The sex-specific effects of neuropeptide receptors in the bed nucleus of the stria terminalis on social behavior

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Abstract

The neuropeptides oxytocin (OT) and vasopressin (AVP) are key regulators of social and emotional behaviors. Accumulating evidence suggesting a role of OT, AVP, and their receptors in the pathophysiology of psychiatric disorders has sparked interest in these systems as therapeutic targets. Nonetheless, the specific neural circuits mediating context and sex specific behavioral effects of OT and AVP are not fully understood. Using the California mouse model of social defeat, we previously found that social defeat stress increases the reactivity of OT neurons in the medioventral bed nucleus of the stria terminalis (BNSTmv) and paraventricular nucleus in female but not male California mice. We also found that intranasal administration of OT has no effect on male social interaction, but reduces this behavior in females, mirroring the effect of social defeat stress. Here we conducted a series of experiments aimed at identifying receptor populations involved in sex specific effects of stress on social interaction behavior and specific sites of action. First we studied the effects of sex and social defeat stress on AVP receptor V1a (V1aR) binding in the forebrain. In females but not males, V1aR binding in the BNSTmv was negatively correlated to social interaction behavior. We hypothesized that activation of this receptor population was mediating stress-induced social deficits, but infusions of V1aR antagonist into the BNSTmv had anxiogenic effects in both males and females. Next, we studied the effects of systemic administration of an OT receptor antagonist (OTA) on social behavior in control and stressed males and females. We found that one dose of OTA reverses the effects of stress on female social behavior, but has the opposite effects in males. To identify potential sites of action, we used immediate early gene immunohistochemistry in mice that received intranasal OT or control infusions. We found that stress increases EGR-1 immunoreactivity in the dorsolateral nucleus accumbens core and anteromedial BNST in females but not males. Based on these results, we performed site-
specific injections of OTA in stressed females. A single dose of OTA within the anteromedial BNST rapidly reversed stress-induced social avoidance in females. Together, these results support the hypothesis that stress-induced hyperactivity of OT neurons contributes to some stress-induced changes in female social behavior by activating OT receptors, and that OTR antagonists may have unappreciated therapeutic potential for stress-induced psychiatric disorders.
Chapter 1. Inhibition of vasopressin V1a receptors in the medioventral bed nucleus of the stria terminalis has sex- and context-specific anxiogenic effects

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Summary
Vasopressin V1a receptors (V1aR) are thought to contribute to the pathophysiology of psychiatric disorders such as anxiety and depression, sparking interest in V1aR as a therapeutic target. Although the global effects of V1aR have been documented, less is known about the specific neural circuits mediating these effects. Moreover, few studies have examined context-specific V1aR function in both males and females. By using the California mouse, we first studied the effects of sex and social defeat stress on V1aR binding in the forebrain. We found that in females but not males, V1aR binding in the bed nucleus of the stria terminalis (BNST) is negatively correlated to social interaction behavior, and that stress increases V1aR binding in the nucleus accumbens (NAc). As the BNST is a key regulator of social behavior and anxiety, we then examined the effects for site specific V1aR inhibition in BNST in control and stressed males and females. Infusions of V1aR antagonist in to the medioventral BNST (BNSTmv) had anxiogenic effects only in animals naïve to defeat. For males, inhibition of V1aR in BNSTmv had anxiogenic effects in social and nonsocial contexts, but for females, anxiogenic effects were limited to social contexts. We also examined effects of V1aR antagonist in the NAc shell in stressed females. Inhibition of V1aR in the NAc shell had no effect on social interaction behavior, but had an anxiogenic effect in an open field test. These data suggest that V1aR in BNSTmv have anxiolytic and prosocial effects in males, and that in females, prosocial and anxiolytic effects of V1aR appear to be mediated independently by receptors in the BNSTmv and NAcsh, respectively. These findings suggest that males have more overlap in neural circuits modulating anxiety in social and nonsocial contexts than females.
Introduction

Vasopressin V1a receptors (V1aR) are key mediators of a myriad of social behaviors including social recognition (Bielsky et al., 2004), social communication (Albers et al., 1986; Lukas and Wohr, 2015), and social approach (Ramos et al., 2013). Pharmacological inhibition of V1aRs can produce anxiolytic effects in mice (Bielsky et al., 2004) while genetic deletion of V1aR in mice produced both anxiolytic effects and reductions in social function (Bielsky et al., 2004). The modulation of both social behavior and anxiety by V1aR has sparked interest in its potential as a novel therapeutic target for stress-related psychiatric disorders, in which anxiety and social deficits can be comorbid (Lee et al., 2013; Meyer-Lindenberg and Tost, 2012). Currently it is unclear whether the effects of V1aR on social and anxiety-like behavior are mediated by independent or overlapping circuits. Although there are important species differences in V1aR binding (Bester-Meredith et al., 1999; Hammock and Young, 2006), dense V1aR binding in the bed nucleus of the stria terminalis (BNST) is observed in a diverse group of rodents (Bales et al., 2007; Bosch et al., 2010) and primates (Freeman et al., 2014a; Freeman et al., 2014b; Young et al., 1999). The BNST has recently been proposed to be a key nucleus for stress-induced psychiatric disorders (Lebow and Chen, 2016), as it is an important regulator of both social behavior (O’Connell and Hofmann, 2011) and affective states (Avery et al., 2015; Hammack et al., 2012). Thus, the BNST is well positioned to modulate effects of neuropeptides on anxiety and social behavior (Coria-Avila et al., 2014; Greenberg et al., 2014; Markham et al., 2009). The BNST is sexually dimorphic both at functional and anatomical levels, which is important because stress-induced psychiatric disorders are more common in women than men (Kessler et al., 1995). Here we examined the effects of social stress on V1aR binding in the BNST and the effects of V1aR inactivation on behavior using the California mouse model of social defeat.

The California mouse is a monogamous species in which both males and females aggressively defend a joint territory (Ribble and Salvioni, 1990). This social organization gives ethological validity to the use of protocols in which males and females experience social defeat (Trainor et al., 2013). Three episodes of social defeat reduce social interaction behavior in females but not males (Greenberg et al., 2015; Trainor et al., 2011). This effect is independent of gonadal steroids (Trainor et al., 2013) and can be reversed with chronic but not acute sertraline treatment (Greenberg et al., 2014). Male California mice exposed to defeat exhibit increased fear behavior in a resident-intruder test (Steinman et al., 2015), similar to the conditioned defeat phenotype described in Syrian hamsters (Clinard et al., 2015; Gray et al., 2015). We first examined the effects of social defeat on V1aR binding in the hypothalamus, lateral septum, BNST, and nucleus
accumbens (NAc). These nuclei are important regulators of social and affective behavior (Davis et al., 2010; Walker and Davis, 2008), and have strong connections with one another (O'Connell et al., 2011). These data showed in the BNSTmv social interaction behavior was negatively correlated with V1aR expression in females but not males. In a previous study we observed that that defeat increased the activity of OT neurons in the BNSTmv of females but not males and that intranasal OT reduced social interaction behavior in females but not males (Steinman et al., 2016). A recent elegant study showed that V1aR can be activated by OT release (Song et al., 2014), so we hypothesized that stress-induced increases in the activity of OT neurons in the BNSTmv reduces social interaction behavior through activation of V1aR. To test this hypothesis, we infused V1aR antagonist in to the BNSTmv. Autoradiographic analyses also showed that in females but not males, V1aR expression is significantly increased in NAc core and shell. Previous studies have implicated an important role of NAc shell on interaction behavior (Campi et al., 2014; Trainor et al., 2011), so we examined whether inhibition of V1aR in the NAc shell in stressed females could increase social interaction behavior. Finally, we examined the effects of V1aR inhibition in the resident intruder test in males based on previous work showing V1aR regulation of aggression. Our results provide important insights into how V1aR modulates anxiety-like behavior in social and nonsocial contexts in males and females.

Materials and Methods

Experiments. Four different experiments were conducted. Experiment 1: Effects of social defeat stress on V1aR binding in males (naïve n=8, stress=7) and females (naïve n=11, stress=9); Experiment 2: effects of V1aR antagonist (V1aR ant) infusion into BNSTmv on social interaction behavior in naïve (aCSF=6, V1aR ant=10) and stressed (aCSF=8, V1aR ant=8) females; Experiment 3: effects of V1aR antagonist infusion into BNSTmv on social interaction behavior and territorial aggression in naïve (aCSF=8, V1aR ant=10) and stressed (aCSF=8, V1aR ant=9) males; and Experiment 4: effects of V1aR antagonist infusion into NAc shell on social interaction behavior in stressed females (aCSF=7, V1aR ant=8). Procedures are detailed below (see Fig. 1 for timelines).

Animals and housing conditions: Three-month old male and female California mice from our breeding colony at UC Davis were group housed (2–3 same-sex animals per cage). Those that
underwent the cannula implantation surgery were singly housed immediately following the procedure. Animals were ear punched for identification and maintained in clear polypropylene cages in a room with controlled temperature (68-74 °F) and 16L:8D light:dark cycle (lights off at 2pm). Humidity was maintained at ambient levels. Water and food (Harlan Teklad 2016, Madison, WI) were provided ad libitum. Each polycarbonate plastic cage was provided with Sanichip bedding and environmental enrichment consisting of nestlets and enviro-dri. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines. All behavioral tests were conducted during lights out (1430–1730) under dim red light (3 lux).

Social defeat: Mice were randomly assigned to social defeat or control handling for 3 consecutive days. Mice assigned to social defeat were placed in the cage of an aggressive same-sex mouse on consecutive days (Trainor et al., 2013). Each episode lasted 7 min or until the resident attacked the focal mouse 7 times (whichever occurred first). Control mice were introduced into a clean cage for 7 min. Immediately after defeat/control mice were returned to their home cage. All behavioral and receptor binding analyses were conducted two weeks after social defeat based on previous observations that the effects of defeat become stronger over time (Trainor et al. 2011).

Social interaction test (SI): Two weeks after defeat or control conditions, all animals were tested in SI as previously described (Greenberg et al., 2014; Trainor et al, 2013). First, each focal mouse was introduced into the open field (89x63x60cm) for 3 min (open field phase). Total distance traveled (Anymaze, Stoelting) was recorded to assess locomotor behavior, and time spent in the center of the arena (within 8 cm of the sides and within a center zone located 14 cm from the sides) was recorded to assess anxiety-like behavior. Next, an empty wire cage was placed against one wall (acclimation phase). The amount of time that the focal mouse spent within 8 cm of the cage (interaction zone) and in the corners opposite to the wire cage was recorded for 3 min. Finally, an unfamiliar intact same-sex mouse was placed into the wire cage for 3 min (interaction phase) and the time spent in the interaction zone and corners was recorded (Fig.2). Mice were returned to their home cage immediately after the end of SI.

Receptor Autoradiography: V1aR binding was measured using receptor autoradiography according to previously established methods (Perkeybile et al., 2015). Immediately following SI
testing mice were euthanized and brains were frozen on dry ice. Brains were sliced at 20 μm using a cryostat and mounted on Super-frost plus slides. Slides were fixed in 0.1 % paraformaldehyde (Sigma Aldrich, St. Louis Missouri, USA) in PBS for 2 min and then rinsed in two 10 min tris buffer washes 50 mM Trizma Base (Sigma, pH 7.4) followed by a 1 hr-room temperature incubation in 50 pM of radiotracer diluted in a tracer buffer (50 mM Trizma Base (pH 7.4), 10 mM MgCl₂, 0.1% bovine serum albumin). We used ¹²⁵I-lin-vasopressin [¹²⁵I-phenylacetyl-D-Tyr(ME)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂] (NEN Nuclear) to detect V1aR. Non-specific binding was identified by immersing adjacent sections in buffer containing both the radioactive specific ligand and 50 μM of unlabeled competitor ligand, (d(CH₂)₅,Tyr(Me)²,Arg⁸)-Vasopressin (Bachem, King of Prussia, PA). Following a 60 min incubation, were washed four times in 50 mm Trizma Base (pH 7.4) with 10 mM MgCl₂ at 4 °C, with a subsequent 30 min wash. Slides then were rinsed in cold water and dried for 15 min. Sections were apposed to Kodak BioMaxMR film (Kodak, Rochester, NY, USA) with ¹²⁵I microscale standards (Perkin-Elmer/NEN) for 72 h. Sections as well as ¹²⁵I microscale standards (Perkin-Elmer/NEN) were placed on Kodak BioMaxMR film (Kodak, Rochester, NY, USA) for 96 h. ImageJ was used to quantify ¹²⁵I-receptor binding. Uncalibrated optical density was converted disintegrations per minute (DPM) using the ¹²⁵I standards. If available, six sections per area were quantified bilaterally and then averaged for analysis. In some animals, one or two sections were damaged, so only four or five were available for analysis. The damaged sections were distributed across all four groups. On each section we also measured a nearby white matter tract that did not show receptor binding and subtracted this from the measurements for the area of interest to account for background (Inoue et al, 2013; Laredo et al, 2015). There was no difference in signal in these regions and sections used to control for non-specific binding.

**Cannula implantation surgery:** Males and females were randomly assigned to control or defeat conditions as described above. Seven days after defeat or control handling, all mice were anesthetized with isoflurane (3–5%) and implanted with bilateral stainless steel guide cannula (Plastics One, C235G-2.2W 1 mm PROJ) aimed at the BNSTmv (AP=0.39 mm, LM=1.1 mm, DV=6.85, fig. 4) (fig.3). The guide cannulae (26 ga, o.d.=0.46 mm; i.d.=0.24 mm; length=5.85 mm), were lowered into burr holes (#105 dremel bit, 1/16″ tip) and attached to the skull with skull screws (plastics one, 00-96 X 1/16) and acrylic dental cement. Bilateral dummy caps (Plastics One, C235DC) were used to maintain patency. Carprofen (5mg/kg) was administered subcutaneously immediately before and once a day during 3 consecutive days after surgery.
Animals were given seven days for recovery, during which the mice were observed and handled daily. An additional set of stressed females was implanted with guide cannulae aimed at the NAc shell as previously described (AP=0.51 mm, LM=1.1 mm, DV=6.85) (Campi et al, 2014). Seven days after recovery, each female received an infusion of V1aR antagonist or aCSF and was tested in the social interaction test as described above. Males and control females were not included in this analysis due to very low V1aR binding levels.

**V1aR antagonist infusion:** In males and females, infusions were made using bilateral internal cannulae (Plastics One, C235I/SPC, 33ga, o.d.=0.21 mm; i.d.=0.11 mm) that projected 1 mm past the cannula guide (6.85 mm total length). The V1aR antagonist [β-Mercapto-β,β-cyclopentamethylenepropionyl11, O-me-Tyr2, Arg8]-Vasopressin (Manning compound, Sigma catalog #:V2255) was dissolved in 2 ml artificial cerebrospinal fluid (aCSF), and frozen in 50 microliter aliquots at a dose of 0.5 mg/ml. Prior to daily injection, one aliquot was further dissolved in aCSF at a 1:20 dilution. Animals were randomly assigned to receive a 200 nl infusion per side containing either aCSF or 5 ng of V1aR antagonist. This dose was adjusted from dose previously shown to have behavioral effects when injected into BNST in rats (10 ng per side) (Veenema et al, 2010). The BNST in California mouse is roughly half the size of rat BNST based on a California mouse brain atlas (brainmaps.org). Hamilton syringes were attached to an automatic micropump delivery apparatus (PHD 2000, Harvard Apparatus, Cambridge, MA) set to deliver 0.1uL/min. Internal guides were kept in place for 1 min after injection. Both males and females were given one infusion, returned to the home cage, and then tested in SI after 30 min (Figs 1 and 2). Females were euthanized after testing to determine injection sites (for hits, see Fig.3). One day after SI, males were tested in the resident-intruder test (described below) 30 min after receiving a second infusion of the same treatment they had received the day before. Males where then euthanized after testing to determine injection sites (Fig.3).

**Resident-Intruder test (RI):** One day after SI, males were tested in RI as previously described (Steinman et al, 2015). Cage bedding had not been changed for three days, which has been shown to increase aggressive behavior (Bester-Meredith et al, 1999). Thirty minutes after the infusion of the V1aR antagonist or aCSF, a group housed, an unfamiliar same-sex intruder was introduced into the home cage of the resident. Tests lasted for 7 min after which the intruder was removed. Tests were video recorded and bouts of aggression, social investigation, and defensive
behaviors (freezing, escape) were quantified as previously described (Steinman et al, 2015; Trainor et al, 2010) (Table 1).

**Histology:** Brains were fixed overnight in 5% acrolein in PBS, after which they were immersed in 20% sucrose in PBS for a day, frozen and sectioned coronally at 40 μm. In order to confirm needle placement, sections were stained using NeuroTrace® 435/455 Blue Fluorescent Nissl Stain (Life Technologies). Mice with injection sites outside of the BNSTmv were excluded from statistical analysis.

**Estrous cycle assessment:** Conducting vaginal lavage before testing disrupts behavior in California mouse (Silva et al, 2010), so estrous cycle was assessed post mortem. There was no systematic bias in the distribution of estrous stage across treatment groups.

**Statistical analysis:** All the statistical analyses were performed with R software. For receptor autoradiography data we used two-way ANOVA (sex and stress). We also performed regression analyses of time spent engaged in social interaction using sex, V1aR binding, and their interaction as parameters (package “ggplot”). Two-way ANOVA was used to analyze behaviors in the SI and RI tests (stress and treatment). For two-way ANOVA analyses that showed a significant interaction between stress and treatment, planned comparisons were used to detect differences between groups (package “lsmeans”, Bonferroni, 0.95 confidence interval). Cohen's d is used to report effect size in this case. Finally, we also performed a 3-way ANOVA to assess possible effects of estrous cycle (stress, treatment and estrous cycle) in the social interaction test.

**RESULTS**

**Effect of stress on V1aR binding in males and females:** There was a stress*sex interaction on V1aR expression in NAc core (Fig.4a, F$_{1,24}$=6.39, p<0.05) and shell (Fig.4b, F$_{1,24}$=4.33, p<0.05). In males, there was no effect of defeat stress on V1aR expression in NAc core (p=0.9, Cohen's $d$ (d)=0.15 ) or shell (p=0.83, d=0.05 ) , while in females, defeat stress significantly increased expression of V1aR in NAc core (Fig.4a, F$_{1,13}$=8.42, p<0.05, d= 1.17) and shell (Fig. 4b, F$_{1,13}$=6.42, p<0.05, d=1.34). In the BNSTmv, dorsal BNST, lateral septum (LS) dorsal, LS
ventral, paraventricular nucleus (PVN), and medial preoptic area (mPOA), there were no main effects of sex or stress (Fig. 4c-h). However, regression modeling of interaction time including the parameters of sex, V1aR binding, treatment, and the interaction between sex and V1aR binding revealed a significant difference between the slopes of V1aR binding in BNSTmv and interaction time in males and females (fig. 5a, regression model F₄,₂₂=5.21, sex * V1aR p=0.004). We also found a significant sex difference between slopes of V1aR binding in NAc core and interaction time (fig. 5b, regression model F₄,₂₂=3.41, sex * V1aR p=0.043), but this was mainly driven by an outlier. Regression models did not indicate any significant interaction between slopes for males and females for any other of the areas analyzed (all p’s > 0.1, table 2).

**Effects of V1aR antagonist in BNSTmv on female behavior:** During the social interaction phase, both defeat (Fig. 6d, F₁,₂₈=5.74 p=0.02) and V1aR antagonist (Fig. 6d, F₁,₂₈=5.08, p=0.03) reduced time spent in the interaction zone. Although the stress*antagonist interaction was not significant (Fig. 6d, F₁,₂₈=0.84, p=0.36), planned comparisons revealed that V1aR antagonist reduced social interaction in control females (Fig. 6d, p=0.01 d=1.29) but not in stressed females (Fig. 6d, p=0.21 d=0.86). This is likely due to a floor effect, because stressed females treated with aCSF had significantly reduced social interaction compared to control females treated with aCSF (Fig. 6d, p=0.02, d=1.33). Interestingly, the effect size of V1aR antagonist in control females is similar to the effect size of social defeat in aCSF treated females. In females the effect of the V1aR antagonist in the BNSTmv appeared specific to social contexts, as there was no effect of V1aR antagonist in the acclimation phase on time in the interaction zone (Fig. 6c). However, defeat stress reduced time spent in the interaction zone during the acclimation phase (F₁,₂₉=12.06, p=0.001, Fig 6c). There were no differences in time spent in the center (fig. 6a) or locomotor behavior (fig. 6b) during the open field phase. When estrous phase was included in to the ANOVA, there was no effect of cycle and no interactions on time spent in the cage during acclimation or time spent in open field (Table 3, all p’s > 0.1).

**Effects of V1aR antagonist in BNSTmv on male behavior:** During open field testing, there was a significant stress*treatment interaction on time spent in center (F₁,₃₁= 7.66, p=0.009, fig. 6e). V1aR antagonist significantly reduced time spent in the center only in naïve animals (p=0.02, d=1.14), but not in stressed animals (p=0.16, d=0.74). There were no differences in total distance traveled (Fig. 6f). During the social interaction test, there were no main effects of stress or treatment on time spent in the interaction area, but there was a trend for a stress*treatment
interaction ($F_{2,34}=3.64, p=0.06$). Planned comparisons revealed that V1aR antagonist significantly reduced interaction time in naïve ($p=0.03, d=1.11$) but not stressed males ($p=0.7, d=0.18$) (Fig. 6h). During acclimation phase, there was a main effect of V1aR to reduce time spent in proximity to the empty cage (Fig.6g, $F_{1,31}=6.78, p=0.01$). However planned comparisons detected an effect of V1aR antagonist in control males ($p=0.02$, Cohen's $d=1.35$) but not stressed males ($0.21, d=0.52$).

In the RI test there was a significant stress*treatment interaction on latency to box (Fig 8a, $F_{1,31}=7.36, p=0.01$), with V1aR antagonist significantly increasing latency in stressed animals ($p=0.04, d=0.94$), and with no effect in naive animals ($p=0.1, d=0.82$). No significant differences were observed in other behaviors quantified during the resident-intruder tests.

**Effects of V1aR antagonist in NAcsh on female behavior:** The V1aR antagonist reduced time spent in the center during open field testing in stressed females (fig. 9a, $F_{1,13}=4.90, p=0.04$). There was no effect of V1aR on social interaction time, time spent in the cage zone during acclimation or distance traveled during open field (fig. 9 b,c, and d).

**DISCUSSION**

Our results suggest that in both males and females, V1aR acting in BNSTmv facilitates social approach in naïve but not stressed animals. Interestingly, while V1aR inhibition in the BNSTmv had anxiogenic effects in both social and nonsocial contexts in males, the effects of V1aR inhibition in the BNSTmv in females were limited to social contexts. Infusions of the V1aR antagonist in the NAcsh, an area which is proximal anatomically to BNSTmv, had an anxiogenic effect in a non-social context in stressed females. Together, these data suggest that effects of V1aR on anxiety-like in males is less context-dependent in males and that in females different populations of V1aR modulate anxiety-like behaviors in social and non-social contexts.

**Anxiogenic effects of social defeat stress and V1aR antagonist**

We previously showed in the California mouse that three episodes of social defeat stress induces social withdrawal in females but not males. In addition stress increased the activity of OT neurons in the BNSTmv (Steinman *et al*, 2016). When we observed that females with more V1aR
binding in the BNSTmv engaged in less social interaction, we hypothesized that increased OT release might reduce social interaction through V1aR (Song et al., 2014). However, the results from our experiments using V1aR infusions do not support this hypothesis. V1aR antagonist in the BNSTmv had no effect in stressed females and actually reduced social interaction behavior in both males and females naïve to defeat. Previous work has demonstrated that global V1aR inhibition in male rodents also reduces social interaction. For example, systemic injection of OT or AVP in adult male rats increased social interaction and these effects were blocked by systemic injections of the V1aR antagonist SR49059 (Ramos et al., 2013). Similarly, male V1aR knockout mice exhibited reduced social interaction behavior compared to wildtype littermates (Egashira et al., 2007). Our results suggest that these effects of V1aR observed in male rodents generalize to females, and may be mediated in part by V1aR in the BNSTmv. Although we did not specifically include an anatomical control in this study, we observed different effects of V1aR antagonist infused in the NAc (see below). Our results highlight the sex- and experience-dependent effects of central V1aR and leave open the question of whether V1aR expressing regions adjacent to BNSTmv may modulate social behaviors.

Autoradiography data also showed that stress increased V1aR binding in the NAcsh and NAc core in females but not males. Previous autoradiography studies in rodents have reported low levels of V1aR binding in the NAc (Johnson et al., 1993), so this stress-induced effect is a novel finding. The NAcsh has been previously implicated in sex specific effects of social defeat in California mouse (Campi et al., 2014; Trainor et al., 2011), so we next hypothesized that stress-induced increases in OT neural activity in females might reduce social interaction behavior by activating V1aR in the NAcsh. However, V1aR antagonist infusions into the NAcsh in stressed females had no effect on social interaction behavior. It’s possible that V1aR in NAc core may work together with V1aR in NAcsh to inhibit social interaction behavior and that more global inhibition of V1aR in NAc might be necessary to see an effect on social interaction behavior. Still, overall our results do not support the hypothesis that increased V1aR activity contributes to stress-induced decreases in social interaction behavior in females.

Effects of V1aR inhibition on behavior: interaction with social defeat stress

While V1aR inhibition within the BNSTmv reduced social interaction behavior males and females naïve to defeat, there were no effects in stressed mice. Intriguingly, the effects of V1aR antagonist on social interaction in control mice were often similar to effects of social defeat in
mice treated with aCSF. It's possible that effects of defeat stress on male behavior could be mediated through decreased availability of AVP. Defeat stress reduces AVP immunoreactivity in both the PVN and BNST while lowering also Avp mRNA expression in the PVN of males (Steinman et al, 2015). Similar effects have been observed in male Mus musculus (Reber et al, 2007). However in our previous studies, social defeat alone was insufficient to reduce social interaction in males (Greenberg et al, 2014; Greenberg et al, 2015; Trainor et al, 2011; Trainor et al, 2013). Only when stressed male California mice receive an acute stressor such as an intranasal infusion (Steinman et al, 2016) or intracranial infusion (this paper) is reduced social interaction observed in stressed males. Handling or immobilization increases the activity of locus coeruleus (LC) neurons (Kawahara et al, 2000) and norepinephrine release (Smagin et al, 1997), which in turn can reduce social interaction in male rats (Cecchi et al, 2002; Varlinskaya and Spear, 2012). It's possible that social defeat exaggerates these responses. For example social defeat increased corticotropin releasing hormone (CRH) in the LC (Reyes et al, 2015) and CRH is known to increase neural discharge rates of LC neurons (Curtis et al, 1997; Jedema and Grace, 2004).

If stressed male California mice had increased noradrenergic responses to handling, this might account for decreased social interaction following infusions.

**Context-dependent effects of V1aR inhibition on behavior**

There were also sex differences in how V1aR modulated behavior in social and nonsocial contexts. For males V1aR inhibition in the BNSTmv had anxiogenic effects in social and nonsocial contexts, but in females V1aR inhibition only affected behavior in social contexts. Thus it appears that in males the effects of V1aR in the BNSTmv on anxiety-like behavior are no dependent on social context. For females V1aR receptors in BNSTmv affect behavior in social contexts but not non-social contexts. Interestingly we found an anxiolytic role for V1aR in NAcsh in females. To our knowledge anxiolytic effects of V1aR in the NAcsh have not been reported. The 3-fold increase in V1aR binding in stressed females but not males is particularly interesting in the context of the open field test. Defeat reduced time in the center of the open field in males but not females. These results suggest that, in socially stressed females, V1aR could be exerting a ‘compensatory’ effect, reducing the effects of stress on exploration in an unfamiliar, non-social environment.

Intriguingly, while V1aR inhibition had no effect on behavior in stressed males in an unfamiliar environment, it did affect behavior in stressed males tested in the resident-intruder test.
When focal males were tested as residents in a familiar home cage, stress significantly decreased latency to box in males, which is considered a defensive behavior (Linfoot et al, 2009; Litvin et al, 2007). These effects were partially reversed by the injection of V1aR antagonist into BNSTmv. Previous work in males has shown that the effects of V1aR are stronger in familiar environments compared to novel environments (Bester-Meredith and Marler, 2001). For the RI test, it’s important to consider V1aR antagonist treated mice were receiving a second injection. Thus it’s possible that prolonged inhibition of V1aR might have a different behavioral effect than acute inhibition.

Conclusions

Our results suggest that a specific population of V1aR in BNSTmv exerts anxiolytic effects in both social and nonsocial contexts for males but that for females the anxiolytic effects of these receptors is limited to social contexts. While prosocial effects of V1aR have been previously reported (Pitkow et al, 2001; Ramos et al, 2013), most studies have found anxiogenic effects of systemic V1aR inactivation in non-social contexts (Bielsky et al, 2004; Mak et al, 2012). Interestingly, overexpression of V1aR in the ventral pallidum of male prairie voles had anxiogenic effects in the elevated plus maze (Pitkow et al, 2001), suggesting that effects of V1aR on anxiety-like behavior are site specific. This is similar to the behavioral effects of brain derived neurotrophic factor which has antidepressant effects in hippocampus (Autry et al, 2011), but pro-depressive effects in NAc or BNST (Berton et al, 2006; Greenberg et al, 2014). Thus it appears that specific populations of V1aR have different effects on anxiety depending on neuroanatomical location, context, and sex. This means that any investigation of potential therapeutic use of V1aR targeting drugs needs to take these factors in to account. Continued study of the effects of V1aR on neural circuits of anxiety in rodents and humans should provide important insights in the potential of V1aR as a novel therapeutic target.

Acknowledgements

Special thanks to Sae Yokoyama who helped with experiments, and to Emilio Ferrer for suggestions on statistical analyses. Supported by NIH F31 MH095253 to MQS, R01 MH103322 to BCT, NSF GRF to SAL, Becas Chile CONICYT to NDW.
Fig. 1. Timeline for experiments 2-4. Males and females were tested in social interaction test (SI) after one injection of V1a receptor antagonist (V1aR) or artificial cerebrospinal fluid into medioventral bed nucleus of stria terminalis (exp. 2,3). Only males received a second injection before resident intruder test (RI) one day after SI (exp. 3). In experiment 4, only females were tested in SI after an injection of V1aR antagonist or saline into nucleus accumbens shell. Arrows represent end of study for females (orange) and males (blue).
**Fig. 2. Diagram of social Interaction test** (3 consecutive stages). Each phase of the social interaction tests, a) open field, b) acclimation, and c) interaction, lasted 3 minutes. Mouse in the small box represents novel conspecific target mouse. In light orange is represented the area considered as the interaction zone.
Fig. 3 Distribution of cannula placement into bed nucleus of the stria terminalis medioventral considered as hits in males and females. Injections that were misses are not shown (n=22). a) Representative picture of needle tracks used in order to determine hits. Ac= anterior commissure. b) California mouse coronal section from brainmaps.org showing most anterior section considered as hit and c) most posterior section considered as hit.
Fig. 4. V1aR binding in naïve and stressed males and females. Mean +/- SEM of V1aR binding in a) NAc core, b) NAc shell, c) BNSTmv, d) dorsal BNST (BNSTd), e) ventrolateral septum (vLS), f) dorsolateral septum (dLS), g) paraventricular nucleus (PVN), and h) medial preoptic area (mPOA). *P<0.05 effect of stress, n=7 female naïve, n=11 female stress, n=9 male naïve, n=8 male stress. Ac= anterior commissure, Fx=fornix, V1aR= V1a receptor, DPM=disintegration per minute.
Fig. 5. Relationship between V1aR binding and social interaction behavior. Fitted regression lines for V1aR binding on interaction time in males and females in a) BNSTmv and b) NAc core.
Fig. 6 Effects of V1aR inhibition in the BNSTmv in naïve and stressed males and females on behavior in the social interaction test. Control and stressed females and males receiving injections of artificial cerebrospinal fluid (aCSF) or V1aR antagonist into BNSTmv. Mean+/-SEM time in center (a,e) and distance traveled (b,f) during open field. Time spent in proximity to the empty cage (c,g) during acclimation trial, and time spent in proximity to a cage containing a novel same sex individual (d,h) during interaction trial. * p<0.05 effect of stress, † p < 0.05 effect of V1aR antagonist. Females: Control aCSF=6, control V1aR antagonist=10, stress aCSF=8, stress V1aR antagonist=8. Males: Control aCSF=8, control V1aR antagonist=10, stress aCSF=8, stress V1aR antagonist=9.
Fig. 7. Effects of V1aR inhibition in the BNSTmv in naïve and stressed males on behavior in the resident intruder test. Mean±SEM time spent showing Latency to box (a), and Anogenital sniffing (b), during resident intruder test in control and stressed males receiving injections of aCSF or V1aR antagonist into BNSTmv. † p < 0.05 effect of V1aR antagonist. n=8 control aCSF, n=10 control V1aR antagonist, n=8 stress aCSF, n=9 stress V1aR antagonist.

Fig. 9 Effects of V1aR inhibition in the NAc shell in stressed females on behavior in the social interaction test. Mean±SEM time in center (a) and distance traveled (b) during open field phase; time spent in proximity to the empty cage during acclimation phase (c) and time spent in proximity to a cage containing a novel same sex individual during interaction phase (d) in stressed females receiving injections of aCSF or V1aR antagonist into nucleus accumbens shell. † p < 0.05 effect of V1aR antagonist. Stress aCSF n=7, stress V1aR antagonist n=8

Table 1. Ethogram used for behavior scoring during resident intruder test. Behavioral observations were always focused on resident. Each activity was recorded in seconds of duration and frequency for a total of 7 minutes.
<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Environment investigation</td>
<td></td>
</tr>
<tr>
<td>Rearing</td>
<td>Resident rears into hind legs and sniffs environment</td>
</tr>
<tr>
<td>Non-social explore</td>
<td>Resident sniffs bedding</td>
</tr>
<tr>
<td>2. Social investigation</td>
<td></td>
</tr>
<tr>
<td>Social exploration</td>
<td>Resident sniffs intruder in any body area but anogenital</td>
</tr>
<tr>
<td>Anogenital sniffing</td>
<td>Residents’ nose in immediate proximity to the anogenital region of intruder</td>
</tr>
<tr>
<td>Move towards</td>
<td>Resident approaches intruder. Usually precedes social investigation</td>
</tr>
<tr>
<td>3. Aggressive behaviors</td>
<td></td>
</tr>
<tr>
<td>Attack latency</td>
<td>Time until resident first attacks intruder</td>
</tr>
<tr>
<td>Chase</td>
<td>Resident follows intruder in a chasing manner</td>
</tr>
<tr>
<td>Bite</td>
<td>Resident bites the intruder</td>
</tr>
<tr>
<td>Keep down</td>
<td>Resident pushes intruder to stay on his back</td>
</tr>
<tr>
<td>4. Defensive behavior</td>
<td></td>
</tr>
<tr>
<td>Submission latency</td>
<td>Time until resident assumes a submissive posture</td>
</tr>
<tr>
<td>Submissive posture</td>
<td>Resident lays down on his back in a submissive posture</td>
</tr>
<tr>
<td>Boxing</td>
<td>Resident stands on its hind legs and moves forepaws towards the intruder</td>
</tr>
<tr>
<td>Move away</td>
<td>Resident moves away from intruder</td>
</tr>
<tr>
<td>5. Anxiety related behaviors</td>
<td></td>
</tr>
<tr>
<td>Freezing</td>
<td>Resident standing on four paws and does not move any part of its body for &gt;2 s</td>
</tr>
<tr>
<td>Escaping</td>
<td>Resident stands along the edge of the home cage and jumps side to side</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>Resident grooms himself</td>
</tr>
<tr>
<td>6. Rest or inactivity (I)</td>
<td>Resident not active behavior but not freezing</td>
</tr>
</tbody>
</table>
Table 2. P values of linear regression model of V1aR binding and interaction time in males and females for all areas analyzed. * = p < 0.05

<table>
<thead>
<tr>
<th>Area</th>
<th>Sex</th>
<th>V1aR</th>
<th>Stress</th>
<th>Sex*V1aR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAc core</td>
<td>0.54</td>
<td>0.06</td>
<td>0.11</td>
<td>0.04*</td>
</tr>
<tr>
<td>NAc shell</td>
<td>0.77</td>
<td>0.48</td>
<td>0.08</td>
<td>0.37</td>
</tr>
<tr>
<td>BNSTmv</td>
<td>0.03*</td>
<td>0.00*</td>
<td>0.00*</td>
<td>0.00*</td>
</tr>
<tr>
<td>dorsal BNST</td>
<td>0.61</td>
<td>0.12</td>
<td>0.02*</td>
<td>0.08</td>
</tr>
<tr>
<td>LS dorsal</td>
<td>0.85</td>
<td>0.23</td>
<td>0.00*</td>
<td>0.37</td>
</tr>
<tr>
<td>LS ventral</td>
<td>0.93</td>
<td>0.17</td>
<td>0.00*</td>
<td>0.15</td>
</tr>
<tr>
<td>PVN</td>
<td>0.93</td>
<td>0.49</td>
<td>0.00*</td>
<td>0.23</td>
</tr>
<tr>
<td>mPOA</td>
<td>0.74</td>
<td>0.38</td>
<td>0.00*</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 3. Experiment 2: Effects of estrous phase on behavior during social interaction test. P values of 3 way ANOVA analysis for dependent variables (a) time in social interaction, (b) time in cage during acclimation, and (c) time in open field including independent variables estrous phase, treatment (trt), stress, and their interaction.

<table>
<thead>
<tr>
<th></th>
<th>Estrous phase</th>
<th>Treatment* estrous</th>
<th>Stress* estrous</th>
<th>Treatment* Stress*estrous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social interaction</td>
<td>0.85</td>
<td>0.12</td>
<td>0.65</td>
<td>0.73</td>
</tr>
<tr>
<td>Time in cage during acclimation</td>
<td>0.67</td>
<td>0.57</td>
<td>0.22</td>
<td>0.11</td>
</tr>
<tr>
<td>Time in open field</td>
<td>0.84</td>
<td>0.84</td>
<td>0.48</td>
<td>0.26</td>
</tr>
</tbody>
</table>
References


Chapter 2. Oxytocin receptors in the anteromedial bed nucleus of the stria terminalis promote stress-induced social avoidance in females

Natalia Duque-Wilckens, Michael Q. Steinman, Bice Chini, Marta Busnelli, Mary Pham, Sae Yokoyama, Sarah A. Laredo, Rebecca Hao, Allison M. Perkeybile, Karen L. Bales, Brian C. Trainor

Summary

The neuropeptide oxytocin (OT) is a key regulator of social and emotional behaviors. Reports in humans that OT has prosocial and anxiolytic effects have sparked interest in the potential use of OT as a therapeutic for stress-induced psychiatric disorders. Nonetheless, it has become clear that the effects of OT are highly context dependent, and that in some cases OT could even contribute to anxiety related states. It was recently proposed that OT increases the salience of social cues, which can explain why OT can either facilitate or inhibit social behaviors. In our previous work we found that social defeat stress increases the reactivity of OT neurons in the medioventral bed nucleus of the stria terminalis (BNST) and paraventricular nucleus in female but not male California mice. We also found that intranasal administration of OT has no effect on male social interaction, but reduces this behavior in females, mirroring the effect of social defeat stress. Here we conducted a series of experiments aimed at testing the hypothesis that stress-induced activation of OT receptors (OTR) contributes to social withdrawal in females, as well as identifying specific sites of action. First we studied the effects of systemic administration of an OTR antagonist (L-368,899, OTA) on social behavior in control and stressed males and females. To identify potential sites of action we used immediate early gene immunohistochemistry in mice that received intranasal OT or control infusions. We found that stress increases EGR-1 immunoreactivity in the dorsolateral nucleus accumbens core and anteromedial BNST in females but not males. Based on these results, we then performed site-specific injections of OTA in stressed females. Finally, we showed that unlike some OTR antagonists (atosiban), L-368,899
does not activate G proteins but prevents OT from activating G-proteins via OTR. Our main finding is that a single dose of OTA, either systemically or within the anteromedial BNST, rapidly reverses stress-induced social withdrawal in females. Our results support the hypothesis that stress-induced hyperactivity of OT neurons contributes to some stress-induced changes in female social behavior by activating OTR and that OTR antagonists may have unappreciated therapeutic potential for stress-induced psychiatric disorders.

INTRODUCTION

Oxytocin (OT) is an evolutionary conserved neuropeptide that is a key regulator of social and emotional behaviors (Dumais and Veenema, 2016; Heinrichs and Gaab, 2007; Johnson and Young, 2017; Neumann and Slattery, 2016). Reports in humans that OT has prosocial (Guastella et al. 2010; Hollander et al. 2007; Hurlemann et al. 2010; Kosfeld et al. 2005; Marsh et al. 2010; Zak, Stanton, and Ahmadi 2007) and anxiolytic effects (Domes et al. 2007; Heinrichs et al. 2003; Kirsch et al. 2005; Petrovic et al. 2008) have sparked interest in the potential use of OT as a therapeutic for stress-induced psychiatric disorders. Nonetheless, it is clear that the effects of OT are context dependent (Bartz et al. 2011; Bethlehem et al. 2014; Shamay-Tsoory and Abu-Akel 2016), and that in some cases OT is anxiogenic. For example, intranasal OT has been reported to enhance perceived social stress (Eckstein et al. 2014), mistrust (Bartz et al. 2011) and aggression (Striepens et al. 2012). Intriguingly, one study found increased number of hypothalamic OT immunoreactive neurons in humans suffering from depression (Purba et al. 1996). These results might reflect OT production as a homeostatic response to stress. An alternate hypothesis is that OT contributes to some aspects of behavioral pathology.

When considering these hypotheses, it is important to consider evidence for sex specific effects of OT. Intranasal OT reduces amygdala reactivity in males (Domes et al. 2007; Kirsch et
al. 2005), but has the opposite effect in women (Domes et al. 2010; Lischke et al. 2012). Sex dependent effects of intranasal OT have also been observed for social perception (Fischer-Shofty, Levkovitz, and Shamay-Tsoory 2013), and resting-state connectivity between amygdala and prefrontal cortex (Ebner et al. 2016). These sex specific effects of OT in humans are consistent with findings in animal research (Carter et al, 2009; de Vries, 2008; Dumais and Veenema, 2016), indicating the importance including both males and females in studies of OT function.

We previously observed that social defeat induces long-term increases in OT neurons activity in the medioventral bed nucleus of the stria terminalis (BNSTmv) and paraventricular nucleus (PVN) in female but not male California mice (Steinman et al. 2016). Similarly, resident-intruder tests induce a stronger increase in OT/c-fos colocalizations in PVN female prairie voles than males (Grippo et al. 2007). We also found that intranasal infusions of OT reduced social interaction in females, mirroring the effect of social defeat stress. In contrast, intranasal OT had no effect on social interaction in males. Based on these data, we hypothesized that stress-induced increases in the activity of OT neurons contribute to social avoidance in females.

Here we focus on the role of OT receptors (OTR) because a previous pharmacology study did not support the hypothesis that vasopressin receptors V1a (V1aR) contribute to stress-induced social withdrawal (Duque-Wilckens et al. 2016). We studied the effects of systemic administration of an OTR antagonist (OTA), the L-368,899, on social and non social behavior in control and stressed males and females. To identify potential sites of action of OTR, we used autoradiography to examine effects of defeat on OTR binding and immediate early gene immunohistochemistry in mice that received intranasal OT or control infusions. Based on these results, we then performed site-specific injections of OTA in either the dorsolateral nucleus accumbens core (NAcdl) or anteromedial bed nucleus of the stria terminalis (BNSTam) of stressed females. Our main finding is that a single dose of OTA, either systemically or within the BNSTam, rapidly reverses stress-induced social withdrawal in females, and that the effects are limited to social contexts. Our results
support the hypothesis that stress-induced increases in the activation OTR induce social withdrawal and imply that OTR antagonists may have unappreciated therapeutic potential for stress-induced psychiatric disorders.

MATERIALS AND METHODS

Animals

Three-month old male and female California mice from our breeding colony at UC Davis were ear punched for identification and maintained in clear polypropylene cages in a room with controlled temperature (68-74°F) and 16L:8D light:dark cycle (lights off at 1400). Humidity was maintained at ambient levels. Water and food (Harlan Teklad 2016; Madison, WI) were provided ad libitum. Each polycarbonate plastic cage was provided with Sanichip bedding and environmental enrichment consisting of nestlets and enviro-dri. All animals were group housed (2 to 3 same-sex animals per cage), since weaning until the end of the experiment, except for those that underwent the cannula implantation surgery, which were singly housed immediately following the procedure and until the end of the experiment. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines. All behavioral tests were conducted during lights out (1430-1730) under dim red light (3 lux).

Social defeat

Male and female mice were randomly assigned to control handling or social defeat for 3 consecutive days. Control mice were introduced into a clean cage for 7 min. Mice of social defeat group were placed in the cage of an aggressive same-sex mouse (Trainor et al. 2013).
Each episode lasted 7 min or until the resident attacked the focal mouse 7 times (whichever occurred first). No animal was physically injured. Immediately after defeat or control handling, mice were returned to their home cage. All behavioral and receptor binding analyses were conducted two weeks after social defeat based on previous observations that the effects of defeat become stronger over time (Trainor et al. 2011).

**Effects of systemic OTR antagonist (OTA) treatment**

To inhibit OTR, we used the high affinity non-peptide OTA L-368,899 (L2540, SIGMA) which passes through the brain-blood barrier (Boccia et al. 2007; Pettibone et al. 1993). Females exposed to social defeat or control conditions were given intraperitoneal injections of either vehicle (sterile PBS) or one of two doses of L-368,899 (1 or 5 mg/kg). These doses were based on previous behavioral studies of L-368,899 (Lee et al. 2015; Olszewski et al. 2013; Wei et al. 2015). Injections were administered 30 min before testing based on pharmacokinetic data (Thompson et al. 1997). After establishing that the 5 mg/kg dose was behaviorally active in females, an additional experiment was conducted in control and stressed males comparing the 5 mg/kg dose vs. vehicle.

**Social interaction test (SI)**

Two weeks after defeat or control conditions, all animals were tested in SI as previously described (Trainor et al. 2013; Greenberg et al. 2013). Briefly, SI test consisted of 3 phases lasting 3 min each. In the open field phase, the focal mouse was introduced into the open field (89 x 63 x 60 cm empty arena). Total distance traveled (Anymaze, Stoelting) was recorded to assess locomotor behavior, and time spent in the center of the arena (within 8 cm of the sides and within a center zone located 14 cm from the sides) was recorded to assess anxiety-like
behavior. In the acclimation phase, an empty wire cage was placed against one wall. The amount of time that the focal mouse spent within 8 cm of the cage (interaction zone) and in the corners opposite to the wire cage was recorded. Finally, in the interaction phase, an unfamiliar intact adult same-sex mouse was placed into the wire cage, and the time spent in the interaction zone and corners was recorded (fig. 1 A,C). Interaction phase videos were later reanalyzed by an observer blind to treatment assignment to include scoring of the following behaviors (for details see table S2): rearing in cage, smelling cage, time spent inactive in interaction zone, auto-grooming, observing cage from a distance, time spent in sides, and time spent in back. “Active investigation” was defined as total time spent smelling + rearing in cage (does not consider time spent inactive in proximity to cage). We also recorded head orientation (towards and away from interaction point). This measure considered orientation of the animal when outside of the interaction zone, which we later called “risk assessment behavior”. Mice were returned to their home cage immediately after the end of SI.

**Odor preference test (OP)**

One day after SI, OP was assessed using same arena used in SI. Two acid-free sheets of paper (9*12 inches) were placed at each side of the arena. In the acclimation phase of the test (4 minutes), 100 μl of DI water were added to both sheets in a pre-defined area of 3*4 inches (interaction area), and animal was allowed to freely explore. Sheet was removed and a new one was placed for odor preference phase. During odor preference phase (4 minutes), 100 μl of 1:10 urine dilution of a known conspecific (cagemate odor) and an unknown conspecific (unfamiliar odor) were added to each of the interaction areas (sides were assigned randomly for each test). The amount of time the focal mouse spent with its nose in interaction area was recorded (fig.1 C). Odor preference was defined as time spent with nose
in unfamiliar odor divided by total time spent with nose in both cage-mate and unfamiliar odors. In addition, total distance traveled, time spent in center of arena, and freezing behavior were recorded for each animal (Anymaze, Stoelting). One hour after OP, brains were collected and immersion fixed in 5% acrolein in PBS. Animals were perfused transcardially with 4% paraformaldehyde solution and brains were collected 60 min after the end of behavior testing.

**OTR autoradiography**

Males and females were exposed to control or defeat conditions and then euthanized two weeks later (Laredo et al. 2015; Duque-Wilckens et al. 2016). Brains were snap frozen on dry ice and then sliced on a cryostat at a 20 μm. Slides were then processed for OTR autoradiography as previously described (Perkeybile et al. 2015, see S1 for details).

**Effects of intranasal OT on Egr-1 immunoreactivity**

To identify potential sites of oxytocin action, immunohistochemistry was used to examine Egr-1 expression in the brains of mice treated with 0.8 IU of OT or saline (Steinman et al. 2016a). We used animals naïve to defeat since we had previously found that intranasal administration of OT reduces SI in naïve females (which mimics the effects that stress has on this behavior). For details of immunohistochemistry see S2).

Photomicrographs of areas of interest were taken under a monochromatic Axiocam MRm camera (Carl Zeiss Meditec) through a Zeiss Axioimager (Carl Zeiss Meditec, Inc., Dublin, CA) in 10x magnification. For counting, boxes were generated for each area of interest using ImageJ software (NIH, Bethesda, MD). The box dimensions were 0.35 X 0.43 mm for paraventricular nucleus (PVN), 0.45 X 0.75mm for anterior BNST (divided in dorsomedial
(BNSTam) and ventromedial), 0.4 X 0.8 mm for NAc core dorsolateral (NAcdl), 0.4 X 0.8 mm for NAc core ventromedial, 0.6 X 0.6 mm for NAc shell, and 0.4 x 0.4 mm for lateral septum (LS) dorsal and ventral. The numbers of Egr-1 positive cells were counted manually using cell counter plugin in ImageJ, and were then divided by box area to express number of egr-1 positive cells by mm2.

**Site-specific injection of OTA**

Seven days after social defeat, females were anesthetized with isoflurane (3-5%) and implanted with bilateral stainless steel guide cannula (Plastics One, Roanoke, VA) aimed at either the NAcdl (AP: + 0.51, ML: +/-1.5, DV: + 6.0, Fig.5F) or BNSTam (AP: + 0.45, ML: +/-1.0, DV: + 5.6, Fig.5E) (Greenberg et al. 2014). The guide cannulae were lowered into burr holes and attached to the skull with skull screws (plastics one, 00-96 X 1/16) and acrylic dental cement. Bilateral dummy caps (Plastics One, Roanoke, VA) were used to maintain patency. Carprofen (5 mg/kg) was administered subcutaneously immediately before surgery and once a day during 3 consecutive days after surgery. Animals were given six days for recovery, during which they were observed and handled daily. After this recovery period, females were randomly assigned to receive a 200 nl infusion of either aCSF or L-368,899 (1 ug per side) into NAcdl or BNSTam using bilateral injectors (Plastics One, Roanoke, VA) that projected 1 mm past the guides. The dose used is within the range of recently reported as having behavioral effects when injected centrally in rodents (0.25-20μg) (Lukas et al. 2011a; Herisson et al. 2016; Calcagnoli et al. 2015; Mullis, Kay, and Williams 2013). Thirty minutes later each mouse was run in the SI test. Hit rate of cannula guide implantation was assessed post mortem with fluorescent Nissl staining (fig.5 G,H, for details see S4).

**Effects of OTA on OT activation of G-proteins and β-arrestins recruitment**
Bioluminescence resonance energy transfer (BRET) assays were used to assess effects of OTA on OT activation of G-proteins and β-arrestins as previously described (Busnelli et al. 2012). Briefly, for G-protein activation HEK293 cells were co-transfected with plasmids containing the specific Ga protein subtypes (Gaq, Gai1, Gai2, Gai3, GoaA, GoaB) fused to Renilla luciferase (Rluc8), Gβ1 and Gγ2-GFP and human OTR. Cells were incubated with OT 10 M, three concentrations of OTA (10 M, 100 nM, 10 nM) or 10 M OT plus 10 M OTA. Two minutes later the BRET between Rluc8 and GFP was measured by adding Rluc substrate coelenterazine 400a using a Tecan Infinite F500 plate reader (for full details see S4). For β-arrestins recruitment HEK 293 cells were transfected with OTR fused to Rluc and β arrestin 1 or β arrestin 1 fused to YFP. Cells were treated with the Rluc substrate coelenterazine h and eight minutes later OT 10 M, OTA 10 M or PBS were added and BRET signal was immediately measured using a Tecan Infinite F500 plate reader (for full details see S4).

**Statistical analysis**

All the statistical analyses were performed with R software. We used two-way ANOVA to analyze receptor autoradiography (sex and stress), behavioral data from systemic OTA administration experiments (stress and drug treatment) and BRET assays (drug and PBS). Two-way ANOVAs were also used to analyze behavior data from site-specific injection experiment (drug and location). For significant two-way ANOVA analyses, planned comparisons were used to detect differences between groups (package “lsmeans”, Bonferroni, 0.95 confidence interval). Cohen’s d was used to report effect size in these cases. Odor preference was calculated as time spent with nose in unfamiliar odor area divided by total time spent with nose in odor area (unfamiliar + cage-mate odors). Thus, odor preference >0.5 indicates preference for the unfamiliar odor, and <0.5 indicates preference for cage-mate odor. For autoradiography data, correlational analyses were done using Pearson correlation
coefficients (two tailed, 0.95 confidence interval). Finally, we also performed a 3-way ANOVA to assess possible effects of estrous cycle (stress, treatment and estrous cycle) in the SI and OP tests. In all experiments, estrous cycle stage was assessed post mortem, since conducting vaginal lavage before testing disrupts behavior in California mice (Silva et al. 2010). There were no main effects of estrous cycle or interaction with stress or treatment (table S1).

RESULTS
Effects of systemic administration of OTA on social interaction behavior

In females, there was evidence that the effects of systemic OTA treatment on social interaction behavior were different in control vs. stressed females (Fig. 1A; F_{2,61} = 12.4, stress by treatment interaction, p=0.06). Stressed females spent less time in the interaction zone than controls if they were treated with saline (p<0.001, d=2.04) or 1mg/Kg of OTA (p<0.01, d=1.29). In contrast, stressed females treated with 5mg/Kg of OTA spent significantly more time in the interaction zone than stressed females treated with saline (p<0.05, d=1.03) and no different from controls (all treatments) (d=0.45). We observed even stronger differences in the amount of time females actively interacted with the cage containing the target mouse (Fig. 1B; stress x drug interaction; p<0.001). Stressed females showed reduced interaction with the target mouse if treated with saline (p<0.001, d=1.6) or the low dose of OTA (p=0.02, d=1.1) but not with the high dose of OTA (d=0.6). In females, stress also significantly increased time spent in corners opposite the interaction zone (fig. 1C; main effect of stress, p<0.001), but only in females receiving saline (p=0.01, d=0.8) or low dose of OTA (p= 0.047, d=1.3), but not high dose of OTA (d=0.25). There weren’t significant differences in any of these variables during acclimation phase (fig. S1). During the open field phase, there was a main effect of stress reducing time spent in center (fig. S1, p<0.001) and no differences in distance traveled.
In males, social defeat stress did not reduce SI, consistent with previous studies (Steinman et al. 2016a; Greenberg et al. 2015; Campi et al. 2014). Instead, males treated with OTA spent significantly less time in the interaction zone in the presence of a target mouse (Fig. 1D; main effect of drug; p=0.03). Unlike females where OTA only affected the behavior of stressed mice, planned comparisons showed a trend for OTA to reduce SI in control (p=0.08, d=1.03), but not stressed males (d=0.52). This suggests the effect of OTA may be stronger in unstressed males. All groups spent similar amount of time in active investigation. There were no effects of stress, treatment, or their interaction on time spent in interaction zone or corners during acclimation phase or distance traveled and time spent in open field during open field phase.

Effects of systemic administration of OTA on odor preference behavior

For females there was a main effect of treatment on odor preference during interaction phase (Fig. 1D main effect of drug; p<0.001). Planned comparisons revealed that stress induced a preference for cage-mate vs. unfamiliar odor in saline treated animals (p<0.001, d=0.94). This preference was reversed with administration of OTA. Intriguingly, while low dose of OTA significantly reversed this preference (p<0.001, d=1.34), the effect of high dose of OTA was less pronounced (p=0.08, d=0.78). Surprisingly, control or stressed males didn’t show preference for any odor, and this was also not affected by treatment.

Effects of OTA on OT activation of G-proteins and β-arrestins recruitment

Incubation with OT significantly reduced energy transfer (BRET) between all 6 G-proteins analyzed, consistent with previous observations that OT is capable of activating Gq and Gi/o pathways (fig. 2) (Busnelli et al., 2012). None of the three concentrations of OTR led to activation of G-proteins. However, OTA treatment full prevented G-protein activation of OT. Similarly, in cells
co-expressing OTR-Rluc and β-arrestin1-YFP, OT increased the BRET ratio, indicating agonist-induced association between the OTR and β-arrestin 1. Similar results were observed using the β-arrestin2 YFP construct. OTA didn’t induce changes in BRET ratio. Thus, L368,899 is a full antagonist at all known OTR dependent second messenger systems.

**Effects of stress on OTR expression in males and females**

In NAcdl (Fig. 3A; main effect of stress; p=0.03), and NAc shell (Fig. 3B; main effect of stress; p=0.03) stressed males and females had significantly less OTR binding than controls. In the PVN, females had significantly less OTR binding than males (Fig. 3E; main effect of sex; p=0.00) and there was no effect of stress. There were no effects of sex or stress in dorsal LS, BNSTam, or central amygdala (CeA) (Fig. 3 C,D,F) or any other areas investigated (fig. S2).

**Effects of intranasal administration of OT on egr-1 immunoreactivity**

In the BNSTam there was evidence for sex specific effects of intranasal OT on egr-1 staining (Fig. 4A; sex*drug interaction; F_{1,30}= 3.174, p=0.08). Planned comparisons showed that intranasal OT increased the number of egr-1 positive cells in females (p= 0.03,d=1.09) but not males (d=0.32). A similar pattern was observed in the NAcdl (Fig. 4B; sex*drug interaction, p=0.06). Planned comparisons showed that intranasal OT significantly increased egr-1 positive cells in females (p= 0.03,d=0.93) but not males (p= 0.58,d=0.31). In the NAc shell, females had more egr-1 positive cells than males (Fig. 4C; main effect of sex; p=0.03). There were no effects of stress, treatment, or their interaction on egr-1 positive cells detected in NAc core ventromedial, LS or PVN (fig. S3).
Effects of OTA infusion into BNSTam or NAcdl in stressed females on SI

The effects of OTA on behavior were dependent on the site of injection (Fig. 5A, injection site*drug, p=0.00). Females that received infusions of OTA into the BNSTam spent significantly more time in the interaction zone with the target mouse than females that receive infusions of saline (p=0.00, d=1.73). In contrast, OTA injections in to the NAcdl (d=0.26) or misses (d=0.46) had no effect on time spent in the interaction zone with the target mice. No effects of treatment or injection site were detected in time spent in corners during interaction (fig. 5B), proximity to empty cage during acclimation (fig. 5C) or distance traveled during open field (fig. 5D).

Effects of OTA on risk assessment behavior

While performing experiments we noticed that stressed females would orient towards the target mouse when outside of the interaction zone. In the systemic OTA experiment, stress increased the amount of time the focal mouse was oriented towards the target mouse (Fig 6A, main effect of stress F$_{1,54}$=6.71, P=0.01), but only for saline and 1mg/Kg OTA treated mice. Both control and stressed females treated with 5 mg/Kg OTA spent less time oriented toward the target mouse (Fig. 6A, main effect of drug, F$_{2,54}$=6.1, P<0.01). No differences in orientation responses were observed in males (Fig. 6B). In site-specific studies, only OTA infusion in the BNSTam reduced the amount of time the focal mouse was oriented toward the target (Fig. 6C, P<0.05). These results suggest that OTR activation in the BNST does not reduce interest in social stimuli, but alters attention to social stimuli.

DISCUSSION

Our results show that a single administration of OTA is sufficient to reverse stress-induced deficits in social interaction behavior. To achieve the similar effects with selective serotonin
reuptake inhibitors, 4 weeks of daily treatment is required. Local infusion of OTA in to the BNSTam, but not NAcDl, mimics the effects of systemic administration of OTA, indicating that the BNSTam is a critical site of action for OTR-dependent social withdrawal in females. Importantly, our data suggest that the effects of OTA are limited to social contexts. Although the concept of OTR producing an “anti-social” phenotype might appear to contradict previous findings, results from the odor preference test indicate that OTR activity does not simply induce an avoidance of all social contexts. Stressed females showed a preference for odors of familiar cagemates and this preference was abolished with OTA treatment. Thus, defeat-induced increases in the activity of OT neurons and subsequent activation of OTR may induce avoidance of unfamiliar social contexts but not familiar social contexts. Furthermore, analyses of orientation responses suggest that OTR in the BNSTam may promote a vigilance response to unfamiliar social contexts that inhibits social approach. The rapid action of OTA on social behavior suggests that further dissection of OTR-dependent behavioral phenotypes could lead to important insights in new treatment strategies for stress-induced psychiatric disorders.

Sex differences in effects of systemic inhibition of OTR

Our results show that OTR inhibition has opposite effects on social behavior in males vs. females in a novel environment. This is consistent with our previous data showing that intranasal OT induces social withdrawal in females but not males, and that stress induces hyperactivation of OT neurons in females but not males (Steinman et al. 2016b). Studies in rodents and humans have repeatedly shown robust sex differences in social behavior regulation by OT. For example, in rats intracerebroventricular (ICV) administration of OT increases social investigation of a novel conspecific in stressed males (Lukas et al. 2011b) but not females (Lukas and Neumann 2014), and injections of OT into the lateral septum reduce social play in juvenile females but not males (Bredewold et al. 2014). Similarly, in prairie voles OT administration during development facilitates...
partner preference behavior in males (Bales and Carter 2003) but not females (Bales et al. 2007). In humans, intranasal OT increases anxiety in women but is anxiolytic in men following a social stress test (Kubzansky et al. 2012). There are several possible explanations for sex-specific actions of OT.

An intuitive explanation for sex differences in OT action could involve sex-specific expression of OT or its cognate receptors including OTR and V1aR, as OT can activate multiple receptor types (Sala et al. 2011; Song et al. 2014, 2016). Although sex differences in vasopressin immunoreactivity are well documented, there is little evidence for sex differences in OT immunoreactivity (DiBenedictis et al. 2017). In California mice, few sex differences in OT immunoreactivity are observed, and only in mice exposed to defeat stress (Steinman et al. 2016). For receptors, males rats have significantly more V1aR binding than females in 8 brain regions (Smith et al. 2017). However, other species report few or no sex differences in V1aR binding (Bales et al. 2007; Insel, Gelhard and Shapiro 1991). In a previous study we considered the hypothesis that sex differences in V1aR expression or function contributed to stress-induced social withdrawal (Duque-Wlickens et al. 2016). There were few sex differences in V1aR binding in California mice and V1aR antagonist infusions in to the BNST reduced social interaction in both males and females while V1aR antagonist infusions in to the NAc had no effects on social interaction. In this study we investigated whether sex differences or stress-induced changes in OTR expression could account for sex differences in behavioral response to defeat. There were no sex differences in OTR expression, consistent with previous reports in other species (Insel, Gelhard, and Shapiro 1991; Bales et al. 2007). When effects of stress were observed, OTR expression was decreased, possibly as a negative feedback response to elevated OT release (Phaneuf et al. 2000). On balance, it does not appear that sex differences in OT or OTR expression can account for the sex-specific effects of OTR we report.
An alternative possibility is that there are sex differences in OTR activation of G proteins. OT facilitates OTR coupling to several different G-proteins and β-arrestins, which provides multiple degrees of freedom for effects on neural activity (Busnelli et al. 2013; Gimpl and Fahrenholz 2001). For example, OT activation of OTR can inhibit inward rectifier (IR) currents of immortalized gonadotropin releasing hormone cells (GN11) through activation of Gq/11 whereas the same IR current can be activated by OT via OTR coupling with Gi/o protein (Gravati et al. 2010). Interestingly, the OTR ligand atosiban, considered to be an OTR antagonist, actually activates Gi-mediated pathways *in vitro* (Reversi et al. 2005; Busnelli et al. 2013) and *in vivo* (Eliava et al. 2016); atosiban was shown to mimic the inhibitory effect of OT on sensory neurons of the spinal cord providing a clear evidence of its unique agonist activity on restricted OTR signaling pathways (Eliava et al. 2016). Using BRET assays we showed that unlike atosiban, OTA blocks OTR activation of Gq, Gi, and β-arrestin by OT. To our knowledge, no study has considered whether sex differences in G-protein activation by a single receptor contributes to sex differences in behavioral effects. This would appear to be possible, as sex differences in the cellular trafficking of corticotropin releasing factor receptors was linked to sex differences in the activity of norepinephrine neurons in the locus coeruleus (Bangasser et al. 2013). While biased agonists such as carbetocin (which selectively activates Gq) have been reported to have different behavioral effects than OT (Klenerova et al. 2009; Klenerova et al. 2010), no study has systematically compared these ligands in males and females. Our results showing that a broad spectrum OTR antagonist has opposite effects on social behavior in males and females suggest that this is a promising direction for further study.

**Specific site of OTR action mediating social avoidance**

We used several approaches to identify a site of action of OTR. The EGR-1 immunoreactivity was effective at identifying nuclei that responded more strongly to intranasal OT
in females compared to males. Since we previously found that intranasal OT reduced social interaction behavior in naïve females but not males (Steinman et al. 2016b), we hypothesized that NAcdl and/or BNSTam could be sites where OTR modulates behavior in the social interaction test. Site specific injections of OTA demonstrated that activation of OTR in BNSTam, but not the NAcdl, induced social withdrawal in stressed females. One injection of OTA in BNSTam rapidly reversed the effects of stress on social interaction in females, which closely mimics effect of systemic administration of OTA. The region of the NAc targeted was more lateral than the NAc core which has identified as an important site of OTR facilitation of pair bonding behavior in female prairie voles (Liu and Wang 2003). The NAc is topographically organized with different subregions of core and shell showing stronger responses to rewarding or aversive experiences (Richard et al. 2013). Our results suggest OTR in the BNSTam generate aversive responses to unfamiliar social contexts.

There has been increased focus on the BNST as a key locus contributing to stress induced psychiatric disorders (Lebow and Chen 2016; Daniel and Rainnie 2016). This sexually dimorphic forebrain structure is involved in the regulation of anxiety (Davis et al. 2010) and social behaviors including aggression (Duque-Wilckens and Trainor 2017; Marsh 2013) and attachment (Coria-Avila et al. 2014). The BNST is a heterogeneous structure containing both excitatory and inhibitory neurons and has strong connections with stress response circuits (Crestani et al. 2013), motivational systems (O’Connell and Hofmann 2011), and social behavior circuits (Newman 1999). The BNST is thought to be a key center for integrating information from social and physical environments to generate avoidance/approach responses (Lebow and Chen 2016). Anteromedial portions of BNST receive inputs from the medial amygdala and posterior BNST, and send direct projections to PVN and CeA (Dong and Swanson 2006; Gomez and Newman 1992). Further study is needed to identify the downstream effects of OTR activation in the BNSTam. Interestingly, social defeat increases levels of brain-derived neurotrophic factor (BNDF) in BNSTam of female but not
male California mice (Greenberg et al. 2014). Furthermore, infusion of a selective TrkB inhibitor in to BNSTam had an identical effect as OTA, increasing social interaction in stressed females. Infusion of TrkB inhibitor in unstressed females had no effect on behavior, suggesting that the role of the BNSTam in modulating social behavior is more important following a stressful experience. It has been reported that OT can increase BDNF expression in hippocampus (Dayi et al. 2015) and neuroblastoma cells (Bakos et al. 2013). Determination of the link between OTR and BDNF could be promising future direction to investigate.

**Stress induces vigilance and avoidance of unfamiliar social contexts**

An intriguing finding was that stressed females spent more time oriented towards the target mouse, but not an empty cage, when outside of the interaction zone. This response that was inhibited by systemic OTA treatment. Male rats confronted with threatening stimuli such as a predator or predator odor exhibit similar orienting responses (Blanchard and Blanchard 1989), which have been described as risk assessment or vigilance behavior (Blanchard et al. 2011). These data raise the possibility that increased OTR activity in stressed females does not simply reduce social motivation, but enhances a social vigilance response toward unfamiliar and possibly dangerous social context. This would be consistent the social salience hypothesis which posits that OT enhances the salience of both positive and negative social experiences (Shamay-Tsoory and Abu-Akel 2016). Our results suggest that anterior subregions of BNST could be an important locus enhancing the salience of potentially threatening social contexts. Results from the odor preference test indicate that the familiarity of the social context is a key factor mediating the behavioral effects of OTR.

In females, social defeat stress induced a preference for the cagemate odor in females but not males, which was rapidly reversed by administration of OTA. This result is similar findings from
human studies reporting that OT increases intragroup prosocial behaviors together with intergroup antisocial behaviors (De Dreu et al. 2010; De Dreu and Kret 2016).

Conclusions

The current findings combined with our previous work (Steinman et al. 2016, Duque-Wilckens et al. 2016) suggest a model that social defeat induces hyperactivity of BNSTmv and PVN OT neurons which leads to activation of OTR in the BNSTam to induce avoidance of unfamiliar social contexts in females. The empirical data support the hypothesis that elevated OT may contribute to social vigilance in novel contexts. Interestingly, intranasal OT has been reported to increase perceived social stress (Eckstein et al. 2014) and mistrust (Bartz et al. 2011) in humans. Together, this evidence suggests that in unfamiliar contexts, OTR antagonists may have unappreciated therapeutic potential for reducing social anxiety. Further study of OT and OTR-sensitive circuits on the behavioral effects of psychosocial stressors could greatly contribute to the understanding of mechanisms underlying social deficits associated with psychiatric disorders.

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A) Interaction zone

B) Active investigation

C) Corners

D) Cagemate odor, Unknown odor

FEMALES

MALES

Odor preference ratio

Active investigation (s)

In interaction zone (s)

In corners (s)
Fig.1. Effects of systemic administration of OTA and social defeat stress on behavior. Mean and SEM time spent (a) in proximity to a cage containing a novel same sex individual (interaction zone), (b) in active investigation, and (c) corners in the SI test. (d) Mean and SEM of odor preference ratio during OP test. Left column shows diagrams representing behavior measured, middle column shows female data, right column shows male data, *=p < 0.05 effect of 5mg/Kg OTA vs. saline in stressed animals, †=p<0.01 effect of stress in saline treated animals, ***=p<0.01 effect of 1mg/Kg OTA vs. saline in stressed animals, =p=0.08 effects of 5mg/Kg OTA vs. saline in stressed animals, #=p<0.05 main effect of OTA.
Fig. 2. Effects of OTA on OTR activation of G-proteins and β-arrestins recruitment: Mean and SEM of BRET ligand effect of OT, PBS, OT+ OTA and three different concentrations of OTA for (A) Gq, (B) Gi1, (C) Gi2, (D) Gi3, (E) GoA, and (E) GoB activation and. β-arrestin 1 (F) and β-arrestin 2 (G) recruitment *=p < 0.05 effect of drug treatment vs. PBS, ***=p < 0.001 effect of drug treatment vs. PBS.

Fig. 3. OTR binding in naïve and stressed males and females. Mean and SEM of OTR binding in (a) NAcdl, (b) NAc shell, (c) dorsal LS, (d) BNSTam, (e) PVN, and (f) CeA. † =p < 0.05 main effect of stress ***=p < 0.01 main effect of sex. Ac=anterior commissure, LV= lateral ventricle, 3rdV= third ventricle, IC= internal capsule.
Fig. 4. Effects of intranasal administration of OT on egr-1 immunoreactivity in naïve males and females Mean and SEM of egr-1 positive cells per mm2 detected in A) BNSTam, and B) NAc dl, * = p < 0.05 effect of intranasal OT vs. saline, † = p < 0.05 main effect of sex, # = trend effect of intranasal OT vs. saline. On the middle column, diagram of areas quantified, on right column, example photos of areas quantified and box placement. Ac = anterior commissure, LV = lateral ventricle.
**Fig. 5. Effects of site-specific administration of OTA on behavior in stressed females.** Mean and SEM time spent (A) in proximity to a cage containing a novel same sex individual (interaction zone), (B) in corners during social interaction, (C) in proximity to an empty cage, and (D) total distance traveled during open field phase. Diagrams showing location of injection considered as hit in (E) BNSTam, and (F) NAc. Under each diagram, the number of animals receiving injection in each site is indicated. Examples of hits in Nissl stained slices in (G) BNSTam and (H) NAc. Arrows indicate site of cannula guide placement, circles indicate injection site. ***=p < 0.01 effect of OTA vs. saline in animals receiving injections in BNSTam.

**Fig. 6. Effects of OTA on risk assessment behavior.** Mean and SEM time spent with head oriented towards unknown conspecific in (A) control and stressed females receiving systemic injections if saline, 1 mg/Kg OTA or 5 mg/Kg OTA, (B) stressed females receiving site specific injections of aCSF or OTA, and (C) control and stressed males receiving systemic injections if saline or 5 mg/Kg OTA. *=p<0.05 main effect of drug, †=p=0.01 main effect of stress.


Laredo, Sarah A., Michael Q. Steinman, Cindee F. Robles, Emilio Ferrer, Benjamin J. Ragen, and Brian C. Trainor. 2015. “Effects of Defeat Stress on Behavioral Flexibility in Males and Females:


Supplementary Methods and Materials

S1. Receptor Autoradiography

OTR binding was assessed using receptor autoradiography as previously described (Bales et al., 2007). Mice were euthanized and brains were snap frozen on dry ice within minutes of social interaction testing completion. Brains were sliced on a cryostat at a 20 μm thickness and mounted on Super-frost plus slides. Slides were fixed for two min in 0.1 % paraformaldehyde (Sigma Aldrich, St. Louis Missouri, USA) in PBS. They were subsequently rinsed in two 10 min 50 mM Trizma Base washes followed by a 1 hr-room temperature incubation in 50 pM of radiotracer diluted in a tracer buffer (50 mM Trizma Base (pH 7.4), 10 mM MgCl2, 0.1% bovine serum albumin). We used [125I]-ornithine vasotocin analog [(125I)OVTA] [vasotocin, d(CH2)5[Tyr(Me)2, Thr4, Orn8, (125I)Tyr9-NH2]; 2200 Ci/mmol] (NEN Nuclear, Boston, MA, USA). Non-specific binding was recognized by immersing adjacent sections in buffer containing both the radioactive ligand and 50 μM unlabeled Thr4, Gly7 OT, a selective OT ligand (Peninsula Laboratories, Belmont, CA, USA). Non-specific binding was recognized by immersing adjacent sections in buffer containing both the radioactive ligand and 50 μM unlabeled Thr4, Gly7 OT, a selective OT ligand (Peninsula Laboratories, Belmont, CA, USA). After radioactive tracer incubation, sections were subjected to four-five min washes in 50 mm Trizma Base (pH 7.4) with 10 mM MgCl2 at 4 °C, followed by a 30 min wash in this buffer while stirring at room temperature. Slides were then rinsed momentarily in cold dH2O and dried for 15 min with a hair dryer on the cold setting. Sections as well as 125I microscale standards (Perkin-Elmer/NEN) were placed on Kodak BioMaxMR film (Kodak, Rochester, NY, USA) for 96 h. ImageJ was used to quantify 125I-receptor binding. The microscale standards were used to generate a curve through which uncalibrated optical density was converted disintegrations per minute (DPM). If available, six sections per region were quantified bilaterally and a mean binding value for a given region in each animal was calculated and used in analyses. In some animals, one or two sections were damaged, so only four or five were available for analysis. The damaged sections were distributed across all four groups. In order to account for background staining we measured radioactivity in white matter tracts such as the corpus collossum, fornix or anterior commissure and then subtracted these values from those found in nearby regions of interest (Inoue et al., 2013). These white matter structures exhibited comparable levels of binding in both the non-specific binding sections and the non-competed sections.

S2. EGR-1 immunohistochemistry protocol
Brains were fixed overnight in 5% acrolein in PBS, transferred to 20% sucrose in PBS, frozen, and then sectioned coronally at 40 μm. Slices were then washed 3 times for 5 min in PBS, treated with 1% sodium borohydride for 10 min, washed in PBS again 2 times, and blocked for 2 hours in 10% normal goat serum (NGS) in PBS. Next, slices were incubated overnight in a primary antibody solution 1:1000 of rabbit antibody anti-EGR1 (4153S, Cell Signaling) at 4°C. On the second day, slices were washed 3 times ashed in PBS and incubated in biotinylated goat anti-rabbit antibody (Vector laboratories) 1:500 in 2% NGS in PBS with 0.5% TX for 2 hours at room temperature, triple washed in PBS and incubated in streptavidin Alexafluor 555 (Thermofisher) 1:250 in PBS TX 0.5%. Next, sections were washed, mounted and coverslipped using Vectashield H-1000 (Vector laboratories).

S3. Fluorescent Nissl stain protocol

Mice receiving site specific injections of OTR antagonist were perfused with 4% paraformaldehyde (PFA) solution. Brains were extracted and immersed overnight in 4% PFA for post fixation. Next, brains were immersed in 10% sucrose in PBS for 24h, then 30% sucrose for another 24h, frozen and sectioned coronally at 40 μm. Then, sections of interest were washed in PBS with 0.1% TX, washed 2 more times in PBS, and incubated for 20 min at room temperature in a solution containing 1:100 Neurotrace 500/525 (Fisher). After incubation, sections were washed in 10 min in PBS-TX 0.1%, and washed two more times for 5 min in PBS. Sections were then mounted and coverslipped using Vectashield H-1000 (Vector).

S4. Bioluminescence resonance energy transfer (BRET) assays

To detect and analyze the interactions between OTR and the different Gα subunits by means of BRET experiments HEK293 cells were co-transfected with OTR, GFP\(^2\)-Gγ2, Gβ1, and one of Gαq-97-Rluc8, Gαi1-91-Rluc8, Gαi2-91-Rluc8, Gαi3-91-Rluc8, GαoA-91-Rluc8, GαoB-91-Rluc8. Finally, to study OTR-mediated β-arrestin recruitment by means of BRET1 experiments, the cells were co-transfected with OTR-Rluc and β-arrestin1-YFP or β-arrestin2-YFP (Busnelli et al 2012). 48 h after transfection, the cells were washed twice, detached, and resuspended with PBS, 0.5 mM MgCl\(_2\), glucose 0.1% (w/v) at room temperature. They were then distributed in a white 96-well
microplate (100 µg of proteins/well) (Optiplate, PerkinElmer Life Sciences) and incubated in the presence or absence of OT (10 µM), different concentration of OTRA (10 µM, 100nM, 10nM), a combination of OT and OTRA (10 µM+ 10 µM) or PBS for 2 min before Rluc substrate addition. The BRET between Rluc8 and GFP2 was measured immediately after the addition of the Rluc substrate coelenterazine 400a (5 µM), using an Infinite F500 reader plate (Tecan, Milan, Italy) that allows the sequential integration of light signals detected with two filter settings (Rluc8 filter, 370– 450 nm; GFP² filter, 510–540 nm). The data were recorded, and the BRET2 signal was calculated as the ratio between GFP² emission and the light emitted by Rluc8.

For β-arrestin recruitment cells were transfected with OTR-Rluc and β arrestin 1 or β arrestin 1 fused to YFP and 48 h later were detached and resuspended in PBS, 0.5 mM MgCl₂, glucose 0.1% (w/v) at room temperature. They were then distributed in a white 96-well microplate (100 µg of proteins/well) (Optiplate, PerkinElmer Life Sciences) and preteated with the Rluc substrate coelenterazine h (5µM) for eight minutes and OT 10µM, OTRA 10µM or PBS were added. Immediately after BRET signal was measured using a Tecan Infinite F500 plate reader. In both cases, the changes in BRET induced by the ligands were expressed on graphs as “ligand-promoted BRET” using the formula,

\[
\text{Ligand-promoted BRET} = \frac{\text{emission GFP}^2/\text{YFP ligand}}{\text{emission Rluc ligand}} - \frac{\text{emission GFP}^2/\text{YFP PBS}}{\text{emission RlucPBS}}
\]

as in (Busnelli et al, 2012)

Table S1. Estrous stage analysis on behavior (3-way ANOVAS including stress, treatment, and estrous stage)

<table>
<thead>
<tr>
<th>Systemic OTRA</th>
<th>Main effect Estrous</th>
<th>Estrous*Treatment</th>
<th>Estrous*Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social Interaction</td>
<td>p=0.8</td>
<td>p=0.2</td>
<td>p=0.4</td>
</tr>
<tr>
<td>Odor preference</td>
<td>p=0.2</td>
<td>p=0.4</td>
<td>p=0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site-specific OTRA</th>
<th>Main effect Estrous</th>
<th>Estrous*Treatment</th>
<th>Estrous*Stress</th>
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</thead>
<tbody>
<tr>
<td>Social Interaction</td>
<td>p=0.8</td>
<td>p=0.6</td>
<td>p=0.9</td>
</tr>
<tr>
<td>Behavior</td>
<td>Description</td>
<td>Scoring method</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Rearing in cage</td>
<td>Animal standing on hind paws leaning against interaction cage with front paws</td>
<td>Manual (key stroke press and hold for duration of behavior)</td>
<td></td>
</tr>
<tr>
<td>Smelling cage</td>
<td>Animal sniffing interaction cage (distance nose to cage &lt;1cm)</td>
<td>Manual (key stroke press and hold for duration of behavior)</td>
<td></td>
</tr>
<tr>
<td>Time spent inactive in interaction zone</td>
<td>Animal is by interaction cage but is not actively investigating (i.e. sitting on the side of the cage or close to cage but pointing head away from cage)</td>
<td>Manual (key stroke press and hold for duration of behavior)</td>
<td></td>
</tr>
<tr>
<td>Auto-grooming</td>
<td>Animal engages in auto-grooming behavior (anywhere in the arena)</td>
<td>Manual (key stroke press and hold for duration of behavior)</td>
<td></td>
</tr>
<tr>
<td>Observing cage from a distance</td>
<td>Animal is in flexibility area and head is oriented towards interaction cage</td>
<td>Manual (key stroke press and hold for duration of behavior)</td>
<td></td>
</tr>
<tr>
<td>Time spent in sides</td>
<td>Animal is in sides of the cage</td>
<td>Automatic (Anymaze, Stoelting)</td>
<td></td>
</tr>
<tr>
<td>Time spent in back</td>
<td>Animal is in back of cage</td>
<td>Automatic (Anymaze, Stoelting)</td>
<td></td>
</tr>
<tr>
<td>Head orientation</td>
<td>Animal’s head oriented away or towards interaction point</td>
<td>Automatic (Anymaze, Stoelting)</td>
<td></td>
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Fig. S1. Effects of systemic administration of OTRA on behavior during acclimation and open field phase. Mean and SEM time spent (a) in proximity to an empty cage (b) in corners, and (c) in open field in the SI test. †p<0.01 main effect of stress.
Fig.S2. OTR binding in naïve and stressed males and females. Mean and SEM of OTR binding in (a) dorsal BNST, (b) BNST medioventral, (c) ventral LS, (d) Prefrontal cortex, (e) Dentate gyrus and (f) Hippocampus CA1.
Fig. S3. Effects of intranasal administration of OT on egr-1 immunoreactivity in naïve males and females. Mean and SEM of egr-1 positive cells per mm2 detected in a) NAc core ventromedial, b) PVN, and c) lateral septum.

Fig. S4. Effects of OTA on head orientation during acclimation phase. Mean and SEM time spent with head oriented towards empty cage in stressed females receiving site specific injections of aCSF or OTA.
Chapter 3. Behavioral Neuroendocrinology of Female Aggression
Natalia Duque-Wilckens and Brian C. Trainor

Summary
Aggressive behavior plays an essential role in survival and reproduction across animal species—it has been observed in insects, fish, reptiles, and mammals including humans. Even though specific aggressive behaviors are quite heterogeneous across species, many of the underlying mechanisms modulating aggression are highly conserved. For example, in a variety of species arginine vasopressin (AVP) and its homologue vasotocin in the hypothalamus, play an important role in regulating aggressive behaviors such as territorial and inter male aggression. Similarly, in the medial amygdala, activation of a subpopulation of GABAergic neurons promotes aggression, while the prefrontal cortex exerts inhibitory control over aggressive behaviors. An important caveat in the aggression literature is that it is focused primarily on males, probably because in most species males are more aggressive than females. However, female aggression is also highly prevalent in many contexts, as it can affect access to resources such as mates, food, and offspring survival. Although it is likely that many underlying mechanisms are shared between sexes, there is sex specific variation in aggression, type, magnitude, and contexts, which suggests that there are important sex differences in how aggression is regulated. For example, while AVP acts to modulate aggression in both male and female hamsters, it increases male aggression but decreases female aggression. These differences can occur at the extent of neurotransmitter or hormones release, sensitivity (i.e. receptor expression), and/or molecular responses.

Introduction

Aggressive behaviors are a critical component of the competition for resources such as food, shelter, and mating opportunities. The expression of aggression may differ within and between individuals, populations, and species (King, 1973; Miczek, Facidomo, Fish, & DeBold, 2007), and different forms of aggression can have different underlying neural and genetic networks (de Boer, Olivier, Veening, & Koolhaas, 2015; Takahashi & Miczek, 2014). However, for most species males engage in more intense and/or more frequent bouts of aggression than females, which may explain why research examining the mechanisms of aggression has
historically been focused on males. For example, Darwin’s writings on aggressive competition focused on the “Law of Battle” and highlighted the evolution of specialized weapons used by males in many species (Darwin, 1859).

Much of what we know about neural and hormonal mechanisms underlying aggression comes from studies of different species of rodents. An important discovery from these studies is that different neuroendocrine mechanisms are engaged to regulate different forms of aggression (Adams, 2006; Blanchard & Blanchard, 1977; Miczek, Fish, & De Bold, 2003; Takahashi & Miczek, 2014). Known forms of aggression include offensive, defensive and escalated aggression. Offensive aggression is associated with competition for resources, and attacks are usually targeted at non-vulnerable body areas of the opponent (Crawley, Schleidt, & Contrera, 1975; Miczek & O’Donnell, 1978). Defensive aggression is a response to fear-inducing stimuli, and as such is characterized by escape and threat behaviors. Here, attacks are usually directed towards vulnerable body areas such as the face of the threatening individual (Blanchard & Blanchard, 2003). More recently, the term escalated aggression has been introduced to describe what appears to be maladaptive behavior (Almeida, Ferrari, Parmigiani, & Miczek, 2005; Haller & Kruk, 2006; Miczek, Faccidomo, Almeida, Bannai, Fish, & Debold, 2004). Like offensive aggression, escalated aggression can be intense, and like defensive aggression attacks are directed towards more vulnerable body parts. However, in escalated aggression social signals are disregarded and attacks may continue even after an opponent has signaled defeat with submissive postures or signals. For these types of aggression, almost all mechanistic studies have been conducted on males. Most of what is known about the mechanisms of female aggression is from the context of maternal aggression, which is expressed by pregnant and early post partum females with the aim of offspring defense (Erskine, Barfield, & Goldman, 1978; Haney, Debold, & Miczek, 1989). This type of aggression is typically directed towards unfamiliar males. However, female aggression can be expressed in a variety of other contexts across taxa, which reflects its adaptive value (Rosvall, 2013a; Stockley & Bro-Jørgensen, 2011). In humans, physical aggression among women is relatively rare (Card, Stucky, Sawalani, & Little, 2008; Crick, Ostrov, & Kawabata, 2007), but verbal aggression or manipulation of interpersonal relationships (Crick et al., 2007) is common (Benenson, 2013; Thornton, Graham-Kevan, & Archer, 2012; Vaillancourt, 2013) and frequently associated with physical and psychiatric problems (Kaltiala-Heino & Fröjd, 2011; Odgers et al., 2008; Pajer, 1998). This has led to a growing appreciation of the significance of aggressive behaviors in females, and a corresponding increase in the number of studies examining the underlying neuroendocrine mechanisms.
This introductory section will briefly summarize the forms of female aggression that have been best described in both non-human and human animals; the following sections will focus on the underlying neuroendocrine mechanisms in males and females. Importantly, while it has been proposed that offensive and defensive aggression subtypes are likely regulated by different mechanisms (Takahashi & Miczek, 2014), different forms of female aggression can include both defensive and offensive components (Lucion & de Almeida, 1996; Parmigiani, Rodgers, Palanza, Mainardi, & Brain, 1989). For males, most of the studies cited here are focused on offensive aggression, unless stated otherwise. Therefore, when talking about mechanisms of male and female aggression, we will focus on the context in which aggression is expressed.

Forms of Female Aggression

Maternal aggression is a defensive behavior in which offspring are protected, usually against conspecific individuals. This evolutionarily ancient form of behavior is present across the animal kingdom (DeVries, Winters, & Jawor, 2015; Figler, Twum, Finkelstein, & Peeke, 1995; Rosvall, 2013b; Sinn, While, & Wapstra, 2008). In mammals, maternal aggression usually takes the form of aggressive confrontation of male intruders by pregnant or lactating females (Almeida, Ferreira, & Agrati, 2014; Palombit, 2012) and appears to have evolved as a strategy to prevent infanticide (Palombit, 2012). Interestingly, increased aggression in this context has been associated to down-regulation of physiological stress response and reduced anxiety (Gammie, D’Anna, Lee, & Stevenson, 2008; Hahn-Holbrook, Holt-Lunstad, Holbrook, Coyne, & Lawson, 2011). Remarkably, in some species in which both sexes make a considerable effort in caring for offspring, males also show increased aggressive behaviors to protect their young (Trainor, Finy, & Nelson, 2008a).

Territorial aggression, in which a resource of physical location is defended from competitors, is typically considered to be a male-typical behavior. A territory usually consists of resources such as food, shelter, and/or breeding sites, and maintaining exclusive access to these resources can increase fitness (Grant, 1993; Maher & Lott, 2000). Territorial aggression by females has been observed in fish (Ziadi-Kuenzli & Tachihara, 2016), reptiles (Jaeger, Kalvarskey, & Shimizu, 1982; Woodley & Moore, 1999), birds (Gowaty & Wagner, 1988), rodents (Ribble & Salvioni 1990), and non-human primates (Pusey & Schroeper-Walker, 2013). Female territorial behavior can consist of defense of an individual territory or the territory of a social group. In most cases both males and females use signals or other types of indirect aggression to settle competitions without resorting to physical conflict (Cant & Young, 2013; Parker & Rubenstein, 1981; Vaillancourt, 2013). If a conflict is not settled through these indirect measures, aggression
between females can be intense and lead to serious injury or even death, as seen in chimpanzees (Pan troglodytes) (Townsend, Slocombe, Emery Thompson, & Zuberbühler, 2007). Competition for mates is usually considered a male typical behavior. Nonetheless, female competition for mates has been described in a variety of taxa, ranging from fish to mammals (Fernandez-Duque & Huck, 2013; Gavish, Sue Carter, & Getz, 1983; Matsumoto & Yanagisawa, 2001; Yasukawa & Searcy, 1982). This type of female competition is common in monogamous species, in which males provide resources other than sperm such as paternal care or access to a territory (Rosvall, 2011). Interestingly, female-female competition for mates can be substantial even in polygynous species. In these, competition can arise when there is a limited number of high quality mates and/or sperm (Preston, Stevenson, Pemberton, & Wilson, 2001; Wedell, Gage, & Parker, 2002), when there is a reduced males to females ratio (Charlat et al., 2007; Rusu & Krackow, 2004), when the breeding season is very short (Forsgren, Amundsen, Borg, & Bjelvenmark, 2004), or to ensure protection from infanticide (Palombit, Cheney, & Seyfarth, 2001; Stockley & Bro-Jørgensen, 2011).

Neuroendocrine Mechanisms of Female Aggression

The neuroendocrine basis of aggression has been mostly described in male rodents, and has been reviewed elsewhere (de Boer, Olivier, Veening, & Koolhaas, 2015; Falkner & Lin, 2014; Nelson & Trainor, 2007; Takahashi & Miczek, 2014). Interestingly, many of the same neural circuits that control male-male aggression are also important for maternal aggression (Gammie, 2005). However, studies of aggression outside the context of maternal defense have revealed important sex differences in the neuroendocrine mechanisms of aggression (Greenberg & Trainor, 2015; Pagani et al., 2015; Scott, Prigge, Yizhar, & Kimchi, 2015; Veenema, Bredewold, & De Vries 2013). Here we will highlight these discoveries and compare and contrast how neural circuits, steroid hormones, and neuropeptides modulate aggressive behaviors in females and males.

Neural Substrates

Aggressive behaviors rely on activity from neurobiological circuits controlling social behaviors (social behavior neural network) as well as motivation (mesocorticolimbic dopamine pathway). The components of these networks have been identified in part through studies assessing expression of immediate early genes, like c-fos and EGR-1, which can be considered as indirect markers of neuronal activity. Studies using techniques such as region specific lesions,
pharmacological manipulations, and/or optical stimulation have been used to directly test how specific microcircuits regulate aggressive behaviors.

The Social Behavior Neural Network

Sarah Newman proposed, in 1999, that a social behavior network (SBN) consisting of the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), lateral septum (LS), periaqueductal gray (PAG), and the medial preoptic area (MPOA)/anterior hypothalamus (AH), work together to modulate social behaviors such as aggression in mammals (Figure 1). These nodes are reciprocally connected, and all express steroid hormones receptors (de Boer et al., 2015). Importantly, this network is evolutionarily conserved across diverse vertebrate taxa (Goodson & Kingsbury, 2013; Greenberg & Trainor, 2015).

Medial amygdala: For rodents, olfaction is the main sensory input regulating social behaviors, and the MeA plays a crucial role in processing sensory information coming from the olfactory bulb. The MEA sends efferent connections to the LS, BNST, and hypothalamus (Canteras, Simerly, & Swanson 1995; Dong, Petrovich, & Swanson, 2001b). The more posterior subregions of the medial amygdala are sexually dimorphic whereas more anterior subregions are not (Cooke, Tabibnia, & Breedlove, 1999). Both subregions appear to play a role in maternal aggression. Highly aggressive lactating female mice show increased c-fos immunoreactivity in anterior MeA compared to lactating females displaying low levels of aggression after being exposed to a male intruder (Gammie & Nelson, 2001). Another study examined the effect of engaging in maternal aggression on EGR-1 expression in the dorsal posterior MeA (MeApd ). Females that engage in aggression have increased expression of EGR-1 in MEApd compared to females that do not engage in aggression (Hasen & Gammie, 2006). Interestingly, in males the MeApd is activated following either aggressive interactions (territorial aggression) as well as sexual behavior (Kollack-Walker & Newman, 1995, 1997; Veening et al., 2005). This suggests a strong connection between reproduction and aggression, at least in males.

Bed nucleus of the stria terminalis: Together with LS, the BNST constitutes an overlapping node between SBN and the mesolimbic dopamine (DA) system. The role of BNST on aggression has been better studied in males, where multiple circuits within BNST have been implicated (Masugi-Tokita, Flor, & Kawata, 2016; Shaikh, Brutus, Siegel, & Siegel, 1986; Veenema, Beiderbeck, Lukas, & Neumann, 2010). Following maternal aggression, the anterolateral BNST
BNSTl) has increased c-fos immunoreactivity (Gammie & Nelson, 2001). Similarly, female California mice that engage in aggression with a female intruder have increased phosphorylated extracellular regulated kinase (ERK) in the BNSTl compared to females that engage in a sham aggression test (Silva, Fry, Sweeney, & Trainor, 2010). This finding is consistent with studies showing increased c-fos in the BNSTl following maternal aggression, as phosphorylation of ERK facilitates transcription of c-fos (Monje, Hernández-Losa, Lyons, Castellone, & Gutkind, 2005). The BNSTl sends projections to LS and lateral hypothalamus, as well as to nuclei of the mesocorticolimic pathway (Dong & Swanson, 2004), so it is likely that the actions of this area on aggression involve more than one pathway. The anteromedial part of BNST (BNSTm) is also highly connected to nuclei involved in aggression, including LS, anterior MeA, lateral VMH, paraventricular nucleus (PVN), Nucleus accumbens (NAC), and ventral tegmental area (VTA). (Dong & Swanson, 2006). The connection between the BNSTv and VTA is especially intriguing as GABAergic projections from BNSTv promote appetitive responses while excitatory glutamatergic projections promote aversive responses. Although the role of this pathway has not been examined in the context of aggressive behavior, both aversion (Resendez, Kuhnmuench, Krzywosinski, & Aragona, 2012) and reward (Fish, De Bold, & Miczek, 2002, 2005) have been described as important properties mediating aggression.

Hypothalamus. The hypothalamus has long been recognized as an important locus mediating aggression (Lammers, Kruk, Meelis, & Poel, 1988; Lipp & Hunsperger, 1978; Siegel, Roeling, Gregg, & Kruk, 1999; Siegel & Pott, 1988). Initial experiments on male rodents (Koolhaas, 1978; Kruk, 1991) and cats (Siegel & Pott, 1988) showed that electrical stimulation of a hypothalamic attack area would trigger intense expression of aggressive behaviors. This attack area was later determined to consist of the lateral hypothalamus (LH) and ventromedial hypothalamus (VMH). After engaging in maternal aggression, female mice have increased EGR-1 immunoreactivity in both VMH and LH (Hasen & Gammie, 2006). Additionally, lesions of the VMH strongly reduce maternal aggression towards male intruders (Hansen, 1989). Curiously, the anterior part of VMH is an important node in a circuit that inhibits other types of maternal behavior such as nursing and pup retrieval (Mann and Babb, 2004; Sheehan et al., 2001; Sheehan and Numan, 1997). The onset of maternal aggression closely tracks the onset of other maternal behaviors in rodents (Mayer & Rosenblatt, 1984), so an important question is how VMH signaling changes so that it can go from inhibiting the combination of maternal behavior and aggression in virgin animals to promoting aggression in lactating females. It was recently shown that a specific
population of progesterone receptor (PR) expressing neurons in the ventrolateral subregion of VMH is key for territorial aggression and sexual behavior in males (Yang et al., 2013a). Interestingly, in females these PR neurons in ventrolateral VMH regulate sexual behavior but have no effect on maternal behavior. It is possible that non-PR expressing neurons in ventrolateral VMH may be more important for modulating maternal aggression or that more lateral subregions of VMH play a more significant role.

The MPOA is another nucleus that is very important for maternal behaviors. Immediate early gene studies have shown that MPOA activity is increased when postpartum females engage in both maternal care (Fleming, Suh, Korsmit, & Rusak, 1994; Numan & Numan, 1995) and maternal aggression (Gammie & Nelson, 2001; Hasen & Gammie, 2006; Motta et al., 2013). Lesion studies have confirmed that MPOA is essential for the display of maternal care (Numan, Corodimas, Numan, Factor, & Piers, 1988), but no study has specifically assessed the effects of MPOA lesion on maternal aggression. The role of the MPOA may generalize to paternal aggression in monogamous species in which males provide parental care. California mouse fathers are more aggressive than virgin males and have significantly more c-fos immunoreactive cells in the MPOA than virgins following a resident-intruder aggression (Trainor, Finy, & Nelson, 2008b).

Finally, the hypothalamic ventral premammillary nucleus (PMv), which has important functions for reproductive behaviors (Cavalcante, Bittencourt, & Elias, 2006; Kollack-Walker & Newman, 1995; Leshan, Louis, Jo, Rhodes, Münzberg, & Myers, 2009), was recently identified as a critical node modulating maternal aggression but not other aspects of maternal care (Motta et al., 2013). The PMv is reciprocally connected with regions important for social behaviors like MeA, VMH and LH (Canteras, Simerly, & Swanson, 1992). Engaging in maternal aggression significantly increases c-fos expression in PMv, and excitotoxic lesions of PMv inhibit aggressive behavior towards a male intruder in female lactating rats without affecting other behaviors including social investigation and pup nursing, licking and grooming (Motta et al., 2013). Interestingly, lesioned rats also show significantly less c-fos expression in anterior BNSTv, MPOA, VMH, and LH compared to unlesioned rats that display strong aggressive behavior, suggesting that the PMv has a key activating role in the maternal aggression neural network.

Lateral Septum. Immediate early gene studies suggest that increased activity in LS is negatively associated with aggression in both males and females (Goodson, Evans, & Soma, 2005; Lee & Gammie, 2007). Consistent with these data, inactivation or lesions of LS increases
intraspecific male aggression in a variety of species (Albert & Chew, 1980; Goodson, Eibach, Sakata, & Adkins-Regan, 1999; Potegal, Blau, & Glusman, 1981; Ramirez, Salas, & Portavella, 1988; Slotnick, McMullen, & Fleischer, 1973). Similarly, activation of GABAA receptors in LS inhibits both maternal aggression (Lee & Gammie, 2009) and male-male (offensive) aggression (McDonald, Markham, Norvelle, Albers, & Huhman, 2012; Wong et al., 2016). A recent study showed that optogenetic activation of the pathway of LS projecting to VMHvl is sufficient to inhibit male territorial aggression without affecting other social and sexual behaviors (Wong et al., 2016). It is unclear whether this circuit is also important for maternal aggression. Further investigation of how the LS-VMH circuit changes with the onset of maternal behavior and aggression is needed.

Periaqueductal gray: The PAG is involved in the motor output of a variety of aggressive behaviors in males (Siegel & Pott, 1988; Siegel & Victoroff, 2009). It receives afferent connections from hypothalamus, BNST, and LS, and is thought to promote species-specific aggressive behaviors (Nelson & Trainor, 2007). In females, the caudal PAG (caPAG) has been associated with modulation of maternal aggression. Increased egr-1 as well as c-fos expression in the caPAG is observed following maternal aggression (Gammie & Nelson, 2001; Hasen & Gammie, 2006), and lesions to caPAG increase maternal aggression in rats (Lonstein & Stern, 1998). Further, injections of GABAA receptor antagonist into this region dose-dependently decrease maternal aggression while promoting maternal care (Lee & Gammie, 2010). Thus, the output from caPAG seems to be crucial for inhibiting maternal aggression in favor of the expression of other parental behaviors.

Mesocorticolimbic Dopamine System

Aggressive behaviors have a strong motivational component (de Almeida & Miczek, 2002; Fish et al., 2002; May, 2011), and as such are modulated by brain regions that define the salience and valence of a stimuli (Kalivas & Volkow, 2005; Love, 2014). The mesocorticolimbic dopamine pathway consists of the ventral tegmental area (VTA) and its efferent projections to the nucleus accumbens (NAC), amygdala, hippocampus, and prefrontal cortex (PFC). This circuit and the SBN interact to regulate social behaviors such as aggression (O'Connell & Hofmann, 2011). In the context of maternal aggression, it has been proposed that the presence of an intruder induces a negative affective state in the lactating dam, which results in the motivation to attack the intruder (Almeida et al., 2014).
Ventral tegmental area. The VTA is a heterogeneous nucleus with important topographical organization (Barker, Root, Zhang, & Morales, 2016; Love, 2014). In the VTA, GABAergic neurons are important inhibitory regulators of dopamine (DA) neurons (Mathon, Kamal, Smidt, & Ramakers, 2003). Early studies showed that infusions of the GABA agonist muscimol into caudal VTA increase aggression in male rats (Arnt & Scheel-Krüger, 1979). More recently, it was shown that optogenetic stimulation of DA neurons in VTA increases isolation-induced aggression in male mice (Yu et al., 2014). These apparently conflicting results might be related to the fact that VTA is a very complex structure. The VTA has different subpopulations of neurons, and both DA and non-DA cells express GABA receptors (Mathon et al., 2003). Further, VTA neurons are capable of signaling using one or more neurotransmitters; for example, some neurons in the VTA can co-release both DA and glutamate (Zhang et al., 2015), or glutamate and GABA (Root et al., 2014). Thus, even what would appear to be a highly specific manipulation (optical stimulation of DA neurons) could result in very complex changes in neurotransmitter release. In general, little is known about the role of VTA on female aggression. Overall, most evidence points to a limited role. Immediate early gene studies observed no changes in c-fos or egr-1 in the VTA following maternal aggression (Gammie & Nelson, 2001; Hasen & Gammie, 2006). Similarly, in lactating rats, inactivation of VTA with microinfusions of 6-hydroxydopamine (6-OHDA) does not have any effect on aggression, although it does affect pup retrieval behavior (Hansen, Harthon, Wallin, Löfberg, & Svensson, 1991). It is unclear whether the VTA plays a more important role in modulating aggression in other contexts.

Nucleus accumbens: For male aggression, there is strong evidence for an important role of the NAc. Indeed, haloperidol, an antagonist of the D2 receptors (highly expressed in NAc) was long used to reduce aggressive behaviors in mentally ill patients (De Deyn et al., 1999). However, systemic D2 inhibition has many additional adverse effects (e.g., decreased arousal and motor problems). Rodent studies have provided more targeted evidence that DA receptors within the NAc have important effects on aggression. Infusion of DA receptor antagonists into NAc significantly reduces territorial aggression in male mice (Couppis & Kennedy, 2008). In females, engaging in one episode of maternal aggression does not affect c-fos immunoreactivity in NAc (Gammie & Nelson, 2001). However, a recent study showed that female Syrian hamsters that engage in repeated displays of territorial aggression show increased spine density in NAc (Staffend & Meisel, 2012). These changes, which are mediated by decreased phosphorylation of fragile X mental retardation protein (FMRP), enhance aggressive behavior in future encounters.
This observation suggests that neuroplasticity within the NAc may play a role in reinforcing aggressive behavior. This is supported by findings in male rodents. Male rats that engage in regularly scheduled bouts of aggression show increased DA release in the NAc in anticipation of aggressive encounters (Ferrari, Erp, Tornatzky, & Miczek 2003). Also, male California mice that win aggressive encounters have increased androgen receptor (AR) immunoreactivity in the NAc (Fuxjager, Forbes-Lorman, Coss, Auger, Auger, & Marler, 2010). It is not clear whether these changes in DA release and AR expression result in neuroplastic changes that affect behavior.

Prefrontal cortex: In general, PFC has important inhibitory effects on aggressive behaviors across species, including humans (Nelson & Trainor, 2007; Raine & Yang, 2006). The PFC receives and sends projections to the hypothalamus, NAc, VTA, and amygdala (Gabbott, Warner, Jays, Salway, & Busby, 2005; Hoover & Vertes, 2011; Peyron, Petit, Rampon, Jouvet, & Luppi, 1998; Rosenkranz & Grace, 2002; Vertes, 2004), and is tightly associated with the serotoninergic system, a main modulator of aggressive behavior. Studies in males have shown that PFC acts primarily to inhibit aggression (Nelson & Trainor, 2007; Takahashi, Nagayasu, Nishitani, Kaneko, & Koide, 2014), although studies examining the role of specific circuitries within subregions of the PFC have shown that this relationship is more complex. For example, activation of medial PFC (mPFC), but not orbitofrontal cortex, inhibits intrasexual (territorial) aggression in males (Takahashi & Miczek, 2014). In the context of maternal aggression, the ventro orbital subregion of PFC (voPFC) has been shown to exert anti-aggressive effects (Veiga, Miczek, Lucion, & Almeida, 2007; Veiga, Miczek, Lucion, & Almeida, 2011).

Hormonal Mechanisms of Female Aggression

Steroid and neuropeptide hormones play an important role in coordinating aggressive behavior with other bodily functions, such as reproduction. In some cases these hormones have similar effects on aggression in both males females. However, in other cases, hormonal mechanisms important for male aggression have no or even opposite effects in females.

Steroid Hormones

Steroid hormones can be produced in the adrenal gland, gonads, and brain. Although the gonads are the most obvious source for sex differences in steroid synthesis, steroid synthesis in adrenal and brain can also differ in males and females. Neuronal effects of steroid hormones are
involved in both the development of aggressive behavior and in its expression during adulthood. Prenatally, steroid hormones contribute to the organization of neural circuits (French, Mustoe, Cavanaugh, & Birnie, 2013), and during adulthood steroids participate in the modulation of aggressive behaviors and the associated physiological responses (French et al., 2013; Soma, Scotti, Newman, Charlier, & Demas, 2008).

Androgens

Gonadal Sources of Androgens.

The role of androgens on aggressive behavior in males is well established (Soma, 2006). Early studies found that castration reduces aggression in males (Payne & Swanson, 1971; Vandenbergh, 1971), although now we know these effects may be mediated by estrogen in addition to testosterone (T) (discussed below). In many seasonally breeding species, increased T levels serve to coordinate increased territorial aggression with mating behavior (Wingfield, Hegner, Dufty, & Ball, 1990; Wingfield, Lynn, & Soma 2001). In females, there is increasing evidence that T is increased in the breeding season (Ketterson, Nolan, & Sandell, 2005), and high levels of T have been related to sexual behavior in women (Anders, Hamilton, Schmidt, & Watson, 2007). In addition, female T levels are higher in colonial bird species with elevated competition for nesting sites compared to solitary species that have less competition for nesting sites (Møller, Garamszegi, Gil, Hurtrez-Boussès, & Eens, 2005).

Dynamic changes in circulating T have been shown to have an important effect on aggression in males. For example, changes in T during intrasexual aggressive encounters can have important long-term effects on aggression in future competitions (Gleason, Fuxjager, Oyegbile, & Marler, 2009). This has been called the challenge effect, which predicts that concentrations of T should be elevated during social competition to promote aggressive behaviors (Wingfield et al., 1990). Interestingly, dynamic changes in T can also affect aggression in females. For example, in the absence of dominant males, females of the African cichlid fish (Astatotilapia burtoni) increase their T levels and show higher levels of territorial aggression (Renn, Fraser, Aubin-Horth, Trainor, & Hofmann, 2012). Similarly, females of the daffodil cichlid (Neolamprologus pulcher) and female marmosets (Callithrix kuhlii) show elevated levels of testosterone after aggressively defending their territories from intruders (Desjardins, Hazelden, Van der Kraak, & Balshine, 2006; Ross & French, 2011). Nonetheless, T elevation in competitive social environment is not always observed in females (Davis & Marler, 2003; Goymann, Wittenzellner, Schwabl, & Makumba, 2008;
Rubenstein & Wikelski, 2005). In some cases, circulating T levels in females actually decreases following an aggressive encounter (Elekonich & Wingfield, 2000; Rubenstein & Wikelski, 2005). Currently, the functional basis for the variability in T responses to aggressive interactions in females is unclear. Although androgens are known to have important metabolic costs (Buchanan, Evans, Goldsmith, Bryant, & Rowe, 2001; Marler & Moore, 1989) and immunosuppressive effects (Hillgarth & Wingfield, 1997), they can have additional side effects in females. These can include breeding delay, altered mate choice behavior, and inhibition of maternal care (Gerlach & Ketterson, 2013; McGlothlin, Neudorf, Casto, Nolan, & Ketterson, 2004; Rosvall, 2013b). Thus, it has been hypothesized that the elevation of T in response to competition should be more common in species with low maternal care, or species in which offspring are relatively less susceptible to parental neglect (Rosvall, 2013a). In turn, species with higher maternal investment are expected to have evolved mechanisms other than elevation of T to mediate aggression. An alternative solution to limit the costs of T would be to employ local synthesis of T.

T Synthetized in the Brain.

Although there is little direct evidence for T synthesis within the brain, recent data suggest that the enzymes necessary to produce T de novo from cholesterol are present in many brain areas (Do Rego et al., 2009). In addition, it is possible that T could be synthesized from dehydroepiandrosterone (DHEA), a steroid precursor that can be synthesized in nervous tissue (Corpéchot, Robel, Axelson, Sjövall, & Baulieu, 1981; Do Rego et al., 2007; Hojo et al., 2004) and adrenal glands (Labrie et al., 2005). DHEA can be metabolized into androgens and/or estrogens in peripheral tissues (Labrie et al., 2005). Data from rodents and songbirds suggest that this conversion of DHEA into active steroids can occur in the brain where it could affect behavior (Dupont, Simard, Luu-The, Labrie, & Pelletier, 1994; Soma, Alday, Hau, & Schlinger, 2004). For example, plasma levels of DHEA are elevated in male sparrows during the non-breeding season (Maddison, Anderson, Prior, Taves, & Soma, 2012; Newman & Soma, 2009), when territorial aggression levels are still elevated but plasma T levels are very low (Soma, Schlinger, Wingfield, & Saldanha, 2003). Interestingly, levels of the enzyme necessary for conversion of DHEA to androgens, 3β-hydroxysteroid dehydrogenase/δ5-δ4 isomerase (3β-HSD), are highest during this time in centromedial and caudal telencephalon (Pradhan, Newman, Wacker, Wingfield, Schlinger, & Soma, 2010). In addition, the activity of these enzymes is further increased during territorial challenges and engaging in aggression. These data suggest that male sparrows have the potential for T synthesis within the brain, and that it can be rapidly modulated. However the
hypothesis that the conversion of DHEA to T increases aggression still needs to be tested directly. In females, DHEA has been linked to territorial aggression in Siberian hamsters. During winter-like days, female Siberian hamsters show increased levels of territorial aggression and have elevated levels of serum DHEA and adrenal DHEA responsiveness (see Figure 2) (Rendon, Rudolph, Sengelaub, & Demas, 2015). Further, aggression reduces serum DHEA levels during short days (Rendon & Demas, 2016). One possible explanation for this result is that DHEA metabolism to androgens during aggressive encounters might reduce circulating DHEA levels, but again, this is not direct evidence that DHEA is being converted to androgens to modulate aggression, as alternative mechanisms of action are possible. For example, DHEA can bind to estrogen and androgen receptors in the brain (Webb, Geoghegan, Prough, & Miller, 2006) and has been shown to modulate action of various receptor systems known to modulate aggression including GABAA and NMDA (Bergeron, Montigny, & Debonnel, 1996; Compagnone & Mellon, 2000; Majewska, 1992). Further studies will be needed to directly test which are the underlying mechanisms mediating the effects of DHEA on aggressive behavior.

Androgens: Mechanisms of Action.

The effects of androgens on behavior can be mediated through direct binding to intracellular androgen receptors (AR), which then migrate to the nucleus to regulate gene expression (Heemers & Tindall, 2007). This so-called genomic effect is relatively slow, with the effects on cell function observed over a period of hours to days. Alternatively, T can rapidly influence aggressive behavior by non-genomic mechanisms in which AR are deposited in the cell membrane (Lösel & Wehling, 2003) or through intracellular AR that remain outside the nucleus. These non-genomic actions are mediated by second messenger pathways that can lead to rapid changes in cell function within seconds or minutes (Foradori, Weiser, & Handa, 2008). Membrane associated AR have been described in dendrites (Tabori et al., 2005) and axons (DonCarlos et al., 2006).

In the context of aggression, both slow and rapid effects of T have been reported. For example, a study in the coral reef fish Stegastes nigricans used implants of flutamide, an AR antagonist, to show that AR are necessary for the expression of territorial aggression in males but not females (Vullioud, Bshary, & Ros, 2013). Here flutamide was administered over the course of 5-6 days, which is consistent with slow acting genomic effects of steroid hormones.. However, T can also have more rapid effects. In white-footed mice (Peromyscus leucopus), one pulse of T can modify territorial marking within 20 minutes, suggesting that non-genomic regulation can
modify this agonistic behavior (Fuxjager, Knaebe, & Marler, 2015). Although these studies did not directly test whether these effects were mediated by AR, other evidence suggests that estrogens are not necessary. For example, male California mice (Peromyscus californicus) injected with T immediately following aggressive encounters are more aggressive in future encounters than males receiving saline, and this effect is not blocked by aromatase (the enzyme responsible for converting T to estrogens) inhibitor treatment (Trainor, Bird, & Marler, 2004). Rapid effects of T on aggressive behavior have also been observed in females. Lythrypnus dalli is a fish species that can show bidirectional sex changes. Dominant females show increased territorial aggression within 2 hours of exogenous administration of 11-ketotestosterone, a teleost analog to dihydrotestosterone, compared to controls (Pradhan, Connor, Pritchett, & Grober, 2014) (Figure 3).

Interestingly, in several cases AR expression in the brain is promoted by T, which in turn can promote sex differences in brain AR expression. In Mus musculus, AR expression is higher in intact males than intact females in BNST, LS, and MPOA; a difference that is abolished by gonadectomy (Lu, McKenna, Cologer-Clifford, Nau, & Simon, 1998). In addition, females treated with T implants show AR expression levels equivalent to males in these nuclei. However, this is not true for every species. For example, although T increases AR gene expression in male rats, no effects of T implants were observed on AR expression in females (Roselli, 1991). Further, in females central AR expression can be higher than in males even with lower circulating T. An intriguing example is the African black coucal (Centropus grillii), a cuckoo bird in which females are more aggressive than males. Females have lower levels of circulating T, but show higher expression of AR mRNA in the taeniae of the amygdala (a brain region analogous to the mammalian MeA) than males (Voigt and Goymann, 2007) (Figure 4). In addition to sex specific expression of steroid receptors, activation of the same receptors in males and females may result in sex-specific gene expression profiles. For example, in the sexually dimorphic dark-eyed junco (Junco hyemalis), treatment with T has different effects on gene expression in the brain for males versus females. Testosterone increased expression of aromatase in hypothalamus in both sexes, but decreased expression of monoamine oxidase A in MeA in females but not males (Peterson et al., 2013). Together, this suggests that different region specific expression pattern of AR, as well as downstream effects of AR in males vs. females, may allow females to harness the beneficial effects of T on certain behaviors while avoiding some of the adverse effects that T may cause on other behaviors, metabolism and reproduction (Wingfield et al., 2001).

Estrogens
Peripheral Sources of Estrogens

Estrogens have very important effects on aggression in both males and females. Early studies in males showed that E administration (Payne & Swanson, 1972a; Vandenbergh, 1971), as well as ovarian implantation (Payne & Swanson, 1971), could increase inter-male aggression. During development, T secreted by the male gonads is converted to estradiol (E2) in the brain, which has long lasting effects on male typical behaviors, including aggression, and inhibition of female like behaviors (Lenz, Nugent, & McCarthy, 2012; Wu et al., 2009).

Acting in the adult brain, estrogens are mainly associated with promoting aggressive behaviors. In females, estrogens promote aggression in reptiles (Rubenstein & Wikelski, 2005), birds (Rosvall et al., 2012), and mammals (Albert, Petrovic, & Walsh, 1989). The role of estrogens on aggression has been particularly well studied in the context of maternal behavior. For example, ovariectomy on gestation day 16 in rats significantly reduces maternal aggression in comparison to controls (sham operated gestating rats), and this effect can be reversed by injections of E2 (Mayer & Rosenblatt, 1987). In males, evidence from several bird and rodent species shows that E2 can increase territorial aggressive behavior (Laredo, Villalon Landeros, & Trainor, 2014). In California mice, estrogens increase territorial aggression if males are housed in cages containing corncob bedding (Trainor et al., 2008a), which increases blood levels of E2-like tetrahydrofuran-diols (Villalon Landeros, Morisseau, Yoo, Fu, Hammock, & Trainor, 2012). However, if California mice are housed on cardboard-based bedding then estrogens decrease aggression (Laredo et al., 2013). These results show that factors such as social experience and diet can have important effects how estrogens modulate aggression. These factors may alter how estrogens interact with estrogen receptors (Byrnes, Babb, & Bridges, 2009; Byrnes, Casey, & Bridges, 2012). Finally, estrogens are involved at multiple levels of processing of social information (Ervin, Lymer, Matta, Clipperton-Allen, Kavalirov, & Choleris, 2015), and can interact with other steroid hormones and neurotransmitter systems to influence aggression (Murakami, 2016; Soma, Rendon, Boonstra, Albers, & Demas, 2015).

Brain Sources of Estrogens

Aromatase is expressed in brain areas that regulate aggressive behaviors across species (Balthazart, Baillien, Charlier, Cornil, & Ball, 2003; Cohen & Wade, 2011; Naftolin, Horvath, & Balthazart, 2001; Shen, Schlinger, Campagnoni, & Arnold, 1995), and its activity has been associated with aggression in males and females. For example, studies in male Japanese quail
(Coturnix japonica) showed that individual differences in territorial aggressive behavior during the breeding season were positively correlated with aromatase in hypothalamus, but no correlation was seen between circulating T and this behavior (Schlinger & Callard, 1989). Further, administration of an aromatase inhibitor, but not an AR antagonist, significantly reduces aggression in this species (Schlinger & Callard, 1990). Similarly, genetic deletion of aromatase completely eliminates territorial aggression in mice (Toda, Saibara, Okada, Onishi, & Shizuta, 2001). In general, aromatase activity in the brain is higher in males than females (Roselli, Horton, & Resko, 1985), a difference mediated by at least two mechanisms. First, aromatase enzyme velocity is higher in males than females in the BNST and VMH (Roselli, Klosterman, & Fasasi, 1996b). Second, androgen receptor is a more effective enhancer of aromatase mRNA expression in male hypothalamus than females (Roselli, Abdelgadir, Jorgensen, & Resko, 1996a). Interestingly, recent data showed that aromatase producing neurons in the MeA had important effects on aggression in both males and females (Unger, Burke, Yang, Bender, Fuller, & Shah, 2015). Aromatase expressing neurons in the MeApd were selectively ablated in adult mice, such that the MeApd was allowed to develop normally in the presence of aromatase. When these aromatase expressing neurons were lesioned, both intermale aggression and maternal aggression were reduced but reproductive behaviors were unaffected (Figure 5, a & b).

Estrogen: Mechanisms of Action

Estrogens exert their action by binding to estrogen receptors (ER) ERα and ERβ, which are expressed throughout the neural circuit of aggression (Cushing, 2016; Kaiser, Kruitjev, Swaab, & Sachser, 2003; Kramer, Simmons, & Freeman, 2008). Similar to androgen receptors, these receptors can be expressed in the nucleus (Heldring et al., 2007; Micevych & Dominguez, 2009), as well as outside of the nucleus including dendrites, axons, and neuronal terminals (Blaustein, Lehman, Turcotte, & Greene, 1992; Milner, McEwen, Hayashi, Li, Reagan, & Alves, 2001; Milner et al. 2005; Towart, Alves, Znamensky, Hayashi, McEwen, & Milner, 2003). Activation of these receptors results in activation second messenger systems that can rapidly modulate cell function (Heimovics, Trainor, & Soma, 2015b). Initial knock out studies assessing global effects of ER in males found that functional ERα facilitate normal expression of inter-male aggression in mice (Ogawa, Lubahn, Korach, & Pfaff, 1997; Scordalakes & Rissman, 2003). Furthermore male aggression in CD-1 mice is positively correlated with the expression of ERα in areas important for aggression including BNST, LS, and LH (Trainor, Greiwe, & Nelson, 2006). ERβ, on the other side, has been associated with the
inhibition of aggression, although this effect seems to depend on age and sexual experience (Nomura et al., 2002; Ogawa et al., 1999). However, studies comparing the effects of selective ERα and ERβ agonists on male aggression have found to both increase or decrease territorial aggression depending on the light cycles used for testing (Trainor et al. 2007). Similar results have been reported in females. Gonadectomized female mice treated with selective ERα agonists have increased aggressive attacks towards intruders (Clipperton-Allen, Almey, Melichercik, Allen, & Choleris, 2011) while females treated with selective ERβ agonists show increased non-attack agonistic behaviors such as social investigation and dominance (Clipperton-Allen, Cragg, Wood, Pfaff, & Choleris, 2010). Interestingly knockout of ERα increases levels of offensive aggression in females (Ogawa, Eng, Taylor, Lubahn, Korach, & Pfaff, 1998). Together these results indicate that in males and females ERα and ERβ have distinct organizational and activational effects on aggressive behaviors.

E can act rapidly to modulate aggression. These effects may originate from the ability of aromatase activity to be rapidly modulated by environmental factors such as stress or social conflict (Balthazart et al., 2003; Dickens, Balthazart, & Cornil, 2012; Dickens, Bournonville, Balthazar, & Cornil 2014). Although the specific directional effects of E on aggression (either increasing or decreasing) depend on factors such as diet (Villalon Landeros et al., 2012), a robust observation is that rapid E action is more likely to be observed in the non-breeding season. In both Peromyscus polionotus (Trainor et al. 2007) and Peromyscus californicus (Trainor et al. 2008a), E rapidly modulates aggression in winter-like short day photoperiods but not in summer-like long day photoperiods. In P. californicus, the specificity of rapid E action under short days has been replicated (Laredo et al., 2014). Similarly, a study in male song sparrows showed that administration of E2 rapidly (within 20 min) increases territorial aggression in non-breeding males only (Heimovics, Ferris, & Soma, 2015a). Currently it is unclear whether rapid activation of ER modulates aggression in females, but rapid effects of E on brain and other behaviors, including memory formation (Gabor, Lymer, Phan, & Choleris, 2015), modulation of pain (An, Li, Yan, & Li, 2014), and anxiety-like behaviors (Holm, Liang, Thorsell, & Hilke, 2014), have been reported in females. This suggests that it is likely that this type of E signaling could be involved in modulation of female aggression.

Progesterone
Although progesterone (P4) production has been classically associated with female gonads, P4 can be also synthetized in male and female adrenal glands and nervous system, and has been shown to affect physiology and behavior in both sexes.

**Gonadal and Adrenal P4**

The effects of circulating progesterone (P4) on aggression have been mainly studied in the context of parental behaviors. In mammals, plasma levels of P4 decrease towards the end of gestation and then rise progressively from post-partum day 3 to 10 (Finley, Zhang, & Fewell, 2015; Taya & Greenwald, 1982). The administration of P4 receptor (PR) antagonist in day 6 post-partum increases maternal aggression (de Sousa et al., 2010) (Figure 6). Since maternal aggression sharply increases during peripartum and remains high for around 2 weeks post-partum, the authors proposed that increasing levels of P4 would have a role in the progressive reduction of maternal aggression after that period. The sharp decline in P4 around parturition has also been shown to be a key component of the onset of pup-directed behavior in females (Bridges, Rosenblatt, & Feder, 1978; Sheehan & Numan, 2002). Interestingly, studies using the biparental species Peromyscus californicus have found that in males, paternal behavior is also associated with a decrease in plasma P4 (Trainor et al. 2003) and PR expression in the BNST (Perea-Rodriguez, Takahashi, Amador, Hao, Saltzman, & Trainor, 2015; Trainor, Bird, Alday, Schlinger, & Marler, 2003). Transgenic mice lacking PR receptors show reduced aggression towards pups (Schneider et al., 2003; Schneider, Burgess, Horton, & Levine, 2009), suggesting that P4 and PR activation may facilitate infanticide.

Some studies have also assessed the role of P4 in the context of territorial aggression, although data here is less clear. Pharmacological manipulations of P4 in rodents have shown that P4 can both reduce and increase territorial aggression. For example, one study showed that administration of P4 reduces aggression in both male and female gonadectomized Syrian hamsters (Fraile, McEwen, & Pfaff, 1987), but another study in the same species showed that in ovariectomized females, daily injections of P4 for ten days increase intrasexual aggression (Payne & Swanson, 1972b). In female bank voles (Clethrionomys glareolus), administration of P4 also increases aggression (Kapusta, 1998). Similarly, studies assessing physiological levels of P4 have yielded conflicting results. One study in California mice (Peromyscus californicus) observed decreases in plasma P4 in females after engaging in aggression (Davis & Marler, 2003b). A similar result was observed in free-living black coucals (Centropus grillii), in which females also have decreased serum P4 after engaging in aggression (Goymann et al., 2008).
Furthermore, females treated with P4 implants show reduced territorial aggression compared to females given empty implants. In contrast, P4 is higher in aggressive compared to non-aggressive female Iguanas (Amblyrhynchus cristatus) (Rubenstein & Wikelski, 2005), and studies in sparrows (Melospiza melodia) have found no association between P4 and female aggression (Elekonich & Wingfield, 2000b). Together, the data available suggests that while P4 seems to be necessary for the expression of maternal aggression, P4 in other contexts can have different effects, and this may be related to the relationship between levels of P and other steroid hormones. During lactation, T and E2 levels are low compared to P4 (Taya & Greenwald, 1982). Interestingly, female California mice showing territorial aggression show the opposite; P4/T ratio is decreased compared to controls (Davis & Marler, 2003a). Further studies are needed to test this hypothesis.

Progesterone: Mechanisms of Action

Similar to estrogens, P4 can exert its effect through genomic and non-genomic mechanisms (Taraborrelli, 2015; Wendler, Albrecht, Wehling, 2012). Nuclear and membrane PR are widely expressed throughout the brain (Schumacher et al., 2014), and have been related to the regulation of a variety of behaviors, including sexual behavior, social recognition and motivation (Bychowski & Auger, 2012; Frye, Koonce, & Walf, 2013; Frye, Walf, Kohtz, & Zhu, 2014; Yang et al., 2013a). To our knowledge, little work has assessed the relationship between female aggression and PR. Deletion of the PR gene has no effect on intermale aggression, but female aggression was not examined (Schneider et al., 2003). On the other hand, ablation of PR-expressing neurons in VMH reduces territorial aggressive and sexual behavior in male mice but has no effect on territorial aggressive behavior in females (Yang et al., 2013a). This line of mice may not be optimal for assessing the role of PR on aggression in females, as C57Bl6 normally do not engage in significant levels of aggression outside of maternal defense. Intriguingly, it is possible for P4 to affect behavior independently of PR. P4 can be metabolized in the brain to allopregnanolone (Dong et al., 2001a; Pinna et al., 2008), which is an efficient positive allosteric modulator of GABAA receptors (Belelli & Lambert, 2005; Herd, Belelli, & Lambert, 2007; Pinna et al., 2000; Puia et al., 2003). The activation of GABAA by allopregnanolone has been mainly associated with an inhibition of aggression. For example, aggression in socially isolated male mice is associated with reduced levels of brain allopregnanolone (Dong et al., 2001a; Pinna, Agis-Balboa, Pibiri, Nelson, Guidotti, & Costa, 2008). Further, administration of fluoxetine, which prevents declines in allopregnanolone, blocks the effects of social isolation on aggression (Pinna
et al., 2003). In females, social isolation does not reduce levels of allopregnanolone, and it also does not increase aggression (Pinna, Dong, Matsumoto, Costa, & Guidotti, 2003). Interestingly, long-term administration of T in female mice results in both increased levels of territorial aggression and reduction of allopregnanolone levels (Pinna, Costa, & Guidotti, 2005), which suggests that T actions on aggression could be mediated in part by down regulation of brain allopregnanolone biosynthesis.

Glucocorticoids

Engaging in aggression frequently generates a significant response from the hypothalamus-pituitary-adrenal (HPA) axis (Bronson & Eleftheriou, 1965; Earley et al., 2006; Ramenofsky, 1985; Schuurman, 1980; Woodley & Moore, 1999). Although an increased glucocorticoid (GC) response is often examined in the context of losing aggressive interactions, GC have important effects on aggressive behaviors.

Glucocorticoids and Aggression

GC production has been involved in both the promotion and inhibition of aggression in humans and animals, and many times this effect is context specific. For example, while acute systemic (Haller, Albert, & Makara, 1997; Mikics, Kruk, & Haller, 2004) and hypothalamic (Hayden-Hixson & Ferris, 1991) injections of GC promote territorial aggression in male rodents, chronic administration of high levels of GC suppresses aggression in a variety of species (Leshner, Korn, Mixon, Rosenthal, & Besser, 1980; Wingfield & Silverin, 1986). Interestingly, GC seems to have a particular important effect on abnormal levels of aggression. Animals selected for high levels of aggression show lower GC responses (Carere, Groothuis, Möstl, Daan, & Koolhaas, 2003; Veenema, Meijer, de Kloet, & Koolhaas, 2003a; Veenema, Meijer, de Kloet, Koolhaas, & Bohus, 2003b), and chronic glucocorticoid deficit induced by adrenalectomy results in escalated aggression in male rats (Haller, Schraaf, & Kruk, 2001). This has also been described in humans. Aggression in habitually violent offenders (Virkkunen, 1985) and patients with antisocial personality disorder (Dolan, Anderson, & Deakin, 2001) is associated with low GC levels. In females, HPA activity has been mainly associated with the reduction of aggression. In female rodents increased corticotrophin releasing hormone (CRH) signaling, as well as administration of dexamethasone, reduces maternal aggression (Gammie, Negron, Newman, & Rhodes, 2004; Gammie, Hasen, Stevenson, Bale, & D’Anna 2005; Vilela & Giusti-Paiva, 2011). Similarly, female crows (Corvus macrorhynchos) show a negative correlation between cortisol
metabolites and dominance rank, which is the opposite of what happens in males (Ode, Asaba, Miyazawa, Mogi, Kikusui, & Izawa, 2015). A study in young women found a negative correlation between circulating cortisol and reactive aggression (Stoppelbein, Greening, Luebbe, Fite, & Becker, 2014). The apparent promotion or inhibition of GC on aggressive behavior may be explained by the fact that GC do not directly activate or inhibit aggression circuits, rather, they modulate neurons properties (Reul & de Kloet, 1985). Thus, the activation of specific neural circuits may be the primary variable modulating aggressive response, which in turn can be secondarily modulated by GC signaling.

Glucocorticoids: Mechanisms of Action

The modulating actions of GC are regulated through two types of receptors, the mineralocorticoid receptors (MR) and the GC receptor (GR) (Herman et al., 2016). MR primarily mediates the action of baseline levels of GC, while lower affinity GR are more important for mediating the effects of stress-induced increases of GC (Myers, McKlveen, & Herman, 2012). Both MR and GR can act as transcription factors, or as mediators of rapid non-genomic signaling (Groeneweg, Karst, de Kloet, & Joëls, 2012). MR are expressed in limbic areas including PFC, LS, and hippocampus, where they co-localize with GR. GR are more widely distributed throughout the brain, but also show lower affinity for GC than MR (Reul & de Kloet, 1985). It has been a challenge to directly assess the specific role of these receptors in regulating aggression, especially GR. This is because the field does not have access to a specific GR antagonist. For example, two groups have reported that RU486 treatment reduces territorial aggression in male rainbow trout (Schjolden, Basic, & Winberg, 2009) and electric fish (Dunlap, Jashari, & Pappas, 2011). The problem for interpreting these results is that RU486 is very strong antagonist of PR receptors also, so it is impossible to rule out a role for PR. Transgenic mice overexpressing or underexpressing GR have been produced (Ridder et al., 2005), but aggressive behavior has not be examined. An ideal strategy would be to examine the effect of inducible knockout of GR in brain regions mediating aggression such as the lateral septum. For MR, the specific antagonist spironolactone has been a useful tool. Intriguingly, a single injection of spironolactone reduced offensive behavior in male rats against a naïve intruder, whereas repeated spironolactone treatment enhanced aggression (Ruiz-Aizpurua, Buwalda, & de Boer, 2013). Thus, while it is clear that GCs can modulate aggression, the specific receptors and site of action mediating these effects have not been identified. Moreover, virtually no studies examining GC regulation of aggression have been conducted in females, despite that fact that, in many rodent species, GC
levels are significantly higher in females than in males (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Trainor, Takahashi, Silva, Crean, & Hostetler, 2010; Weiser & Handa, 2009).

Peptide Hormones: Vasopressin and Oxytocin

Arginine vasopressin (AVP) and oxytocin (OT) are highly conserved neuropeptides. They are mainly synthetized in hypothalamus, and can be released peripherally (Sivukhina & Jirikowski, 2016; Yang, Wang, Han, & Wang, 2013b) and centrally through either local synaptic or somatodendritic release (Bosch & Neumann, 2012; Dumais & Veenema, 2016; Gobrogge, Liu, Young, & Wang, 2009; Love, 2014). These neuropeptides regulate a variety of social behaviors (Albers, 2015; Caldwell & Albers, 2015; Veenema & Neumann, 2008). Although it is usually assumed that the behavioral effects of OT are mediated by OT receptor (OTR), and AVP through AVP receptor (AVPR) subtypes, recent work has highlighted the promiscuity of these receptors (Manning et al., 2012). The effects of OT can be mediated by AVPR, while AVP can also activate OTR (Anacker, Christensen, LaFlamme, Grunberg, & Beery, 2016; Ramos et al., 2013; Song, Larkin, Malley, & Albers, 2016). OTR and AVPR are expressed throughout the structures regulating aggression (Caldwell & Albers, 2015). Importantly, the expression of the neuropeptides and their receptors are regulated by gonadal hormones (Amico, Thomas, & Hollingshead, 1997; Bale, Pedersen, & Dorsa, 1995; Delville, Mansour, & Ferris, 1996; De Vries, Wang, Bullock, & Numan, 1994; Young, Muns, Wang, & Insel, 1997a). The actions of gonadal hormones may play an important role in determining the sex-specific effects of OTR and V1aR activation on behavior (Dumais & Veenema, 2016).

Vasopressin

The effects of AVP on aggression in rodents are highly sex-specific. In male rodents, AVP typically promotes aggressive behavior when the individual is in a familiar context. This effect is best described in Syrian hamsters (Mesocricetus auratus), in which AVP acting in the AH is necessary for the display of territorial aggressive behaviors (Ferris, Albers, Wesolowski, Goldman, & Luman, 1984; Ferris, Meenan, Axelson, & Albers 1986; Ferris & Potegal, 1988). Microinjections of AVP into ventrolateral hypothalamus (VLH) (Delville et al., 1996), MeA, and LS (Koolhaas, Moor, Hiemstra, & Bohus, 1990) have also been found to increase male territorial aggression in rodents. The effects of AVP on aggression are thought to be mediated mainly through its actions on AVPR subtype V1a (V1aR). For example, in male hamsters, oral administration of the V1aR antagonist SRX251 (Ferris et al., 2006) significantly reduces male
aggression in a resident intruder test. Similarly, infusion of Manning compound (a highly specific V1aR antagonist) into the lateral ventricle reduces aggression in male California mice (Bester-Meredith, Martin, & Marler, 2005). Microinjections of Manning compound directly into medioventral BNST (BNSTmv) also increased attack latency for males tested in a resident-intruder test (Duque-Wickens et al. 2016)). The effects of V1aR on aggression can be species specific and likely mediated by species differences in social systems as well as V1aR receptor distribution (Goodson & Bass, 2001). For example, intracerebroventricular (i.c.v) infusion of either AVP or Manning had no effect on aggression in the white-footed mouse (Peromyscus leucopus) (Bester-Meredith et al., 2005). Both hamsters (Ferris & Potegal, 1988) and California mice (Ribble & Salvioni, 1990) are highly territorial species, while the white-footed mouse is less territorial (Metzgar, 1971). In addition to territorial aggression, AVP has been found to be an important molecule regulating selective aggression in monogamous species. Selective aggression refers to the aggression directed towards opposite sex individuals after the formation of a pair bond (Carter & Getz, 1993). After forming a pair bond with a female, male prairie voles (Microtus ochrogaster) treated with an i.c.v. injection of V1aR antagonist showed reduced aggression towards unfamiliar females (Winslow, Hastings, Carter, Harbaugh, & Insel, 1993). Selective aggression is absent in the polygynous montane vole (Microtus montanus) (Young, Winslow, Nilsen, & Insel, 1997b).

In females, there is strong evidence that AVP has important effects on maternal aggression. For example, Brattleboro rats that are congenitally deficient of AVP show markedly reduced maternal aggression (Fodor et al., 2014). Similarly, studies using rat dams selected for high (HAB) and low (LAB) anxiety behaviors show that AVP signaling is key for the expression of maternal aggression (Bosch & Neumann, 2010, 2012). The increased anxiety levels showed by HAB rats is a result of augmented hypothalamic AVP activity, which may be due to a polymorphism in the AVP gene promoter region (Murgatroyd et al., 2004; Wigger et al., 2004). Interestingly, HAB rats also show increased levels of maternal care and aggression (Bosch, Meddle, Beiderbeck, Douglas, & Neumann, 2005a; Neumann, Krömer, & Bosch, 2005). Blockade of V1aR in the MPOA reduces maternal aggression and maternal care in HAB rats (Bosch & Neumann, 2008). Besides the MPOA, the BNST (Bosch, Pförtsch, Beiderbeck, Landgraf, & Neumann, 2010) (Figure 7) and central amygdala (Bosch & Neumann, 2010) are key sites of local signaling for V1aR regulation of maternal aggression. Finally, chronic administration of AVP to LAB can enhance maternal care and aggression to levels more similar to HAB rats (Bosch & Neumann, 2008). Activation of V1aR during lactation, but not other stages, may have an important role in modulating sensory processing in order to coordinate both motivation to engage in
maternal care and aggressive defense in response to intruders. An i.c.v. injection of V1aR antagonist prior to resident intruder testing in lactating dams increases activation as measured by BOLD responses in areas associated with somatosensory processing (important for social investigation), while it reduces activation in areas associated with aggression (Caffrey, Nephew, & Febo, 2010). Importantly, the V1b receptor for AVP has been shown to also affect maternal behavior, but this receptor doesn’t seem to modulate maternal aggression (Bayerl, Klampfl, & Bosch, 2014).

While V1aR activation robustly promotes maternal aggression, very different results have been observed for non-lactating females. Microinjections of Manning compound into the AH increased territorial aggression in non-lactating female hamsters (Gutzler, Karom, Erwin, & Albers, 2010). This is also surprising given that V1aR antagonist injections in AH have the exact opposite effect in male hamsters (Caldwell & Albers, 2004; Ferris & Potegal, 1988). However, similar sex-specific effects of AVP signaling within AH have been reported in hamsters. For example, injections of AVP in AH results in increased flank marking behavior in males tested in the presence or absence of another males (Ferris, Mellon, Koppel, Perry, Fuller, & Delville, 1997), while in females the same treatment increases this behavior in a non-social context (Hennessey, Huhman, & Albers, 1994). Interestingly, AVP microinjections into the anterior hypothalamus had no effect in a social context (Gutzler et al., 2010). Context-dependent effects of V1aR have also been observed in California mice. Infusion of V1aR antagonist into the BNSTmv had anxiogenic effects in both social and nonsocial contexts for males, whereas for females, V1aR antagonist had anxiogenic effects in social contexts only (Duque-Wilckens et al. 2016). Currently, the mechanisms underlying sex differences in V1aR function are unknown. One possibility is that there may be sex differences in the cell types expressing V1aR receptor. So far, most analyses of V1aR expression have been conducted with autoradiography, which does not allow for cell type analysis. The determination of which cells express V1aR in the anterior hypothalamus and BNST could provide important insights on how activation of this receptor can have such different behavioral effects in males and females.

Oxytocin

The effects of OT on aggression are highly dependent on context in males and females. For example, acute administration of OT either i.c.v (Calcagnoli, de Boer, Althaus, den Boer, & Koolhaas, 2013) or intranasally (Calcagnoli, Kreutzmann, de Boer, Althaus, & Koolhaas, 2015a) reduces intermale territorial aggression in rats. Male aggression in resident intruder tests is also

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enhanced after i.c.v. injection of OTR antagonist (Calcagnoli et al., 2013). These effects of OT appear to be at least partly mediated by CeA, as site-specific infusions of OT are sufficient to reduce offensive aggression (Calcagnoli, Stubbendorff, Meyer, de Boer, Althaus, & Koolhaas, 2015b). On the other side, mice with a homozygous mutation in the OT gene show significantly reduced territorial aggression (DeVries, Young, & Nelson, 1997), although in this experiment it is not possible to know if the effects of OT are organizational or activational.

In females, OT appears mostly to inhibit aggression, but only if they are not lactating. For example, female OT knockout mice are more aggressive towards other females than wild type females (Ragnauth et al., 2005). Once again it's not clear whether the effects of OT are developmental or activational. In female Syrian hamsters, site-specific injections of OT into MPOA or AH significantly reduce aggression, while the injection of OTR antagonist had the opposite effect (Figure 8) (Harmon, Huhman, Moore, & Albers, 2002). Finally, i.c.v. injections of OT inhibited aggression directed toward unrelated young in female mice, and this effect was independent of ovarian hormones or prolactin (McCarthy, 1990). In contrast, OT mostly enhances maternal aggression. For example, correlational studies have found that OT is positively associated with maternal aggression in rats (Bosch et al., 2005), and direct infusion of OT into CeA or PVN increases maternal aggression in this species Bosch et al., 2005). Similarly, infusion of OT into CeA increases aggression in lactating hamsters (Ferris, Foote, Meltser, Plenby, Smith, & Insel, 1992). A previous study reported that infusion of antisense oligonucleotides designed to inhibit OT in PVN increases maternal aggression (Giovenardi, Padoin, Cadore, & Lucion, 1998). However, experimental knockdown of OT was not verified in these rats, so it is unclear whether the OT expression was inhibited as expected. Intriguingly, it has been suggested that effects of OT on maternal aggression are mediated by V1aR, as i.c.v. infusion of OTR antagonist has no effects on maternal aggression (Neumann, Tosch, Ohl, Torner, & Krömer, 2001) and OTR antagonist infused into CeA increased maternal aggression (Lubin, Elliott, Black, & Johns, 2003). The apparent contradictory effects of OT on female aggression might be explained partly by the recent hypothesis that a primary role of OT is to signal the salience of social stimuli regardless of the valence (Shamay-Tsoory & Abu-Akel, 2016). Thus, one possibility is that OT enhances the salience of the intruders’ behaviors. In the case of non-lactating females, OT could be reducing aggression towards opponents that are not a direct threat to survival (other females, infants), which would be beneficial considering the potential costs of engaging in aggressive encounters. On the other side, since infanticide by males is a direct threat to fitness, OT would promote
aggressive behavior by enhancing salience of the male intruders’ behavior. Further investigation is needed to better understand the role of OT in different social contexts.

Conclusions

Although historically male and female aggression has been studied independently, much of the neural circuitry controlling aggression is shared between sexes. Furthermore, recent studies are revealing that brain circuits originally viewed as exerting sex-specific control of aggression are really context dependent. For example, the MPOA has usually been considered to be less important for male aggression. However, recent data suggest the MPOA may be important for parental aggression in both males and females. Sex differences arise more frequently in the neurochemical mechanisms used by brain circuits. This is likely a result of sex-specific adaptations to different social and reproductive roles. For example, costs associated with high levels of certain hormones like T are generally higher in females. This may have resulted in females developing alternatives to high T to facilitate the expression of aggressive behaviors. Sex differences in the neurochemical control of aggression can also occur beyond the level of the receptor. We see this with V1aR, which has pro-aggressive effects in males, but anti-aggressive effects in virgin females. Understanding the mechanisms through which these sex-specific effects are generated will be a challenging but important task for future research.
Figure 1. Simplified neural circuits and connections associated with aggressive behavior. Green: Social behavior neural network. Orange: Mesocorticolimbic dopamine system. PFC = prefrontal cortex, LS = lateral septum; NAc = nucleus accumbens; MeA = medial amygdala; BNST = bed nucleus of the stria terminalis; MPOA = hypothalamic medial preoptic area; LH = lateral hypothalamus; VMH = ventromedial hypothalamus; PMv = ventral premammillary nucleus; VTA = ventral tegmental area; PAG = periaqueductal gray.
Figure 2. ACTH challenge time course for cortisol and DHEA across photoperiods. Cortisol levels for (a) long-day females, (b) short-day females; DHEA levels for (c) long-day females and (d) short-day females receiving either an ACTH or control treatment. White bars, long days; grey bars, short days. Bar heights represent means ± SEM *p < 0.05. Reproduced with permission from “The Agonistic Adrenal: Melatonin Elicits Female Aggression Via Regulation Of Adrenal Androgens,” by N. M. Rendon, L. M. Rudolph, D. R. Sengelaub, and G. E. Demas, 2015, Proceedings of the Royal Society of Biological Sciences, 282(91819).
Figure 3. Rates of agonistic interactions between alpha and beta Lythrypnus dalli relative to the time before or after alphas were treated with either 11-ketotestosterone or cholesterol. (A) Alpha approaches beta; (B) alpha displaces beta; (C) beta approaches alpha; and (D) beta displaces alpha. *p < 0.05 (n = 8 cholesterol and n = 10 ketotestosterone). Differences in aggressive behavior of both alphas and betas are seen within 2 h of implanting the alpha. Dotted lines represent the transient window of social instability that follows male removal. Reprinted from
Figure 4. Mean (± SEM) expression level of AR, ERα (ER) and aromatase (ARO) mRNA in nucleus taeniae (TnA) of male and female black coucals. The expression of AR mRNA in TnA was significantly higher in females than males (c), particularly in central and caudal part of the nucleus (d). Reprinted with permission from “Sex-Role Reversal Is Reflected in the Brain of African Black Coucals (Centropus grillii),” by C. Voigt and W. Goymann, 2007, Developmental Neurobiology, 67, 1560–1573.
Figure 5. (A) Ablation of aromatase + meA neurons reduces specific components of male and maternal aggression. No difference between aroCre/IPIN and aro+/IPIN males in mating with an estrous female. Comparable percent of aroCre/IPIN and aro+/IPIN males sniff and attack an intruder male. Aro Cre/IPIN males take significantly longer to attack and tail rattle, and they attack intruder less. (Unger et al., 2015). Reprinted from “Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes,” by E. K. Unger, K. J. Burke, C. F. Yang, K. J. Bender, P. M. Fuller, and N. M. Shah, 2015, Cell Reports, 10(4), 453–462.
Figure 5. (B) Ablation of aromatase + meA neurons reduces specific components of male and maternal aggression. The vast majority of arocre/IPIN and aro+/IPIN females retrieved all pups to the nest, and they did so with similar latencies. Comparable percentages of aroCre/IPIN and aro+/IPIN females sniffed and attacked an intruder male. There was a significant decrease in number of attacks directed to the intruder male by aroCre/IPIN females. Reprinted from “Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes,” by 2015, Cell Reports, 10(4), E. K. Unger, K. J. Burke, C. F. Yang, K. J. Bender, P. M. Fuller, and N. M. Shah, 453–462.
Figure 6. (A) Maternal aggressive behavior of lactating females [6th day postpartum] who underwent SHAM surgery or ovariectomy [OVX] on the first day postpartum. A Student t test was used between the two experimental groups [with a p < 0.005 level of significance]. Indicates a significant difference between the groups [SHAM and OVX]. (B) Maternal aggressive behavior of lactating females [sixth day postpartum] that received OIL, RU 486 (0.2, 1.0, 2.0, and 4.0 mg/kg); and FIN injected SC, 2 h before test one-way ANOVA was used between the three experimental groups [with a p < 0.05 level of significance]. Indicates a significant difference between the groups [OIL, RU 0.2 mg and FIN]. The data are expressed as mean [± SEM] of the frequencies of behaviors studied. The number of animals [n] is given between parentheses. Reprinted from “Progesterone and Maternal Aggressive Behavior in Rats,” by F. L. de Sousa, V. Lazzari, M. S.

Figure 7. Effect of blocking V1aR within BNST on maternal aggression in lactating rats. Maternal aggression was tested after bilateral local injections of vehicle (VEH) or of a V1aR antagonist (AVP-A) during the 10-min maternal defense test. Reprinted with permission from "Maternal Behaviour Is Associated With Vasopressin Release in the Medial Preoptic Area and Bed Nucleus of the Stria Terminalis in the Rat," by O. J. Bosch, J. Pförtsch, D.I. Beiderbeck, R. Landgraf, and I. D. Neumann, 2010, Journal of Neuroendocrinology, 22, 420–429.
Figure 8. (A) Duration of aggression, social, or non-social behaviors (mean ± SEM) if resident hamsters tested for 7 min in their home cage with a non-aggressive intruder (A) immediately after a microinjection of OT in the medial preoptic area (MPOA). (B) 30 min after a microinjection of OT antagonist in MPOA. Reprinted with permission from “Oxytocin Inhibits Aggression in Female Syrian Hamsters,” by A. C. Harmon, K. L. Huhman, T. O. Moore, and H. E. Albers, 2002, Journal of Neuroendocrinology, 14, 963–969.
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