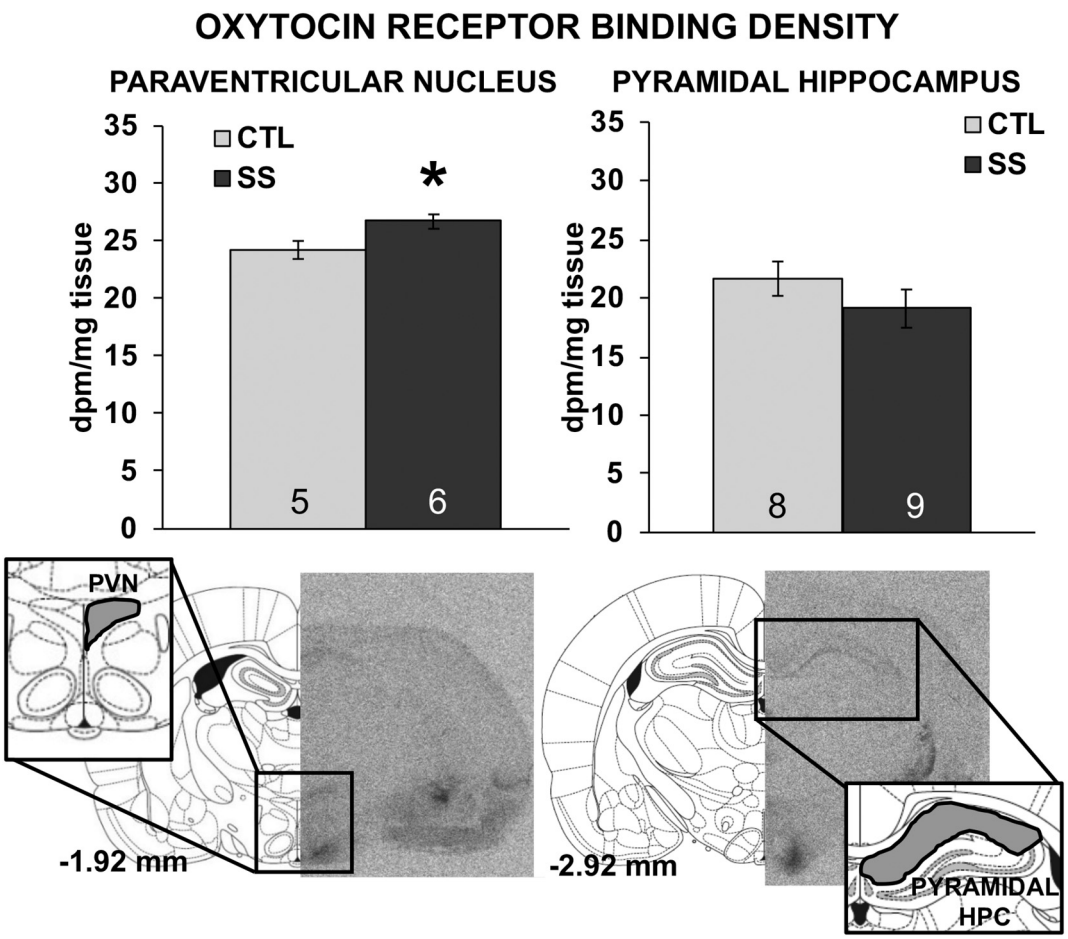

Fig. 12. Mean (± S.E.M.) oxytocin receptor (OTR) binding density in the paraventricular nucleus (PVN) and pyramidal part of the hippocampus (HPC) in CTL (control) and SS (social instability stress) rats after an injection of saline or an oxytocin receptor antagonist (OTR-A). Atlas images depicting the PVN and HPC (images used with permission from Paxinos and Watson, 2005) and corresponding representative autoradiograms of OTR binding are found on the bottom. Highlighted in gray on the atlas images are the areas measured for OTR binding density. *higher in SS rats, $p < 0.05$.

control rats when they were tested in adulthood, although the reduced social interaction with unfamiliar same-age peers in SS rats compared with controls rats is evident in adulthood (Green et al., 2013) and in adolescence in our previous studies (Hodges et al., 2017, 2018) and in the present study. There was no effect of OTR-A on social approach. Nevertheless, consistent with our prediction of a greater effect of reduced sociability in SS rats after OTR-A treatment, systemic OTR blockade enhanced social avoidant behaviour in SS rats. Others have reported that OTR-A treatment reduced social approach in adult male rats and mice, but OTR-A had no effect on time spent in social avoidance zones in adult male mice (Duque-Wilckens et al., 2018; Lukas et al., 2011). A reduction in social avoidance was reported, however, after OTR-A treatment in socially-defeated adult female mice (Duque-Wilckens et al., 2018). Defeated female mice showed increased social avoidance compared to non-defeated females when treated with saline, and the OTR-A was without effect in the non-defeated females (Duque-Wilckens et al., 2018). Thus, the role of OTR-A in social avoidance may be limited to whether or not rodents were previously exposed to a stressor.

We also replicated our previous finding that the reduction in social interaction with an unfamiliar rat in adolescent male SS rats compared with controls is the result of reduced time spent grooming and sniffing the face and body of the unfamiliar rat rather than anogenital sniffing (Hodges et al., 2018). Moreover, we found that SS rats displayed more anogenital sniffing with an unfamiliar peer than did controls. Anogenital sniffing increases and social grooming behaviours decrease from adolescence to adulthood in male rats (Meaney and Stewart, 1981). Thus, adolescent SS may disrupt the normal development of social behaviours with peers in adolescence by facilitating the maturation of anogenital sniffing and social grooming same-age and same-sex peers. Our prediction of reduced sociability in adolescent male SS rats after OTR blockade did not reach statistical significance. It is possible that a higher dose of the OTR-A L-368,899 hydrochloride or a more localized dose (i.e. administered to specific brain regions) would have resulted in reduced social interaction with an unfamiliar peer to a greater extent in adolescent male SS rats than in adolescent male controls. In male voles, microfusions of an OTR antagonist into the nucleus accumbens reduced social interactions with same-sex peers (Yu et al., 2016). Direct injection of an OTR-A into the lateral septum or nucleus accumbens may better reveal reduced sociability in SS rats at a lower dose compared to controls given that SS rats have higher OTR binding densities in these regions than do controls.

4.2. Adolescent social instability blunts plasma corticosterone responses to oxytocin receptor antagonism in male rats

The reduced levels of plasma corticosterone across all time points in SS rats relative to controls was driven by OTR-A treatment, and this was in contrast to our prediction of a greater corticosterone response in OTR-A-treated SS rats than in control rats in response to being socially active. An increase in plasma corticosterone concentration in OTR-A-treated control rats compared with saline-treated control rats is consistent with previous studies (Neumann et al., 2000; Smith and Wang, 2014). For example, one study in adult rats found increased levels of

Table 1
Summary of the results.

	Stress effect (SS relative to CTL)	Drug effect (OTR-A relative to Saline)	Stress X drug interaction
Social behaviour			
Social approach	↑	=	↑Social approach: SS-OTR-A vs SS-Sal and SS-OTR-A vs CTL-OTR-A
Social avoidance	=	=	
Total social interaction	↓		Social interaction ↓ by OTR-A in SS only ($p = 0.09$)
Anogenital sniffing	↑		
Other physical interaction	↓		
Corticosterone	↓	↑	
OT-IR in PVN	↓	=	
OT-IR & FOS-IR in PVN	↓	=	
FOS-IR			
PVN	↓	=	
ARC	=	↓ ($p = 0.08$)	
dLS	↑	=	
NAcC and NAcS	=	=	
4 subregions of MEA	=	=	
PFC-CG	↑	↓	
PFC-PL	↑	=	
PFC-IL	↑	=	
Hippoc-CA1	↑	=	
Hippoc-CA2	=	=	
Hippoc-CA3	↑	=	
iDG	↑	=	
sDG	↑	=	
OTR binding density			
PVN	↑	n.a.	n.a.
Hippocampus	=		

Large arrows indicate differences of $p < 0.05$. Differences of $p < 0.1$ and $p > 0.05$ are included. N.A. not applicable; OT, oxytocin; IR, immunoreactive PVN, paraventricular nucleus; CG, cingulate gyrus; PL, prelimbic cortex; IL, infralimbic cortex; ARC, arcuate nucleus; dLS, dorsal lateral septum; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; MeAD, anterodorsal medial amygdala; MeAV, anteroventral medial amygdala; MePD, posterodorsal medial amygdala; MePV, posteroventral medial amygdala; CA1, CA2, CA3, cornu ammonis regions 1, 2, and 3 of the hippocampus (hippoc); iDG, infrapyramidal layer of the dentate gyrus; sDG, suprapyramidal layer of the dentate gyrus; OTR, oxytocin receptor.

plasma corticosterone in males and females before and after exposure to a forced swim test after an intracerebroventricular injection with an OTR-A (Neumann et al., 2000). The reduced effect of OTR antagonism on plasma corticosterone concentrations in adolescent SS rats may be because of their reduced oxytocin-IR cell numbers in the PVN and perhaps a reduced number of oxytocin cells to regulate plasma corticosterone relative to controls. Moreover, adolescent SS exposure was associated with fewer activated oxytocin neurons in the PVN in response to a social encounter. Our data demonstrate that the oxytocin-induced regulation of HPA responses is reduced after adolescent social instability in male rats.

Because of our previous finding of greater levels of plasma corticosterone when paired with an unfamiliar peer after 1 h of isolation in adolescent SS rats compared with controls that underwent 1 h of isolation and returned to their familiar partner (Hodges and McCormick, 2015), we speculated that a possibility for SS rats spending less time interacting with unfamiliar peers is because of high corticosterone inducing increased anxiety. Increased generalized anxiety was found in adult male rats after repeated subcutaneous treatment (Mitra and Sapolsky, 2008) or intraperitoneal treatment (Li et al., 2017) with high corticosterone. In contrast to our speculation, adolescent SS rats spent less time interacting with unfamiliar peers even though saline-treated adolescent SS rats did not show an increase in plasma levels of corticosterone and OTR-A-treated adolescent SS rats showed reduced levels of plasma corticosterone compared to controls. The effect of stressor exposure and corticosterone treatment on sociability in rats is mixed. Repeated social defeat in adolescent male rats was found to increase social interactions with same-age peers in adulthood in one study (MacKay et al., 2017) and increase social avoidance in adult male rats (Carnevali et al., 2017). Corticosterone treatments between postnatal days 28 and 42 were found to increase social interactions with same-age peers in adolescent male rats (Veenit et al., 2013), and corticosterone treatment was found to restore time spent in social interaction to normal levels in adrenalectomized male rats (File et al., 1979). Thus, altered HPA responsiveness in adolescent SS rats may have contributed to increased social anxiety during physical social interactions, but this will need to be investigated further.

4.3. Oxytocin receptor antagonism did not restore altered activation of the paraventricular nucleus of the hypothalamus, lateral septum, prefrontal cortex, and hippocampus in response to interacting with an unfamiliar peer in male rats exposed to adolescent social instability

Our finding of reduced activation of the PVN of adolescent male SS rats compared with controls in response to being paired with an unfamiliar rat is consistent with our findings in Hodges et al. (2018). One study reported fewer Fos-IR cells in the PVN of adult male rats that underwent their 10th exposure to social defeat compared to rats that underwent their 1st exposure to social defeat (Martinez et al., 1998). Multiple exposures to unfamiliar rats may explain reduced activity in the PVN of adolescent SS rats compared to controls in response to interacting with an unfamiliar peer. As previously mentioned, adolescent SS rats also had reduced oxytocin IR cells and reduced activation of oxytocin neurons in the PVN in response to interacting with an unfamiliar peer. Reduced activation of oxytocin neurons in the PVN may cause adolescent SS rats to experience high anxiety and reduced time spent interacting with unfamiliar peers. The bilateral administration of oxytocin to the PVN was found to reduce anxiety-like behaviour on an elevated plus maze in adult male rats (Blume et al., 2008). Adolescent SS might increase OTR binding density in the PVN to compensate for the reduction in oxytocin cell activity. Nevertheless, the higher OTR binding densities in SS rats than in CTL rats did not render our intraperitoneal OTR-A treatment more effective at increasing corticosterone in SS rats. Direct injections of an OTR-A into the PVN of adolescent SS rats might reduce social interactions to a greater extent than in controls, and further studies will be required to determine if reduced activity of oxytocin neurons may represent reduced oxytocin the PVN in response to social interaction in adolescent SS rats

The finding of increased Fos-IR in the dorsal lateral septum in response to social approach and social interaction (and, possibly the blood sampling) in SS rats compared with controls is in contrast to our previous finding of no difference in Fos-IR between SS and control rats in response to only social interaction (Hodges et al., 2018). The lateral septum contributes to social recognition and activity of the lateral septum facilitates the investigation of novel objects over familiar objects in male rats (Lukas et al., 2013; Veenema et al., 2012; Myhrer, 1989). Although we have evidence that SS rats can discriminate between familiar and unfamiliar peers when these are behind mesh, when physical interaction is possible, SS rats spent the same amount of time interacting physically with familiar and unfamiliar same-age, whereas controls would spend more time in interaction with unfamiliar peers (Hodges et al., 2017). Thus, the greater activity of the lateral septum may reflect a dysfunction in SS rats. SS rats also had increased Fos-IR in all analyzed subregions of the prefrontal cortex and in all analyzed subregions of the hippocampus except for the CA2 in response to social approach and social interaction compared with controls. Our finding of no difference in Fos-IR in the CA2 between SS and CTL rats in response to social approach and social interaction is consistent with our previous

finding in the CA2 in response to social interaction only (Hodges et al., 2018). The prefrontal cortex and the hippocampus are both involved in the ability of rats to perform the correct sequence of social behaviours with a peer during social interaction (Bell et al., 2009; Himmler et al., 2014; Kolb and Nonneman, 1974; Maaswinkel et al., 1997). Increased activity of the prefrontal cortex and hippocampus of SS rats suggest a stronger recruitment of these regions in response to interacting with an unfamiliar peer. Whether a stronger recruitment of these regions is involved causally with the atypical social behaviour of SS rats or is the result of their atypical social behaviour has yet to be determined.

OTR antagonism was found to reduce neuronal activation of the cingulate gyrus and the arcuate nucleus in response to being paired with an unfamiliar peer in both SS and control rats. The cingulate gyrus is involved in the ability to display correct social behaviours with a peer (Bell et al., 2009; Schneider and Koch, 2005), however, no effect of OTR antagonism was observed for time in social interaction. A more fine-grained analysis of the quality of social interactions may be warranted. The arcuate nucleus regulates circulating plasma corticosterone (Leon-Mercado et al., 2017; reviewed in Herman and Cullinan, 1997). For example, plasma corticosterone levels decrease in response to corticosterone or glucocorticoid receptor agonists administered into the arcuate nucleus (Leon-Mercado et al., 2017) and plasma corticosterone levels increase when the arcuate nucleus is lesioned (Larsen et al., 1994) in male rats. Reduced activation of the arcuate nucleus after OTR-A treatment may be causally involved in increased plasma corticosterone levels in OTR-A treated rats compared with saline-treated rats.

The sample size and number of dependent variables did not allow for a multivariate statistical network analysis, and we were thus restricted to a more descriptive approach in investigating the pattern of activity across brain regions. When controlling for drug treatment, there were negative associations between Fos-IR in the prefrontal cortex and hippocampus regions in SS rats, which were positively associated in control rats. There were positive associations between Fos-IR cell counts in hippocampal regions and counts in the medial amygdala, lateral septum, hypothalamus, and nucleus accumbens in SS rats, which were negative in controls. The nucleus accumbens is involved in the preference for social novelty (Smith et al., 2017a) and the rewarding properties of social interactions (Dölen et al., 2013; Hung et al., 2017; LeGates et al., 2018) in adolescent and adult male rodents. We previously demonstrated increased oxytocin receptor binding in the nucleus accumbens of SS compared CTL rats (Hodges et al., 2018) and differences in dendritic structure in subregions of the medial amygdala and lateral septum of SS rats compared with CTL rats (Hodges et al., 2019). The medial amygdala is implicated in many social behaviours, including socially investigating same- and opposite-sex conspecifics (Adekunbi et al., 2018; Donato et al., 2010; Newman, 1999). Our Fos-IR data suggest differences in co-ordinated neural activity between social brain regions in adolescent SS rats and controls in response to social behaviour testing.

Another limitation in this experiment was that the Fos-IR was in response to both social approach and social interaction. Thus, it is difficult to interpret what the Fos-IR cell counts mean for increased social approach and reduced social interaction in SS rats. Fos-IR cell counts in social brain regions in response to social approach behaviours are likely to be resulting in either higher or fewer Fos-IR cell counts in those regions in response to social interactions with unfamiliar peers. The intraperitoneal injection in this study may have also influenced differences in Fos-IR findings between this study and the Hodges et al. (2018) study (Fos-IR is increased in response to intraperitoneal injection; Ryabinin et al., 1999). Moreover, even though rats did not display any agitated behaviour toward our blood sampling procedure and it is a very quick procedure, blood sampling may have influenced corticosterone release and Fos-IR in all brain regions. Thus, group differences in Fos-IR only suggests the possible involvement of certain brain regions in social interaction because Fos can be increased by any number of stimuli and events other than those under consideration in the present experiment.

5. Conclusion

Our findings provide evidence that oxytocin function in social brain regions is vulnerable to altered social experiences in the adolescent period, such that adolescent SS disrupts the influence of oxytocin on social behaviour, HPA function, neural function in response to social behaviour, and alters OTR binding in the PVN. Altered connectivity between social brain regions and OTR binding in adolescent SS may combine to render social behaviours, such as social avoidance, more susceptible to the effects of oxytocin. Moreover, differences in the activity of oxytocinergic neurons and in OTR binding in the PVN may combine to alter the HPA function of SS rats in response to social behaviour and social context. Differential cross-talk between and within social brain subregions impacts multiple social behaviours and can result in altered glucocorticoid exposure, and these types of changes during adolescence can act disrupt proper social and neural development (reviewed in Burke et al., 2017; Pellis and Pellis, 2017), highlighting the detrimental effects of changing the quality of social experiences in adolescence.

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