# BEHAVIORAL ENDOCRINOLOGY - EDITOR'S PICK 2021

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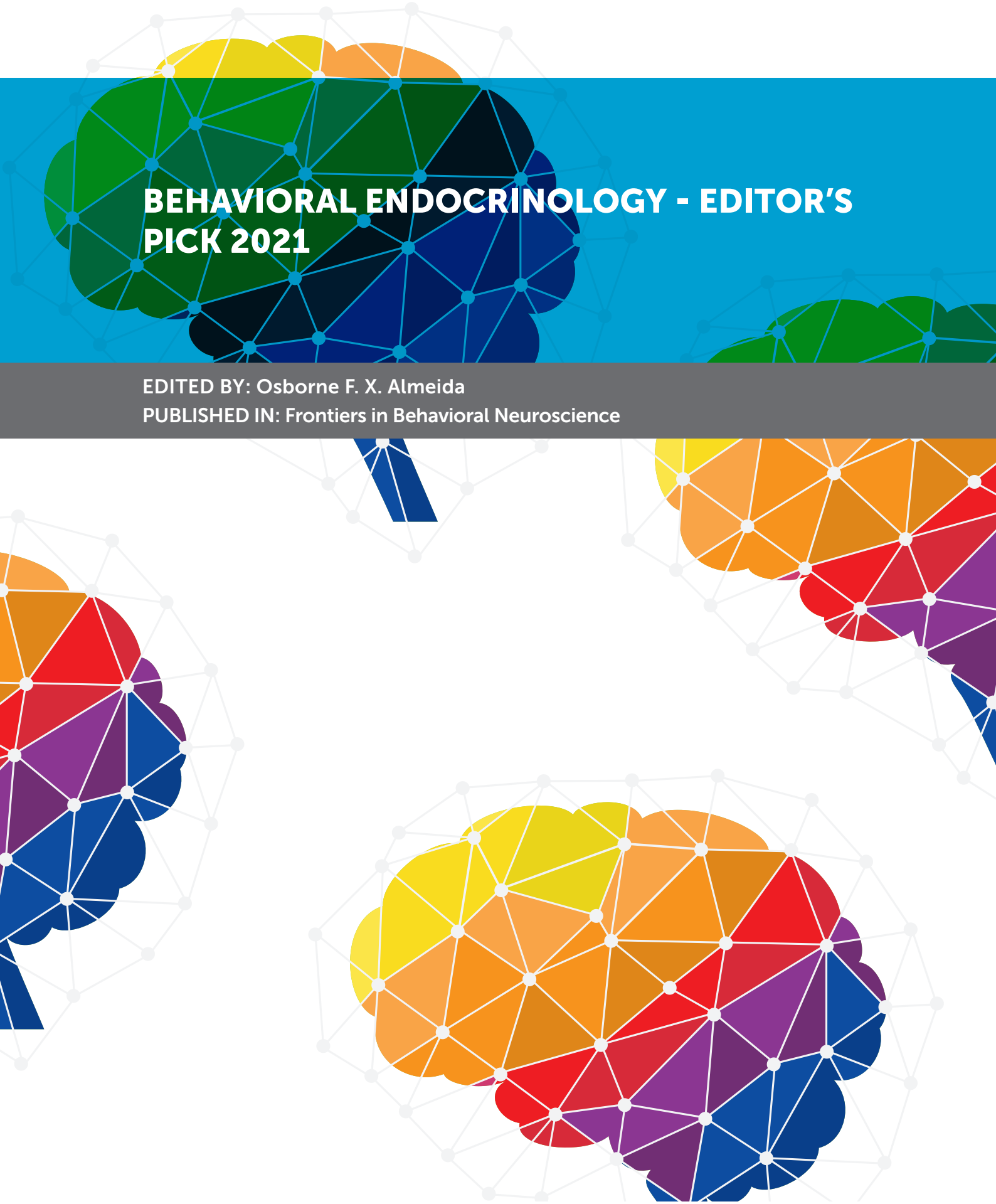

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# BEHAVIORAL ENDOCRINOLOGY - EDITOR'S PICK 2021

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ORIGINAL RESEARCH
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# Orexin Receptor Blockade-Induced Sleep Preserves the Ability to Wake in the Presence of Threat in Mice

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Retention of the ability to wake from sleep in response to dangerous situations is an ideal characteristic of safe hypnotics. We studied the effects of a dual orexin receptor antagonist-22 (DORA-22) and the GABA-A receptor modulator, triazolam, on the ability to wake in response to aversive stimuli. We examined four modalities of sensory inputs, namely, auditory (ultrasonic sound), vestibular (trembling), olfactory (predator odor), and autonomic (hypoxia) stimuli. When the mice fell asleep, one of the four stimuli was applied for 30 s. In the case of auditory stimulation, latency to arousal following vehicle, DORA-22, and triazolam administration was 3.0 (2.0–3.8), 3.5 (2.0–6.5), and 161 (117–267) s (median and 25–75 percentile in the parentheses, $n$ = 8), respectively. Latency to return to sleep after arousal was 148 (95–183), 70 (43–98), and 60 (52–69) s, respectively. Similar results were obtained for vestibular and olfactory stimulation. During the hypoxic stimulation, latencies for arousal and returning to sleep were not significantly different among the groups. The findings of this study are consistent with the distinct mechanisms of these sleep promoting therapies; GABA-A receptor activation by triazolam is thought to induce widespread central nervous system (CNS) suppression while DORA-22 more specifically targets sleep/wake pathways through orexin receptor antagonism. These data support the notion that DORA-22 preserves the ability to wake in response to aversive and consciousness-inducing sensory stimuli, regardless of modality, while remaining effective in the absence of threat. This study provides a unique and important safety evaluation of the potential for certain hypnotics.

**Keywords: orexin, hypocretin, hypnotics, dual orexin receptor antagonist, triazolam, aversive stimuli**

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## INTRODUCTION

Although living in the modern world allows us to encounter dangerous situations far less frequently than what wild animals may encounter, it is still important for humans to be able to awaken quickly during natural disasters such as earthquakes, volcanic explosions, and fires. Stress and anxiety resulting from worrying about sleeping through these occurrences may lead to conditions such as insomnia.

Even during sleep, the brain continuously processes sensory information. This has been demonstrated by brainstem auditory evoked potential recordings (Perrin et al., 1999) and neuroimaging (Portas et al., 2000). The threshold required for the sensory input to reach

**Abbreviations:** CNS, central nervous system; DORA, dual orexin receptor antagonist.

the cerebral cortex, however, is higher during sleep than when awake due to thalamic sensory gating (McCormick and Bal, 1994). Central nervous system (CNS) depressants, such as benzodiazepines, also affect the threshold required in order for sensory input to evoke arousal. For example, the short-acting benzodiazepine triazolam impaired the ability in sleeping humans to wake up upon exposure to a loud fire alarm (Johnson et al., 1987).

The orexin/hypocretin-signaling pathway was discovered in 1998 (de Lecea et al., 1998; Sakurai et al., 1998) and plays an important role in regulating arousal and sleep (Sakurai, 2007) as well as vigilance state-dependent changes in autonomic functions (Kuwaki, 2015; Carrive and Kuwaki, 2016). Dual orexin receptor antagonists (DORAs) that block orexin receptors 1 and 2 have recently been developed and promote sleep through a decrease in arousal signaling (Gotter et al., 2014). GABA-A modulators, the most widely used hypnotic (Roehrs and Roth, 2000; Wang and Liu, 2016), and DORAs have different sleep promoting mechanisms, so it stands to reason that their effect on sensory-input induced arousal may also be different.

Tannenbaum et al. (2014) previously showed that one of the DORAs, DORA-22, did not impair the ability to wake in response to emotionally salient acoustic stimuli in dogs. In their study, the authors used an acoustic tone classically conditioned to be associated with a food reward. In almost all of the trials, DORA-22-treated dogs woke up in response to the salient positive stimulus but not to the neutral stimulus in a similar way to when they received no drug. The same group of authors later showed similar results with monkeys (Tannenbaum et al., 2016). Unfortunately, however, the authors mentioned only tested positive but not negative emotion-associated stimulus. Another weak point of their studies was that they used cue-conditioned test paradigm but not innate salient stimuli. Therefore, possible effect of DORA-22 on sensory processing circuit is still an open question even though it may not affect memory retrieval process.

The purpose of the present study was to examine the possible effects of a DORA-22, on negative valence stimuli-induced arousal which is independent from learning and memory, and compare them with GABA-A receptor modulators, eszopiclone and triazolam. We also analyzed the latency to return to sleep after the stimuli ceased in order to evaluate any possible retention of sleep promoting effects from the drugs.

# MATERIALS AND METHODS

## Animals

Experiments were conducted on male C57BL/6 mice (25–35 g, Clea Japan). Animals were maintained under normal laboratory conditions (controlled 23°C temperature and food/water *ad libitum*) under a regular 12-h light/dark cycle (19:00 lights off and 07:00 lights on). All experiments were performed during the dark phase when nocturnal mice are most active. Experiments were performed in accordance with the guidelines outlined by the Physiological Society of Japan (2015) and were approved by the Experimental Animal Research Committee of Kagoshima University (MD16051).

## Compounds

All pharmacological agents were diluted in 20% d-alpha tocopherol polyethylene glycol 1,000 succinate (Vitamin E-TPGS) vehicle to a dose volume of 0.1 ml/10 g, and were administered orally using standard stainless steel gavage needles affixed to a 1 ml syringe (p.o.). Hypnotics tested included a DORA-22 (100 mg/kg; Gotter et al., 2014; a kind gift from Merck & Co., Inc., Kenilworth, NJ, USA), triazolam (1.25 mg/kg; Sigma-Aldrich Corporation, St. Louis, MO, USA), and eszopiclone (15 mg/kg; Carbosynth Ltd., Compton, Berkshire, UK). Doses were determined according to previously published articles (Gotter et al., 2014) and clinical dosage information for humans (10 mg for suvorexant, a derivative of DORA-22, 0.125 mg for triazolam, and 1 mg for eszopiclone).

All mice received treatment with all drugs/vehicle in randomized order. An interval between administrations of at least 3 days was used according to the previous article (Winrow et al., 2012) to prevent any possible influences of repeated procedures and residual drug effects. Half-life off-rate of DORA-22 to orexin 2 receptor binding was reported to be 37.8 min (Gotter et al., 2013) and no next-day effects was reported at least in monkeys (Gotter et al., 2013).

## Sleep Recordings

Under isoflurane (1.5%–2.0%, inhalation through face mask) anesthesia, electrodes were implanted for EEG/EMG recording. Two holes were drilled in the skull, and the arms of the electrode for the EEG were implanted at sites approximately 1.5 mm lateral to the Bregma. EMG recording wires made of stainless steel (Cooner Wire, Chatsworth, CA, USA) were inserted into the neck muscles bilaterally. Each electrode was fixed rigidly to the skull with dental cement. After surgery, mice were given an antibiotic, penicillin G (40,000 U $kg^{-1}$), and an analgesic (buprenorphine, 0.05 mg $kg^{-1}$). Animals were individually housed and allowed to recover for at least 7 days. The implanted electrode of each mouse was connected to a cable for signal output. Signals were amplified (AVH-11, Nihon Kohden, Tokyo, Japan) and digitally recorded on a computer with signal processing software (Chart, ADInstruments Inc., Bella Vista, NSW, Australia). Sleep stages were judged according to the method previously published (Nakamura et al., 2003). In brief, wakefulness was defined by a high frequency (8–30 Hz) low-amplitude EEG with a high EMG tone. Slow wave sleep (SWS) was defined by a low-frequency (0.25–4 Hz) high-amplitude EEG. Non-SWS sleep or rapid eye movement (REM) sleep was defined by a mixed-frequency (4–8 and 8–30 Hz) low amplitude EEG associated with weak or absent EMG activity.

## Test for Sleep-Promoting Effects of the Hypnotics

Before the aversive stimuli-experiments, the length and magnitude of sleep-promoting effects of the hypnotics were tested in our experimental setting without any stimulation. Mice ($n$ = 5) in their home cage were connected to the cable for EEG/EMG measurement at 19:00 when the dark phase

begins. After 1 h of baseline measurement, either vehicle, DORA-22, triazolam, or eszopiclone was orally administered to the mice. Mice eventually received all four drugs in a random order. The sleep-wake rhythm was measured until 02:00 (6 h after administration). Night dosing was selected because in nocturnal mice the baseline time spent sleeping is smaller than it is in daytime and thus any sleep-promoting effects are easier to observe. Sleep time was calculated for every 30 min. Mice's natural sleep is not continuous as humans but relatively short repetition of sleep and wake. Therefore, we thought that latency to arousal by aversive stimuli and latency to return to sleep should be evaluated in comparison with natural sleep/awake duration. For this purpose, we calculated episode duration of sleep and awake in this (without stimulation) experiment.

## Aversive Stimuli-Induced Arousal Testing

Stimulation experiments were conducted between 21:00 and 24:00, which correspond 1–4 h period after the drug administration, in the different sets of the animals to the above-stated without stimulation group. When the mouse fell asleep for more than 1 min and in SWS, one of the stimuli (see below) was applied and any possible effects of the stimulus on the sleep-wake cycle were observed for 30 min. The same stimulus was applied 2–3 times in one animal during the experimental period of 3 h and the average value was used as the representative value for the experiment. Only one type of stimulus was tested over the course of one experimental night.

The following four aversive stimuli were tested using different set of the animals. First, for auditory stimulation, ultrasonic sound (25 kHz, 100 dB, 0.5 s × 7 times, interval 4.5 s; Moriya et al., 2018) was applied from a position 20 cm above the sleeping mouse ($n$ = 8). Ultrasonic sound was generated by PET-AGREE (apparatus used for training pets; K2 Enterprises, NY, USA). Second, for vestibular stimulation, trembling (180 rpm) was applied to a measuring cage containing a mouse ($n$ = 8) for 30 s. Trembling was done by a shaker (mini-shaker PSU-2T; WakenBtech, Kyoto, Japan) on which the measuring cage was placed. Third, for olfactory stimulation, a cotton swab containing 10 μL of TMT (2,4,5-trimethyl-3-thiazoline, a predator odor which is extracted from fox feces) was placed at the distance of 1 cm from the tip of the mouse's nose ($n$ = 10) for 30 s (Tashiro et al., 2016). Fourth, for hypoxic stimulation, 10% $O_2$ gas (1,000 ml/min) was introduced into a gas tight chamber (750 ml) in which the mouse ($n$ = 8) was placed. Oxygen concentration in the chamber was monitored (model JKO-25LJ II CM, JIKCO, Tokyo, Japan) at the output port. Oxygen concentration in the chamber became 10% within 120 s, was maintained there for 180 s, and then returned to the normal room air concentration (21% $O_2$).

For the sleep state analysis, we calculated the latency to wake up from the aversive stimulus and latency to return to sleep after

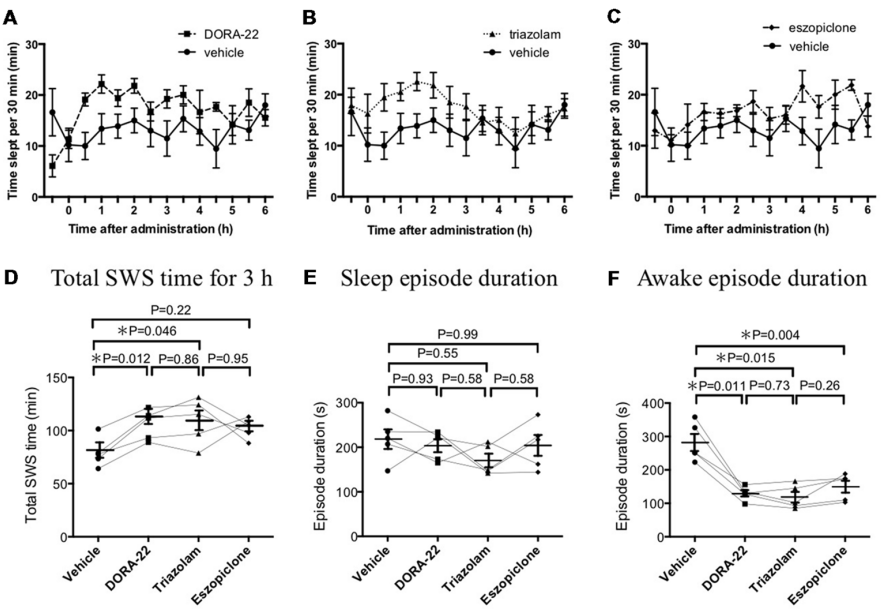


**FIGURE 1 |** Effects of dual orexin receptor antagonist-22 (DORA-22), triazolam, and eszopiclone on sleep time and sleep architecture. Time slept per every 30 min is shown for **(A)** DORA-22, **(B)** triazolam, and **(C)** eszopiclone for 6 h after p.o. administration of the drugs. The same values for the vehicle are shown repeatedly for comparison purposes. Each animal received DORA, triazolam, eszopiclone, and vehicle in a randomized order on spaced days. Data are shown as Mean ± SEM. **(D)** Comparison among the four treatments ($F_{(3,12)}$ = 5.101, $p$ = 0.017) revealed that DORA-22 and triazolam, but not eszopiclone, significantly increased total sleep time during a 3-h period starting at 1 h after administration. These drugs did not affect sleep episode duration ($F_{(3,12)}$ = 0.906, $p$ = 0.467; **E**) but did decrease awake episode duration ($F_{(3,12)}$ = 33.0, $p$ < 0.001; **F**). In **(D–F)**, data from the same animal are connected with lines to show possible interactions between drugs in individual mice. Horizontal lines indicate mean value for each treatment. Statistical results using repeated measure ANOVA followed by Tukey's multiple comparisons test are indicated in the graph.

arousal. Every mouse was tested for the same aversive stimulus after receiving vehicle, DORA-22, and triazolam.

## Statistical Analysis

In the test for the sleep promoting effects of the hypnotics, data were expressed as mean ± SEM. Statistical comparisons were performed using repeated measure ANOVA followed by Tukey's multiple comparisons test. In the stimuli-induced arousal testing, data were expressed as median and 25–75 percentile because data were not normally distributed (see "Results" section) and non-parametric statistics were more suitable. Statistical comparisons were performed using the Friedman test, a repeated measure nonparametric multiple comparisons test. When appropriate, it was followed by Dunn's *post hoc* test. All statistics were calculated using Prism6 software (GraphPad Software, Inc.). Differences were considered significant at $p < 0.05$.

# RESULTS

## Sleep Time Without Aversive Stimuli

Before the aversive stimuli-experiments, the length and magnitude of any sleep-promoting effects from the various hypnotics were tested in our experimental setting without any stimulation (**Figure 1**). During the 6 h of the observation period, mice spent 160 ± 11 min, 221 ± 6 min, 210 ± 13 min, and 209 ± 7 min sleeping (SWS and REM sleep) under the effects of either vehicle, DORA-22, triazolam, or eszopiclone, respectively. Although all three drugs seemingly had a sleep promoting effect, the detailed characteristics were different. As to SWS duration, DORA-22 (206 ± 5 min, $p = 0.006$, $n = 5$, Tukey's multiple comparison test) and triazolam (209 ± 13 min, $p = 0.016$) significantly increased and eszopiclone (205 ± 7 min, $p = 0.057$) tended to increase as compared to vehicle (154 ± 10 min). While on REM sleep, DORA-22 (15.3 ± 1.7 min, $p = 0.033$) significantly increased, triazolam (1.5 ± 0.2 min, $p = 0.089$) tended to decrease, and eszopiclone (3.9 ± 0.7 min, $p = 0.784$) showed no effect as compared to vehicle (5.1 ± 0.9 min). In addition, effect of eszopiclone appeared to have a later onset than DORA-22 and triazolam (compare **Figures 1A–C**). Since the main purpose of this study was to compare aversive stimuli-evoked responses among the hypnotics, we thought similar magnitude and similar time course of sleep-promoting effect of hypnotics would be desirable. From this consideration, we selected the 3 h starting from 1 h after the injection until 4 h after the injection for statistical analysis. In addition, we focused on SWS since duration of REM sleep was too short to evaluate aversive stimuli-evoked responses. Comparison among the 4 treatments by repeated measure ANOVA ($F_{(3,12)} = 4.944$, $p = 0.018$) and subsequent multiple comparison with Tukey's test revealed that DORA-22 ($p = 0.012$) and triazolam ($p = 0.046$), but not eszopiclone ($p = 0.22$), significantly increased total SWS time during a 3-h period as compared to vehicle treatment (**Figure 1D**). These drugs did not affect sleep episode duration ($F_{(3,12)} = 0.906$, $p = 0.467$; **Figure 1E**) but did decrease awake episode duration ($F_{(3,12)} = 33.0$, $p < 0.001$; **Figure 1F**).

Thus, we confirmed that DORA-22 and triazolam had similar sleep promoting effects over a similar time course for the selected dosages. From these results, we decided to compare vehicle, DORA-22, and triazolam, but not eszopiclone, in the next step of aversive stimuli-induced arousal testing. The testing took place during the 1–4 h period after the drug injection because the lag period for drug absorption and distribution appeared to be approximately 1 h. The confirmation period required to define sleep before stimulation was performed was set as 60 s because each sleep episode typically lasted for approximately 200 s (**Figure 1E**).

## Aversive Stimuli-Induced Arousal and Return to Sleep After the Cessation of the Stimuli

Next, we examined whether the animal was able to promptly wake up from sleep induced by DORA-22 and triazolam in response to aversive stimuli. We also examined the latency to return to sleep after arousal. Latency to arousal and latency to return to sleep in drug-treated groups were compared with those in the vehicle-treated "natural" sleep group.

For auditory stimulation (**Figure 2A**), the latency to arousal following vehicle, DORA-22, and triazolam administration was 3.0 (2.0–3.8), 3.5 (2.0–6.5), and 161 (117–267) s (median and 25–75 percentile in the parentheses, $n = 8$), respectively. After mice received the vehicle and DORA-22, they woke up during the stimulation period of 30 s but after triazolam mice woke up after secession of the stimulus. Latency to return to sleep after arousal was 148 (95–183), 70 (43–98), and 60 (52–69) s, respectively for vehicle, DORA-22, and triazolam. Returning to sleep was never observed during the stimulus period in all the drug treatments. Statistical analysis using the Friedman test and Dunn's *post hoc* test showed that the latency to arousal was significantly prolonged by triazolam ($p = 0.005$) but not by DORA-22 ($p > 0.99$) when compared to vehicle. Latency to return to sleep was significantly shorter in DORA-22 ($p = 0.018$) and triazolam ($p = 0.004$) when compared to vehicle and there was no significant difference between DORA-22 and triazolam ($p > 0.99$). Similar results were obtained for vestibular stimulation (**Figure 2B**, $n = 8$) and olfactory stimulation (**Figure 2C**, $n = 10$). The only exception was that there was no significant difference between latency to return to sleep in the vehicle treatment and DORA-22 treatment ($p = 0.22$) after olfactory stimulation. This is probably because one out of 10 animals showed a longer latency to return to sleep under DORA-22 than they did with vehicle.

In contrast to the above-mentioned auditory, vestibular, and olfactory stimulation, there were no significant differences in the latencies to arousal and return to sleep among vehicle, DORA-22, and triazolam when hypoxia was used as a stimulus (**Figure 2D**, $n = 8$). There was a time lag of about 120 s before $O_2$ concentration in the chamber reached 10% and most animals woke up within that 120 s. For example, the $O_2$ concentration in the chamber when the mice woke up from sleep with vehicle, DORA-22, and triazolam was 16.3 (15.4–18.4), 16.4 (15.0–17.9), and 15.6 (14.4–17.6) % (median and 25–75 percentile in the

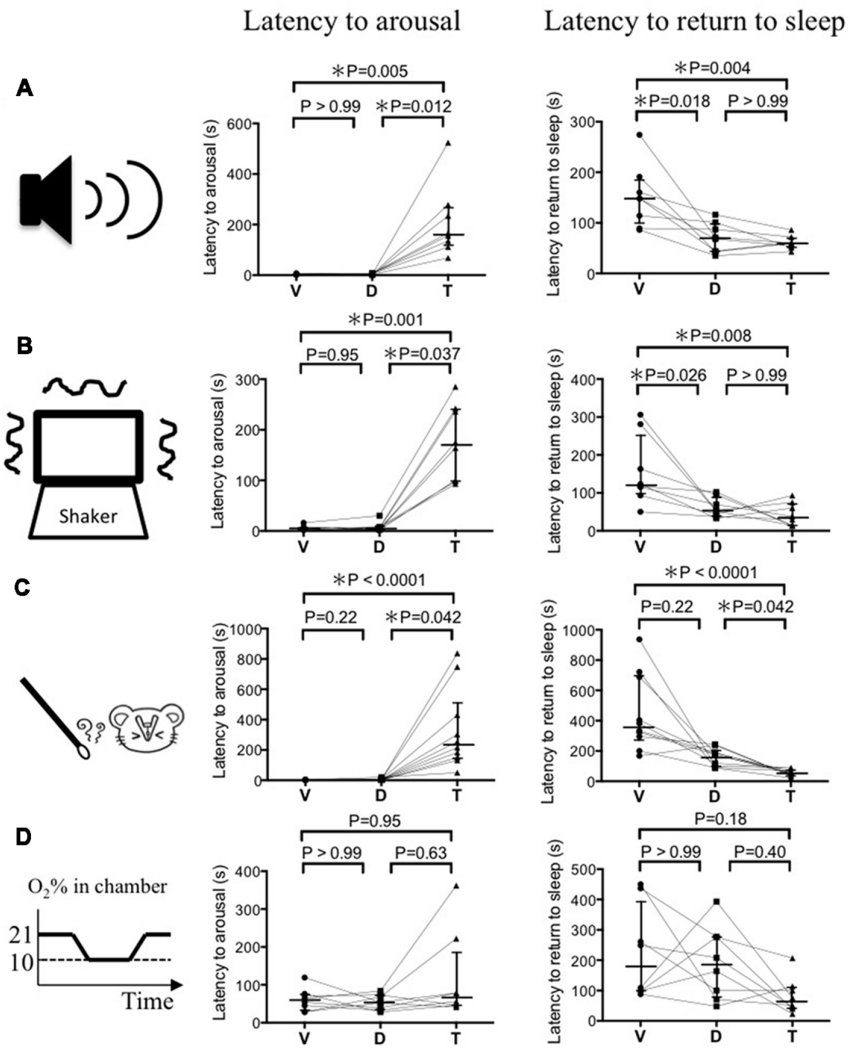


**FIGURE 2 |** Latency to arousal and latency to return to sleep induced by four modalities of sensory stimulation. **(A)** Auditory stimulus by ultrasonic sound ($n$ = 8). **(B)** Vestibular stimulus by cage shaking ($n$ = 8). **(C)** Olfactory stimulus by a predator odor ($n$ = 10). **(D)** Autonomic stimulus by hypoxia ($n$ = 8). Each animal received vehicle (V), DORA-22 (D), and triazolam (T) in a randomized order on spaced days and were tested for one of the stimuli 2–3 times. The value from those tests were averaged and are represented as a single dot. Data from the same animal are connected with lines to show possible drug interactions within an individual animal. Horizontal lines indicate, from top to bottom, 75 percentile, median, and 25 percentile, respectively. Statistical results using the Friedman test, a repeated measure nonparametric multiple comparisons test, are indicated in the graph. Note that latency to arousal in DORA-22 treated mice is not different from those in vehicle treated mice and significantly shorter than those in triazolam treated mice, with the exception of hypoxia. Also note that latency to return to sleep in DORA-22 treated mice is significantly shorter than those in vehicle treated mice and not different from those in triazolam treated mice at least for auditory and vestibular stimuli.

parentheses), respectively. There was no significant difference among the drugs. Some animals re-slept even when the hypoxic $O_2$ concentration of 10% continued. Out of the eight animals undergoing the hypoxic stimulation, the number that re-slept under the vehicle, DORA-22, and triazolam conditions were 5, 5, and 7, respectively. A chi-square test revealed no statistical differences ($p$ = 0.446) among the treatments.

## DISCUSSION

This study demonstrates that DORA-22 (100 mg/kg) and triazolam (1.25 mg/kg) had similar sleep promoting effects (30%–40% increase in SWS time as compared to vehicle) in a similar time course (approximately 4 h after oral administration) in mice. During this period, aversive stimuli-induced arousal and the return to sleep after arousal were examined. As expected, auditory stimulus-induced arousal was delayed significantly in the triazolam treatment ($p$ = 0.005) but not in the DORA-22 treatment ($p$ > 0.99) when compared to the vehicle treatment. Even though the DORA-22 treatment showed a short latency to wake up, the sleep-promoting effect of DORA-22 seemed to remain because the latency to return to sleep after arousal was significantly shorter than vehicle treatment and not different from triazolam. Similar results were obtained for vestibular and

olfactory stimuli-induced arousal and return to sleep. In contrast, hypoxic stimulus-induced arousal and return to sleep were not different among groups.

We had expected that eszopiclone (15 mg/kg) would show a sleep promoting effect similar to triazolam and DORA-22 because eszopiclone as well as triazolam and suvorexant (another DORA) are effective clinically in humans (Matheson and Hainer, 2017). In a preliminary experiment, we tried a higher dose of eszopiclone (100 mg/kg, $n = 2$) but the result was similar to the selected dose of 15 mg/kg. Gotter et al. (2014) reported that the sleep-promoting effects of eszopiclone are highly species specific. In their study, they showed that treatment with eszopiclone resulted in consistent effects in rats and rhesus monkeys, variable effects in mice (60 mg/kg), and paradoxical hyperarousal in dogs. The reason behinds the differences in species has not yet been revealed, but the authors speculated that possible differences in the GABAergic pathways in the brains of these species may be the cause. It may be interesting to point out that activation of extrasynaptic GABA-A receptor in the pontine reticular formation promotes wakefulness (Vanini and Baghdoyan, 2013). In any case, we stopped further experimentation using eszopiclone because our main purpose was to examine any possible effects DORA-22 may have on negative valence stimuli-induced arousal and compare it with at least one of the GABA-A receptor modulators. We were able to confirm that triazolam was suitable for this purpose. Nevertheless, we admit that possible species difference in the effectiveness of different drugs is a limitation of this study.

The prolonged latency to wake up during triazolam treatment did not seem to be caused by a general inhibition of waking systems in the brain because there was no significant change in the latency to wake in response to hypoxic stimulus (**Figure 2D**). Rather, it seemed to be caused by inhibition of sensory input pathways. An increase in latency to wake from triazolam treatment in the auditory and vestibular stimuli tests indicated an importance of the thalamus where the GABA-A receptor is involved in sensory gating before the signal reaches the cerebral cortex (McCormick and Bal, 1994). The unchanged latency to wake in response to hypoxic stimulus may be explained by the fact that autonomic reflex-like responses do not depend on the thalamo-cortical pathway. A possible contribution of the medullary GABAergic pathway, however, has been proposed to be responsible for arousal in response to intermittent hypoxia (Darnall et al., 2012). One of the more surprising results was that the latency to wake in response to olfactory stimulus was also prolonged with triazolam treatment. It is interesting because olfactory information directly reaches the cerebral olfactory cortex from the glomerulus in the olfactory bulb and thus is not gated by the thalamus. The periglomerular cells of the olfactory system that contain the GABA-A receptor (Panzanelli et al., 2007) may be responsible for the gating effect.

We noticed that latency to return to sleep after arousal by aversive stimuli (150–300 s in vehicle and 50–100 s in DORA-22 and triazolam, **Figures 2A–C**) was similar to and did not exceed awake episode durations in our tests where no stimuli were given (∼300 s in vehicle and ∼100 s in DORA-22 and triazolam, **Figure 1F**). This result indicates that the aversive stimuli used in this experiment were mild enough to not elicit a continuous alerting effect on the mice. Due to the observation that DORA-22 and triazolam were effective for approximately 4 h, at least without stimulation (**Figure 1**), and aversive stimulus-induced arousal testing was performed within this time window, it was not surprising to observe a short latency to return to sleep in the drug-treated groups.

We used within-subject design to compare drugs' effect for each aversive stimulus. This design gives us higher statistical power than independent design but at the same time possible habituation effect to the stimulus may distort the results. However, such habituation effect seemed minimal in the current experimental setting and randomized order of dosing. If the habituation effect took place, then some animals that were treated with vehicle after DORA-22 and/or triazolam should had longer latency to arousal than those that was treated with vehicle as the first drug. This seemed not the case since the data distribution for vehicle showed very narrow range (**Figure 2**). In addition to statistical high power, within-subject design needs fewer animals than independent design. Therefore, when habituation effect is enough smaller than the effect of interest, within-subject design is a good choice of experimental design.

Orexin exerts its wake promoting/stabilizing effect through activation of monoaminergic systems such as noradrenaline in the locus coeruleus, serotonin in the dorsal raphe, and histamine in the tuberomammillary nucleus (Sakurai, 2007). Thus, the sleep-promoting effect of orexin receptor blockade is believed to be elicited by an inhibition of orexin signaling in these nuclei. Although orexin receptors are also expressed in some thalamic nuclei (Marcus et al., 2001), possible effects of orexin receptor blockade on sensory input gating have not yet been reported. It is of interest to note that orexin is involved in stress-induced analgesia (Watanabe et al., 2005; Inutsuka et al., 2016) as well through possible activation of descending pain-inhibitory pathways (Ho et al., 2011). If orexin does also plays a role in sensory input gating, it seems plausible for it to also be anti-nociceptive. Therefore, any effect that orexin receptor blockade might have on sensory gating may occur in a direction opposite to that of any sleep-related sensory gating.

## CONCLUSION

The findings of this study are consistent with the distinct mechanisms of these sleep promoting therapies; GABA-A receptor activation by triazolam is thought to induce widespread CNS suppression, which includes sensory gating systems, while DORA-22 more specifically targets sleep/wake pathways through orexin receptor antagonism. These data support the notion that DORA-22 preserves the ability to wake in response to aversive and consciousness-inducing stimuli, regardless of modality, while remaining effective in the absence of the threat. This study provides a unique and important point of view for the evaluation of the safety of these hypnotics.

## AUTHOR CONTRIBUTIONS

SI and TK designed the study. All the authors conducted the study and analyzed the data. SI and TK wrote the manuscript. All authors approved the final version of the manuscript.

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# Low Daytime Light Intensity Disrupts Male Copulatory Behavior, and Upregulates Medial Preoptic Area Steroid Hormone and Dopamine Receptor Expression, in a Diurnal Rodent Model of Seasonal Affective Disorder

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Seasonal affective disorder (SAD) involves a number of psychological and behavioral impairments that emerge during the low daytime light intensity associated with winter, but which remit during the high daytime light intensity associated with summer. One symptom frequently reported by SAD patients is reduced sexual interest and activity, but the endocrine and neural bases of this particular impairment during low daylight intensity is unknown. Using a diurnal laboratory rodent, the Nile grass rat (*Arvicanthis niloticus*), we determined how chronic housing under a 12:12 h day/night cycle involving dim low-intensity daylight (50 lux) or bright high-intensity daylight (1,000 lux) affects males' copulatory behavior, reproductive organ weight, and circulating testosterone. We also examined the expression of mRNAs for the aromatase enzyme, estrogen receptor 1 (ESR1), and androgen receptor (AR) in the medial preoptic area (mPOA; brain site involved in the sensory and hormonal control of copulation), and mRNAs for the dopamine (DA) D1 and D2 receptors in both the mPOA and nucleus accumbens (NAC; brain site involved in stimulus salience and motivation to respond to reward). Compared to male grass rats housed in high-intensity daylight, males in low-intensity daylight displayed fewer mounts and intromissions when interacting with females, but the groups did not differ in their testes or seminal vesicle weights, or in their circulating levels of testosterone. Males in low-intensity daylight unexpectedly had higher ESR1, AR and D1 receptor mRNA in the mPOA, but did not differ from high-intensity daylight males in D1 or D2 mRNA expression in the NAC. Reminiscent of humans with SAD, dim winter-like daylight intensity impairs aspects of sexual behavior in a male diurnal rodent. This effect is not due to reduced circulating testosterone and is associated with upregulation of mPOA steroid and DA receptors that may help maintain some sexual motivation and behavior under winter-like lighting conditions.

**Keywords: hormones, light, nucleus accumbens, medial preoptic area, seasonal affective disorder, sexual behavior**

## INTRODUCTION

Seasonal affective disorder (SAD) is a recurrent major depressive disorder with a seasonal pattern that in most cases worsens in fall and winter, but remits in spring and summer (Rosenthal et al., 1984; American Psychiatric Association, 2013). Up to 5%–10% of people living in latitudes far from the equator are thought to be above the clinical threshold to be diagnosed with SAD, and subsyndromal symptoms are experienced by a much larger percentage of the population (e.g., Potkin et al., 1986; Rosen et al., 1990; Magnusson and Partonen, 2005; Grimaldi et al., 2009; Wirz-Justice et al., 2019). The symptoms of SAD are numerous and include not only depressed mood but also anxiety, irritability, reduced physical activity, hyperphagia, sleep disruption, and low libido (Rosenthal et al., 1984; Jacobsen et al., 1987).

Given the clinical definition of SAD, it is not surprising that most research on seasonal changes in human affect and behavior has focused on the etiology and treatment of depressed mood. There has been considerably less attention paid to the other seasonal changes, and almost none to the biological basis of winter-time decreases in libido, sexual activity, and sexual satisfaction (Kasper et al., 1989; Roenneberg and Aschoff, 1990; Bronson, 1995; Avasthi et al., 2001; Demir et al., 2016; Arendt and Middleton, 2018). Many studies have reported seasonal variation in circulating gonadal steroids in humans (Smals et al., 1976; Ronkainen et al., 1985; Kauppila et al., 1987a,b; Kivelä et al., 1988; Dabbs, 1990; Meriggiola et al., 1996; Valero-Politi and Fuentes-Arderiu, 1998; Garde et al., 2000; Wisniewski and Nelson, 2000; van Anders et al., 2006; Stanton et al., 2011; Demir et al., 2016), but there is no evidence that SAD patients have atypical gonadal hormone levels at any time of year (although they do for some pituitary and adrenal hormones—Jacobsen et al., 1987; Avery et al., 1997; Martiny et al., 2004; Thorn et al., 2011). In addition, the seasonal changes in testosterone particularly seen in men most often involve a peak in fall/winter (Smals et al., 1976; Dabbs, 1990; Valero-Politi and Fuentes-Arderiu, 1998; Wisniewski and Nelson, 2000; van Anders et al., 2006; Stanton et al., 2011), which does not temporally correspond with what would be expected for a wintertime decline in sexual interest and activity. Thus, the low winter-time libido and sexual function in SAD patients and other individuals in the general population is unlikely to be due to a drop in circulating gonadal hormone levels. Interestingly, there is some evidence that the winter-time reduction in libido also does not depend on the presence of a mood disorder (Bossini et al., 2009).

The underlying mechanisms may instead be due to seasonal modifications in the central nervous system sites underlying sexual behaviors. The neural network involved in mammalian sexual behaviors includes the medial preoptic area (mPOA) lying just rostral to the hypothalamus. In many animals, the mPOA is critical for the sensory and gonadal steroid regulation of partner choice, sexual motivation, and/or the expression of copulatory behaviors (for reviews see Hull and Dominguez, 2015; Micevych and Meisel, 2017; Pfaff and Baum, 2018). Relevant to SAD, the hormonal sensitivity of the mPOA is affected by changes in season or photoperiod. Winter-like short day length reduces mPOA aromatase activity (the enzyme that converts testosterone into estradiol) in seasonally breeding male Golden hamsters (Callard et al., 1986), causes a drop in androgen receptor (AR) binding in their mPOA (Bittman and Krey, 1988), decreases AR immunoreactivity in the mPOA of male Siberian hamsters (Tetel et al., 2004), and lowers both estrogen receptor (ESR) and progestin receptor immunoreactivity in the mPOA of female Syrian hamsters (Mangels et al., 1998). Similar effects of the season can be found for the steroid hormone sensitivity of the mPOA in sheep (Skinner and Herbison, 1997) and European starlings (Riters et al., 2000).

Not only is the mPOA's response to steroid hormones critical for its role in the display of sexual behaviors, but activity of the neurotransmitter, dopamine (DA), in the mPOA is also essential. DA is released in the mPOA of male rats and Japanese quail when they are exposed to female sensory cues, and this DA response appears to determine their subsequent behavioral interactions with the female (Hull et al., 1995; Kleitz-Nelson et al., 2010). Disrupting DA receptor signaling in the mPOA by infusing the D1/D2 receptor antagonist, cis-flupenthixol, impairs both sexual motivation and performance in male rats (Pehek et al., 1988). Furthermore, selective antagonism or agonism of D1 and D2 receptors in the mPOA reveals that low D1 signaling and high D2 signaling is especially disruptive for males' latency to begin copulating, but hastens ejaculation (Hull et al., 1989). D1 and D2 signaling in the mPOA also modulates sexual behaviors in female rats, with high D1 or D2 activity promoting sexual solicitation depending on the subjects' ovarian hormone milieu (Graham and Pfaus, 2010, 2012). Lastly, the consolidation of sexual experience in male rats requires mPOA D1 receptor activity during interactions with the female (McHenry et al., 2012), while others have shown that previous sexual experience not only increases the number of D2 receptor-immunoreactive cells in the male rat mPOA but also that Fos expression in these D2R-immunoreactive cells is positively correlated with a number of facets of their copulatory behaviors (Nutsch et al., 2016).

The mPOA is not the only site in the brain where changes in DA signaling may be associated with seasonal changes in libido and sexual activity. Similar to other depressive disorders, SAD involves decreased interest or pleasure in most activities (i.e., anhedonia; American Psychiatric Association, 2013), which is associated with the mesolimbic DA system dysfunction (Nestler and Carlezon, 2006). Mesolimbic DA signaling is essential for high sexual motivation and behaviors in laboratory rodents (Yoest et al., 2014; Hull and Dominguez, 2015), and natural or experimental changes in ambient light do alter DA synthesis, metabolism, and receptor content in many areas of the laboratory rodent and human brain (e.g., Neumeister et al., 2001; Eisenberg et al., 2010; Tsai et al., 2011; Cawley et al., 2013; Deats et al., 2015; Goda et al., 2015; Itzhacki et al., 2018).

It is reasonable to hypothesize that decreased libido in male SAD patients results from altered gonadal steroid and DA sensitivity of the mPOA and nucleus accumbens (NAC). In the present experiment, we tested this hypothesis in a male diurnal rodent—the Nile grass rat (*Arvicanthis noliticus*). We

have found that, similar to SAD patients, winter-like lighting regimen (involving either short daylength or reduced daytime light intensity) increases depression- and anxiety-like behaviors and produces cognitive impairments in Nile grass rats (Leach et al., 2013a,b; Deats et al., 2014; Ikeno et al., 2016; Soler et al., 2018). Those behavioral responses are accompanied by changes in central DA, serotonin, orexin, neurotrophin, and stress-mediating systems (Leach et al., 2013a,b; Deats et al., 2014; Ikeno et al., 2016; Soler et al., 2018). Humans are diurnal and neurobehaviorally stimulated by light, so studying diurnal grass rats offers advantages for understanding how light affects the human brain and behavior over studying most other laboratory rodents (which are nocturnal; Yan et al., 2019). Also similar to humans, but not some laboratory rodents like hamsters that are seasonal breeders, grass rats will copulate all year around (McElhinny et al., 1997; Blanchong et al., 1999) so are an excellent model for studying seasonal influences on diurnal male copulatory behavior, testosterone levels, and brains.

Instead of studying the effects of day length or daylight duration, we here studied the effects of winter-like daylight intensity. This is because it is the seasonal differences in daylight intensity that are most salient to modern humans. Most humans around the world now use artificial lights, so the duration of light across seasons is much less variable than the intensity of the light we receive across seasons (Hébert et al., 1998). We predicted that housing in winter-like, low-intensity daylight would: (1) impair males' copulatory behaviors when paired with a conspecific female; (2) decrease aromatase, ESR1, AR, D1 and D2 mRNA expression in the mPOA; and (3) reduce D1 and D2 mRNA expression in the NAC.

# MATERIALS AND METHODS

## Subjects

Subjects were from a stock of Nile grass rats (*Arvicanthus nilotocus*) originally captured in sub-Saharan Africa by Dr. Laura Smale in 1993 and maintained for almost two decades at Michigan State University using outbred breeding (McElhinny et al., 1997). Animals were housed in transparent polypropylene cages (43 × 23 × 20 cm) containing wood chip bedding and an 8 × 6 cm PVC pipe for shelter/enrichment. They were maintained under 12 h light-12 h dark conditions (lights on at 06:00 h), typical of their equatorial habitat. After reaching adulthood, males were singly housed. Pre-experimental colony room light was supplied by cool white fluorescent lights mounted on the ceiling, with light intensity at the center of the room at ∼300 lux. Animals had food (Prolab 2000 #5P06, PMI Nutrition) and water *ad libitum*. This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). The protocol was approved by the Institutional Animal Care and Use Committee of Michigan State University.

## Lighting Conditions

At the onset of the experiment, males were moved out of the colony and singly housed in smaller environmental chambers. In these chambers, they were subjected to a 12 h bright daylight (1,000 lux)-12 h dark (1 lux) condition (bright Light/Dark; brLD) or a 12 h dim daylight (50 lux)-12 h dark (1 lux) condition (dim Light/Dark; dimLD) for 5 weeks (for sexual behavior tests) or for 4 weeks (for testosterone, reproductive organ, and brain measures). Different groups of animals were used for the behavioral and biological measures in order to avoid any effects of group-level differences in sexual experience complicating the biological data interpretation. Our prior work has shown that male grass rats housed in brLD and dimLD for 4–5 weeks differ in their anxiety- and depression-related behaviors, stress responsiveness, spatial memory, and numerous neural characteristics (Leach et al., 2013a,b; Deats et al., 2014; Ikeno et al., 2016). Light was provided by cool white fluorescent bulbs (Jesco Lighting, SP4-26SW/30-W), with the same full spectrum maintained in both the brLD and dimLD conditions.

## Copulatory Behavior

Before being placed into brLD or dimLD conditions, males were screened to ensure they copulated during a 30-min interaction with unfamiliar female grass rats from our colony that were primed with subcutaneous injections of 10 or 20 μg estradiol benzoate once a day for two consecutive days, followed 24 h later by a subcutaneous injection of 250 or 500 μg progesterone (Sigma, USA). Females were used for behavior testing starting 3 h later. Males were removed from their home cages and placed in larger glass aquaria (61 × 32 × 29 cm) that contained wood chips for bedding and a hormone-primed female under ∼300 lux (i.e., colony room) illumination. Males that successfully copulated were then randomly assigned to be housed in either the brLD ($n$ = 11) or the dimLD ($n$ = 14) condition for 5 weeks. After 5 weeks, they were then tested again in the glass aquaria for copulatory behaviors with unfamiliar ovarian hormone-primed female grass rats. If males did not make contact with a stimulus female within 2 min after the beginning testing, or if a stimulus female did not show lordosis in response to a male's mounts, the stimulus female was replaced with another hormone-primed female grass rat and the test started over. Male-female interactions were recorded for 15 min and males' latencies to begin sniffing the females, latencies to first mount, their frequencies of mounts, and their frequencies of intromissions were later scored. "Hit rate" as a measure of copulatory efficiency was determined by the number of intromissions divided by the number of mounts plus intromissions × 100. Ejaculations were not reliably observed in most males within the 15-min observations.

## Reproductive Organ Weights and Plasma Testosterone Levels

A set of experimentally naïve animals from the colony were housed in either brLD ($n$ = 7) or dimLD ($n$ = 8) for 4 weeks and sacrificed between 09:00 and 10:00 h with an overdose of sodium pentobarbital. Animals were weighed, their testes and seminal vesicles collected and stripped of fat, and the fresh tissues weighed to the nearest mg. Trunk blood was obtained from another set of experimentally naïve male grass rats housed in each condition for 4 weeks and sacrificed during the day

at Zeitgeber T2 (nine dimLD, nine brLD) or in the evening at T14 (eight dimLD, eight brLD). Blood was centrifuged at 15,000 rpm for 15 min, and the plasma stored at −80°C until being assayed for testosterone using a commercially available EIA kit per the manufacturer's protocol (Enzo Life Sciences, Farmingdale, NY, USA).

## Brain Processing and qtPCR

Brains from experimentally naïve male grass rats (brLD $n = 11$ for mPOA, $n = 10$ for NAC; dimLD $n = 8$ for mPOA; $n = 12$ for NAC) were collected, quickly frozen on dry ice, and stored at −80°C until later analysis of five mRNAs relevant to sexual and other motivated behaviors. Brains were cut into 200-μm sections and bilateral 1-mm diameter micropunches (Harris Micropunch, Electron Microscopy Sciences, Pennsylvania, PA, USA) were made to obtain the mPOA and NAC. Tissue was processed and qtPCR run as described previously (Grieb et al., 2018). Briefly, tissue punches were homogenized by pulsed sonication in RLT Plus buffer (Qiagen, Germantown, MD, USA) containing β-mercaptoethanol. mRNAs were extracted using an RNeasy Plus Mini Kit (Qiagen, Germantown, MD, USA) and quantified with a GeneQuant100 machine (Harvard Bioscience, Holliston, MA, USA). RNA purity was determined by the ratio of absorbances at 260:280 nm wavelengths, and ratios of ∼2.0 was considered pure. Equal concentrations of mRNAs were converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was conducted with 2.5 ng/μl of converted cDNA (based on starting concentration of RNA converted to cDNA) and samples were run in triplicate. Runs included cDNA, primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a 25-μL reaction involving an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A no-template control was run alongside the samples to ensure that no primer-dimer amplification had occurred. In addition, mRNA samples not run through the reverse transcription kit were also run at the same time to ensure no gDNA contamination. Amplification efficiencies were calculated for each primer set, and each was within the accepted range (1.90–2.10) to use the ΔΔCT method to calculate fold change between groups (Schmittgen and Livak, 2008).

Preoptic area mRNAs analyzed were for the androgen receptor (forward—ACTACTTCTCTGCAGTGC CT; reverse—CCAGGAAATAGAACTGGGGAAC), ESR1 (forward—CCAGCTCCACTTCAGCACAT; reverse—GAGCC TGGGAGTTCTCAGAT), and aromatase (forward—CTACT GTCTGGGAATCGGGC; reverse—GTTGCAGGCACTTCC AATCC). mPOA and NAC mRNAs analyzed were for the DA D1 receptor (forward—GTGGGCGAATTCTTC CCTGA; reverse—GGGCAGAGTCTGTAGCATCC) and D2 receptor (forward—GGACATGAAACTCTGCACCG; reverse—ATCCATTCTCCGCCTGTTCAC). All were compared to the "housekeeping" gene HPRT1 (forward—CTCATGGAC TGATTATGGACAGGAC; reverse-GCAGGTCAGCAAAGAA CTTATAGCC). The primers (Integrated DNA Technologies, Coralville, IA, USA) were designed based on the corresponding gene sequences in laboratory mice and rats, and the identities of all the PCR products we obtained in grass rats were confirmed by sequencing at the MSU Genomic Core.

## Data Analyses

Data sets were confirmed to be normally distributed and have homogeneous variance among groups. Data were subjected to Grubb's single-outlier tests and any outliers were removed. The data were then analyzed using two-tailed independent Student *t*-tests comparing groups of brLD males to dimLD males. Statistical significance was indicated by $p$s $< 0.05$.

# RESULTS

## Copulatory Behaviors

All brLD and dimLD male grass rats included in the study copulated with ovarian hormone-primed stimulus females during the 15-min observations. The groups did not significantly differ in their latency to begin sniffing the females ($t_{(23)} = 0.18$, $p = 0.986$), but there was a trend for dimLD males to take longer to mount ($t_{(23)} = 1.704$, $p = 0.10$). DimLD males also mounted the females less frequently ($t_{(23)} = 2.28$, $p < 0.033$) and intromitted less often ($t_{(23)} = 2.10$ $p = 0.047$) compared to brLD males (**Figure 1**). Males' "hit rate" was similar between the brLD and dimLD groups ($t_{(23)} = 0.30$, $p = 0.766$).

## Reproductive Organs and Plasma Testosterone

Daytime light intensity did not affect males' testes weights (1.40 ± 0.03 vs. 1.45 ± 0.44 g/g bodyweight × 100; $t_{(13)} = 0.96$, $p = 0.35$) or seminal vesicle weights (0.91 ± 0.12 vs. 0.89 ± 0.07 g/g bodyweight × 100; $t_{(13)} = 0.18$, $p = 0.85$). It also did not affect their levels of circulating testosterone ($F_{(1,34)} = 1.83$, $p = 0.18$), although there was a significant effect of when blood was taken, such that male grass rats sacrificed during the day had higher circulating testosterone compared to males sacrificed at night ($F_{(1,34)} = 4.23$, $p = 0.049$; **Figure 2**). There was no significant interaction between light intensity group and time of day on testosterone levels ($F_{(1,34)} = 0.528$, $p = 0.47$).

## Medial Preoptic Area and Nucleus Accumbens mRNAs

Male grass rats housed in brLD and dimLD conditions did not differ in their mPOA aromatase mRNA expression ($t_{(17)} = 1.635$, $p = 0.120$), but dimLD males had significantly higher ESR1 ($t_{(17)} = 2.175$, $p = 0.044$) and AR ($t_{(16)} = 2.31$, $p = 0.035$) mRNA expression compared to brLD males (**Figure 3A**). dimLD males also had significantly higher D1 ($t_{(17)} = 4.21$, $p = 0.001$), but not D2 ($t_{(16)} = 1.52$, $p = 0.149$), receptor mRNA expression in the mPOA. In the NAC, dimLD and brLD males did not significantly differ in their DA D1 ($t_{(20)} = 0.874$, $p = 0.393$) or D2 ($t_{(19)} = 0.129$, $p = 0.878$) receptor mRNA expression (**Figure 3B**).

# DISCUSSION

Winter-time decrease in libido, sexual activity, and sexual satisfaction are commonly associated with SAD, and also

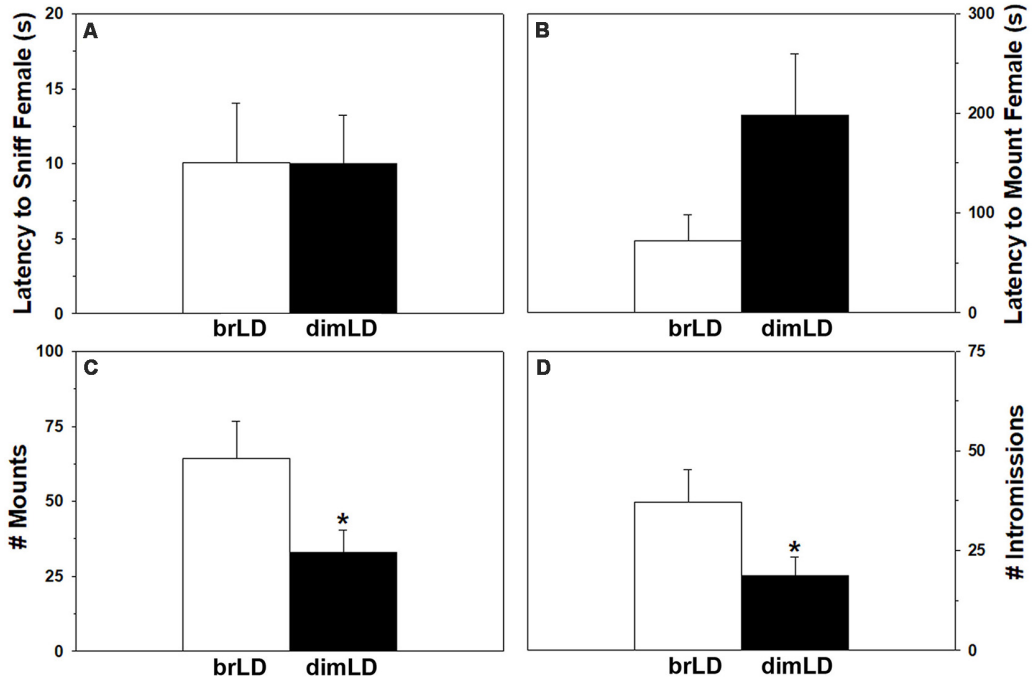


**FIGURE 1 |** Behavioral response to dim daylight. Male grass rats were housed in bright daylight intensity (brLD) or dim daylight intensity (dimLD) and their **(A)** latency to sniff an estrus female, **(B)** latency to mount an estrus female, **(C)** frequency of mounts, and **(D)** frequency of intromissions is shown (Means ± SEMs). *$p < 0.05$.

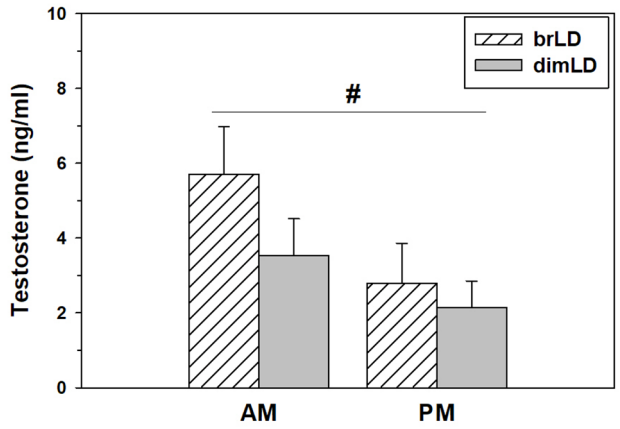


**FIGURE 2 |** Hormonal response to dim daylight. Circulating levels (Mean ± SEM) of testosterone in male grass rats housed in bright daylight intensity condition (brLD) or dim daylight intensity (dimLD) condition, and sacrificed during the day (hatched) or night (gray). #Significant main effect of time of day, $p < 0.05$.

experienced by many people whose seasonal symptoms would not reach the clinical threshold for this disorder (Schlager et al., 1993; Harmatz et al., 2000; Avasthi et al., 2001; Demir et al., 2016; Arendt and Middleton, 2018). It has been suggested that the seasonal decrease in sexual function (and thus mating) may have evolutionary origins, such that births would be less likely to occur during the increasingly resource-poor autumn, and instead be biased toward early spring (Eagles, 2004; Davis and Levitan, 2005). In support, SAD is more common in people of reproductive age than those younger or older (Magnusson et al., 2000). Despite a possible evolutionary benefit, for most modern humans around the globe which use artificial light and live in resource-rich environments, seasonal changes in sexual function can be quite distressing and very little is known about the neurobiological underpinnings.

## Influence of Daytime Light Intensity on Male Copulatory Behavior and Relevance to SAD

Using diurnal male grass rats as a model, we hypothesized that housing in low-intensity light during the daytime would recapitulate the effects of winter on male sexual activities. Our results generally supported this hypothesis. When compared to males housed in the bright, high-intensity daylight condition (brLD), males in the dim, low-intensity daylight condition (dimLD) had a trend toward longer latencies to begin mounting estrus females and showed about half as many mounts and intromissions. This suggests that, in male grass rats, being housed in winter-like daylight intensity may have only minor negative effects on sexual approach/motivation (reflected by the latencies to sniff and mount the females), but more considerable disruptive effects on copulatory performance (mounts and intromission). This is consistent with the winter-time reduction in men's sexual activity and satisfaction reported in a number of studies (Schlager et al., 1993; Harmatz et al., 2000; Avasthi et al., 2001; Demir et al., 2016; Arendt and Middleton, 2018).

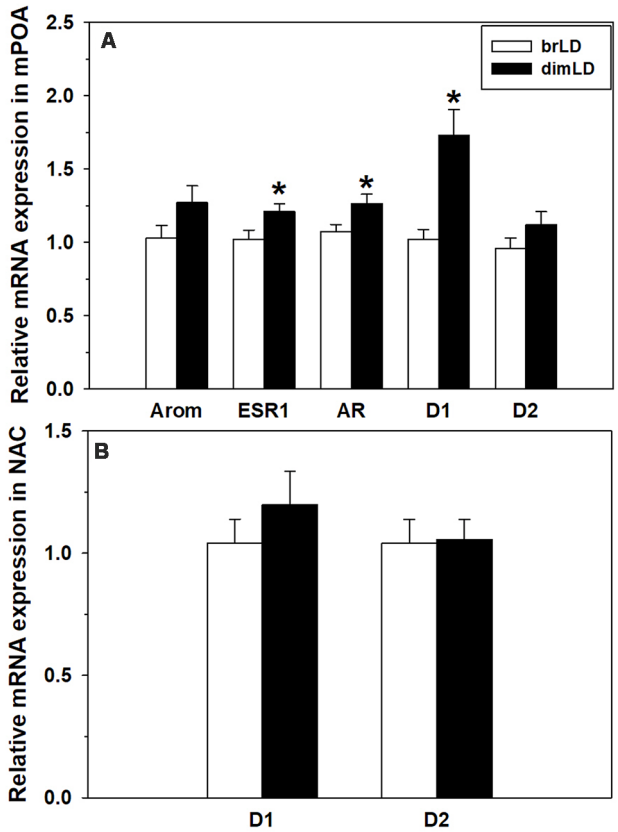


**FIGURE 3 |** Neural response to dim daylight. **(A)** Relative levels (Mean ± SEM) of mRNAs for aromatase (Arom), estrogen receptor 1 (ESR1), androgen receptor (AR), D1 and D2 in the medial preoptic area (mPOA) of male grass rats housed in bright daylight intensity condition (brLD) or dim daylight intensity (dimLD) condition. **(B)** Relative levels (Mean ± SEM) of mRNAs for D1 and D2 in the nucleus accumbens (NAC) of male grass rats housed in brLD or dimLD conditions. *$p < 0.05$.

At first, however, our results appear somewhat inconsistent with the reduced libido (i.e., sexual interest and motivation) often thought to be associated with SAD (Rosenthal et al., 1984). While no animal model is likely to reflect all symptoms of a given disorder, we also believe there is a lack of clarity in the "low libido" often cited as a symptom of SAD. First, the diagnostic criteria for SAD in the DSM-5 do not specifically involve anything related to sexual motivation or performance. Although some instruments frequently used to examine the depressive symptoms associated with SAD do ask a question about sexual functioning, they either offer libido as the only example of a concern related to sexual activity for patients to endorse (HRDS/HAM-D), only ask specifically about libido (BDI), or only ask about sexual satisfaction (Zung's SDS). Other common instruments do not ask about sexuality at all (CES-D, HADS-Dl QUIDS-SR16, MADRS, SPAQ). Thus, it is unclear if seasonal changes in the frequency or satisfaction of sexual activity are or are not usually captured by the high endorsement of "low libido" in SAD patients. Greater discrimination among the motivational, behavioral, and emotional aspects of human sexual functioning in SAD would be useful for understanding the validity of our and other laboratory rodent models of this affective disorder.

## Influence of Daytime Light Intensity on Gonadal Function

Short winter-like day length increases the duration of melatonin released from the pineal gland each day. Particularly in animals that are seasonal breeders, this elevated melatonin signal inhibits pituitary gonadotropin synthesis, causes gonadal regression, and eventually the cessation of mating (Carter and Goldman, 1983; Arendt, 1986; Kriegsfeld et al., 2015; Weems et al., 2015; Simonneaux, 2019). Of note, short day lengths do not reduce circulating testosterone in some laboratory rodents that are not highly seasonal breeders including rats (Prendergast and Kay, 2008), CD1 mice (Nelson, 1990), and California mice (*P. californicus*; Trainor et al., 2008; also see Trainor et al., 2006). Short day lengths also do not affect testosterone levels or testes mass in male grass rats housed within the laboratory (Nunes et al., 2002). As far as we are aware, there have been no previous studies on the effects of winter-like daylight intensity on gonadal function in any diurnal or nocturnal rodent. We found that male grass rats housed in brLD or dimLD conditions had similar circulating testosterone levels that were within the range previously reported for laboratory-housed and wild male grass rats (Sicard et al., 1994; Nunes et al., 2002). Testes and seminal vesicle weights were also similar between our two groups. We did not have an *a priori* expectation for these measures because it is unclear if grass rats are somewhat seasonal breeders or simply opportunistic breeders that mostly rely on recent environmental conditions to determine their reproduction (Neal, 1981; Delany and Monro, 1986; Sicard et al., 1994). It should be kept in mind for interpreting our results that the behavioral data and the blood (and brains) were obtained from different groups of dimLD and brLD male grass rats, which was done to avoid confounding effects of group differences in copulatory behaviors on circulating testosterone. Also, the sets of dimLD and brLD males used for behavior and blood analyses differed by 1 week in how long they were in their lighting conditions, although there are no obvious reasons why a difference of 4 vs. 5 weeks would have mattered for males' circulating gonadal hormones.

In any case, our results suggest that the impairments in copulatory behavior in dimLD-housed males are independent of their gonadal function. Other studies have found that changes in laboratory rodent behavior due to day length are also not positively related to gonadal hormones. For example, aggression in female Siberian hamsters housed in short day lengths is independent of their circulating ovarian hormones (Scotti et al., 2007), and aggression in male Siberian hamsters is inversely related to their circulating testosterone (Jasnow et al., 2000). In male California mice, housing in short days does not affect circulating testosterone (Nelson et al., 1995), but still increases aggression (Trainor et al., 2008). In laboratory rats, short-day lengths increase depression- and anxiety-like behaviors (Prendergast and Kay, 2008; although see Dulcis et al., 2013) without affecting circulating testosterone or testes size (Wallen et al., 1987; Prendergast and Kay, 2008). It has

also long been known that in some subpopulations of male laboratory rodents there is no positive relationship between their circulating testosterone and copulatory behaviors (Davidson, 1966; Damassa et al., 1977). Additionally, as mentioned above there is no evidence that gonadal function is abnormal in men who experience winter-time reductions in libido, sexual activity, or sexual satisfaction; our results from male grass rats suggest that these reductions are likely unrelated to circulating testicular hormones.

While we found no effect of daytime light intensity on gonadal function, we did find significantly higher circulating testosterone in male grass rats sacrificed in the morning compared to those sacrificed at night. A circadian rhythm in circulating gonadal hormones is commonly found in mammals (Kriegsfeld et al., 2002), and the circadian rhythm suggested by our results is similar to the morning peak in testosterone found in diurnal humans (e.g., Diver et al., 2003; Brambilla et al., 2009). It is also consistent with the early-morning peak and evening nadir in hypothalamic gonadotropin releasing hormone (GnRH) cell activity, and circulating luteinizing hormone (LH), in female grass rats from our colony (McElhinny et al., 1999). In contrast to diurnal grass rats, the nocturnal laboratory rat and mouse show their peak circulating testosterone levels in the early evening (Kriegsfeld et al., 2015).

## Influence of Daytime Light Intensity on mPOA Steroid Hormone and DA Receptor mRNAs

The mPOA is a critical node in the neural network underlying the hormonal and sensory control of male sexual behavior (Hull and Dominguez, 2015). There is a large population of cells in the mPOA where endogenous testosterone can be aromatized into estradiol, which then acts on cytosolic ESRs as well as other substrates to regulate male sexual motivation and performance (Balthazart et al., 2004). Testosterone also acts directly on ARs in the mPOA to promote male sexual activity (McGinnis et al., 2002; Harding and McGinnis, 2004). We found that mPOA levels of aromatase mRNA did not differ between males housed in dimLD and brLD conditions, suggesting a similar capacity for local estradiol synthesis. The groups differed in ESR1 and AR mRNA expression, though, with both transcripts higher in dimLD males than brLD males. This was unexpected because the impaired copulation in the dimLD-housed males would have intuitively been consistent with lower ESR1 and AR expression in the mPOA. Thus rendering it less sensitive to steroid hormone influences on sexual behavior.

Winter-like short day length downregulates ARs in the mPOA of seasonally breeding male Golden and Siberian hamsters (Bittman and Krey, 1988; Tetel et al., 2004), as well as downregulates ESR1 in the mPOA of female Syrian hamsters (Mangels et al., 1998), concomitant with cessation of their copulatory behavior. In non-seasonal breeders, however, the consequences of short day length on central AR and ER expression are more complex, being both species- and site-specific (Trainor et al., 2007, 2008). Similar to our findings related to reduced daytime light intensity, short day length increases ESR1 mRNA in the mPOA of male Oldfield mice (*P. polionotus*). However, housing in short days does not affect ESR1 mRNA expression in the mPOA of male Deer mice (*P. maniculatus*; Trainor et al., 2007) or estrogen receptor immunoreactivity in the mPOA of Siberian hamsters (Kramer et al., 2008). Short day length also increases ESR1 mRNA and/or estrogen receptor protein in the posterior bed nucleus of the stria terminalis (BNST) of male Oldfield mice, Deer mice, and Siberian hamsters, but not in male California mice (*P. californicus*; Trainor et al., 2007, 2008; Kramer et al., 2008). Because these non-seasonally breeding animals show no changes in circulating testosterone or sexual behavior in response to short day length, the seasonal changes in their behavior (e.g., increased aggression and anxiety) are independent of circulating steroids. In our male grass rats, reduced copulatory behavior in response to low-intensity daylight is also independent of circulating testosterone and more likely associated in some manner with the upregulated AR and ESR1 expression in their mPOA. Given the direction of the results, we propose that the upregulated expression may be part of a compensatory mechanism that maintains sexual behavior, albeit at lower levels, in the dimLD males. An alternative hypothesis could be that upregulation of these receptors in the mPOA actively suppresses male copulatory behavior in dimLD males. While we can find no evidence from the literature that upregulating AR or ESR1 in the mPOA would impair copulation or any other sociosexual behavior in male rats, overexpressing estrogen receptors in other sites such as the medial amygdala or BNST does reduce prosocial behaviors in male prairie voles (Cushing et al., 2008; Lei et al., 2010).

We also found upregulated D1 mRNA in the mPOA of dimLD-housed male grass rats, which was unexpected based on what is known about DA receptors in this brain site and male copulation. High mPOA D1 activity relative to D2 activity is associated with faster sexual motivation and more mounts and intromissions before ejaculation in male laboratory rats (Hull et al., 1989; Moses et al., 1995), not the somewhat longer latencies to mount females and lower mount and intromissions frequencies in our dimLD grass rats. Similar to the upregulated AR and ESR1 expression in the mPOA, perhaps upregulated D1 receptor expression in the mPOA of dimLD male grass rats helps maintain some level of sexual activity in these animals.

## Influence of Daytime Light Intensity on NAC DA Receptor mRNAs

DA receptor signaling in the NAC is essential for incentive salience, motivation to approach, and/or continued responding for a rewarding stimulus (Salamone et al., 2005; Castro and Berridge, 2014). It would make sense if that extends to natural rewards such as sexual activity, but the literature so far has shown that neither D1 or D2 antagonism nor D2 agonism in the NAC affects copulatory behavior in male laboratory rats tested under high-motivation conditions (Hull et al., 1986; Moses et al., 1995; Guadarrama-Bazante and Rodríguez-Manzo, 2019). Nonetheless, D2 agonism in the NAC (but not mPOA) can reinstate mounting by sexually sated male rats that have low motivation to copulate (Guadarrama-Bazante and Rodríguez-Manzo, 2019).

We found no significant difference between our two groups of male grass rats in levels of D1 or D2 receptor mRNAs in the NAC. This does not mean that other aspects of their mesolimbic systems do not differ in ways that affect their sexual behavior, and this would be worthy of future investigation. A number of studies report relationships among light, DA, and mood. Pharmacologically induced catecholamine depletion during the summer causes relapse in SAD patients (Lam et al., 2001) and significantly lowers mood in healthy women living for just 2 days under a normal circadian cycle but with very dim daytime light intensity (10 lux; Cawley et al., 2013). A study of people naturally experiencing winter-time short day length along with low daytime light intensity found that the ventral tegmental area and other DA-rich midbrain cell groups have much less tyrosine hydroxylase immunoreactivity compared to midbrains obtained from people during the summer (Aumann et al., 2016). On the other hand, human cerebrospinal fluid levels of DA and its metabolites are higher (Hartikainen et al., 1991), striatal presynaptic DA synthesis and storage are elevated (Eisenberg et al., 2010), and DA transporter binding in the striatum is lower (Neumeister et al., 2001) in winter. Even the small differences in potential sunshine exposure in a subtropical location (i.e., Taiwan) is associated with lower D2/D3 receptor availability in the human striatum (Tsai et al., 2011). These results collectively suggest a winter-time downregulation of midbrain DA cells, but upregulation of forebrain terminal and synaptic mechanisms that may permit some degree of compensation in humans. The effects of winter-like conditions on central DA systems in diurnal rodents is less clear. Male Nile grass rats in either winter-like photoperiod or low daytime light intensity have fewer hypothalamic cells containing tyrosine hydroxylase (Deats et al., 2015) and winter-like reduction in both daylight length and intensity downregulate DA turnover in the NAC of Sudanian grass rats (*Arvicanthis* ansorgei), which could be restored to control levels by daily bright light "therapy" (Itzhacki et al., 2018). In diurnal chipmunks, short days decreased DA content in the striatum but increased DA levels in the hypothalamus and amygdala (Goda et al., 2015).

## CONCLUSIONS AND FUTURE DIRECTIONS

Seasonal changes in human sexual motivation and function are quite common but are rarely studied. As such, the biological bases are poorly understood. The present findings from diurnal male grass rats, along with other research on diurnal and non-seasonally breeding rodents, indicates that the locus of such effects of winter-time light conditions is not at the level of the gonads but in brain sites such as the mPOA that are vital for sexual activity. It will be valuable in future studies to determine if such effects of dim daylight on sexual behavior in grass rats are reversible by daily "light therapy." It will also be valuable to determine the effects of winter-like dim daylight on copulation, ovarian hormone levels, and relevant mRNAs in the mPOA of female grass rats. Women suffer from SAD 2–4 times more often men (Kasper et al., 1989; Lee and Chan, 1998), and consistently show some inhibition of ovarian function during winter (Ronkainen et al., 1985; Kauppila et al., 1987a,b; Kivelä et al., 1988). Perhaps female grass rats housed in dim daylight would show less copulatory behaviors compared to females in bright daylight, but the effects are due to both a drop in ovarian function and reduced steroid and DA sensitivity of their mPOA and other hypothalamic sites involved in female copulation (e.g., ventromedial nucleus).

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). The protocol was approved by the Institutional Animal Care and Use Committee of Michigan State University.

## AUTHOR CONTRIBUTIONS

JL and LY conceived and designed the experiments. KL-D conducted the experiments. JL, LY, and KL-D conducted data analyses and wrote the manuscript.

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# Low Salivary Testosterone Level Is Associated With Efficient Attention Holding by Self Face in Women

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Capacity to recognize one's own face (hereinafter referred to as self face) is a fundamental component of various domains of social cognition such as empathy in humans. Previous research has demonstrated that a high level of androgen suppresses empathic behavior and social brain function. Taking these into consideration, we hypothesized that people with high androgen level show reduced response to self face. The present study examined this hypothesis by investigating the association between attentiveness towards self face, as assessed by a psychophysiological experiment, and salivary testosterone concentration. The attentional responses to self face was measured by a modified Go/NoGo task. In this task, self face or unfamiliar other's face was presented simultaneously with Go or NoGo signal. In go trials, participants had to divert their attention from the face to a peripheral target. The reaction time (RT) for peripheral target detection in each condition was measured. In addition to behavioral data, saliva samples were collected to assay salivary testosterone concentration. The index of potency of self face to hold viewer's attention that was computed based on RT data was regressed against salivary testosterone concentration in men and women separately. The analyses revealed that self face holds visuospatial attention more effectively in women with low than high salivary testosterone level, but no such trend was observed in men. This pattern of results indicates that low testosterone level is associated with a pronounced response to self face as we hypothesized and raises the possibility that multiple aspects of self-face processing are under the influence of endocrinological function.

**Keywords: self, face, attention, testosterone, sex difference**

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## INTRODUCTION

Self-awareness is considered a cornerstone of social cognition (Gallup, 1970; Keenan et al., 2000; Humphreys and Sui, 2016). The distinction between self and other is indispensable in the theory of mind and perspective taking (Happé, 2003; Bradford et al., 2015). Reflecting this special status self holds in social cognition, one's visual system processes self face in a manner different from an unfamiliar or a highly familiar other's face. Tong and Nakayama (1999) demonstrated that the representation of self face is highly viewpoint invariant. In addition,

many neuroimaging studies have revealed increased activation of neural regions, such as the medial prefrontal cortex and anterior cingulate cortex, to self faces compared with other's faces (Keenan et al., 2000; Kircher et al., 2001; Heatherton et al., 2006), which indicates that exposure to self face induces introspection and emotional reaction effectively. Interestingly, part of these neural regions is recruited in inference of other's mental status and perspective taking as well (Mitchell et al., 2005; Healey and Grossman, 2018), which gives further credence to the view that self-face processing comprises the basis of social cognition (Happé, 2003).

One phenomenon reflecting the special status of self face is its effectiveness to hold one's attention (Humphreys and Sui, 2016). Psychophysical and eye-tracking studies have shown that self face holds adult's attention more effectively than an unfamiliar or a familiar other's face (Brédart et al., 2006; Devue et al., 2009; Humphreys and Sui, 2016; Wójcik et al., 2018).

There is great interindividual variation in the ability of social cognition. Such individual differences presumably stem from various factors, including environmental and biological factors. Among these, many studies point out that a high level of androgen is associated with poorer function in many domains of social cognition (van Wingen et al., 2011). Direct evidence for the link between social cognition and androgen comes from testosterone administration studies. This line of study has shown that a single administration of testosterone reduces empathy and mentalizing (Hermans et al., 2006; van Honk et al., 2011; but see Nadler et al., 2019). Correlational studies also linked a high level of testosterone to impaired social function, indicating the possibility that testosterone can impair social cognition ability even within the physiological range (Ronay and Carney, 2013; Zilioli et al., 2015).

Taken together, these pieces of evidence indicate that a high level of testosterone leads to a lower level of social cognition ability. Taking this into consideration, together with the proposed link between social cognition and self-processing (Happé, 2003), it is highly conceivable that a high level of testosterone is associated with reduced behavioral and neurophysiological responses to self-related information, including self face. However, the association between neuroendocrinological function and self-face processing has not been examined fully with only a few exceptions that investigated the association between representation of self face and levels of hormones (Colonnello et al., 2013; Welling et al., 2016). Specifically, Colonnello et al. (2013) revealed that intranasal administration of oxytocin increases one's ability to discriminate self and other's faces, while Welling et al. (2016) demonstrated that testosterone administration makes one's representation of self face more masculine than the actual self face. Thus, despite the abundance of studies that indicate effectiveness of self face to hold viewer's visuospatial attention (Devue et al., 2009; Humphreys and Sui, 2016; Wójcik et al., 2018), no study to date has investigated the link between the indicators of endocrinological function and attentional responses to self face.

The present study attempts to fill in the gap in knowledge by investigating the association between salivary testosterone concentration and strength of attention holding by self face to examine the hypothesis that a high level of testosterone is associated with weaker attention holding by self face. We collected data from men and women during their 20–30 s because many previous studies found link between testosterone level and sociocognitive function in population within similar age range (Welling et al., 2007, 2016; van Honk et al., 2011; Volman et al., 2011).

# MATERIALS AND METHODS

## Participants

The present study included 44 males (mean age = 20.7 years old, *SD* = 2.9; age range = 18–32) and 36 females (mean age = 21.3 years old, *SD* = 3.3; age range = 18–35) participants with normal or corrected to normal visual acuity after they gave written informed consent. Most of them were in their early 20 s. There was no significant between-group difference in age, $t_{(78)} = 0.82$, $p = 0.42$, $d = 0.18$. Participants with history of psychiatric and neurological conditions or being currently on medication were excluded from the final sample.

## Procedure

### Behavioral Experiment

After the participants arrived at the lab, we took a picture of each participant's face against cream-white background. The participants were instructed to maintain a neutral expression with their mouths closed. The image was cropped and adjusted in size so that the resultant image fit an 8.6 cm × 8.6 cm square that served as the face stimulus in the Self condition. Face stimuli presented in the Other condition were created by averaging 30 faces of people with roughly the same age (20–30 s) as the participants. The unfamiliar face for female participants was created from 30 female faces while that for male participants was created from 30 male faces. The identical same-sex average face was presented for all the participants in each sex in the Other condition because the participants' age was not widely distributed. The size of the face image in the Other condition was equal to that in the Self condition. The stimuli were presented on a 17-inch monitor viewed from ∼65 cm away.

After instructions were given to the participants, the experiment started. At the start of each trial, a fixation cross appeared at the center against white background for 500 ms. Then, a face image was presented. In two-thirds of the trials, a small green square subtending 0.67 cm was presented at the height of the nose (Go condition), and a small red rectangle was presented in the remaining trials (NoGo condition). One-hundred and fifty milliseconds after the presentation of the face image, two 1.3 cm × 2.3 cm black rectangles were presented at the periphery of screen, ∼11.8 cm from the center, simultaneously with the face image. One was presented in vertical, and the other in horizontal orientation. In the Go condition, the participants identified the location of the horizontal target using a key press as soon as possible. In the NoGo condition, they were instructed to refrain from making any responses. The targets stayed on the screen for 1,250 ms in the NoGo trials. We included Go and NoGo conditions so that the participants would pay attention to the face image; we wanted to make sure that participants

direct overt attention to the facial image because there was a good chance that they might process the faces only in parafoveal vision due to relatively large size of the facial images used. After the key press or 1,250 ms passes after the appearance of face image, feedback about the correctness of response was presented for 300 ms. There was no intertrial interval. Thus, after the disappearance of feedback, the experiment immediately proceeded to the next trial. The sequence of the stimulus presentation is schematically shown in **Figure 1**. There were 32 Go and 16 NoGo trials each for the Self and Other conditions, yielding in total of 96 trials. The trials in these conditions were pseudorandomly ordered with the restriction that trials of the four conditions (Go/NoGo × Self/Other) were evenly distributed throughout the experiment.

### Salivary Sample Collection

The saliva sample was collected between 12:00 and 14:00 h to mitigate the influences of circadian fluctuation (Dabbs, 1990). Each saliva sample was deposited into a polystyrene tube by passive drool and stored at −80°C until the assay. The participants refrained from eating, drinking, smoking, brushing their teeth, and exercising for 1 h before the experiment. They also rinsed their mouths with water ∼15 min before the sample collection.

### Self-administered Questionnaires

After the behavioral experiment, participants were asked to complete the Japanese translation of Rosenberg's self-esteem scale (Rosenberg, 1965) and the self-consciousness scale (Sugawara, 1984). We collected data of these questionnaires because attitude to and one's evaluation of self might influence attentional responses to self face. Self-esteem scale is comprised of ten 5-point items that measure the level of positive evaluation of one's worth and abilities (range = 5−50; Yamamoto et al., 1982). Self-consciousness scale includes 7-point items that measure private (range = 7−70) and public self-consciousness (range = 7–77; 10 items for private self-consciousness and 11 items for public self-consciousness). Private self-consciousness refers to the tendency to pay attention to own inner states, while public self-consciousness is the tendency to pay attention to own appearance and how one's behavior is evaluated by others. The self-consciousness scale is a modified version of Feininger's inventory (Fenigstein et al., 1975) but includes items more familiar to the Japanese population. Still, it shows a reliable two-dimensional structure of public and private self-consciousness (Fenigstein et al., 1975).

## Testosterone Concentration Analysis

After all participants had completed the experimental tasks, the concentration of salivary testosterone in each sample was assayed by enzyme immunoassay (EIA) using a commercially available kit (Salimetrics Europe Limited, Suffolk, UK). Testosterone level in saliva samples is known to correlate with serum testosterone level in men but not necessarily in women. At the same time, salivary concentration of testosterone is supposed to reflect the level of free-testosterone and testosterone only weakly binding to sex hormone-binding globulin and hence is considered to be a reliable indicator of the level of bioactive testosterone (Papacosta and Nassis, 2011). The sample was first centrifuged and the aqueous layer was aliquoted for assay. Information about the recovery and specificity of the kit can be found online in the EIA kit manual. In short, testosterone concentration in 25 μl of undiluted saliva samples was measured by competitive immunoassay. The optical densities of each well of the plate was read by a microplate reader at 450 nm and then converted to testosterone concentration values on the basis of simultaneously measured standard curve. The percent cross-reactivity with estradiol, progesterone, and cortisol is reported to range from ND (none detected)

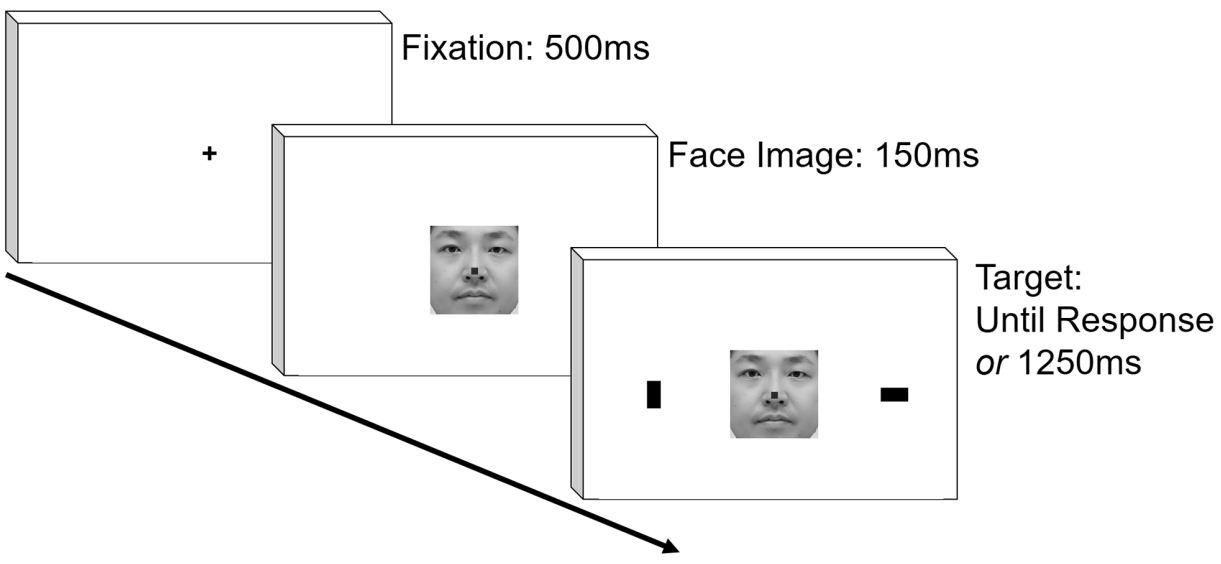


**FIGURE 1 |** Schematic representation of temporal sequence in stimulus presentation. The face image is not exactly the same with those presented in the actual experiment.

to <0.03 (see for more details, https://salimetrics.com/wp-content/uploads/2018/03/testosterone-saliva-elisa-kit.pdf).

## Statistical Analysis

The potency of self face to hold a participant's visuospatial attention was quantified as the standardized difference between reaction time (RT) in Self-Go and Other-Go conditions using the following equation:

$$RT_{diff} = \frac{RT_{self} - RT_{other}}{RT_{self} + RT_{other}}$$

where $RT_{self}$ and $RT_{other}$ are mean RT in successful trials in Self-Go and Other-Go conditions, respectively. Higher $RT_{diff}$ indicates less efficient disengagement of visuospatial attention from self than other's face, thus more efficient holding of attention by self face. We obtained essentially the same results when analyzing $RT_{self} - RT_{other}$ without standardization. Thus, in the following, we report only the results of $RT_{diff}$.

We carried out linear and quadratic regression analyses separately for male and female participants because previous studies found sex differences in androgenic effects on higher-order cognition (Moffat and Hampson, 1996; Sapienza et al., 2009; Doi et al., 2015, 2018). We also carried out two-way between-participant analysis of variance (ANOVA) and *t*-tests for group comparisons. All the statistical analyses were carried out using R 3.5.2 (R Development Core Team). The power was computed by G*Power 3.1 (Faul et al., 2007) using medium effect size (Cohen, 1988).

# RESULTS

## Sex Difference

We first examined sex differences in hormonal and behavioral measures. The mean and standard deviations of these variables are summarized in **Table 1**. The range of salivary testosterone concentration was comparable to the previous studies (Deady et al., 2006; Welling et al., 2007; Cobey et al., 2015). As expected, the salivary testosterone concentration was significantly higher in male than in female participants, $t_{(78)} = -16.2$, $p < 0.001$, $d = 3.79$. No other comparison reached statistical significance, $ts < 1.54$, $ps > 0.12$.

## Association Between Testosterone and Attentiveness Toward Self Face

$RT_{diff}$ was regressed against the salivary testosterone concentration. The scatterplots between $RT_{diff}$ and salivary testosterone concentration are shown in **Figure 2** for male and female participants separately. There was a significant negative correlation between $RT_{diff}$ and testosterone concentration in female participants, $r_{(34)} = -0.49$, $p = 0.003$, but not in male participants, $r_{(42)} = -0.05$, $p = 0.76$, power = 0.71.

To clarify the nature of this pattern of correlational analysis, three additional analyses were conducted. In the first analysis, we carried out multiple regression analysis for data of female participants with $RT_{diff}$ as the dependent variable. The predictors included testosterone concentration, age, and questionnaire results of self-esteem, public self-consciousness, and private self-consciousness. The results are summarized in **Table 2**. As can be seen in the table, the correlation between testosterone concentration and $RT_{diff}$ persisted even after the influences of the other predictors were controlled for.

From the visual inspection of scatterplot (**Figure 2**), there seems to be a curvilinear trend in the relationship between testosterone concentration and $RT_{diff}$ in male participants. Considering this together with the previous study indicating curvilinear relationship between androgen and behavior (Moffat and Hampson, 1996; Tan and Tan, 1998; Sapienza et al., 2009; Doi et al., 2015; for a review see Swift-Gallant and Monks, 2017), in the second analysis, we carried out a quadratic regression analysis with $RT_{diff}$ as the dependent variable and testosterone concentration and squared testosterone concentration as the independent variables. The quadratic model did not show significant fit to $RT_{diff}$, $F_{(2,41)} = 1.47$, $p = 0.24$, $r^2 = 0.07$, power = 0.59.

In the third analysis, participants were first classified into high and low testosterone groups within each sex. The median value of testosterone concentration within each sex was used as the criteria of participant grouping. For example, female participants whose testosterone level was higher than the median testosterone concentration in all the female participants were included into high-female group. Then, we submitted $RT_{diff}$ to a two-way between-participant ANOVA with factors of sex (2) and testosterone (2; high–low). The mean and standard deviation in each group are shown in **Figure 3**.

ANOVA revealed a significant main effect of testosterone, $F_{(1,76)} = 5.97$, $p = 0.017$, $\eta_p^2 = 0.073$, but the main effect of sex failed to reach significance, $F_{(1,76)} = 0.35$, $p = 0.56$, $\eta_p^2 = 0.004$. These effects were qualified by a significant interaction between sex and testosterone, $F_{(1,76)} = 6.21$, $p = 0.015$, $\eta_p^2 = 0.076$. Simple main effect analysis revealed $RT_{diff}$ as significantly higher in female participants with low rather than high salivary testosterone concentration, $F_{(1,76)} = 11.07$, $p = 0.0014$, $\eta_p^2 = 0.13$. No such effect was found in male participants, $F_{(1,76)} = 0.001$, $p = 0.97$, $\eta_p^2 < 0.001$, power = 0.38. For explanatory purpose, we tested whether the averaged $RT_{diff}$ differed from zero. *T*-tests revealed significant deviation of $RT_{diff}$ from zero in female participants with low testosterone, $t_{(17)} = 3.55$, $p = 0.002$, but not in the other three groups, $ts < 1.9$, $ps > 0.08$, powers $> 0.51$.

**TABLE 1** | The means and standard deviations of hormonal and behavioral results.

| | Testosterone (pg/ml) | $RT_{Self}$ (ms) | $RT_{Other}$ (ms) | Self-Esteem | Public | Private |
|---|---|---|---|---|---|---|
| Male | 262.7** (65.3) | 533.4 (76.7) | 535.9 (81.4) | 31.8 (7.6) | 52.6 (10.2) | 46.0 (8.5) |
| Female | 77.2 (22.7) | 562.4 (92.0) | 562.4 (101.7) | 29.6 (6.7) | 55 (10.8) | 46.4 (8.5) |

*The standard deviations are in the parenthesis. $RT_{Self}$, reaction time in Self-Go condition; $RT_{Other}$, reaction time in other-Go condition; Public, public self-consciousness; Private, private self-consciousness, **$p < 0.01$ in group comparison.*

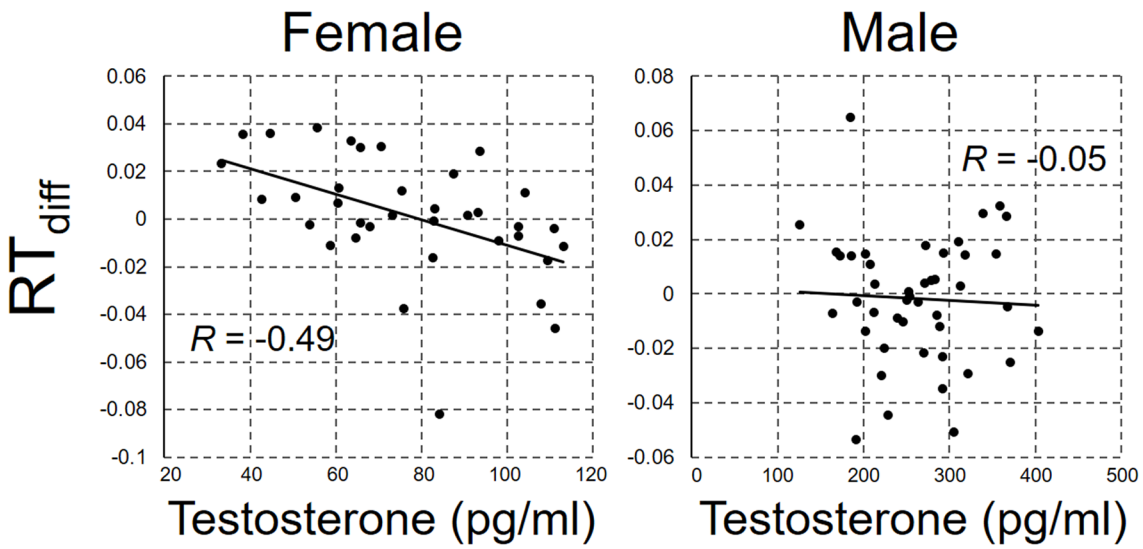


**FIGURE 2 |** The scatterplot between $RT_{diff}$ and salivary testosterone concentration for female and male participant.

**TABLE 2 |** Summary of the statistical values of the multiple regression analysis.

| | *β* | SE | *t*-value | *p*-value |
|---|---|---|---|---|
| Testosterone | −0.512 | 0.167 | −3.072 | 0.005 |
| Age | 0.141 | 0.175 | 0.808 | 0.426 |
| Self Esteem | −0.185 | 0.186 | −0.994 | 0.329 |
| Public | −0.201 | 0.183 | −1.102 | 0.28 |
| Private | −0.029 | 0.184 | −0.158 | 0.876 |
| Intercept | −0.012 | 0.163 | −0.076 | 0.94 |

*β, standardized coefficient of each predictor; SE, standard error of each predictor; Public: public self-consciousness; Private, private self-consciousness.*

## DISCUSSION

Many studies have shown the association between androgenic function and cognitive/perceptual abilities such as spatial perception, financial decision making, and aggression (Moffat and Hampson, 1996; Mazur and Booth, 1998; Sapienza et al., 2009; Doi et al., 2015). The ability of social cognition is no exception to this, and an accumulating number of studies has linked a higher level of testosterone with poorer ability in many domains of social cognition (Welling et al., 2016; van Honk et al., 2011; Ronay and Carney, 2013; Zilioli et al., 2015; but see Nadler et al., 2019). Given the close linkage between processing of self-related information and sociocognitive functions (Happé, 2003; Bradford et al., 2015), it seems plausible to postulate an association between testosterone level and self-related information processing.

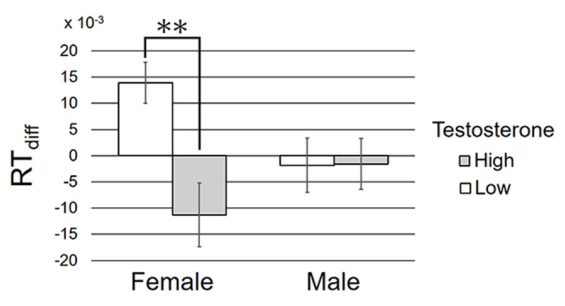


**FIGURE 3 |** The mean and standard deviation of $RT_{diff}$ in each condition. $^{**}p < 0.01$ in the simple main effect analysis.

The present study revealed that female participants with low salivary testosterone show inefficient disengagement of attention from self face compared with those with a relatively high testosterone level. In other words, self face holds attention more efficiently in female participant with low than high testosterone level. Actually, female participants with low testosterone level was the only group that showed self-face advantage in attention holding in the present study in line with the previous findings (Devue et al., 2009; Humphreys and Sui, 2016; Wójcik et al., 2018). Furthermore, efficiency of attentional disengagement from self face was not related to any variables tested other than testosterone level in female participants. Taken together, these observations seemingly indicate that a high testosterone level is associated with a reduced response to self face as hypothesized. Although many studies have revealed androgenic influences on social cognition, to the best of our knowledge, this is the first to empirically show the relationship between systemic androgen levels and the attentional response to self face.

Some neural regions recruited in self-face processing, such as the medial prefrontal cortex and amygdala, are rich with androgen receptors in mammals (Simerly et al., 1990; Finley and Kritzer, 1999), and a previous study has found functional decoupling of these regions by testosterone administration (Volman et al., 2011). Thus, downregulation of functional connectivity in this neural network probably explains the reduced attentiveness toward self face in the present study. A previous study showed that men administered with exogenous

testosterone tend to have an inner representation of their own face that is more morphologically masculine than what is actually true (Welling et al., 2016). Taken together with this, the present study indicates that androgen modifies multiple aspects of self-face processing.

Together with the previous findings associating high level of testosterone with impaired socio-cognitive abilities (Hermans et al., 2006; van Honk et al., 2011; Ronay and Carney, 2013), the overall pattern of the present results seemingly supports the proposed link between self-face processing and social cognition (Happé, 2003). However, we did not collect measures of the other aspects of social cognition such as empathy and perspective taking because the primary aim of the present study was not to validate the link between self-face processing and social cognition in general. As a result, it remains unclear whether testosterone affects all aspects of social cognition including self-face processing in the same way. With regard to this point, the extreme male brain theory of autism (Baron-Cohen, 2010) claims that hypermasculinization of brain induced by exposure to excessive level of androgen shower during fetal period leads to later impairment in empathic behavior while promoting systemizing tendency. Furthermore, many researchers argue that high level of fetal androgen exposure decreases second/fourth digit length ratio (2D:4D; Manning et al., 1998; Hönekopp et al., 2007). On the basis of these, if self-face processing is intrinsically linked to social cognition, stronger attention holding by self face should be observed in people with high compared to low 2D:4D. Thus, multiple measures of social cognition and 2D:4D should be incorporated to get a more comprehensive picture of the relationship between self-face processing, social cognition, and androgen. In relation to this, it would also be of interest to see if individual difference in self-face processing is related to the empathizing–systemizing cognitive styles (Baron-Cohen, 2009).

Interestingly, we found no robust relationship between behavioral performance and salivary testosterone concentration in men. RTs in women were numerically longer than men irrespective of conditions. Short RT in men may have resulted in a kind of floor effect that masked any association between testosterone level and behavioral effect. A sex difference in sensitivity to the activational effect of androgen has often been reported in previous studies (Moffat and Hampson, 1996; Sapienza et al., 2009; Doi et al., 2015, 2018), but its cause remains largely unknown. One possible explanation is that self-face processing of male young adult, whose brain had already been masculinized/defeminized to some extent during the fetal period (Baron-Cohen, 2010), is not modified further by subtle difference in the level of circulating testosterone within physiological range. The social brain is saturated with androgens in this population at this stage of life, so differences in endogenous testosterone level may not have observable effect on self-related information processing. However, at this point, this is mere speculation and should be validated with empirical results. An alternative explanation is the often-reported curvilinearity or plateauing due to ceiling effect in the relationship between androgen and behavior (Swift-Gallant and Monks, 2017). This explanation is partly refuted in the present dataset because a quadratic regression model including squared testosterone concentration as the predictor failed to show correlation with self-face advantage ($RT_{diff}$). However, this may be because of relatively weak power of statistics in the present study.

In contrast to women with low testosterone, those with high testosterone level showed tendency to be more attentive to unfamiliar other's face than self face. A previous study found that testosterone administration reduces perceived trustworthiness of others' faces in women (Bos et al., 2010). Given that threatening images are the most potent stimuli to capture attention (Mogg and Bradley, 2010), the observed tendency in women with high testosterone seemingly stems from reduced trust in unfamiliar others.

Lack of clear self-face advantage in men was totally unexpected. There are several explanations for this null result. First, our previous study (Doi and Shinohara, 2018) has shown that male young Japanese do not show clear attentional prioritization of self face over other's face after mid-adolescence. Taking into consideration the previous finding indicating that developmental change of face processing continues into late adulthood (Anastasi and Rhodes, 2005; Boutet et al., 2015), we might get a different picture if, we recruit younger or older population. The second potential cause is that the stimulus onset asynchrony (SOA) between face image and target stimuli was not optimal to detect self-face advantage in attentional responses in men. Previous studies on the influences of facial information on visuospatial attention have revealed that the effect of facial information on behavioral response is sensitive to SOA (Liu et al., 2016; Carlson et al., 2019). Thus, it is necessary to test the association between testosterone and attentional responses to self face using more varying SOAs in the future study.

We presented averaged face of same-sex persons as unfamiliar other's face. Average faces are generally perceived to be attractive (Little et al., 2011) From the perspective of evolutionary psychology, attractive face signals health and high reproductivity (Rhodes, 2006). Considering this, it is possible that participants have implicitly deemed averaged same-sex face as potential competitor for resources and sexual mates (Ellis, 2006). Such intrasex competition might have increased attentiveness to other's face especially in men, who are reported to show stronger tendency of intrasexual competition (Ellis, 2006), which might explain the lack of self face advantage in men. Several studies have shown that the attributes (age, sex) of viewers interact with those of viewed faces in determining the pattern of neural and behavioral responses to other's face (Doi et al., 2010; Hills and Lewis, 2011; Kret and De Gelder, 2012; Rhodes and Anastasi, 2012). For example, Doi et al. (2010) revealed that the amplitude of an event-related potential component reflective of emotional and attentional responses to face increases in response to the same-sex compared to opposite-sex faces with neutral expression. In the present study, we used average faces of same-sex people within age range similar to participants to match the attributes of self and other's face closely. However, we cannot deny the possibility that this specific choice introduced some complications to the results in the present study.

There are several limitations that qualify the interpretation of this study. First, this is a mere correlational study, and thus,

we cannot ascertain the causal linkage or direction between testosterone and attentiveness to self face. To establish the presumed causal linkage, a testosterone administration study on attentiveness to self face is warranted. Second, we did not collect information on menstrual cycle in female samples. Testosterone can exert influences on neural function through aromatization into estrogen (Roselli et al., 2010). Thus, the effect of testosterone might be confounded by fluctuations in secretion levels of other hormones such as estrogen and progesterone, as might be the levels of testosterone itself. These hormones could confound the results for men as well. Furthermore, the level of testosterone itself fluctuates during menstrual cycle. Thus, simultaneous measurement of multiple hormones should be required in the future study to see whether the attentional response to self face is specifically linked to testosterone level or not.

## CONCLUSION

In summary, the present study investigated the association between attentiveness to self face and salivary testosterone concentration. The results revealed that self face holds visuospatial attention of female individuals with low testosterone level more effectively than those with high testosterone concentration. This finding gives support to the view that self-face processing, a fundamental component of social cognition, is also under the influences of androgenic function.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Graduate School of Biomedical Sciences, Nagasaki University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

HD conceived the study, carried out data collection and analysis, and wrote the manuscript. KS assisted in data collection and hormone assay and approved the manuscript.

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# Lower Digit Ratio (2D:4D) Indicative of Excess Prenatal Androgen Is Associated With Increased Sociability and Greater Social Capital

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Positive social interactions are crucial for human well-being. Elevated prenatal exposure to testosterone as indicated by a low second-to-fourth finger length ratio (2D:4D) relates to more aggressive/hostile behavior in men of low 2D:4D, especially in challenging situations. How much people enjoy interacting with others is determined by the personality trait sociability. Given its role in approach and avoidance behavior, sociability might also be influenced by prenatal sex hormones, but studies are inconclusive so far. Here, we investigated the association between 2D:4D and the personality trait sociability complemented by personal social capital and personal social network size, in a population-based cohort of 4998 men. Lower 2D:4D correlated significantly with higher trait sociability, bigger personal social capital, and larger personal social network size. These effects were consistent across both hands separately and their mean value. Furthermore, both factors of sociability (1) liking party and company of friends and (2) isolation intolerance, correlated significantly with the prenatal testosterone marker. The exploratory analysis revealed no link between 2D:4D and responses to the personality trait aggression items or items of anti-social-personality disorder. Our data suggest that prenatal androgen exposure organizes the brain with lasting effects on social behavior.

**Keywords: 2D:4D, digit ratio, sociability, aggression, opioid receptor, social behavior, isolation intolerance**

## INTRODUCTION

During the early prenatal window, androgens and estrogens influence the development with long-lasting effects on the structure and composition of the body and on behavior. Prenatal stress relates to increased androgen load; accordingly, intervention programs to reduce maternal stress during pregnancy are being developed (Lenz et al., 2018b). Animal models (mice, sheep) have shown permanent organizational effects of prenatal testosterone on the brain (Brown et al., 2015; Huber et al., 2018). These early effects also contribute to sex differences in adult behavior.

Aggression and social relationships are subject to gender dimorphisms. In comparison to men, women show less direct aggressive behavior (Archer, 2004), but in conduct disorder, women show

hurtful manipulation of relationships (relational aggression) more often than men (Ackermann et al., 2019). Men less often report having a close confident other than the spouse (Antonucci, 1994), spend less time involved in responding to requests from others (Kessler et al., 1985; Troisi, 2001) and on online social networks (Bouna-Pyrrou et al., 2015, 2018), have a smaller risk to use them pathologically (Bouna-Pyrrou et al., 2015), and are less often the target of online communication (Griffiths et al., 2004). The sex differences may suggest that prenatal exposure to androgens influences aggressive and social behaviors in adulthood. Due to ethical reasons and the long time interval between the prenatal window and adulthood, it is hardly possible to directly investigate the effects of intrauterine sex hormones. Hence, biomarkers have been established. The second-to-fourth finger length ratio (2D:4D) is widely used to study prenatal sex hormone exposure. Reinforced prenatal androgen signaling causes lower 2D:4D in mice (Zheng and Cohn, 2011) and indirect effects of such organizational properties have been also found in humans (Manning et al., 2014), for critical review see Berenbaum et al. (2009), Del Giudice et al. (2018). E.g., human maternal plasma testosterone during pregnancy shows a negative correlation with new borns' digit ratio in both sexes (Ventura et al., 2013), amniotic fluid testosterone is negatively related to 2 year olds' 2D:4D (Lutchmaya et al., 2004), and females with exposure to excessive prenatal testosterone levels due to congenital adrenal hyperplasia (CAH) have lower 2D:4D values than normal controls (Brown et al., 2002; Buck et al., 2003). Hence, lower 2D:4D is indicative of higher prenatal androgen load in humans.

A meta-analysis reported that lower 2D:4D relates to more aggression in men (Hönekopp and Schuster, 2010), although these effects have been found to be small. Furthermore, situational factors, and adult hormone levels play a moderating role (Hönekopp and Watson, 2011). From an evolutionary perspective, one could also expect that social behaviors involving approach and bonding might be related to biological factors such as prenatal sex hormone exposure. Studies investigating social behavior and prenatal testosterone exposure have been conducted under varying contexts and with the use of different methods, ranging from economic games to observation of interactions. The findings have been inconsistent, perhaps due to the complexity of human behavior and its interplay with environmental factors (Millet and Buehler, 2017). Indeed, contextual factors such as the presence of aggressive (Kilduff et al., 2013) or sexual cues (Van den Bergh and Dewitte, 2006), adult hormone levels (Millet and Dewitte, 2008; van Honk et al., 2012; Manning et al., 2014; Portnoy et al., 2015; Millet and Buehler, 2017), cognitive reflection (Millet and Aydinli, 2019), and time-pressure (Bird et al., 2019) moderate the relationship between 2D:4D and prosocial behavior in economic games. Furthermore, the relationships might differ across sex (Hönekopp and Watson, 2011). However, the evidence seems to be more consistent at least at the level of achievements in adults. Within men, more prenatal androgen (lower 2D:4D) is associated with higher academic grade (Nye et al., 2017; Tektas et al., 2019), larger reproductive success (Manning et al., 2000), and higher trading outcome in financial traders (Coates et al., 2009). Thus, in contrast to what one might expect due to the above reported sex differences, men with lower (more masculine) 2D:4D perform better in tasks that require networking or bonding. Accordingly, in men, lower 2D:4D has been related to more fairness (Millet and Dewitte, 2006), stronger cognitive reflection (Bosch-Domenèch et al., 2014), and higher betweenness centrality, i.e., they connect separated parts of the social structure (Kovářík et al., 2017). Moreover, males with lower 2D:4D show more courtship behavior in social interactions with women (Roney and Maestripieri, 2004).

How much people enjoy interacting with others or need to be in company (two factors of sociability) and how many people they know to rely on (social capital) are important determinants of human well-being and health. For example, a low social capital has been associated with negative health outcomes (Murayama et al., 2012) including depression, pain, and psychosomatic symptoms (Åslund et al., 2010). Thus, associations between 2D:4D and health further highlight the importance to understand the role of prenatal androgen exposure in adult social behavior. For example, in males, lower 2D:4D has been associated with lower anxiety (Evardone and Alexander, 2009), a higher risk for conduct problems during childhood (Eichler et al., 2018), addictive and substance use disorders (Kornhuber et al., 2011, 2013; Canan et al., 2017, 2019; Lenz et al., 2017, 2018a, 2019a; Siegmann et al., 2019), suicide (Lenz et al., 2016, 2019b), and reduced life expectancy in adulthood (Lenz and Kornhuber, 2018).

Given the complexity of behavior in experimental tasks or hypothetical trading situations, relatively stable indicators of social behavior, like the personality trait sociability, personal social capital, and personal social network size provide a suitable approach to investigate the link between social behavior and organizational effects of prenatal androgens. Furthermore, the Alternative Five Model (measuring sociability as one of five factors) has been established for traits with a strong biological-evolutionary basis and increases the comparability of our results with animal models (Zuckerman et al., 1993).

Here, we tested whether 2D:4D relates to sociability, personal social capital, and personal social network size in a large population based cohort of 4998 young males. We also explored whether 2D:4D is associated with aggression and anti-social personality characteristics.

# MATERIALS AND METHODS

## Study Sample

The data analyzed here originate in the third survey wave of the longitudinal Cohort Study on Substance Use Risk Factors (C-SURF)[1]. From 2010 to 2012, 7556 young males, who attended their mandatory recruitment for the Swiss army, gave written informed consent and 5987 participated in the first wave. Data for this study were derived from the third wave which has been conducted between April 2016 and

[1] www.c-surf.ch

March 2018 and which has included 5516 males (see[2] for Questionnaire No. 3).

## Behavioral Phenotyping

To measure *sociability*, we used the subscale sociability of the Alternative Five Factor Model (Zuckerman-Kuhlman Personality Questionaire, ZKPQ-50-cc) (Zuckerman et al., 1993) questionnaire, consisting of 10 binary items and its summation score (Aluja et al., 2006). The scale was further divided in the two subscales representing (1) liking lively parties and friends and (2) intolerance of social isolation. *Personal social capital with the subscales bridging and bonding* was quantified by an adaptation of the Personal Social Capital Scale (Archuleta and Miller, 2011; Chang and Zhu, 2012; Wang et al., 2014) with only the 5 most relevant items per subscale selected in C-SURF and a Likert Scale 1–5 to respond. Bonding social capital refers to how well a person is embedded within their various networks of different types of people (e.g., family members, friends, and former colleagues), and bridging social capital refers to how well a person is embedded within different types of social organizations. *Personal social network size* was estimated in C-SURF by two items referring to social network size from the Personal Social Capital Scale (Archuleta and Miller, 2011; Wang et al., 2014). The first item refers to perceived number of friends (from the bonding capital subscale) and the second to the perceived number of cultural, recreational, and leisure groups/organizations in the subject's community (from the bridging capital subscale).

*Aggression* was quantified using the 10 items scale of the ZKPQ-50-cc (Aluja et al., 2006). The score on the *Anti-Social Personality Disorder* scale was probed with items from the Mini-International Neuropsychiatric Interview (M.I.N.I.) with ASSIST-WHO (Sheehan et al., 1998; Hergueta et al., 2015).

## 2D:4D

The participants were instructed to document the lengths of their second and fourth fingers in millimeters separately for their right and left hands (see[2], Questionnaire No. 3 ID: J18) similar to the methods described by Reimers (2007) and Lenz et al. (2018a). The instruction was "Hold your left hand in front of you. Look at where your index finger joins the palm of your hand. Find the bottom crease. Go to the middle of this crease. Put the 0 of your ruler exactly on the middle of the bottom crease (see 2a in the picture below). Make sure the ruler runs straight up the middle of your finger. Measure to the tip of your finger (not your nail see 2b in the picture) in millimeters." Finger lengths under 10 mm or over 100 mm (Reimers, 2007) and, additionally, 2D:4D values outside of the 2.5 and 97.5 percentiles (Hell and Päßler, 2011; Lenz et al., 2018a) separately for the right and left hand were excluded. Subsequent, we calculated the mean of right-hand 2D:4D and left-hand 2D:4D (M2D:4D) which served as our primary predictor. Whereas some studies report that target traits are more strongly related to 2D:4D of the right hand (Manning et al., 1998; Hönekopp and Watson, 2010; Kornhuber et al., 2011; Masuya et al., 2015; Bilgic et al., 2016), other report stronger associations with 2D:4D of the left hand (Muller et al., 2012; Kornhuber et al., 2013; Hong et al., 2014; Lenz et al., 2017, 2019a). As far as we know, there is no reliable explanation for different associations of right- and left-hand 2D:4D with prenatal androgen load. There is also no support for superiority of either side in a meta-analysis on aggression (Hönekopp and Watson, 2011). Separate values for right-hand 2D:4D (R2D:4D), left-hand 2D:4D (L2D:4D), and the difference between R2D:4D and L2D:4D (2D:4Dr-l) were defined as exploratory predictors. Moreover, regarding quality control, we refer to a previous analysis of the same cohort (except for 9 patients with missing data on alcohol-related questions) which showed median values of 2D:4D similar to other studies (Lenz et al., 2019a).

## Statistical Analyses

Continuous data are presented as the median and interquartile range (IQR) and nominal data as frequencies (FREQUENCIES function in SPSS). For missing data points, the corresponding study subjects were excluded from the specific analyses and the number of individuals included in these analyses is reported. Correlations were calculated using Spearman's method, because normal distribution was rejected for all variables. We used the Mann–Whitney *U* test to compare independent groups. For two-sided tests, $p < 0.05$ was considered to be statistically significant. All reported *p*-values are uncorrected for multiple comparisons. Data were analyzed using IBM SPSS Statistics Version 21 for Windows (SPSS Inc., Chicago, IL, United States) and Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, United States).

# RESULTS

## Sample Characteristics

Due to missing values or eliminations resulting from quality control of R2D:4D and L2D:4D, 518 individuals were excluded from the statistical analyses. This resulted in a total cohort of 4998 study subjects and M2D:4D, L2D:4D, and R2D:4D sub-cohorts of 4778, 4898, and 4878 individuals. The total cohort was characterized as follows: age 25 years (IQR 25–26; $N = 4998$); body mass index 23.5 kg/m$^2$ (IQR 21.8–25.5; $N = 4990$); 79.6% gainfully employed ($N = 4997$); 3.0% secondary education, 1.2% basic vocational education, 34.4% secondary vocational/technical education, 4.3% community college, 11.2% vocational high school, 11.8% high school, 23.4% bachelor (university), 6.1% master (university), 4.6% other ($N = 4985$); 82.9% single, 5.2% married, 0.1% divorced, 11.6% not married, not separated, not divorced but living together with my partner (e.g., in registered partnership), 0.1% married but separated, 0.1% widowed ($N = 4989$).

## Trait Sociability

Lower M2D:4D (indicative of higher levels of prenatal androgen exposure) correlated with higher trait sociability ($\rho = -0.043$, $N = 4755$, $p = 0.003$), and both L2D:4D and R2D:4D correlated similarly with trait sociability ($\rho = -0.045$, $N = 4875$, $p = 0.002$; $\rho = -0.032$, $N = 4855$, $p = 0.024$). 2D:4Dr-l did not correlate with

[2]www.c-surf.ch/img/questionnaires_pdf/q3_follow_up2_en.pdf

trait sociability ($p > 0.05$). As shown in **Table 1**, both subscales of sociability correlated significantly with 2D:4D.

Statistics for the M2D:4D differences for the 10 individual binary items (*post hoc* analysis) are shown in **Supplementary Table S1**. Specifically, the items "At parties, I enjoy mingling with many people whether I already know them or not." and "I am a very sociable person." were significantly associated with lower M2D:4D.

## Personal Social Capital

Lower M2D:4D correlated with bigger personal social capital ($\rho = -0.040$, $N = 5762$, $p = 0.005$), and both L2D:4D and R2D:4D correlated similarly with bigger personal social capital ($\rho = -0.036$, $N = 4882$, $p = 0.012$; $\rho = -0.013$, $N = 4861$, $p = 0.039$). 2D:4Dr-l did not correlate with personal social capital ($p > 0.05$). **Table 2** shows the results of the *post hoc* analysis on subscale level.

Item level analysis revealed significant correlations with the items "interacting with people makes me feel like a part of a large community," "the people I interact with would be good job references for me" and "if I needed an emergency loan, I know someone I can turn to", for details see **Table 3**.

## Personal Social Network Size

2D:4D correlated negatively with the personal social network size (**Figure 1**).

**TABLE 1 |** *Post hoc* analysis Sociability: Spearman correlations at facet level.

| Sociability | | M2D:4D | L2D:4D | R2D:4D | 2D:4Dr-l |
|---|---|---|---|---|---|
| Parties/Friends | ρ | −0.036 | −0.031 | −0.034 | −0.008 |
| | *p* | **0.012** | **0.029** | **0.019** | 0.600 |
| | *N* | 4763 | 4883 | 4863 | 4763 |
| Isolation Intolerance | ρ | −0.035 | −0.041 | −0.020 | 0.018 |
| | *p* | **0.017** | **0.005** | 0.172 | 0.221 |
| | *N* | 4760 | 4880 | 4860 | 4760 |

*2D:4D, second-to-fourth-finger length ratio; primary predictor: M2D:4D, mean of R2D:4D and L2D:4D; exploratory predictors: L2D:4D, left-hand 2D:4D; R2D:4D, right-hand 2D:4D; 2D:4Dr-l, difference between R2D:4D and L2D:4D. P < 0.05 (uncorrected) in bold. Cronbach's alpha: Parties/Friends 0.48, Isolation Intolerance 0.57.*

**TABLE 2 |** *Post hoc* analysis Personal social capital: Spearman correlations at subscale level.

| Personal social capital | | M2D:4D | L2D:4D | R2D:4D | 2D:4Dr-l |
|---|---|---|---|---|---|
| Bridging | ρ | −0.032 | −0.032 | −0.030 | 0.011 |
| | *p* | **0.026** | **0.026** | **0.035** | 0.443 |
| | *N* | 4764 | 4884 | 4863 | 4764 |
| Bonding | ρ | −0.038 | −0.031 | −0.032 | −0.001 |
| | *p* | **0.009** | **0.030** | **0.025** | 0.968 |
| | *N* | 4768 | 4888 | 4867 | 4768 |

*2D:4D, second-to-fourth-finger length ratio; primary predictor: M2D:4D, mean of R2D:4D and L2D:4D; exploratory predictors: L2D:4D, left-hand 2D:4D; R2D:4D, right-hand 2D:4D; 2D:4Dr-l, difference between R2D:4D and L2D:4D. P < 0.05 (uncorrected) in bold. Cronbach's alpha: Bridging 0.79, Bonding 0.83.*

## Aggression and Anti-social Personality

M2D:4D, L2D:4D, R2D:4D, or 2D:4Dr-l did not correlate with aggression or anti-social personality disorder score ($p > 0.05$, **Supplementary Table S2**).

# DISCUSSION

Here, we report that higher sociability and bigger personal social capital are correlated with lower 2D:4D in a population-based cohort of young Swiss men. Notably, both factors of sociability, liking lively parties and friends and intolerance of social isolation (Zuckerman et al., 1993), correlated independently with 2D:4D across both hands. Furthermore, we provide preliminary evidence for an association between bigger personal social network size and lower 2D:4D. These results suggest that, in men, higher prenatal androgen exposure improves sociability and leads to a bigger social capital and social network size in adulthood. Our observation is consistent with a study showing that prenatal testosterone as measured in amniotic fluid during 13–20 weeks of gestation is associated with approach behavior and reactivity to happy faces in brain reward areas of boys (Lombardo et al., 2012). The large sample size of nearly 5000 study participants analyzed here is a major strength of this project. It is limited by the 2D:4D self-measurement method which is related to reduced reliability in comparison to expert measured 2D:4D (Hönekopp and Watson, 2010).

Sociability involves the opioid system of the brain (Knowles et al., 1989; Kalin et al., 1995). In animal experiments, prenatal androgen receptor inhibition by flutamide down-regulates cerebral expression of the μ opioid receptor 1 in adulthood (Huber et al., 2018). In line with this association between prenatal sex hormone effects and opioid signaling, R2D:4D in men has been related to genetic polymorphisms in opioid receptors (Pearce et al., 2018). During social laughter – related to the sociability factor "party and friends" – endogenous opioids are released, and the depletion during social isolation motivates to seek company – related to the sociability factor "isolation intolerance" (Knowles et al., 1989; Kalin et al., 1995). The minor G-allele of the μ-opioid receptor 1 polymorphism rs1799971 is associated with more pleasure experienced in social situations (Troisi et al., 2011), and mice with this variant have increased motivation for non-aggressive social interactions and show less avoidance after social defeat (Briand et al., 2015). Taken together, prenatal androgen exposure may organize cerebral opioid signaling with behavioral effects on sociability. Future research should investigate how prenatal influences might interact with genetics to affect sociability.

We found lower 2D:4D to be associated with higher sociability. Our findings are in line with previous reports on higher betweenness centrality in men with lower 2D:4D, i.e., these subjects connect separated parts of the social structure (Kovářík et al., 2017). Furthermore, academic, reproductive, and trading success, all negatively correlated with 2D:4D (Manning et al., 2000; Coates et al., 2009; Nye et al., 2017; Tektas et al., 2019), have networking as an essential common mechanism to success.

**TABLE 3 |** *Post hoc* analysis Personal social capital: Spearman correlations at item level.

| Personal social capital | M2D:4D | | | L2D:4D | | | R2D:4D | | | 2D:4Dr-l | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | *p* | *N* | ρ | *p* | *N* | ρ | *p* | *N* | ρ | *p* | *N* |
| Interacting with people makes me want to try new things | −0.026 | 0.077 | 4771 | −0.021 | 0.138 | 4891 | −0.023 | 0.107 | 4870 | 0.009 | 0.514 | 4771 |
| Interacting with people makes me interested in what people unlike me are thinking | −0.018 | 0.206 | 4771 | −0.019 | 0.189 | 4891 | −0.013 | 0.355 | 4870 | 0.009 | 0.535 | 4771 |
| Interacting with people makes me feel like a part of a large community | −0.038 | **0.009** | 4770 | −0.034 | **0.019** | 4890 | −0.036 | **0.011** | 4869 | 0.009 | 0.551 | 4770 |
| Interacting with people makes me feel connected to the bigger picture | −0.008 | 0.567 | 4765 | −0.014 | 0.322 | 4885 | −0.010 | 0.492 | 4864 | 0.016 | 0.281 | 4765 |
| I come into contact with people all the time | −0.012 | 0.390 | 4770 | −0.016 | 0.258 | 4890 | −0.006 | 0.693 | 4869 | 0.012 | 0.400 | 4770 |
| There are several people I trust to solve my problems | −0.024 | 0.099 | 4769 | −0.026 | 0.074 | 4889 | 0.013 | 0.380 | 4868 | 0.013 | 0.365 | 4769 |
| If I needed an emergency loan, I know someone I can turn to | −0.037 | **0.010** | 4769 | −0.027 | 0.061 | 4889 | −0.033 | **0.022** | 4868 | −0.006 | 0.700 | 4769 |
| There is someone I can turn to for advice about making very important decisions | −0.016 | 0.261 | 4770 | −0.008 | 0.589 | 4890 | −0.020 | 0.170 | 4869 | −0.019 | 0.193 | 4770 |
| I know several people well enough to get them to do anything important | −0.020 | 0.165 | 4770 | −0.017 | 0.224 | 4890 | −0.017 | 0.235 | 4869 | 0.001 | 0.963 | 4770 |
| The people I interact with would be good job references for me | −0.031 | **0.031** | 4770 | −0.027 | 0.057 | 4890 | −0.025 | 0.082 | 4869 | 0.010 | 0.503 | 4770 |

*2D:4D, second-to-fourth-finger length ratio; primary predictor: M2D:4D, mean of R2D:4D and L2D:4D; exploratory predictors: L2D:4D, left-hand 2D:4D; R2D:4D, right-hand 2D:4D; 2D:4Dr-l, difference between R2D:4D and L2D:4D. P < 0.05 (uncorrected) in bold.*

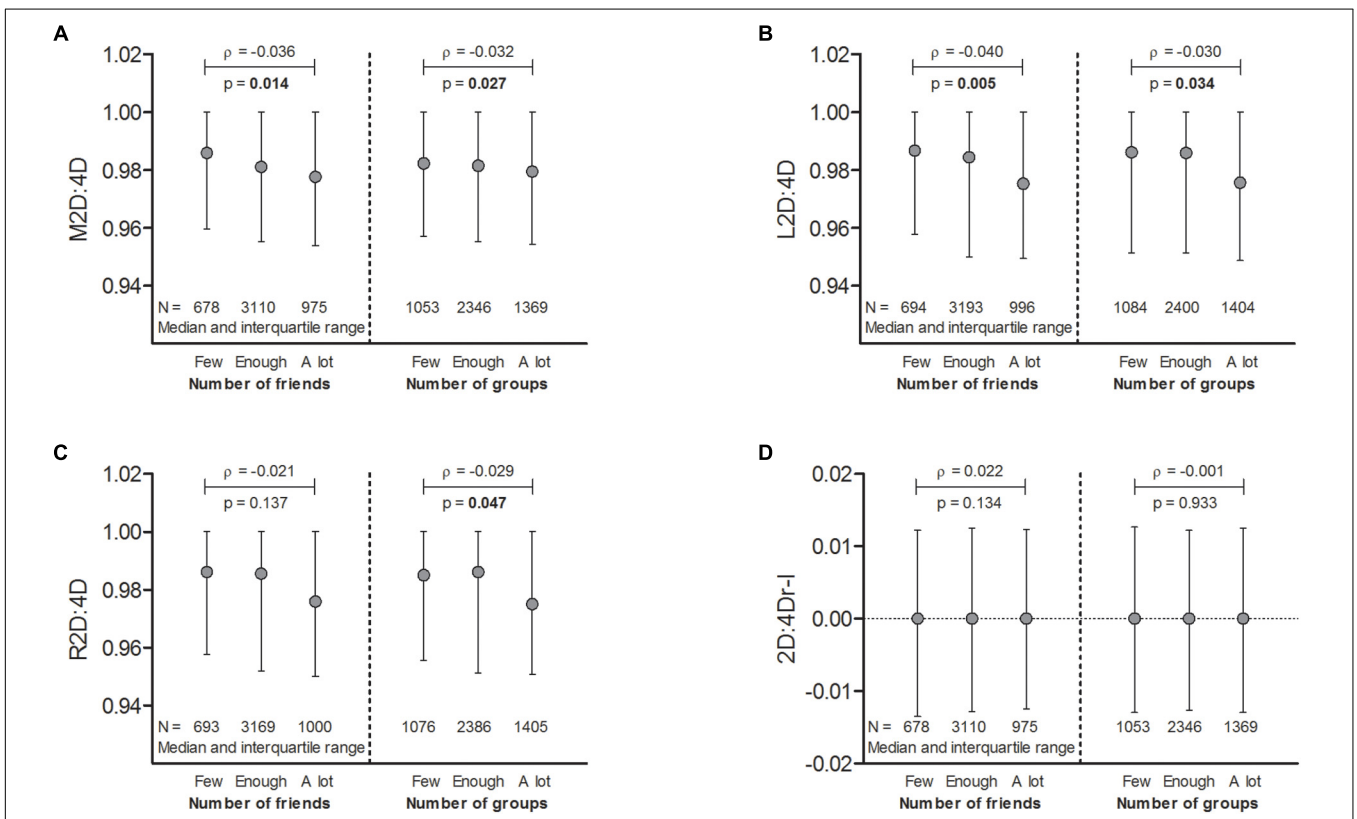


**FIGURE 1 |** The M2D:4D **(A)**, L2D:4D **(B)**, and R2D:4D **(C)** ratios [but not 2D:4Dr-l **(D)**] were negatively correlated with self-reports for the number of friends and the number of cultural, recreational, and leisure groups/organizations/associations in one's community. 2D:4D, second-to-fourth-finger length ratio; primary predictor: M2D:4D, mean of R2D:4D and L2D:4D; exploratory predictors: L2D:4D, left-hand 2D:4D; R2D:4D, right-hand 2D:4D; 2D:4Dr-l, difference between R2D:4D and L2D:4D. $P < 0.05$ (uncorrected) in bold.

Hence, higher sociability might mediate the relationship between lower 2D:4D and successfulness in men.

In our adult cohort, we did not find any significant correlation between 2D:4D and aggression, which might be explained by the low precision due to the employed self-measurement technique and the fact that correlations of aggression and 2D:4D in adults are mainly found in challenging situations (Hönekopp and Watson, 2011) and in other situations are small at the best (Hönekopp and Watson, 2011).

By contrast, we found lower 2D:4D to be associated with higher sociability. At the first glance, our findings may contradict that lower 2D:4D (indicative of higher prenatal androgen exposure) relates to behavioral symptoms in boys (Williams et al., 2003; Eichler et al., 2018), which entails problems in social interaction. Aggression, fighting, and lacking obedience are characteristics of conduct disorder. However, the frontal lobe and cognitive reflection are still developing in children. Frontal lobe development and cognitive reflection inhibit aggressive outbursts and the shift of neural regulation to prefrontal areas takes place during puberty (Cubillo et al., 2012; Rubia et al., 2013; Tyborowska et al., 2016). In adulthood, cognitive reflection is higher in individuals with lower 2D:4D (Bosch-Domenèch et al., 2014) and probably explains the moderating role of sexual and aggressive cues on the relationship between 2D:4D and aggressive behavior (Hönekopp and Watson, 2011). Without a situationally triggered testosterone surge, aggression as a trait is less evident in daily life and cognitive reflection might counteract aggressive trends in men with low 2D:4D. Boys with higher sociability (following higher prenatal androgen load) may be involved into fights more often due to the increased total frequency of interactions with others and given the fact that physical aggression is used instrumentally in healthy young children.

In support of this developmental view on aggression, we also did not find a correlation of prenatal testosterone with anti-social personality disorder (ASPD) items. Whereas conduct disorder increases the risk for ASPD (Olsson and Hansson, 2009), other factors like intelligence, parent psychopathology, parent-child relation, and peer-rejection are known to moderate this risk essentially (Olsson and Hansson, 2009).

In this study, lower 2D:4D correlated with bigger personal social capital and a larger personal social network. Here, we will argue that negotiation strategies, which are conceptually related to social networking, change from children to adulthood into more functional behavior in people with lower 2D:4D. In adult men, lower 2D:4D is associated with more uncooperative behavior, but only when they act intuitively or less reflected (Millet and Buehler, 2017; Millet and Aydinli, 2019) and as already mentioned, men with lower 2D:4D have stronger cognitive reflection skills (Bosch-Domenèch et al., 2014). In general, adult men with low 2D:4D prefer fair from either altruistic or egoistic choices (Millet and Dewitte, 2006), even though their faces appear more dominant to others (Neave et al., 2003). In children, however, a lower 2D:4D is still unrelated to fair choices and correlates with less altruistic choices instead (Millet and Dewitte, 2006). In adults, social status relevance (potentially leading to a surge in testosterone) within a given context moderates the impact of 2D:4D on cooperative behavior, aggression, and dominance in economic games (Millet and Buehler, 2017). Taken together, evidence on negotiation strategies of lower 2D:4D subjects supports our findings on the relationship between 2D:4D, social capital, and network size.

Furthermore, children with a higher status – as measured in number of friends/interaction partners – choose the prosocial option less often (Horn et al., 2018). In contrast to our data from adults, in which a bigger social capital and a larger social network are associated with lower 2D:4D, in boys the strategies to gain status may still be dysfunctional, as a link with number of friends/interaction partners and 2D:4D was not found (Horn et al., 2018).

The relationship between sociability, aggression, and behavioral strategies to gain status or bond might change from childhood to adulthood, when cognitive reflection and the frontal lobe have fully developed. As a consequence, normative behavior, learned cooperation, and fairness may be utilized by adult men with low 2D:4D, at least in unchallenging situations. Furthermore, experiences from frequent social interactions (sociability) and from testing the limits with others during childhood (instrumental aggression) might in the end help to bond with others and make these subjects more resilient, explaining the long term positive outcomes of men with lower 2D:4D in academia (Nye et al., 2017; Tektas et al., 2019), reproduction (Manning et al., 2000) and trading (Coates et al., 2009).

Although we found that low 2D:4D in men is associated with higher trait sociability and possibly more social bonds to rely on, there is evidence for a more avoidant attachment style (Del Giudice and Angeleri, 2016) and lesser quality of relationships in people with low 2D:4D (Knickmeyer et al., 2005). Furthermore, intimate partner violence is actually higher in low 2D:4D men (Romero-Martínez et al., 2013). Thus, sociability and a bigger social capital in men do not necessarily mean that intimate or close relationships are better on the long term. They might even be worse as subjects are more directed at social status than intimacy.

G-allele carriers of the μ opioid receptor 1 polymorphism rs1799971 experience more pleasure in social situations (Troisi et al., 2011) and alcohol-dependent G-allele carriers show increased cue-reactivity to alcohol stimuli in certain brain regions which correlates with craving (Bach et al., 2015). As endogenous opioids contribute to the punishing effects of social isolation and rejection (Knowles et al., 1989; Kalin et al., 1995; Briand et al., 2015), it is interesting that an interaction between 2D:4D and the rs1799971 polymorphism has been reported for alcohol dependence (Gegenhuber et al., 2018). Both aspects of sociability, the interest in parties and friends und isolation intolerance, which correlated with 2D:4D in our study, might influence the development of alcohol dependency. This study's results indicate that the pleasure to bond with others and enjoy social laughter is increased in people with low 2D:4D which might lead to more reward (opioid release) experienced during these situations. This mechanism might potentiate the rewarding effect of consumption (again opioid release) by increased chances of social laughter and bonding. Finally, also isolation intolerance

might play a role, as it might induce drinking behavior to cope with loneliness. However, further research is needed to test these hypotheses.

At first glance, the observed negative correlation between 2D:4D and social network might contradict the fact that lower 2D:4D has been associated with suicide completion (Lenz et al., 2016) because social connectedness has been shown to be protective against suicidal behaviors (Fässberg et al., 2012). However, for suicide completion, it has been argued that correlations of lower 2D:4D with stronger cognitive reflection (Bosch-Domenèch et al., 2014; Millet and Aydinli, 2019) might play a role, leading to better planned and more successful suicide attempts, as 2D:4D measured independently from cognitive reflection is unrelated to suicidal thoughts and attempts (Lenz et al., 2019b).

## LIMITATIONS

Self-measured 2D:4D is less reliable than expert-measures and is said to reach only 46% of its reliability (Hönekopp and Watson, 2010). Furthermore, finger deformation was not assessed in this project, which has reduced precision. We are aware of current criticism on 2D:4D as a proxy for prenatal androgen exposure, as the experimental evidence used to support the validity of 2D:4D as a biomarker of prenatal androgen exposure has not been replicated consistently (Berenbaum et al., 2009; Huber et al., 2017; Del Giudice et al., 2018).

Besides correlating our primary independent variable with our dependent variables, we extended the analysis to exploratory testing of left and right hand 2D:4D and asymmetries of left and right hand 2D:4D, but did not correct for multiple hypothesis testing which might have resulted in false positive findings in the exploratory analysis.

Personal social capital was assessed in C-SURF only with a selection of items from the Personal Social Capital Scale, using only the 5 most relevant items per subscale. Even though we found a good internal consistency of 0.85 Cronbach's alpha, construct validity remains unknown for this subset of items.

Personality disorder diagnoses like anti-social personality disorder should be assessed by experienced clinicians using structured clinical interviews (Paap et al., 2017). Here, we correlated the summation score of a self-report screening instrument with unknown discriminability for this clinical disorder.

We did not find a correlation between 2D:4D and aggression as a personality factor. In a meta-analysis on 2D:4D and aggressive behavior, it was reported that any correlation found appear to be very small and findings are context dependent (Hönekopp and Watson, 2011). We investigated the personality factor aggression with a questionnaire and did not use an experimental setup with provocative cues or interaction partners. Furthermore, we face a lower reliability of self-measured 2D:4D measures in comparison to expert ratings. Moreover, sex differences in aggression appear to be larger in children than in adults (Campbell, 2006; Archer, 2009) and our adult cohort is rather homogeneous in age.

Exploratory analysis of social network size was only probed by two self-reported items and future research should use more reliable and objective measures to investigate the relationship between 2D:4D and social network size.

Finally, our cohort consisted of mostly Caucasian young men and the results cannot be transferred to other ethnicities, gender, or age groups.

## CONCLUSION

To summarize, our data show that low 2D:4D is associated with higher trait sociability, bigger personal social capital, and larger personal social network size. Given the complexity of human behavior and environmental/nurture effects on personality, it is not surprising that the correlations are small though. Our study provides a better understanding of the link between prenatal influences and social behavior in adulthood. It also leads to an interesting hypothesis on the mediating role of sociability between prenatal environment and life achievements, behavioral problems in adolescence, and other health related aspects.

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## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This study was approved by the Ethics Committee for Clinical Research of Lausanne University Medical School (Protocol No. 15/07). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

VB and BL conceived and designed the research, analyzed the data, and wrote the manuscript. GG, MM-K, SM, SF, and JS performed the experiments. CM and JK commented on the manuscript and provided the intellectual input.

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## SUPPLEMENTARY MATERIAL

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# The Relationships Among Testosterone, Cortisol, and Cognitive Control of Emotion as Underlying Mechanisms of Emotional Intelligence of 10- to 11-Year-Old Children

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Emotional intelligence is an important factor contributing to social adaptation. The current study investigated how salivary testosterone (T) and cortisol (C) levels, cognitive control of emotional conflict processing were associated with children's emotional intelligence (EI). Thirty-four 10- to 11-year-old children were enrolled and instructed to complete questionnaires on emotional intelligence as well as empirical tasks of emotional flanker and Stroop with event-related potential (ERP) recordings. Saliva collection took place on another day without ERP tasks. Results showed that lower T and C levels were associated with higher accuracy in emotional conflict tasks, as well as better emotional intelligence (managing self emotions). In the Stroop task, higher T/C ratios were associated with greater congruency effects of N2 latencies, and lower cortisol levels correlated with stronger slow potential activities (SP). For girls, the correlation between cortisol and emotional utilization was mediated by the SP amplitudes on fearful conflicts in the flanker task (95% CI: −8.64, −0.54, $p < 0.050$). In conclusion, the current study found the relationship between cortisol and an emotional intelligence ability, emotional utilization, might be mediated by brain activities during emotional conflict resolution processing (SP responses) in preadolescent girls. Future studies could further investigate testosterone-cortisol interaction and its relation with cognitive control of emotion as underlying mechanisms of emotional intelligence.

**Keywords: emotional conflict control, cortisol, testosterone, emotional intelligence, preadolescence, event-related potentials**

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## INTRODUCTION

The perception, processing, regulation, and utilization of emotional information is well-known as emotional intelligence (EI) (Nelis et al., 2009), which intrinsically includes self-control of emotions (Davis and Rachel, 2016). EI is another phrase for emotional competence (Mayer and Salovey, 1993), which is critical for solving problems like conflict. There is a lot of evidence suggesting that EI contributes to successful social adaptation (Martins et al., 2010; Punia and Sangwan, 2011; Davis and Rachel, 2016). However, EI may also contribute to negative emotional manipulation

(Davis and Rachel, 2016). Today training and intervention of EI are of interest to scientists and the public, yet there is limited evidence of the underlying neural and hormonal mechanisms. Positive relationships were reported between EI and cognitive control (Checa and Fernández-Berrocal, 2015). Cognitive control can be influenced by sub-cortical emotional (bottom–up) processing, and this communication is mediated by testosterone and cortisol (Terburg et al., 2009). High testosterone was reported to down-regulate the interaction between cognitive and emotional systems and therefore diminishes the impact of cognitive control (Schutter and Van Honk, 2004). Basal cortisol also seems to correlate with cognitive control (Schutter and Van Honk, 2005). Few studies have took testosterone, cortisol and cognitive control processings as mechanisms underlying emotional intelligence, although there were some studies concerning other hormones and emotional perception in the context of emotional intelligence (Cardoso et al., 2014; Koven and Max, 2014; Milivojevic et al., 2014; do Vale et al., 2015). It is possible that the ratios of the basal levels of testosterone and cortisol are closely related to emotion regulation (Van Honk and Schutter, 2006). In this study we concentrate on the correlations among the hormones testosterone and cortisol, the cognitive control of emotion and EI in children.

Top–down modulation of emotional processing has been investigated as the cognitive control of emotion (Ochsner and Gross, 2005). According to the dimensional overlap theory (Kornblum et al., 1990, 1999), conflicts can be further categorized based on the overlap between the response (R), the task-relevant stimulus (SR), and the task-irrelevant stimulus (SI). The emotional flanker task contains the stimulus-stimulus (S-S) conflicts that SR (the target emotional facial expression) overlaps with SI (the bilateral distractor emotional faces) (Fenske and Eastwood, 2003; Liu et al., 2013). The emotional Stroop task contains both S-S and S-R conflicts, with the affective word ("FEARFUL" or "HAPPY") on an emotional (happy or fearful) face, and participants are required to report the expression on the face (Etkin et al., 2006, 2010; Egner, 2008; Liu et al., 2010; Chechko et al., 2012; Soutschek and Schubert, 2013). It is reported that S-S and S-R conflicts involve distinct conflict control processes (Egner et al., 2007; Akcay and Hazeltine, 2011; Li et al., 2015) and rely on different neural substrates, since S-R conflict tasks activated the anterior cingulate cortex (ACC), precuneus and supplementary motor areas and S-S conflict tasks activated the inferior parietal cortex (Liu et al., 2004). The empirical study adopting color-object Stroop task to investigate the developmental changes of stimulus (S) interference and response (R) interference in 6–10 years old children found that the response interference control matured later (at age 10–12 years) than the stimulus interference control (at age 6–7 years), which further suggested the distinctive conflict control processes on the S-S and S-R conflicts from the child development evidences (Jongen and Jonkman, 2008).

Electrophysiological studies showed that conflict control processes are composed of two subprocessings: the N2 component of event-related potentials (ERPs), peaking at approximately 200 ms after stimulus onset, is associated with detection on both emotional (Shen et al., 2013; Fan et al., 2016; Xue et al., 2016) and non-emotional conflicts (Liu et al., 2010; Larson et al., 2014). The P3 and/or slow potential (SP) components, with a central-parietal neural distribution, are related to conflict resolution on both emotional (Fan et al., 2016; Xue et al., 2016) and non-emotional conflicts (Jonkman, 2006; Abundis-Gutiérrez et al., 2014). Both the cognitive control of emotional and non-emotional conflicts has been shown to activate the ACC, the dorsolateral prefrontal cortex (DLPFC), the parietal regions, the insula and the visual cortex (Chechko et al., 2012; Soutschek and Schubert, 2013).

Development of the interaction between emotional brain and cognitive brain could impact cognitive control of emotional processing throughout childhood and adolescence (Heller et al., 2016). The triple balance model of emotion (Van Honk and Schutter, 2006) hypothesized that the balance between the emotional brain and the cognitive brain would influence emotional processing at three levels which were linked to testosterone and cortisol. It has been reported that the first significant increase of testosterone occurs at 10 (SizoNenko and Paunier, 1975). Puberty peaks in brain functional activity showed to be related to testosterone (Braams et al., 2014). Previous studies demonstrated a possible negative association between testosterone levels and cortical response to word-face Stroop conflicts in 10- to 15-year-old adolescents (Cservenka et al., 2015), but during adults' cognitive control of emotional processing, lower testosterone levels were associated with both stronger (Volman et al., 2011) and weaker response (Van Strien et al., 2009). There were also inconsistent results of the associations between the cortisol levels and executive functions (Raffington et al., 2018). Conflict detection might be influenced by testosterone and cortisol, since cortisol might enhance fear sensitivity (Watling and Bourne, 2013) and testosterone might enhance reward sensitivity (Van Honk and Schutter, 2006). According to the three balance model of emotion and the existing evidence, the testosterone/cortisol ratios can be related to cortical activities and behavioral tendencies in emotional processing (Terburg et al., 2009), but there are little evidence about the associations between testosterone/cortisol ratios and neural dynamic processing of emotional cognitive control.

To our knowledge, this is the first study to investigate the possible mediation effects of cognitive control of emotion on the relationships between hormones and EI components. The aims of the current study were to investigate the relationships among EI, the emotional conflict processing, and daily circulating testosterone and cortisol in 10- to 11-year-old children. We mainly focused on 10- to 11-year-old children in the current study for the following reasons. First, it is found that testosterone show their first significant rise at 10 years old and further lead the important influence on the brain reactivity (SizoNenko and Paunier, 1975; Nguyen et al., 2013). Second, most studies found that it is a crucial age for the neurodevelopment of cognitive control abilities (Waxer and Morton, 2011; Larson et al., 2012; Erb et al., 2017). Different types of conflict control develop at different speeds and with varied patterns (Jongen and Jonkman, 2008; Bryce et al., 2011) and may be indistinguishable in children up to 9 years of age, but may be related yet separable by 10–11 years of age (Brydges et al., 2014).

Third, but not the least, at this age, the neural function of frontal and limbic areas gets more mature and starts to strengthen the interconnections to facilitate children's perception and regulation on emotional information and states (Derntl et al., 2009; Sapienza et al., 2009; Stanton et al., 2011; Terburg et al., 2012). We adopted the emotional flanker task and emotional Stroop task to measure the emotional conflict control processing. We hypothesized that children's high testosterone/cortisol ratios would diminish the efficiency of brain activities in emotional conflict control processes, and the efficiency of emotional conflict control processing would be positively associated with EI.

# MATERIALS AND METHODS

## Participants

Thirty-six right-handed children participated in the experiment, and the data of two children were removed from analysis because of too much head movement. The data of remaining 34 children (16 girls [10.56 ± 0.32 years old] and 18 boys [10.90 ± 0.29 years old]) were further analyzed. The parents of child participants accomplished the written questionnaire, in which they were asked that whether the participant and his/her family had the neurological and/or psychiatric disorders. None of the participants reported that he/she or his/her family had neurological or psychiatric disorders. All the participants had normal or corrected-to-normal visual acuity, and they were naïve to the purpose of the experiment. This study was approved by the Ethics Committee of the Institute of Psychology, Chinese Academy of Sciences and School of Psychology, Capital Normal University. The work described has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and Uniform Requirements for manuscripts submitted to biomedical journals. The ERP, behavioral measurements and saliva collection were all undertaken with the understanding and written consent of the participants' parents.

## Procedure

The brief outline of the time course of the tests: first, the salivary collection for all participants was on a school day before the ERP tasks; second, the completion of the emotional flanker and Stroop tasks with EEG recording; then, the completion of paper-pencil version of Emotional Intelligence Scale questionnaire (EIS).

## Salivary Collection and Analysis

Salivary testosterone and cortisol samples were collected within the same hours from all participants to minimize seasonal and diurnal variation as much as possible. Salivary collection was scheduled on a school day when no EEG recordings were scheduled. Participants were asked to rinse their mouths thoroughly 1 h before the saliva collection to avoid contamination from food debris and prevent sample dilution. During the interval between the mouth rinse and the saliva collection, the participants were asked to abstain from food or drink. Saliva samples were collected twice during a half-hour period from 8:40 a.m. to 9:10 a.m. Participants were instructed to pull the sponge out of the Salivette and place it into their mouth. They were told to chew the sponge very gently and roll it around in their mouth for 2 min. Saliva samples were then stored at −20°C as soon as possible. Testosterone and cortisol levels were determined using testosterone and cortisol ELISA kits (DRG, Germany). For testosterone, the reported inter- and intra-assay coefficients of variance were < 9.6 and 13.8%, respectively, with an analytical sensitivity of 1.9 pg/ml. For cortisol, the reported inter- and intra-assay coefficients of variance were < 7.5 and 4.5%, respectively, with an analytical sensitivity of 0.537 ng/mL.

## Emotional Flanker and Stroop Tasks

Each participant was instructed to participate in two computerized emotional conflict control tasks with EEG recordings, including the emotional flanker and the emotional Stroop task. The presentation sequence of these two tasks was counterbalanced among participants.

### Emotional Flanker Task

The facial images in the revised emotional flanker task (Fenske and Eastwood, 2003; Liu et al., 2013) were of the face of six models (three males, three females) displaying both happy and fearful faces. The face stimuli were from our lab's collection, and they were collected and used under the standardized procedure. Another 30 volunteers (male, 16, female, 14; age range 22.3–28.7 years) were instructed to assess the valence and arousal of each facial image by using normative nine-point scale. For the valence rating, happy images (Mean = 7.71, Standard deviation [SD] = 0.34) featured higher valence scores than fearful images (Mean = 2.11, $SD = 0.31$) ($p < 0.001$). For the arousal rating, there were no significance between happy (Mean = 6.82, $SD = 0.35$) and fearful images (Mean = 7.01, $SD = 0.33$) on arousal scores ($p > 0.05$). Each stimulus consisted of a central target face and two bilateral rows of two faces each, with the five faces in each stimulus from a single model. The visual angle of each stimulus was 1° vertical and 3.8° horizontal. According to the target facial expressions and the congruency between the target facial expression and the bilateral facial expressions, there were four types of trials: target fearful face in congruent trials (FFFFF), target fearful face in incongruent trials (HHFHH), target happy face in congruent trials (HHHHH), and target happy face in incongruent trials (FFHFF).

### Emotional Stroop Task

The stimuli in the revised emotional Stroop task (Etkin et al., 2006) were the same with the emotional flanker task. Each stimulus consisted of a gray facial expression image overlaid with the red Chinese word "愉快" (happy) or "恐惧" (fearful). The visual angle was approximately 1° × 1.8°. Participants were instructed to concentrate on the facial expression and ignore the word over each presented stimulus. In the congruent condition, the facial expression was compatible with the meaning of the word. For the incongruent condition, the facial expression was incompatible with the meaning of the word.

For these two tasks, all the stimuli were presented on a computer monitor with a black background (17 inches, 1024 × 768 at 100 Hz), and the participant's viewing distance was

65 cm. Participants were required to press the left or right button to judge whether the facial expression was happy or fearful, and the mappings of the left and right index fingers to the happy and fearful stimuli were counterbalanced among participants. In both tasks, each trial began with a grey fixation cross " + " displayed for 250 ms and the presentation of a stimulus for 1500 ms, followed by randomly varied inter-trial intervals (ITIs) between 800 and 1000 ms. In the practice section, a total of 16 trials were displayed to allow participants to become familiar with the response rules, and the formal experiment section consisted of 4 blocks with 65 trials in each block. Each task consisted of 65 congruent trials with happy faces, 65 congruent trials with fearful faces, 65 incongruent trials with happy faces, and 65 incongruent trials with fearful faces. The participants were permitted to rest for 2–3 min after each block, and the whole task lasted approximately 18 min. In the two tasks in our study (see **Figure 1**), fearful conflict refers to cognitive conflict in trials with fearful target face. Happy conflict refers to cognitive conflict in trials with happy target face.

## EIS Questionnaire Measurement

Participants were required to complete the Chinese version of the EIS, which has been found to be suitable for measuring emotional intelligence of children and adolescents (Wang, 2002). EIS contains 33 items and is used to measure four EI abilities with the Emotion Perception (EP), Managing Self Emotions (MSE), Managing Others' Emotions (MOS), and Emotional Utilization (EU) subscales (Wang, 2002; Sevdalis et al., 2007).

## EEG Recording and Data Analysis

The electroencephalogram (EEG) was recorded from sixty-four electrodes embedded in a Neuroscan cap with the electrodes placed according to the 10–20 system locations. Four bipolar electrodes were placed on the outer canthi of both eyes and the inferior and superior areas of the left eye to monitor the vertical and horizontal electrooculogram (VEOG and HEOG, respectively). The EEG signal with a nose reference was continuously recorded with online filters at 0.05–100 Hz and was amplified at a sampling rate of 1000 Hz. The electrode impedance was kept below 5 k$\Omega$. The signal was epoched into trials with 100 ms prior to (for baseline correction) and 1000 ms after the stimulus onset, and epochs exceeding $\pm$ 100 $\mu$V at any electrode were excluded. The averaged ERPs were further digitally filtered off-line (zero phase shift; bandwidth: 1 and 30 Hz; slope: 24 dB/octave). The N2 and SP components were further analyzed according to previous literature (Larson et al., 2014) and current ERP data. The N2 components were analyzed over the fronto-central brain areas (average from F3, FC3, Fz, FCz, F4, and FC4) during the 220–370 ms time window after stimulus onset. The SP component was analyzed over the parieto-central areas (average from CP3, P3, CPz, Pz, CP4, and P4) in a time window of 510–680 ms.

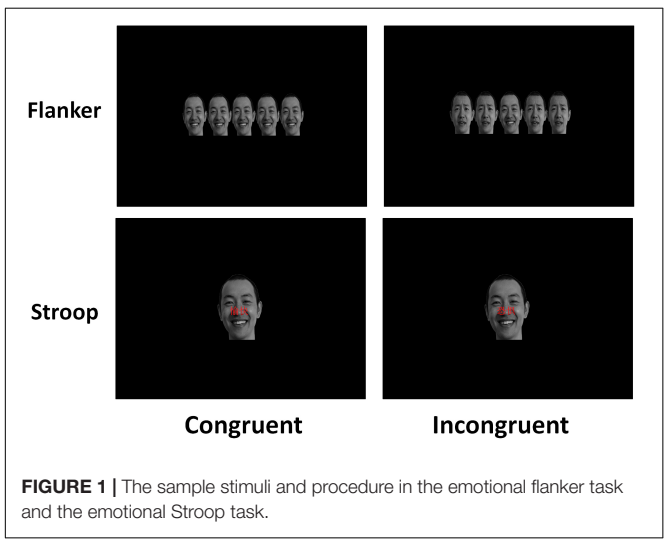


**FIGURE 1 |** The sample stimuli and procedure in the emotional flanker task and the emotional Stroop task.

For the behavioral and electrophysiological data analyses of the conflict control tasks, the response accuracy, reaction time (RT), and mean amplitudes and peak latencies of the N2 and SP components were analyzed with $2 \times 2 \times 2 \times 2$ repeated ANOVAs with within-subject independent variables (IVs) of Task (flanker, Stroop), Expression (happy, fearful) and Congruency (congruent, incongruent) and the between-subject IV of Gender (boy, girl). The Greenhouse–Geisser correction for violations of sphericity was used where appropriate, and the significant interactions were tested by Sidak test for multiple comparisons.

Consistent with other studies (Nguyen et al., 2013), a natural logarithm transformation of the levels of testosterone (ln_T) and cortisol (ln_C) and the T/C ratio (ln_ T/C) was used to avoid analytical bias. Since the Shapiro-Wilk test indicated that ln_T was non-normally distributed in the samples from the boys ($p < 0.05$), Spearman's correlation analysis was used to examine the interrelationships among hormone levels, behavioral data and brain activities in boys and girls separately. Pearson correlation analysis was used to examine the non-sex-specific associations.

In addition, a mediation model was built to establish the roles of the neural activities during emotional conflict control processes in the correlation between the hormone levels and the EI abilities of individuals. According to Baron and Kenny's (1986) conventions, the total effect was considered to be the association of the independent variable (IV) with the dependent variable (DV), the direct effect was the association of the IV with the DV after adjusting for the mediating variable (MV), and the indirect effect was path a (relation between IV and MV) $\times$ path b (relation between MV and DV after controlling for IV). The significance of the indirect effects was measured by a bootstrapping procedure (Preacher and Hayes, 2008). We used 10,000 samplings to generate 95% confidence intervals (CIs). If the CIs did not contain zero, then the association between the IV and DV was significantly explained by the MV ($p < 0.05$).

# RESULTS

## Hormone Levels and Emotional Intelligence

There were no significant differences between boys and girls on the hormonal assay results ($p > 0.05$) and on EI scores results ($p > 0.05$) (**Table 1**).

**TABLE 1 |** Descriptive statistics and associations between hormones and EIS scores.

| | | | Association with hormone (boys and girls together) | | | |
|---|---|---|---|---|---|---|
| **Measure** | **Boys** | **Girls** | ***EIS*-EP** | ***EIS*-MSE** | ***EIS*-MOS** | ***EIS*-EU** |
| T(pg/ml) | 20.70 (6.84) | 20.44 (7.87) | 0.03 | −0.40* | −0.14 | −0.161 |
| C(ng/ml) | 2.83 (1.26) | 2.86 (0.75) | −0.04 | −0.42* | −0.032 | 0.006 |
| T/C ratio ($\times 10^{-3}$) | 7.96 (2.77) | 7.16 (1.99) | 0.08 | −0.17 | −0.122 | −0.190 |
| Age (years) | 10.90 (0.29) | 10.56 (0.32) | | | | |
| *EIS-EP* | 37.16 (4.84) | 36.56 (4.07) | | | | |
| *EIS*- MSE | 34.22 (6.74) | 34.56 (3.28) | | | | |
| *EIS*- MOS | 31.11 (4.48) | 31.40 (4.25) | | | | |
| *EIS*- EU | 15.67 (3.01) | 15.58 (2.94) | | | | |

*Data are presented as mean and standard deviation. Total N = 34. Components in the EIS questionnaire: EP, Emotion Perception; MSE, Managing Self Emotions; MOS, Managing Others' Emotions; EU, Emotional Utilization. *$p < 0.05$.*

## Behavioral Performances on the Two Tasks

For accuracy in the two tasks (**Table 2**), ANOVA showed a significant main effect of Gender ($F_{(1,32)} = 13.1$, $p < 0.001$, $\eta^2 = 0.29$), with girls providing more accurate responses than boys. Congruency also showed a significant main effect ($F_{(1,32)} = 31.2$, $p < 0.001$, $\eta^2 = 0.49$), with participants exhibiting a higher accuracy in congruent trials than incongruent trials.

In the analysis of the RT, a significant main effect of Task was observed ($F_{(2,64)} = 10.5$, $p < 0.001$, $\eta^2 = 0.25$), with participants responding faster in the flanker task than in the Stroop task ($t_{(32)} = -2.8$, $p < 0.05$). The main effect of Expression was also significant ($F_{(1,32)} = 9.5$, $p < 0.01$, $\eta^2 = 0.23$), and RTs were shorter in response to happy faces than to fearful faces. The interaction between Task and Congruency was detected ($F_{(2,64)} = 23.9$, $p < 0.001$, $\eta^2 = 0.43$), and RTs were shorter in congruent trials than incongruent trials in the flanker ($t_{(32)} = -3.7$, $p < 0.001$) and the Stroop tasks ($t_{(32)} = -8.2$, $p < 0.001$).

## ERP Waveforms

The mean peak latencies and amplitudes of N2 and SP components are shown in **Table 3**. The grand average waveforms of N2 and SP are displayed in **Figures 2**, **3**, respectively.

### N2 Components

For N2 amplitudes, the interaction among Task, Expression, and Gender was marginally significant ($F_{(2,64)} = 3.0$, $p = 0.06$), and boys exhibited more negative N2 responses to happy faces than fearful faces in the flanker task ($t_{(64)} = 2.79$, $p < 0.01$). There were no significant main effects or interaction effects for N2 latencies.

### SP Components

SP latencies showed a significant main effect of Expression ($F_{(1,32)} = 5.87$, $p < 0.05$, $\eta^2 = 0.16$), with happy faces inducing shorter SP latencies than fearful faces. A significant main effect of Congruency was also observed ($F_{(1,32)} = 22.1$, $p < 0.001$, $\eta^2 = 0.41$), with shorter SP latencies in congruent trials than in incongruent trials. Additionally, the interaction among Task, Expression, and Congruency was significant ($F_{(2,64)} = 4.4$, $p < 0.05$, $\eta^2 = 0.12$). In the flanker task, SP latencies were shorter in congruent trials than in incongruent trials when the target faces were fearful ($t_{(32)} = 3.7$, $p < 0.001$). In the Stroop task, SP latencies were shorter in congruent trials than in incongruent trials when faces were fearful ($t_{(32)} = 3.7$, $p < 0.001$) and happy ($t_{(32)} = 3.6$, $p < 0.001$). SP latencies were also shorter in response to happy faces than to fearful faces in incongruent trials of the flanker task ($t_{(32)} = 3.5$, $p < 0.01$). There were no significant main effects or interaction effects for SP amplitudes.

## The Correlations Among Emotional Abilities, Emotional Conflict Control, and Hormone Levels

For the whole sample, negative associations were found between testosterone and Managing Self Emotions ($r = -0.40$, $p < 0.05$), and between cortisol and Managing Self Emotions ($r = -0.42$,

**TABLE 2 |** Mean and standard deviation of response accuracy and reaction time (RT) in the two tasks.

| | | Flanker | | Stroop | |
|---|---|---|---|---|---|
| | | **Congruent** | **Incongruent** | **Congruent** | **Incongruent** |
| Boys | Accuracy | 0.83 (0.07) | 0.81 (0.08) | 0.88 (0.06) | 0.77 (0.10) |
| | RT | 695 (125) | 703 (130) | 706 (135) | 746 (149) |
| Girls | Accuracy | 0.92 (0.05) | 0.88 (0.07) | 0.95 (0.04) | 0.85 (0.07) |
| | RT | 688 (114) | 704 (114) | 723 (100) | 803 (109) |

**TABLE 3 |** Mean peak latencies (ms) and amplitudes (μV) of N2 and SP components in the two tasks.

| | | Flanker | | Stroop | |
|---|---|---|---|---|---|
| | | **Congruent** | **Incongruent** | **Congruent** | **Incongruent** |
| Boys | N2 latency | 284 (28) | 281 (30) | 290 (38) | 293 (36) |
| | N2 amplitude | −4.84 (4.61) | −5.59 (4.3) | −3.57 (3.6) | −3.74 (3.99) |
| | SP latency | 578 (40) | 562 (32) | 555 (33) | 585 (34) |
| | SP amplitude | 15.5 (8.55) | 15.66 (8.22) | 13.95 (8.45) | 13.73 (7.44) |
| Girls | N2 latency | 285 (33) | 280 (31) | 286 (33) | 294 (33) |
| | N2 amplitude | −4.80 (3.58) | −4.94 (3.4) | −4.90 (3.79) | −5.93 (5.56) |
| | SP latency | 584 (40) | 587 (37) | 565 (28) | 598 (40) |
| | SP amplitude | 14.56 (8.17) | 14.98 (7.74) | 13.4 (8.31) | 13.96 (6.62) |

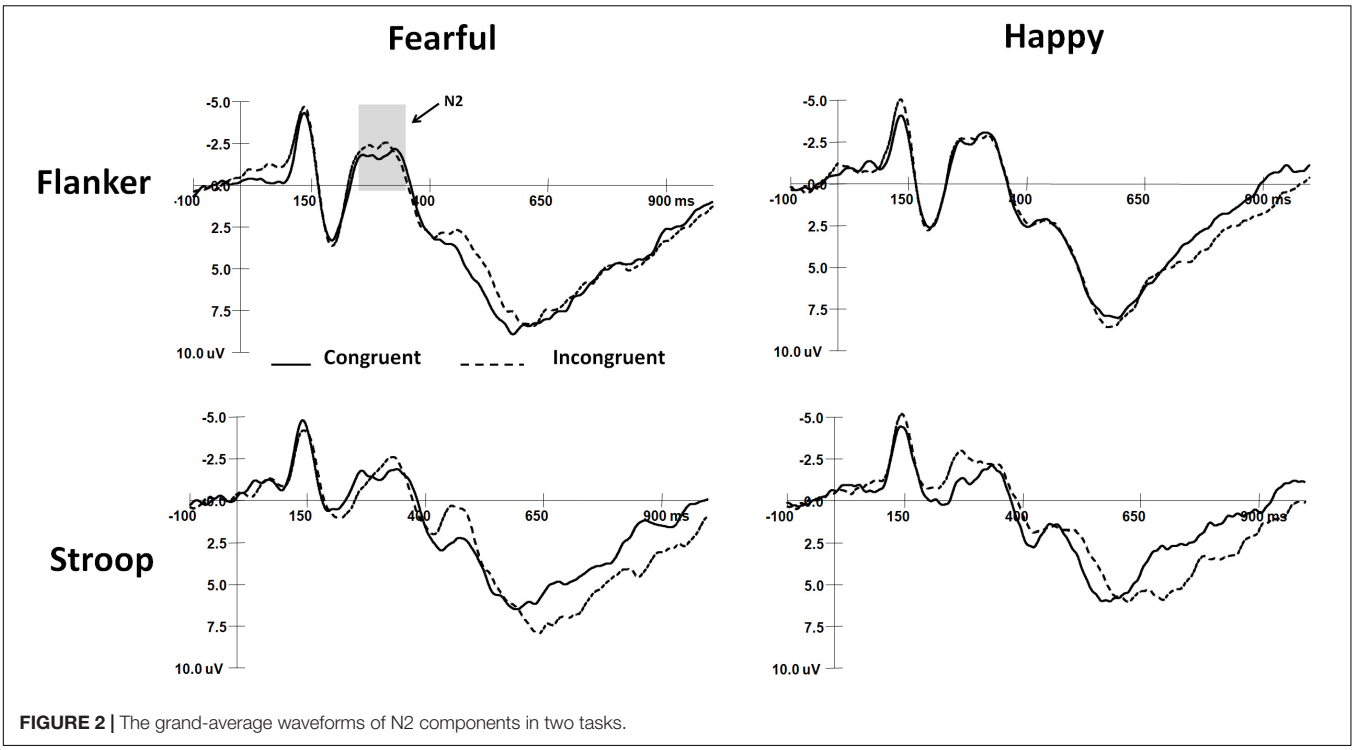


**FIGURE 2 |** The grand-average waveforms of N2 components in two tasks.

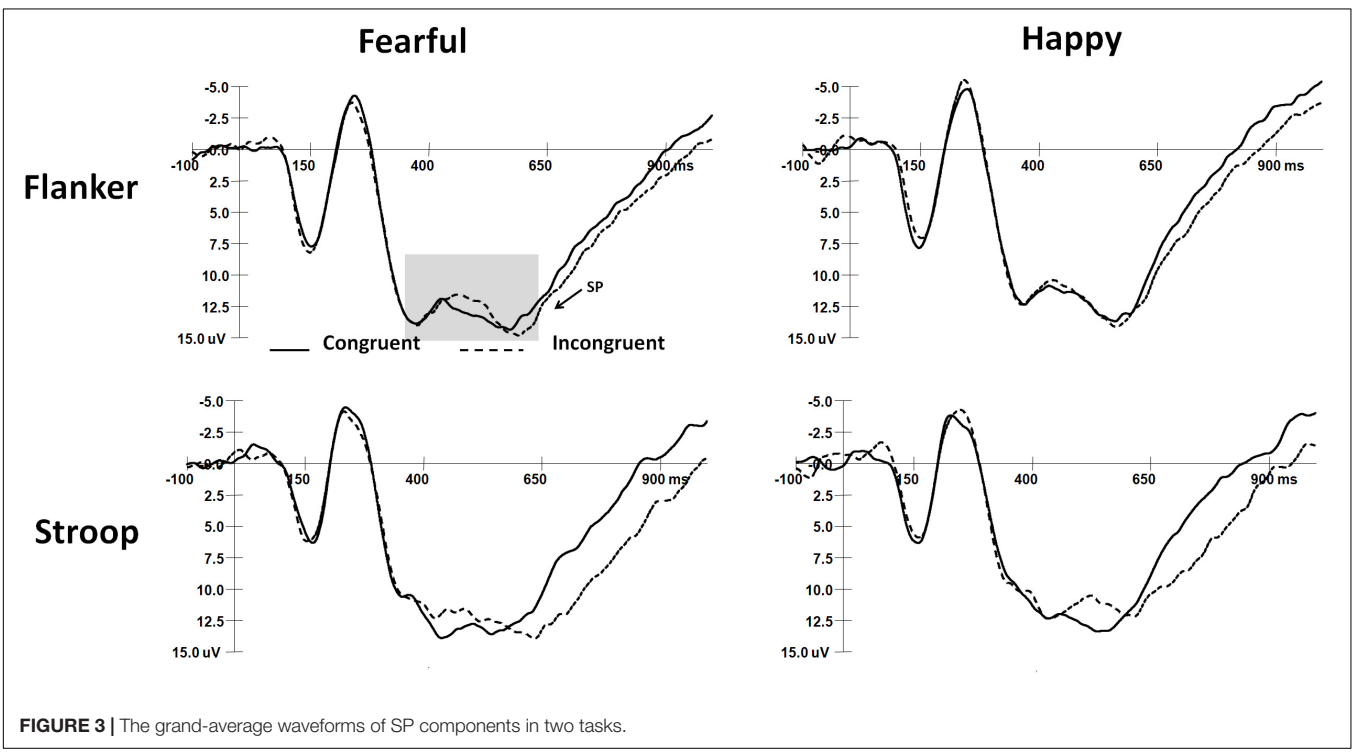


**FIGURE 3 |** The grand-average waveforms of SP components in two tasks.

$p < 0.05$), and no significant correlations between brain activities and Managing Self Emotions (all $p > 0.05$) were found.

There were significant negative correlations between testosterone and accuracy ($r = -0.36$, $p < 0.05$), and between cortisol and accuracy ($r = -0.36$, $p < 0.05$) in the congruent trials in the emotional flanker task when target faces were fearful. There were also significant negative correlations between cortisol and accuracy ($r = -0.45$, $p < 0.01$) in the congruent trials in the emotional Stroop task when target faces were happy.

**TABLE 4 |** Associations between brain activities and testosterone-cortisol balance.

| | Testosterone | Cortisol | T/C ratio |
|---|---|---|---|
| **Flanker** | | | |
| N2 latency_left_fearful[1] | 0.46** | 0.45** | −0.01 |
| [D]N2 latency_left_fearful | −0.37* | −0.40* | 0.05 |
| [D]N2 latency_middle_fearful | −0.22 | −0.37* | 0.12 |
| **Stroop** | | | |
| [D]N2 latency_middle_fearful | −0.01 | −0.26 | 0.37* |
| [D]N2 amplitude_left_happy | −0.23 | −0.36* | 0.18 |
| **Stroop** | | | |
| SP latency_left_fearful[1] | −0.15 | 0.07 | −0.35* |
| SP amplitude_middle_fearful[2] | −0.09 | −0.40* | 0.38* |
| SP amplitude_middle_happy[1] | −0.13 | −0.45** | 0.39* |
| SP amplitude_middle_happy[2] | −0.16 | −0.60** | 0.53** |
| SP amplitude_right_fearful[2] | −0.21 | −0.49** | 0.37* |
| SP amplitude_right_happy[1] | −0.17 | −0.51** | 0.42* |
| SP amplitude_right_happy[2] | −0.21 | −0.59** | 0.46** |
| SP amplitude_overall_fearful[2] | −0.15 | −0.43* | 0.35* |
| SP amplitude_overall_happy[1] | −0.20 | −0.53** | 0.40* |

*[D]Congruency effects; [1]Congruent trials; [2]Incongruent trials. *p < 0.05, **p < 0.01.*

In the flanker task, N2 latencies in congruent trials were positively correlated with testosterone and cortisol levels when the target faces were fearful (testosterone: $r = 0.46$, $p < 0.01$; cortisol: $r = 0.45$, $p < 0.01$) (**Table 4**). T/C ratios were positively correlated with the differences in N2 latencies between incongruent and congruent trials in the Stroop task over the midline area ($r = 0.37$, $p < 0.05$). In the Stroop task, SP amplitudes were positively correlated with T/C ratios (see **Table 4**).

### Girls

Emotional Utilization scores in EI negatively correlated with cortisol levels ($r = -0.57$, $p < 0.05$). Cortisol levels were also associated with the SP amplitude in incongruent trials of the flanker task ($r = 0.63$, $p < 0.01$) and the SP amplitude difference ($r = 0.51$, $p < 0.01$) when the target face was fearful. Hormone levels were negatively correlated with accuracy in congruent trials of the flanker task when the facial expression was fearful ($r = -0.52$, $p < 0.05$, testosterone; $r = -0.68$, $p < 0.05$, cortisol). Cortisol levels were positively correlated with RT in the incongruent trials of the Stroop task when the facial expression was fearful ($r = 0.51$, $p < 0.05$), and RT in the congruent trials of the Stroop task when the facial expression was happy ($r = 0.50$, $p < 0.05$). Cortisol levels correlated with accuracy in congruent trials of the Stroop task when the facial expression was fearful ($r = -0.56$, $p < 0.05$) and happy ($r = -0.56$, $p < 0.05$) and SP amplitude in incongruent trials of the flanker task when the target face was fearful ($r = 0.54$, $p < 0.05$). Finally, cortisol levels also correlated with SP amplitude differences when the facial expression was happy in the Stroop task ($r = -0.51$, $p < 0.01$).

### Boys

Managing Self Emotions in EI was positively correlated with accuracy in the incongruent trials of the Stroop task ($r = 0.55$, $p < 0.05$). Emotion Utilization positively correlated with the SP amplitude difference in the flanker task when the target face was fearful ($r = 0.48$, $p < 0.05$). Testosterone levels were negatively correlated with Managing Self Emotions in EI ($r = -0.51$, $p < 0.05$). Testosterone levels were also negatively correlated with response accuracy ($r = -0.51$, $p < 0.05$) and positively correlated with N2 latency ($r = 0.49$, $p < 0.05$) in incongruent trials of the flanker task when the target face was happy. Testosterone levels were also negatively correlated with the difference in accuracy between congruent trials and incongruent trials of the Stroop task when the facial expression was fearful ($r = -0.50$, $p < 0.05$).

## The Mediation Effects Among Emotional Abilities, Emotional Conflict Control, and Hormone Levels

Based on the correlation results, we further assessed a mediation model including brain activity. We included the scores associated with Emotional Utilization of the girls as the DV, their cortisol levels as the IV, and the SP amplitudes in incongruent trials of the flanker task when the target face was fearful as the MV. The SP amplitude in incongruent trials of the flanker task when the target face was fearful mediated the influence of the cortisol levels on the utility of emotion in EI [95% $CI = (-8.64, -0.54)$, $p < 0.05$].

# DISCUSSION

To our best knowledge, this is the first study investigating the correlations among hormones (testosterone and cortisol), EI and neuronal activities during emotional conflict control processes in preadolescent children. Overall, the results suggested a more complicated picture of relationships than we expected. Although there were no significant correlations between hormone ratios and EI, lower hormone (testosterone or cortisol) levels were found to be related with better abilities of managing self emotions (a component of EI) in preadolescent children. Furthermore, we also found that the associations between cortisol and emotional utilization (another component of EI) were mediated by neural activities in conflict resolution on emotional conflicts in girls. Another finding was that T/C ratio correlated with conflict processing when processing fearful faces.

The comparison of different conflict control processes with varied conflict types in our study supported the dimensional overlap model (Liu et al., 2010) and further indicated that the varied combination of different dimensions of stimulus-response mappings may induce varied conflict control processes (Liu et al., 2004; Egner et al., 2007).

The current behavioral conflict control processes were not modulated by the valence of the target facial expression, which was inconsistent with previous findings in adults, potentially revealing that the brain functions of preadolescent children might not be sufficiently mature to support the interplay between these two processes. Our study revealed distinct response patterns dependent on emotional prosody. RTs were shorter in response to happy faces than fearful faces, which is consistent with previous evidence showing that happy expression was the first expression that children could identify (Watling and Bourne, 2013).

Testosterone levels may mediate cognitive function through attentional control processes (Martin et al., 2009). The current study showed that emotional conflict control processes in both boys and girls were associated with testosterone. For boys, lower testosterone levels were related to greater accuracy in conflict control tasks with happy conflicts (in the flanker task) and fearful conflicts (in the Stroop task) and were associated with shorter frontal responses to happy conflicts during conflict detection processing (in the flanker task). For girls, lower testosterone levels correlated with greater accuracy in congruent trials of the flanker task when the target faces were fearful, consistent with the findings of Van Strien et al. (2009). Similarly, for behavioral responses, Tyborowska et al. (2016) found that lower testosterone levels were associated with faster responses to happy faces than to angry faces; however, they also found that lower testosterone levels were associated with more errors with angry faces than with happy faces. They analyzed the behavioral data with boys and girls together. Previous studies have provided puzzling results upon examination of brain activities during emotional control. Cservenka et al. (2015) adopted an emotional Stroop task to study the effects of hormones on emotional conflict control processes and brain activity in adolescence and found significant gender effects, as testosterone was negatively correlated with frontal and striatal activities in male adolescents and cerebellar and precuneus activities in female adolescents. These findings revealed that testosterone could play important roles during emotional cognitive control processes and indicated that sex differences should be further examined.

Previous studies showed that children's salivary cortisol levels were tightly associated with their inhibition control abilities and fear perception (Klimes-Dougan et al., 2001; Gunnar et al., 2009). Our present findings revealed a tight relationship between cortisol levels and emotional conflict control processes in both boys and girls. Upon examination of the neuronal activity during conflict resolution, we found that lower cortisol levels in girls were associated with better conflict resolution in the parietal cortex (greater SP amplitude) in fearful conflicts in the flanker task. Consistently, Schutter et al. (2002) revealed cortisol-related reductions in transmission between the left prefrontal and right parietal cortex in healthy subjects aged 20–28 years.

Interestingly, the relationships between hormones and brain activities in the flanker task showed that it might take longer to detect whether there were conflicts in the flanker task when all the five faces were fearful (congruent trials) when children bore higher hormone levels. At the same time, higher hormone levels correlated with smaller congruency effects of N2 latency when the target face was fearful. We speculated that these hormone-latency associations mainly indicated high concentration of hormone was related to the delay of processing fearful faces in congruent trials in the flanker task. The reason was that the congruency effects in N2 latency were outcomes of subtracting latency of congruent trials from latency of incongruent trials, However, there might be distinct brain mechanisms for the association between fearful expression processing and testosterone (Van Honk and Schutter, 2006) and cortisol (Watling and Bourne, 2013). Furthermore, a recent study reported that higher daily cortisol concentrations inhibited functional connectivity between the prefrontal cortex and the amygdala when processing fearful faces compared to neutral faces (Hakamata et al., 2017).

In addition, our findings might also indirectly indicate the association between fearful face processing and T/C ratio. This is the first study providing evidence for T/C ratios and brain activities in emotional processing in children. When faces were fearful, T/C ratios were correlated with SP latency and congruency effects on N2 latency, while neither testosterone or cortisol levels were correlated with them. This is similar with a previous study. Glenn et al. (2011) found that psychopathy was associated with an increased T/C ratio, but was not associated with testosterone or cortisol independently. In addition, the faster SP responses related to higher T/C ratios might indicate T/C-related behavioral impulsivity (Terburg et al., 2009; Romero-Martínez et al., 2016; Manigault et al., 2019). A recent study also found that high testosterone relative to cortisol, was associated with aggressive behavior in 16-year-old adolescents (Platje et al., 2015). However, since T/C ratio is not correlated with any component of EI, further studies should be carried out with larger sample size and different measures.

The lack of sex differences in testosterone levels, as reported in our results, is consistent with previous studies of children (Quaiser-Pohl et al., 2016; Nguyen et al., 2018). Distinguishing between the biologically active free fraction of gonadal hormone levels (as measured in saliva) and the amount of available hormone levels in total (as measured in blood) might be important (Quaiser-Pohl et al., 2016). As for cortisol levels, it has been proposed that sex-related differences in HPA regulation emerge at puberty (Wang et al., 2018).

Previous studies of adults found that EI is positively associated with affective executive function processes (Sevdalis et al., 2007). Our findings implied that different aspects of EI were related to varied neuronal activities in different brain areas during conflict control processes. Self-management of emotion was associated with both frontal and parietal activities during neuronal processes of conflict monitoring and conflict resolution of affective conflicts. However, emotional utilization was only associated with parietal activities during conflict resolution of affective conflicts.

We found that lower testosterone levels in boys were associated with better self-management of emotion in EI, and lower cortisol levels in girls were associated with better emotional utilization in EI. Bechtoldt and Schneider (2016) found that the testosterone and cortisol levels in male adults did not correlate with EI. One reason for this difference may be due to differences in the collection times of the saliva samples. Samples collected between 3 p.m. and 8 p.m. have been shown to contain relatively lower levels of testosterone and cortisol during the daytime (Nelis et al., 2009), whereas samples collected around 9 a.m., as in the current study, contain relatively higher levels. Another possible reason for the discrepancy is the criterion validity of the EI scale. Bechtoldt and Schneider (2016) specifically stressed that the ability-based tests of EI that they chose assessed emotion management by evaluating maximum performance measures in

hypothetical contexts, which may be inadequate for predicting emotion-regulating behavior in real contexts. A significant mediation effect of emotional conflict control on the association between EI and hormones was found in girls. Lower cortisol levels were associated with better utilization of emotion in girls, and the association was mediated by a stronger parietal response to emotional conflicts. Our findings revealed an essential role of neural activities conflict resolution in mediating the hormonal effects on emotional abilities in girls.

There were several limitations in the current study. First, a single-point measure of hormones is a significant weakness of our study, and it would be better to measure salivary hormones on two or more consecutive days with multiple time-points on each day. Second, the small sample size is also a weakness; therefore, we adopted strict Bonferroni corrections for multiple comparisons. Third, the emotional words used in the current study may be not that emotional in terms of the emotion-inducing effect compared with facial expression images, and further studies should adopt some emotional words with higher arousal levels.

## CONCLUSION

The study shows that testosterone correlates with conflict detection and managing self emotions, while cortisol correlates with conflict detection and resolution as well as managing self emotions. Besides, in girls, neural activities during conflict resolution in the S-S conflicts mediate the correlation between cortisol levels and emotional utilization. In addition, the relationships between hormones and neural activities vary depending on the type of emotional conflict control task and emotional stimuli. We thereby provide supportive preliminary evidence for hormonal and neural mechanisms underlying emotional intelligence in preadolescence. Future studies can further investigate the involvement of hormone-mediated emotional processing during the development of emotional intelligence in children and adolescents, and latent state-trait modeling could be applied to model individual differences in salivary testosterone, cortisol, and their interaction in the future studies.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Institute of Psychology, Chinese Academy of Sciences and School of Psychology, Capital Normal University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

TL and FS wrote the first draft of the manuscript together and designed the study and interpreted the results, then revised the draft. DL undertook the EEG data processing and statistical analysis. JS supervised the project and revised the draft. All authors contributed to and have approved the final manuscript.

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# Masculinized Second-to-Fourth Digit Ratio (2D:4D Ratio) Is Associated With Lower Cortisol Response in Infant Female Rhesus Monkeys (*Macaca mulatta*)

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The second-to-fourth digit ratio (2D:4D ratio) is considered a postnatal proxy measure for the degree of prenatal androgen exposure (PAE), which is the primary factor responsible for masculinizing the brain of a developing fetus. Some studies suggest that the organizational effects of PAE may extend to the hypothalamic-pituitary-adrenal (HPA) axis response to stress. This study investigates the relationship between 2D:4D ratio and HPA axis functioning using a rhesus monkey (*Macaca mulatta*) model. Subjects were $N$ = 268 (180 females, 88 males) rhesus monkey infants (3–4 months of age). Plasma cortisol concentrations were assayed from two blood samples obtained during a 25-h experimental social separation stressor at 2- and 7-h post-separation. Subjects' 2D:4D ratio was measured later in life ($M_{age}$ = 6.70 years). It was hypothesized that infant rhesus monkeys that exhibited a more masculine-like 2D:4D ratio would show lower levels of circulating cortisol after a social separation and relocation stressor. The results showed that there was a sex difference in the left-hand 2D:4D ratio. The results also showed that there was an overall sex difference in cortisol concentrations and that female, but not male, monkeys that exhibited a more masculine-like right- and left-hand 2D:4D ratio exhibited lower mean stress-induced cortisol concentrations early in life. These findings suggest that higher levels of prenatal androgens in females, as measured by 2D:4D ratio, may be related to an attenuated HPA axis stress-response, as measured by plasma cortisol levels. To the extent that these findings generalize to humans, they suggest that the organizational effects of PAE extend to the infant HPA axis, modulating the HPA axis response, particularly in females.

**Keywords: 2D:4D ratio, cortisol, HPA axis, prenatal androgen exposure, rhesus monkeys, stress**

## INTRODUCTION

Prenatal androgen exposure (PAE) is thought to be the main source of morphological and central nervous system masculinization, and, consequently, is responsible for many of the phenotypic behavioral differences observed between males and females (Phoenix et al., 1959; Hughes, 2001; Thornton et al., 2009). During gestation, androgens bathe the brain, initiating enzymatic cascades

that masculinize the developing fetus (Hughes, 2001) through a variety of epigenetic mechanisms (Gegenhuber and Tollkuhn, 2019). The degree of PAE varies between the sexes (males typically have a higher degree of PAE; Wilson et al., 1981), but there are also wide individual differences within each of the sexes. Studies show that variation in PAE contributes to stable individual differences in brain function and behavior (Hines et al., 2016; Spencer et al., 2017; Del Giudice et al., 2018). Second-to-forth digit ratio (2D:4D ratio) is associated with sex differences in behavior, for example, one well-replicated line of research shows the same-sex attraction in women is associated with a more masculinized 2D:4D ratio (Williams et al., 2000; Kraemer et al., 2006; Watts et al., 2018), although the inverse relationship is not always found in men (Williams et al., 2000; Voracek et al., 2005). Thus, PAE plays a complex and important modulating role in the development of typical sex differences through its organizational effect on the brain. Studies also show that PAE is implicated in the development of several psychopathological disorders with extant sex differences in incidence rates, such as autism spectrum disorder (Cherskov et al., 2018), schizophrenia (Paipa et al., 2018), attention deficit hyperactive disorder (Martel et al., 2008) and, particularly relevant to this study, anxiety disorders (de Bruin et al., 2006).

Given its organizational effects on the brain and periphery, PAE may modulate the hypothalamic-pituitary-adrenal (HPA) axis. Numerous studies suggest that there are sex differences in the functioning of the HPA axis (for a review, see Handa et al., 1994). For example, using the same paradigm described in the present study, Capitanio et al. (2005) assessed sex differences in HPA axis response. Briefly, this paradigm consists of a 25-h stress-inducing social separation of infant rhesus monkeys from their mothers. During this period, infant subjects are assessed on a variety of biobehavioral metrics, including undergoing blood sampling at 2-h and 7-h post-separation, respectively. Obtained plasma cortisol is then assayed for cortisol concentrations using radioimmunoassay (for a detailed description of this methodology, see Capitanio et al., 2005). Using this paradigm, Capitanio et al. (2005) showed that male rhesus monkey infants exhibit lower plasma cortisol and are less responsive to dexamethasone and adrenocorticotropic hormone (ACTH) during an experimental social separation and relocation stressor, when compared to females, replicating plasma cortisol and ACTH findings in human subjects (Kudielka et al., 2004). Studies also suggest that the plasma cortisol levels of females may be more sensitive to other variables that affect the response of the HPA axis to stress (Uhart et al., 2006), with research suggesting that the greater prevalence of women with depression, when compared to men, may be related to the tendency of females to show an elevated HPA axis response to stress when compared to males (for a review, see Bale and Epperson, 2015). Given these tendencies, more PAE may lead to a more masculinized HPA axis in males, when compared to females, although few studies have assessed this possibility. As stress reactivity is a complex and emergent phenomenon, influenced by both the organizational and activational effects of testosterone, there is a greater need to understand the individual contributions of PAE in an organizational capacity.

While direct measures of PAE can be made by extracting amniotic fluid (Spencer et al., 2017; Beking et al., 2018; Wang et al., 2019), there are decided limitations to this methodology. For example, the composition of extracted amniotic fluid represents androgen levels at a single time point, which may not capture the day-to-day variability of PAE or the sustained effect of higher mean levels of PAE. While some have attempted to measure PAE by other means, such as assaying venous or arterial umbilical cord blood, these data often do not correlate with concomitant androgen levels in the amniotic fluid (van de Beek et al., 2004). Moreover, none of these methods, including assaying amniotic fluid for androgen levels using a single point in time, provide a long-term chronic pooled estimate of PAE exposure. Given the difficulty in collecting representative samples across pregnancy, many researchers have opted for proxy measurements of PAE, such as the 2D:4D ratio, first proposed by Manning et al. (1998), and widely used by others following the initial publication (Hönekopp et al., 2007). While somewhat controversial, the 2D:4D ratio has been widely used to investigate biological influences on gender differences. Comparisons of 2D:4D ratios and phenotype have not always shown clear sex differences and its use as a proxy to demonstrate prenatal contributions to gender differences are sometimes inconsistent, with recent commentaries (see Swift-Gallant et al., 2020) suggesting myriad reasons including small sample sizes, variation in behavior, that 2D:4D ratio is intended as an imperfect proxy for PAE, and that PAE is only partially responsible for variation in 2D:4D ratio. Indeed, while 2D:4D ratio provides a useful measure of chronic pooled PAE, other influences on 2D:4D ratio, such as genetic influences, cannot be ruled out, nor are they mutually exclusive. The bulk of the studies suggest that the 2D:4D ratio is sexually-dimorphic in humans (for a review, see Manning, 2011) and non-human primates (Nelson and Shultz, 2010), though the direction of the dimorphism may be species-specific, with male rhesus monkeys typically exhibiting a higher 2D:4D ratio, while female humans typically exhibit a higher 2D:4D ratio (Baxter et al., 2018).

PAE is thought to be, at least in part, responsible for 2D:4D ratio, with sex differences in 2D:4D ratio already apparent prenatally (Galis et al., 2010). For example, a recent study showed a significant relationship between urinary testosterone levels in pregnant female monkeys and subsequent 2D:4D ratio of offspring (Baxter et al., 2019). Studies in nonhuman primates also show that experimentally increasing circulating prenatal androgens, thus increasing PAE, masculinizes 2D:4D ratios (Abbott et al., 2012). Similarly, studies investigating congenital adrenal hyperplasia, a condition that leads to abnormally high PAE, show that female humans with this condition exhibit a more masculinized 2D:4D ratio (Brown et al., 2002; Rivas et al., 2014). Studies also suggest that the effects of PAE extend to behavior and temperament. For example, one study showed that a feminized 2D:4D ratio in women (but not men) is associated with increased temperamental harm avoidance (Jeon et al., 2016), which may be related to the organizational effects of PAE on the HPA axis. Another study in young adult females showed that experimental administration of testosterone led to

reductions of cognitive empathy, and this testosterone-induced low empathy was related to PAE, as measured by 2D:4D ratio (van Honk et al., 2011). Such 2D:4D relationships with harm avoidance and empathy suggest that prenatal organizational effects likely modulate gender differences in mood and may explain, at least in part, male-female differences in the risk for mood disorders.

Researchers have noted the utility of rhesus monkeys (*Macaca mulatta*) for studying organizational effects of PAE on the brain (Thornton et al., 2009; Baxter et al., 2018), due to their genetic (Gibbs et al., 2007), temperamental (Weinstein and Capitanio, 2008), and social (Capitanio, 1985) similarities to humans. Particularly relevant to this study, the rhesus monkey response to stress has been widely studied and is well-characterized (see Sanchez, 2006). One important strength of utilizing a rhesus monkey, rather than a human, model to investigate the relationship between 2D:4D ratio and development is that the rhesus monkey environment is closely controlled, eliminating extraneous variables that may impact human development (for example, socioeconomic status or race; Henry et al., 2019). This, plus the relative ease of obtaining direct measurements, increases the ability to assess potential causal mechanisms with a higher degree of certainty. The present study investigated the relationship between PAE, as measured by 2D:4D ratio, and early-life HPA axis response to stress, as measured by circulating cortisol concentrations in infant male and female rhesus monkeys during an ecologically-meaningful, well-validated stressor. The purpose of this study is to investigate the organizational effects of PAE on the HPA axis. To the extent that women are at greater risk for mood disorders that are HPA-axis-related (Rainville and Hodes, 2019), investigating whether higher PAE in females has a protective effect while lower PAE in males is a risk factor for dysregulated HPA axis function may provide important information concerning both the etiology of sex differences in the HPA axis and the organizational effects of PAE on subsequent risk for anxiety and depression. Based on earlier findings showing sex differences in plasma cortisol response to a social stressor (Capitanio et al., 2005), it is hypothesized that infant rhesus monkey females will have a greater cortisol response to social separation from their mother and their social group when compared to males. Given earlier findings of a sex difference in 2D:4D ratio of rhesus monkeys (Baxter et al., 2018), it is hypothesized that there will be a sex difference in 2D:4D ratio, such that male rhesus monkeys will exhibit a higher 2D:4D ratio pattern, when compared to female rhesus monkeys. Furthermore, given findings suggesting the relationship between female-typical 2D:4D ratio and personality/temperament in women (Jeon et al., 2016) and men (Evardone and Alexander, 2009) and other studies showing that females tend to exhibit greater sensitivity to environmental moderators of the HPA axis (Barr et al., 2004; Uhart et al., 2006), it is hypothesized that females with a more male-typical 2D:4D ratio will exhibit lower plasma cortisol concentrations in response to stress when compared to other females, while males with a more female-typical 2D:4D ratio will exhibit higher cortisol concentrations in response to stress when compared to other males.

# MATERIALS AND METHODS

Subjects were $N = 268$ rhesus monkeys (180 females, 88 males) housed at the California National Primate Research Center (CNPRC) in Davis, California in outdoor, 0.2-hectare field cages. Subjects lived in large social groups (approximately 60–100 animals of all age and sex classes), which is about the same size as typical rhesus monkey groups, in conditions approximating the natural social composition (matrilineally organized extended-family groups with multiple adult males, infants, and juveniles). This study was carried out following the recommendations of the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and with the guidelines established by the California National Primate Research Center (CNPRC). All procedures were reviewed and approved by the Animal Care and Use Committee of the University of California-Davis.

## Cortisol Sampling

Cortisol samples were obtained when the subjects were infants, during standardized experimental testing outlined by Capitanio et al. (2005). Briefly, at 3–4 months of age, infants were separated from their mothers and their larger social groups and underwent a standardized, 25-h biobehavioral assessment, in which they participated in a wide variety of biobehavioral tests. As part of the testing battery, blood samples were obtained *via* femoral venipuncture at 2-h following separation (11:00 h) and again approximately 5-h later (16:00 h). All blood samples were drawn using unheparinized syringes and immediately transferred to EDTA tubes. The samples were centrifuged at 4°C at 1,277 *g* for 10 min. Plasma was pipetted into tubes and stored at −80°C until they were assayed for cortisol concentrations. All cortisol data were collected between 2001–2016. The samples collected before 2014 ($n = 151$), were assayed using a commercial radioimmunoassay kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Samples collected after 2014 ($n = 117$), were assayed using a quantitative competitive immunoassay (Siemens Healthcare Diagnostics, Tarrytown, New York, NY, USA). For a description of each assaying procedure, see Vandeleest et al. (2019). There were no significant differences in the mean cortisol concentrations assayed using the two methods ($t_{(264)} = 0.56$, $p = 0.580$). As preliminary analyses showed that the two cortisol samples were significantly and positively correlated across time points ($r = 0.74$, $p < 0.0001$), mean cortisol concentrations were used in analyses. To account for any variance due to cohort year, cortisol concentrations were statistically standardized across cohort years and the resulting standardized values were used in all further analyses.

## Digit Ratio Measurements

Digits were measured between 1–17 years after cortisol sampling during routine biannual health examinations. As part of these routine examinations, subjects were sedated with ketamine (15 mg/kg, intramuscular). Two technicians measured the fingers by working together, using the same procedure described in Baxter et al. (2018). Briefly, monkeys were laid in a recumbent

position on a table, and, to increase accuracy, the first technician used a wooden craft stick to depress and restrain the monkeys' palms and fingers flat against the table. A second technician measured the restrained fingers using a digital caliper, measuring from the finger-crease most proximate to the palm to the most distal point of the finger, following guidelines from Manning (2011). Using a caliper to directly measure fingers may yield more accurate and reliable measures than indirect measurements from photos or scans (Fink and Manning, 2018). Subjects' second and fourth fingers of their right and left hands were measured at least twice, until at least two measurements were obtained within ± 1.5 mm, and the average length of the finger was calculated by averaging the two closest measurements. Subjects' 2D:4D ratios were calculated by dividing the average length of the second finger by the average length of the fourth finger for each hand. All digit ratio data were collected between 2016–2018 (inter-rater reliability >0.90). Preliminary analyses showed that right-hand 2D:4D ratio and left-hand 2D:4D ratio were significantly and positively correlated ($r = 0.27$, $p < 0.001$). Right- and left-hand 2D:4D ratios were statistically standardized across cohort years and the resulting standardized right- and left-hand 2D:4D ratio values were used in all further analyses. For ease of interpretation, all figures depict unstandardized values.

## Data Analysis

As an earlier study showed a significant relationship between 3–4 month infant monkeys' cortisol concentrations and age (Capitanio et al., 2005), infant age (days old) at the time of cortisol sampling was controlled in all analyses. ANOVAs were used to test for sex differences in 2D:4D ratio, with sex as the independent variable and left- or right-hand 2D:4D ratio as the dependent variable. An ANOVA was also used to test for sex differences in plasma cortisol, with sex as the independent variable, mean plasma cortisol concentrations as the dependent variable, and infant age entered as a covariate.

Multiple regression was used to test the relationship between cortisol and 2D:4D ratio, with right- or left-hand 2D:4D ratio and infant age as the independent variables and mean plasma cortisol concentrations as the dependent variable. Because preliminary analyses showed sex differences in cortisol concentrations ($p < 0.0001$), the multiple regression analyses were performed separately for males and females. All analyses were performed using SPSS, version 25.

# RESULTS

As hypothesized, results from ANOVA showed a significant sex difference in mean cortisol concentrations between males ($M = 69.17 \pm 1.93$) and females ($M = 81.80 \pm 1.84$); $F_{(1,263)} = 18.20$, $p < 0.0001$; see **Figure 1**). There was also a significant sex difference in the left-hand 2D:4D ratio ($F_{(1,248)} = 6.837$, $p = 0.009$), with males exhibiting a higher left-hand 2D:4D ratio ($0.81 \pm 0.003$), when compared to females ($0.80 \pm 0.002$). There was not a detectable sex difference in the right-hand 2D:4D ratio ($p = 0.22$).

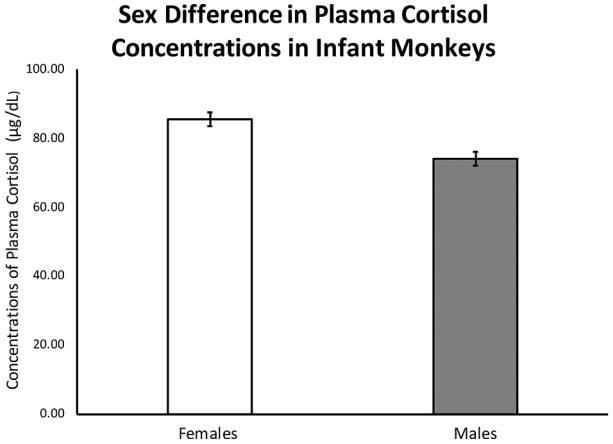


**FIGURE 1 |** Results from an ANOVA with sex as the independent variable and infant stress-response cortisol concentrations as the dependent variable and infant age entered as a covariate showed a significant effect of sex on stress-induced cortisol concentrations in 3-to-4-month-old infants ($F_{(1,263)} = 18.20$, $p < 0.0001$). Error bars represent standard error of the mean.

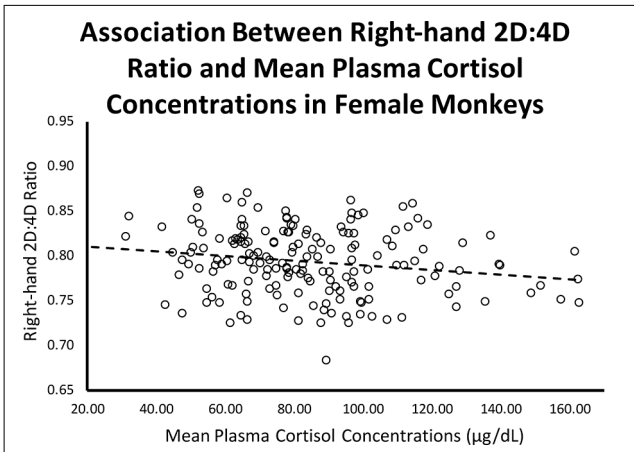


**FIGURE 2 |** Controlling for infant age, there was a significant (β = −0.204, $p = 0.007$; Overall model: $R = 0.203$, $F_{(2,173)} = 3.70$, $p = 0.027$) negative relationship between right-hand 2D:4D ratio and stress-induced plasma cortisol concentrations for female rhesus monkeys.

## Females

Controlling for infant age at cortisol sampling, results from a multiple regression analysis showed a significant negative relationship between right-hand 2D:4D ratio and mean plasma cortisol concentrations for females (β = −0.204, $p = 0.007$; Overall model: $R = 0.203$, $F_{(2,173)} = 3.70$, $p = 0.027$; see **Figure 2**).

Controlling for infant age at cortisol sampling, results from a multiple regression analysis showed a significant negative relationship between left-hand 2D:4D ratio and mean plasma cortisol concentrations for females (β = −0.199, $p = 0.009$; Overall model: $R = 0.198$, $F_{(2,173)} = 3.54$, $p = 0.031$; see **Figure 3**).

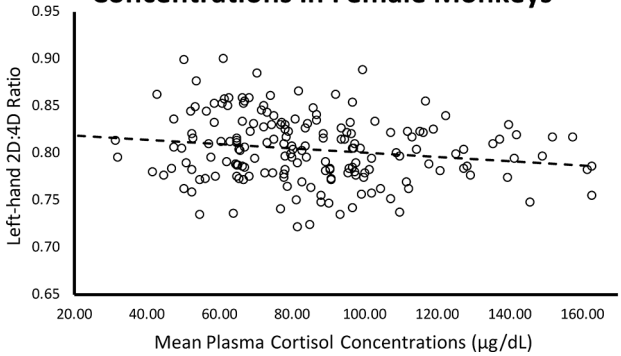


**FIGURE 3 |** Controlling for infant age, there was a significant (β = −0.199, $p = 0.009$; Overall model: $R = 0.198$, $F_{(2,173)} = 3.54$, $p = 0.031$) negative relationship between left-hand 2D:4D ratio and stress-induced plasma cortisol concentrations for female rhesus monkeys.

## Males

The relationship between right-hand 2D:4D ratio and infant cortisol concentrations was not significant for males (β = 0.023, $p = 0.837$; Overall model: $R = 0.076$, $F_{(2,82)} = 0.24$, $p = 0.789$), nor was the relationship between left-hand 2D:4D ratio and infant cortisol concentrations (β = 0.010, $p = 0.929$; Overall model: $R = 0.073$, $F_{(2,82)} = 0.22$, $p = 0.803$).

# DISCUSSION

Partial support was found for the hypotheses: There was a relationship between circulating stress-response plasma cortisol and 2D:4D ratio in females, with females that possessed a more male-typical 2D:4D ratio exhibiting lower plasma cortisol concentrations as infants during a mother-infant social separation stressor. To illustrate this point, 61% of females with 2D:4D ratios that were at or above the male 2D:4D ratio average had cortisol concentrations that were comparable to males. For the males, however, there was no relationship between 2D:4D ratio and infant plasma cortisol concentrations. To the extent that the 2D:4D ratio is a biomarker for the degree of PAE, these results suggest that PAE has a prenatal organizational effect on the HPA axis, which appears to attenuate the stress response of the HPA axis in female rhesus monkeys. To our knowledge, this is the first report of a relationship between PAE and stress-induced plasma cortisol levels.

One explanation for the finding that there is a relationship between infant plasma cortisol concentrations and 2D:4D ratio in females, but not males, is that high levels of PAE may lead to organizational changes that masculinize the HPA axis, leading to a more masculinized response in females. Specifically, infant females that were likely exposed to relatively higher levels of prenatal androgens (as indicated by their 2D:4D ratio) showed an attenuated, male-like cortisol response to a social separation stressor, at least at the level of the HPA axis. This interpretation is corroborated by our finding that infant female rhesus monkeys have higher plasma cortisol concentrations than infant male rhesus monkeys, replicating other studies showing that infant rhesus monkey females have higher stress-induced concentrations of plasma cortisol when compared to infant rhesus monkey males (Capitanio et al., 2005), and studies in humans showing that depressed females have more feminized 2D:4D ratios, when compared to non-depressed females (Smedley et al., 2014; De Kruijff et al., 2016). Perhaps the strongest experimental evidence that testosterone has an organizational effect that attenuates the female response to stress comes from rodent studies. These studies show that when androgens are administered during the organizational phase, the exposed females exhibit an attenuated glucocorticoid response to stress (Seale et al., 2005). In line with this research, individuals with congenital hyperplasia, a condition where the fetus is exposed to high levels of PAE, exhibit dysregulated cortisol biosynthesis as well as a blunted plasma cortisol response to stress (Merke and Bornstein, 2005; Turcu and Auchus, 2016). Furthermore, McHenry et al.'s (2014) comprehensive review of cortisol and the glucocorticoid response to stress and anxiety in animals suggests that, whether exogenous or naturally occurring, PAE decreases anxiety- and depression-like behaviors later in life. Similarly, human males with a more female-like 2D:4D ratio show an increased risk for depression (Bailey and Hurd, 2005), although this appears to have a low effect size and is not always seen in smaller samples (Martin et al., 1999; Li et al., 2019). One possible explanation for the failure of this and some other studies to find a relationship between PAE and cortisol concentrations in males is that males are exposed to substantially higher levels of prenatal androgens than are females (Knickmeyer and Baron-Cohen, 2006), which may reduce interindividual variability (i.e., a ceiling effect), potentially resulting in a failure to detect the same relationship in males. Taken together, these and other findings suggest that a more masculinized 2D:4D ratio is related not only to an attenuated cortisol response in females but also with lower rates of anxiety and depression in both sexes. One possible ramification of these findings and the data presented is that females whose brains are masculinized as a result of higher PAE may be at lower risk for subsequent affective psychopathology.

Consistent with the hypotheses, results showed that females exhibited higher stress-induced plasma cortisol concentrations when compared to males. Studies of sex differences in adult cortisol response have mixed findings, with one meta-analysis concluding that men show higher plasma cortisol concentrations than women (Kudielka and Kirschbaum, 2005). Still, other studies show that sex differences in stress-response plasma cortisol may vary with the type of stressor (Uhart et al., 2006; Goel et al., 2011). Other studies suggest that puberty status may further modulate sex differences in plasma, salivary, and urinary cortisol (Gifford and Reynolds, 2017; Van der Voorn et al., 2017). Human studies investigating sex differences in blood and salivary cortisol in prepubertal children similarly show mixed results (Dahl et al.,

1992; Gifford and Reynolds, 2017; Hollanders et al., 2017; Van der Voorn et al., 2017), which may reflect population differences, paradigm, or methodological differences between studies. Perhaps because of the homogeneous rearing and the experimentally manipulated stressors, studies of nonhuman primate infants are more consistent in showing that infant females have higher stress-response plasma cortisol than male infants, particularly when the investigation of sex differences is the primary variable under consideration (Capitanio et al., 2005; Vandeleest et al., 2013). Given the similarity between humans and rhesus monkeys in HPA axis functioning (Sanchez, 2006), this disparity in variability when comparing humans and nonhuman primates may be a consequence of the more controlled early environments, situational testing, and, consequently, increased homogeneity in treatments and early experiences of nonhuman primates although species differences cannot be ruled out. One advantage of the nonhuman primate model is the homogeneous early environment, which increases the capacity to detect effects. While correlation cannot establish causation, one possible explanation is that early PAE has a masculinizing effect on both the 2D:4D ratio and the HPA axis. Future studies should investigate 2D:4D ratio and the HPA axis response perhaps using other measures, such as corticotropin releasing hormone or ACTH, which may give a better estimate of central mechanisms that may be affected by PAE, leading to the sex differences observed.

One possible limitation of these findings is that cortisol concentrations were obtained when subjects were infants, while 2D:4D ratio was measured later in life, spanning a wide range of time (1–17 years later). Nevertheless, studies show that inter-individual differences in stress-induced plasma cortisol concentrations are stable from early in life into adulthood (Higley et al., 1992), and inter-individual differences in 2D:4D finger ratio also appear to be constant across development (Trivers et al., 2006), stabilizing early in life and showing trait-like individual differences across time. For example, Trivers et al. (2006) first measured 2D:4D ratios in $N = 108$ 9-year-old Jamaican children and then measured them a second time 4 years later, finding modest inter-individual stability, even though many of the children had gone through puberty between measure one and measure two. Although we did not have repeated 2D:4D measures on all of our subjects, we did repeatedly measure the 2D:4D ratios of a separate representative sample ($N = 63$) across 2 years. Results from bivariate correlations showed a statistically significant positive correlation between the two measurements ($r = 0.51$, $p < 0.0001$), suggesting that, for the present sample, 2D:4D ratio remained stable across time. Subsequent studies are underway to assess the relationship between adult cortisol concentrations and 2D:4D ratios to verify whether the cortisol and 2D:4D relationship is present in adult rhesus monkeys. Studies show that testosterone inhibits cortisol secretion in human adults (for example, see Terburg et al., 2009). It is also of note that, beginning in the first month and ending at the third month of life, there is a postnatal surge in testosterone levels in male, but not female, rhesus monkeys (Robinson and Bridson, 1978; Frawley and Neill, 1979). While this surge is unlikely to have affected cortisol levels in females, it is possible that for the males, measuring cortisol at a later time point may have produced different results, although that remains speculative.

There was a significant sex difference in the left- but not right-hand 2D:4D ratio, with males exhibiting a higher 2D:4D ratio, when compared to females. That the males had a higher 2D:4D ratio, when compared to females, is partially consistent with our earlier study in rhesus monkeys (Baxter et al., 2018), but in that study, the difference was seen in both hands. It is of note that in the previous study, the left hand showed greater sexual dimorphism and the study had a larger sample size, and, as noted in the methods section of this paper, there was a positive correlation between right- and left-hand 2D:4D ratio, suggesting that we may have been underpowered to detect this difference in the right hand.

Finding a relationship between PAE and stress-induced levels of plasma cortisol in female rhesus monkeys is an important first step in investigating the organizational effects of PAE on postnatal HPA axis functioning. Given that much of the early work investigating the masculinizing effect of PAE was performed in rhesus monkeys (Goy et al., 1988), future research should investigate the relationship between PAE and HPA axis functioning through experimental manipulation of PAE in rhesus monkeys, which would lead to evidence for a cause and effect relationship. If such efforts provide further support of the relationship between PAE and HPA axis functioning, 2D:4D ratio may be an important noninvasive biomarker for studies assessing the masculinizing effect of androgens on the HPA axis, as well as studies investigating the early risk for developmental affective disorders, particularly anxiety-related disorders and other HPA axis abnormalities.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Davis, Davis, CA, United States.

## AUTHOR CONTRIBUTIONS

EKW, PJ, and JDH contributed to the conception and design of the study, assisted with data analysis and interpretation of findings, and wrote the first draft of the manuscript. EKW, PJ, AB, JPC, and JDH contributed to the acquisition of the data. EKW, PJ, EC, AB, JPC, and JDH wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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# Developmental Fluoxetine Exposure Alters Behavior and Neuropeptide Receptors in the Prairie Vole

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Developmental exposure to selective serotonin reuptake inhibitor (SSRI) increases the risk of Autism Spectrum Disorder (ASD), however, the underlying neurobiology of this effect is not fully understood. Here we used the socially monogamous prairie vole as a translational model of developmental SSRI exposure. Paired female prairie voles ($n$ = 20) were treated with 5 mg/kg subcutaneous fluoxetine (FLX) or saline (SAL) daily from birth of the second litter until the day of birth of the 4th litter. This design created three cohorts of FLX exposure: postnatal exposure in litter 2, both prenatal and postnatal exposure in litter 3, and prenatal exposure in litter 4. Post-weaning, subjects underwent behavioral testing to detect changes in sociality, repetitive behavior, pair-bond formation, and anxiety-like behavior. Quantitative receptor autoradiography was performed for oxytocin, vasopressin 1a, and serotonin 1a receptor density in a subset of brains. We observed increased anxiety-like behavior and reduced sociality in developmentally FLX exposed adults. FLX exposure decreased oxytocin receptor binding in the nucleus accumbens core and central amygdala, and vasopressin 1a receptor binding in the medial amygdala. FLX exposure did not affect serotonin 1A receptor binding in any areas examined. Changes to oxytocin and vasopressin receptors may underlie the behavioral changes observed and have translational implications for the mechanism of the increased risk of ASD subsequent to prenatal SSRI exposure.

**Keywords: oxytocin receptor, vasopressin receptor, serotonin receptor, 5-HT, autism, antidepressant, SSRI, autoradiography**

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## INTRODUCTION

In humans, antidepressant medication, most frequently a selective serotonin reuptake inhibitor (SSRI), is commonly prescribed to pregnant and lactating women with major depression (Boukhris et al., 2016). Use of SSRIs during pregnancy has increased dramatically over the last several decades, with estimates ranging from 6 to 13% of pregnancies in the United States (Cooper et al., 2007; Andrade et al., 2008; Alwan et al., 2011). Pharmacological treatment of maternal depression is typically recommended during the prenatal period, primarily because of the well-established negative effects of maternal depression (Davalos et al., 2012; Jarde et al., 2016). However, there

may be side effects of SSRIs leading to preterm labor, altered gestational length and early delivery (Hayes et al., 2012), congenital heart malformations (Knudsen et al., 2014; Gentile, 2015a), persistent pulmonary hypertension (Grigoriadis et al., 2014), and adverse neurodevelopmental outcomes (El Marroun et al., 2014; Glover and Clinton, 2016). There is reason for concern about the effects of early exposure to SSRIs on the developing brain. SSRIs can cross the placental barrier (Hendrick et al., 2003; Rampono et al., 2009) and enter into breast milk (Kristensen et al., 1999; Rampono et al., 2000). Exposed infants show altered brain activity measured via EEG (Videman et al., 2017).

A growing body of research indicates increased rates of Autism Spectrum Disorder (ASD) in prenatally SSRI-exposed children (Croen et al., 2011; El Marroun et al., 2014; Gidaya et al., 2014; Gentile, 2015b; Boukhris et al., 2016; Andalib et al., 2017). While others have found no relationship when controlling for maternal factors (Hviid et al., 2013; Kobayashi et al., 2016) recent meta-analyses indicate that SSRI-exposure does increase autism diagnosis when pooling across studies (Man et al., 2015; Kaplan et al., 2017). Disentangling the effects of the underlying psychiatric condition of the mother from the effects of SSRIs on fetal development is difficult, and causality remains to be established.

Decades of research have indicated a link between ASD and serotonin, starting with the finding of hyperserotonemia in a subset of individuals shortly after the disorder was first described (Schain and Freedman, 1961). Hyperserotonemia has remained a consistent finding in a large subgroup of individuals diagnosed with ASD, with roughly one third of individuals presenting with high whole blood serotonin levels (Schain and Freedman, 1961; Anderson et al., 1987; Hranilovic et al., 2007; Gabriele et al., 2014; Muller et al., 2016). This finding has led researchers to suggest that hyperserotonemia underlies differences in the brain which are responsible for the appearance of autistic behavior (Whitaker-Azmitia, 2005; Yang et al., 2014). Animal models corroborate that hyperserotonemia leads to behavioral and neuroendocrine changes consistent with those seen in autism (Whitaker-Azmitia, 2005; McNamara et al., 2008; Veenstra-VanderWeele et al., 2012; Madden and Zup, 2014; Tanaka et al., 2018). Developmental hyperserotonemia decreases the number of oxytocinergic cells in the paraventricular nucleus of the hypothalamus in both rats (McNamara et al., 2008) and prairie voles (Martin et al., 2012), while decreasing affiliative behavior and increasing anxiety.

The effects of hyperserotonemia on the brain are rooted in serotonin's critical role during early development as a trophic factor, long before it begins to function as a neurotransmitter. As a growth factor, it regulates development of its own and related systems and guides cell division, differentiation, migration, myelination, synaptogenesis, and dendritic pruning (Lauder, 1993; Azmitia, 2001; Wirth et al., 2017). Because serotonin exposure at this time also functions to autoregulate its own innervation throughout the brain via a negative feedback mechanism, developmental hyperserotonemia can cause organizational change which may enduringly alter serotonergic neurotransmission (Whitaker-Azmitia, 2001). Despite the relative paucity of serotonin neurons, they innervate almost all parts of the brain, making this system a powerful mediator of brain activity in many regions. Thus, alterations in serotonin during development may be particularly influential.

Significant overlap exists in psychiatric conditions associated with serotonin dysfunction and ASD. For instance, heightened rates of anxiety and depression may be seen in ASD populations (Lugnegård et al., 2011) and serotonin-based treatments, including SSRIs, show efficacy in treating some symptoms of ASD (Kolevzon et al., 2006; Hollander et al., 2012). Furthermore, depletion of tryptophan, the serotonin precursor, worsens repetitive behavior symptoms in ASD (McDougle et al., 1993, 1996). In addition, gastrointestinal problems are prevalent in ASD (Adams et al., 2011; Chaidez et al., 2014; McElhanon et al., 2014), and serotonin is highly involved in gut motility (Sikander et al., 2009). These comorbidities suggest that disrupted serotonin signaling may underlie the neurobiology of autism.

The serotonin system has important interactions with other systems in the brain. One such example is the interaction seen in the serotonin and oxytocin (OT) systems, both during development and in adulthood. Animal models indicate these systems are anatomically interconnected. Fibers from the dorsal and median raphe project to the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, where oxytocin receptors (OTR) are distributed around them (Emiliano et al., 2007). Serotonin acts on OT neurons via serotonin receptors located in the PVN and SON, where OT is produced (Osei-Owusu et al., 2005). Likewise, OT acts via OTR on serotonin neurons in the raphe nuclei, where serotonin is produced, which may mediate the release of serotonin and have a role in the anxiolytic effects of OT (Yoshida et al., 2009). While evidence suggests that these two neurochemical systems may be working in tandem, it is not yet clear how early SSRI use may affect neural OT.

Vasopressin (AVP) is structurally and genetically similar to OT, and both play a central role in modulating the development of normal social behavior (Carter, 2014). Direct approaches to target the oxytocinergic and vasopressinergic systems are aimed at treating social dysfunction in disorders such as ASD. Although clinical results remain contradictory regarding whether effects are prosocial or antisocial (De Dreu et al., 2010; Guastella et al., 2010), recent advances in our understanding of the complex neurobiology of OT and AVP signaling, release, and degradation present promising avenues for understanding social function in ASD.

Animal models are useful in establishing causal links to long-term effects of perinatal SSRI exposure on social behavior in offspring (Zucker, 2017). Results are complicated by age, sex, and context-specific effects. Pre- and postnatal FLX exposure resulted in loss of a preference for a social partner vs. an empty cage, and a deficit in social recognition, in mice (Bond et al., 2020). When rats were tested as pre-adolescents, prior exposure to perinatal FLX prevented effects of maternal stress on play behavior in both sexes, but also resulted in an increase in aggressive play in males only (Gemmel et al., 2017). When tested as adults, perinatal exposure resulted in sex-specific increases in social behaviors (Gemmel et al., 2019). Another study of perinatal exposure found decreases in social interaction in male rats when tested as adults

(Silva et al., 2018). In addition, some types of social behavior (i.e., pair bonding) are not present in rats and mice, necessitating a different animal model.

In the present study, we used the prairie vole as a translational model of developmental SSRI exposure. Prairie voles are socially monogamous microtine rodents that form lasting adult heterosexual pair bonds characterized by the formation of a partner preference, intrasexual aggression, and bi-parental care. Prairie voles are highly social and have a well described neurohypophyseal nonapeptide system (for review see Young et al., 2011) and can be tested in standardized assays of social behavior and anxiety-like behavior (e.g., partner preference, elevated plus maze). Here we use the prairie vole to examine how developmental exposure to a SSRI affects adult behavior and neural OTR, vasopressin 1a (V1aR), and serotonin 1A (5-HT1a) receptors and to determine if these changes replicate aspects of the symptomology of ASD.

# MATERIALS AND METHODS

## Subjects

Subjects were laboratory-housed prairie voles (*Microtus ochrogaster*) from the breeding colony at the University of California, Davis. This colony was derived from a lineage of stock which was wild-caught near Champaign, IL. Animals were housed on a 14:10 light dark cycle with lights on at 0600. Food (Purina high-fiber rabbit chow) and water were available *ad libitum* in the home cage. Breeding pairs and offspring prior to weaning were housed in large polycarbonate cages (44 cm × 22 cm × 16 cm) and were given compressed cotton nestlets for bedding. Offspring were weaned on postnatal (PND) 20 and housed in small polycarbonate cages (27 cm × 16 cm × 16 cm) throughout testing with a same-sex sibling when available and a similarly aged non-sibling when not. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

## Drugs

Fluoxetine hydrochloride (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in isotonic saline in a concentration of 1 mg/ml. It was then filtered into sterile solution and injected subcutaneously at the nape of the neck in a dose of 5 mg/kg. This dose was chosen based on the literature and the results of our own prior dose finding study. Both 5 and 10 mg/kg doses of FLX are commonly used in other rodent studies for perinatal administration (Gemmel et al., 2017, 2019; Grieb and Ragan, 2019). In the prairie vole dose-finding study, we examined the effect of 5 mg/kg FLX, 10 mg/kg FLX, or saline (SAL) vehicle on forced swim behavior and sucrose preference in socially isolated adult female prairie voles. At 5 mg/kg, females struggled significantly less (when compared to SAL, $t_{36} = -2.92$, $p = 0.005$), and spent approximately 40% less time immobile (although this was not statistically significant). In contrast, at 10 mg/kg struggle behavior did not differ from SAL, and time spent immobile trended toward an increase (when compared to saline, $t_{37} = 1.64$, $p = 0.106$). We therefore determined that 5 mg/kg was a more appropriate dose for the current study (data are available in **Supplementary Figure S1**).

## Design and Procedures

Virgin prairie voles (20 male, 20 female) were paired and allowed to raise a litter of pups together undisturbed. On the day of birth of the second litter, females were hand caught and pups were briefly removed. Litters were culled to two male and two female pups when possible. Females were given a subcutaneous injection of 5 mg/kg FLX or SAL at the nape of the neck and returned to the home cage along with her pups. On subsequent days, the female was hand caught and FLX or SAL was injected without removing the pups from the nipples. Females were dosed daily in this way with either FLX or SAL until the day of birth of the fourth litter. This design created three cohorts of FLX exposure: postnatal exposure in litter 2 (POST), both prenatal and postnatal exposure in litter 3 (PRE + POST), and prenatal exposure in litter 4 (PRE) (**Figure 1**). The average interbirth interval for litter 2–3 was 22.7 ±0.34 days (range 21–26), and for litter 3–4 was 22.9 ±0.19 days (range 21–24).

## Parental Care of Prenatally Exposed Offspring

Parental care is minimally altered following treatment with FLX (Villalba et al., 1997), however the effects of withdrawal prior to weaning has not been examined in prairie voles. Parental care of prenatally FLX-exposed subjects (litter 4) was quantified in the home cage to determine whether FLX withdrawal would significantly alter parental behavior. Undisturbed parental care was observed in the home cage for 20 min once during the morning and once in the afternoon on 2 days between PND 1-3. Behaviors were quantified in real-time using Behavior Tracker 1.5 (behaviortracker.com) using methods previously validated to measure the type and amount of parental care (Perkeybile et al., 2013). Both maternal and paternal behavior was measured, including huddling, non-huddling contact, licking/grooming, pup retrieval, nest building, and maternal nursing postures.

## Behavioral Tests

After weaning, subjects underwent behavioral testing. Half of each litter, one male and one female when possible, underwent behavioral testing during periadolescence, between PND21 and PND39. Periadolescent subjects underwent alloparental care, elevated plus maze, and open field testing in that order. The other half of each litter, one male and one female when possible, underwent behavioral testing as adults, between PND45 and PND120. Adult subjects were tested for alloparental care, elevated plus maze, and open field; in addition, they also underwent intrasexual adult affiliation and partner preference testing. All behaviors were quantified using Behavior Tracker 1.5 (behaviortracker.com). Behavioral tests occurred from 1 to 5 days apart.

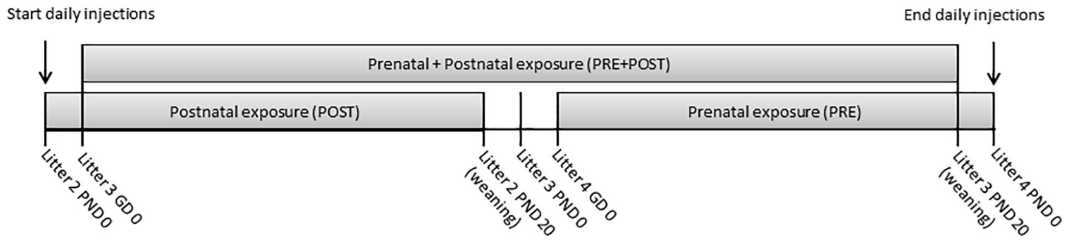


**FIGURE 1 |** Timeline of maternal daily dosing and subject exposure. GD, gestational day; PND, postnatal day.

## Alloparental Care

A minimum of 24 h after weaning, subjects were tested with a novel pup to measure alloparental care behavior as previously described (Bales et al., 2004a). Subjects were placed into an arena consisting of two polycarbonate cages (27 cm × 16 cm × 16 cm) connected by a short clear tube for a 45-minute acclimation period. This period was followed by a 10 min test in which a novel pup (PND 0-4) was placed into the arena. The subject was free to move about the arena and interact with the pup. Tests were video-recorded and later scored by a trained observer blind to condition. Behaviors quantified included frequency and latency of approach, sniffing, licking and grooming the pup, autogrooming, physical contact with the pup, huddling, pup retrievals, non-injurious biting, attacks, digging, and location in the arena relative to the pup. Digging and autogrooming were considered potential stereotypical behaviors. When attacks occurred, the test was immediately stopped and the subject removed from the arena. If possible, injuries were treated and the pup returned to the home cage. If necessary, the pup was euthanized. Each pup was used for no more than two test sessions. Following testing, animals were returned to their home cage.

Sex differences in prairie voles in this test are well-established, with males responding with higher levels of alloparental care than females. This sex difference, although already present in peri-adolescents, becomes more marked as animals become adult (Roberts et al., 1998).

## Elevated Plus Maze

The elevated plus-maze was used as a measure of anxiety and exploration (Insel et al., 1995) based on the rodent predisposition to prefer dark enclosed spaces (Campos et al., 2013). The maze consisted of two open and two enclosed opaque arms, each 67 cm long and 5.5 cm wide. The arms were elevated 1 m above the floor. Each vole was placed into the center of the maze and its behavior was scored for 5 min. Any animals that jumped off the open arms of the maze were captured and placed back into the center of the maze. If a subject jumped off the maze three times, the test was stopped. Throughout the course of the study, only four animals jumped off the maze, and data from only two animals had to be removed due to jumping. Trained observers blind to conditions scored behavior live for duration of time in the open and closed arms, freezing, and autogrooming with an inter-rater reliability greater than 90%. Autogrooming was considered a potential stereotypical behavior. Following testing animals were returned to their home cage.

It is worth noting that at baseline, prairie voles spend a higher amount of time in the open arms of the elevated plus-maze than mice typically do (Komada et al., 2008). While across 90 genetically engineered strains, mice spent an average of 9.19 ± 0.36% time in the open arms of the maze, prairie voles often spend 35–75% of their time in the open arms (Bales et al., 2004b; Greenberg et al., 2012). Male prairie voles tend to spend more time in the open arms, or exhibit higher frequencies of open arm entries, than females (Bales et al., 2004b; Greenberg et al., 2012).

## Open Field

The open field test was used as a second measure of anxiety and exploration (Ramos and Mormède, 1997). The open field consisted of a 40 cm × 40 cm × 40 cm plexiglass arena with a grid marked on the floor. The subject was placed in the center of the arena and behavior was digitally recorded for 10 min. Time spent in the center and the periphery was measured, as well as the frequency of rearing. Tests were video recorded and later scored using Behavior Tracker by trained observers with an inter-rater reliability greater than 90%. Following testing animals were returned to their home cage. Sex differences for prairie voles are not well established and are absent in some studies (Greenberg et al., 2012); we did not therefore predict any sex differences at baseline for this test.

## Intrasexual Adult Affiliation

Subjects were placed into a novel arena (27 cm × 16 cm × 16 cm) with a stimulus animal of the same sex and body size for 5 min as a low-threat, low-aggression social interaction task (Perkeybile and Bales, 2015). Behavior was video recorded and later scored by an observer blind to the treatment condition. The ethogram used to score behavior included affiliative behaviors (sniffing, physical contact, allogrooming, and play), anxiety related behaviors (rearing, digging, abrupt withdrawal), and aggressive behaviors (lunging, wrestling, chasing). Digging and autogrooming were considered potential stereotypical behaviors. Prior to testing, stimulus animals were screened for aggressive behavior with a novel animal, and were not used if they displayed high levels of aggression. Stimulus animals were collared prior to the start of testing to allow for identification during later behavioral scoring. Stimulus animals were used for a maximum

of 2 tests, and were not reused if they experienced an aggressive interaction. Tests were continuously monitored for high levels of aggression and were stopped if necessary. Intense aggression was rarely seen. Following testing, animals were returned to the home cage. At baseline, we expected males to be more aggressive and less affiliative than females (Bales and Carter, 2003b).

## Partner Preference

This test is commonly used as an operational index of the formation of a pair-bond in the prairie vole (Williams et al., 1992; Bales and Carter, 2003a; Bales et al., 2013). Male subjects were housed with a female "partner" for 24 h prior to testing and female subjects were housed with a male partner for 6 h prior to testing. These durations have been previously shown to be sufficient time for the formation of a partner preference and account for the sex difference in time to pair bond formation (Williams et al., 1992; DeVries and Carter, 1999). Following this cohabitation, the opposite-sex mate of the subject (partner) and a non-related opposite-sex animal matched on age and weight to the mate (stranger) were tethered in opposing ends of a three-chamber testing apparatus. The subject was placed untethered in the empty middle chamber and was free to move about all three chambers and interact with either the partner or stranger for 3 h. The test was digitally recorded, and the duration of time in each of the three locations was quantified, as was the duration of side by side contact with the stranger and partner.

## Brain Extraction and Tissue Sectioning

Brains were taken from behaviorally tested animals of both ages (juvenile and adult), but only brains from the PRE + POST exposure cohort were analyzed for receptor binding (see below). Twenty-four hours after completion of all behavioral testing, subjects were euthanized via cervical dislocation and rapid decapitation under deep anesthesia. Brains were removed quickly and placed in powdered dry ice and then stored at −80°C until sectioning. Brain tissue was sectioned coronally in 20 μm slices at 20°C on a cryostat (Leica) and thaw mounted on Fisher Superfrost Plus slides. Slides were stored at −80°C until the time of assay.

## OTR and V1aR Autoradiography

Because they showed the largest effects on behavior, quantitative receptor autoradiography for OTR, V1aR, and 5-HT1aR was performed for the PRE + POST exposure cohort. Analyses were carried out on the right side of the brain only, as tissue punches were taken from the left side for additional analyses. Tissue was allowed to thaw in slide boxes containing desiccant packets. OTR and V1aR autoradiography was performed as previously reported (Perkeybile and Bales, 2015) with minor adjustments. For OTR binding, the ligand $^{125}$I-OVTA [$^{125}$I-ornithine vasotocin [d(CH$_2$)$_5$[Tyr(Me)$^2$, Thr$^4$, Orn$^8$, ($^{125}$I)Tyr$^9$-NH$_2$] analog], 2200Ci/mmol (Perkin Elmer, Waltham, MA, United States) was used. For V1aR binding, the ligand $^{125}$I-LVA [$^{125}$I-lin-vasopressin [$^{125}$I-phenylacetyl-D-Tyr(ME)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$] analog], 2200Ci/mmol (Perkin Elmer, Waltham, MA, United States) was used. After assay completion, slides along with $^{125}$I-autoradiographic standards (American Radiolabeled Chemicals, St. Louis, MO, United States) were exposed to Biomax MR film (Kodak, Rochester, NY, United States) for 72 h and then developed. We have previously reported a sex difference in the nucleus accumbens shell, with males displaying higher OTR binding than females at baseline (Guoynes et al., 2018).

## 5-HT$_{1A}$ Autoradiography

For 5-HT$_{1A}$ binding, 3.0 nM [$^3$H]WAY-100635, 74Ci/mmol (Perkin Elmer, Waltham, MA, United States) was used. Tissue was rinsed in 50 mM Tris–HCl buffer (pH 7.5) followed by a 120 min incubation in the tracer buffer at room temperature. 10 nM of L-485,870, a dopamine antagonist, was included to prevent binding of WAY-100635 to Dopamine D4 receptors. Following the incubation period, tissue was rinsed twice in 50 mM Tris buffer at 4°C and then dipped in dH$_2$O and air dried. Tissue was exposed to Carestream BioMax MR Film (Kodak, Rochester, NY, United States) for 6 weeks with $^3$H microscale standards (American Radiolabeled Chemicals, St. Louis, MO, United States). We had no *a priori* predictions as far as 5-HT$_{1A}$ binding sex differences at baseline for this species.

## Quantification

Experimenters were blind to conditions during autoradiogram quantification. ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used to quantify OTR optical binding density (OBD) in previously reported (Insel and Shapiro, 1992) regions of interest (ROI) including the nucleus accumbens core and shell, anterior central amygdala, and the lateral septum, and for V1aR in the medial amygdala, lateral septum, and ventral pallidum. 5-HT1aR OBD were quantified in the anterior and posterior lateral septum, dorsal hippocampus, dorsal raphe, and frontal cortex using MCID Core Digital Densitometry system (Cambridge, United Kingdom). The ten standard OBD values were used to generate a standard curve. Three separate measurements for ROIs and background OBD were averaged to yield normalized values and account for individual variation in background across samples.

## Data Analysis

Statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC, United States). All analyses were carried out using generalized linear mixed models (GLMM) utilizing backward selection to eliminate non-significant variables from the model. Significance level was set at $p < 0.05$ for all analyses and all tests were two-tailed. Data were checked for normality, and if not normally distributed, square root, quad root, or reciprocal transformation was used. If data was not transformable to normality, a GLMM was still used as recommended by Feir-Walsh and Toothaker (1974). *Post hoc* analyses utilized least squares means when the omnibus test was significant. The random factor used in all analyses was a pair ID (for the subject's parents) to account for differences due to parenting or genetic background for subjects within the same litter or across litters. Drug condition was nested within this term, as each female maintained a consistent drug condition throughout the study and thus all offspring of a given pair had the same drug condition.

When a three-way interaction was statistically significant, all two-way interactions which included the variables in the three-way interaction were left in the model even if not significant.

## Parental Care

A multivariate mixed model was used for analysis of parental care behavior. All three types of nursing were included in one model, as were behaviors that were examined concomitantly in both mothers and fathers that were not independent, such as huddling. Factors included in the model were pair ID and drug condition of the mother prior to cessation of treatment, as well as age of pups at observation and time of day as covariates.

## Alloparental Care Test

For the alloparental care analyses, variables were summed for duration of time in the same location (with the pup) or different location (without the pup) in the testing arena. A ratio was created to examine relative proportion of time spent in the same location as the pup relative to duration in a different location than the pup using the equation: ratio = with the pup/(with the pup + without the pup). Factors included in the model were pair ID, drug condition, sex, exposure cohort, age group, and interactions of these factors. Also analyzed were time spent in contact to the pup, time spent retrieving the pup, time spent in proximity to the pup, latency to approach, duration of social investigation, duration of licking, and duration of huddling over the pup.

## Elevated Plus Maze

For the elevated plus maze analysis, a ratio was created to examine the proportion of time spent on the open arms relative to total time on the maze using the equation: ratio = time on open arms/(time on open arms + time on closed arms). Factors included in the model were pair ID, drug condition, sex, exposure cohort, age group, and interactions of these factors. Autogrooming, entries onto the arms of the maze, and duration of freezing, were also analyzed.

## Open Field Test

For the open field test analyses, a ratio was created to examine proportion of time spent in the center of the arena relative to total time using the equation: ratio = time in center/(time in center + time in periphery). Factors included in the model were pair ID, drug condition, sex, exposure cohort, age group, and interactions of these factors. Rearing was also analyzed.

## Intrasexual Adult Affiliation

For the intrasexual adult affiliation analyses, the frequency of aggressive behavior was calculated by summing the frequencies of lunging and wrestling. Factors included in the model for each behavior (including affiliative, anxiety-like, and aggressive behaviors, as described above) were pair ID, drug condition, sex, exposure cohort, and interactions of these factors.

## Partner Preference Test

For between-group partner preference test analyses, a difference score was created to examine duration of time spent in the same cage as the partner relative to time spent with the stranger using the equation: difference = time with partner - time with stranger. The same procedure was used to examine physical contact with the partner relative to contact with the stranger using the equation: difference = time in contact with the partner - time in contact with the stranger. Duration of time spent in the empty chamber was analyzed separately, and square root transformed for analyses to make the residuals for this model normally distributed. Factors included in the model were pair ID, drug condition, sex, exposure cohort, and interactions of these factors.

Within-group partner preference analyses for the SAL and FLX groups were performed using matched t-tests for time spent in contact with the partner vs. time spent in contact with the stranger.

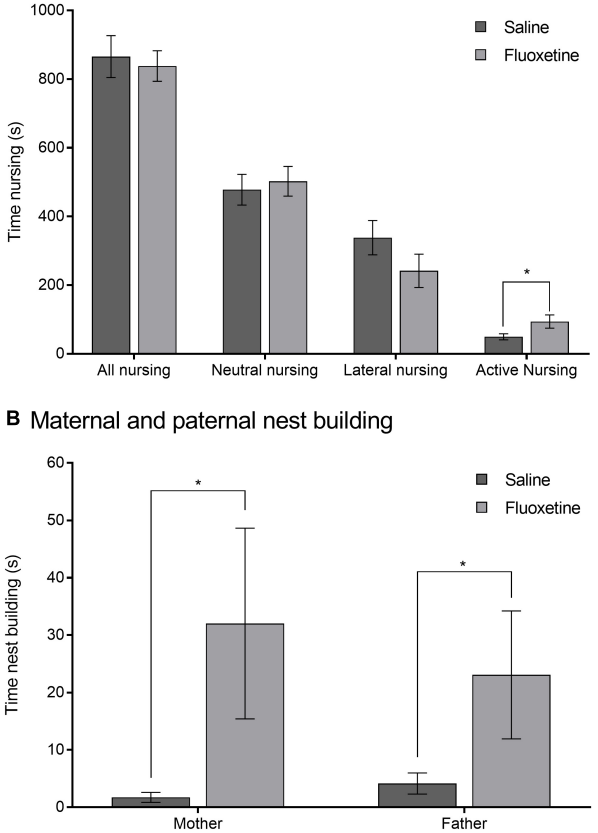


**FIGURE 2 |** Parental care of prenatal exposure subjects. **(A)** Mean (±SEM) total, neutral, lateral, and active nursing duration comparing mothers previously exposed to saline to mothers previously exposed to fluoxetine. **(B)** Mean (±SEM) duration of nest building in mothers previously exposed to saline and their male pair-mates (fathers) compared to mothers previously exposed to fluoxetine and their pair-mates. *$p < 0.05$.

## Oxytocin, Vasopressin 1a, and Serotonin 1a Receptor Binding

For all binding analyses, density of binding in three sequential areas of each ROI were averaged for each individual. The model

included pair ID, drug condition, sex, age group, and interactions of these factors.

Pearson correlations were calculated for the 4 ROIs quantified for OTR and the 3 ROIs quantified for V1aR with difference in time in physical contact and duration of time in the empty chamber in the partner preference test. Correlation of OTRs in the central amygdala and proportion of time on the open arms of the elevated plus maze was also examined. When multiple comparisons were made within a single behavioral or neuroanatomical test, a Benjamini-Hochberg false discovery rate adjustment for multiple comparisons was used (Benjamini and Hochberg, 1995).

# RESULTS

## Parental Care

Parental care of the PRE cohort was minimally altered by the drug condition of the mother, either FLX withdrawal or no withdrawal from SAL at the time of parenting. Drug condition did not alter total duration of nursing, nor did it alter duration of neutral nursing postures or lateral nursing postures. However, duration of active nursing was altered by drug condition ($F_{1,51} = 5.11$, $p < 0.05$), with FLX-withdrawing dams spending more time in active nursing than those who had been treated with SAL (**Figure 2A**). Nest building duration was also greater in FLX-withdrawing mothers ($F_{1,51} = 4.06$, $p < 0.05$) as well as their untreated male pair-mates ($F_{1,51} = 4.79$, $p < 0.05$) compared to pairs in which mothers were previously treated with SAL (**Figure 2B**). Because of the high amount of variability in this behavior, we also analyzed nest-building with a non-parametric Kruskal-Wallis test. The duration of nest-building in FLX-withdrawing mothers, compared to SAL mothers, remained significant ($\chi^2_1 = 4.62$, $p < 0.05$), however, the effect was non-significant in their male mates ($\chi^2_1 = 1.14$, $p > 0.05$). All other behaviors observed were not affected by drug condition including maternal huddling, paternal huddling, maternal non-huddling contact, paternal non-huddling contact, maternal licking and grooming, paternal licking and grooming, maternal pup retrieval, paternal pup retrieval, maternal autogrooming, or paternal autogrooming.

## Behavior of Developmentally Exposed Offspring

### Alloparental Care Test

Duration of overall pup physical contact was greater in males than in females ($F_{1,167} = 8.28$, $p < 0.01$). A three-way interaction of condition, sex, and age group ($F_{1,167} = 3.77$, $p < 0.05$) indicated that among FLX subjects, adult females were in contact with the pup less than periadolescent females ($t_{41} = 2.88$, $p < 0.05$) and that among SAL subjects, periadolescent females were in contact with the pup less than periadolescent males ($t_{49} = 2.06$, $p < 0.05$). Adult females spent less time in contact with the pup compared to adult males exposed to either SAL ($t_{52} = 1.97$, $p < 0.05$) or FLX ($t_{44} = 2.83$, $p < 0.01$) (**Figure 3A**). Put another way, females were in contact with the pup less than males under matching conditions, with the exception of FLX periadolescent females, which spent more time in contact with the pup than did FLX periadolescent males.

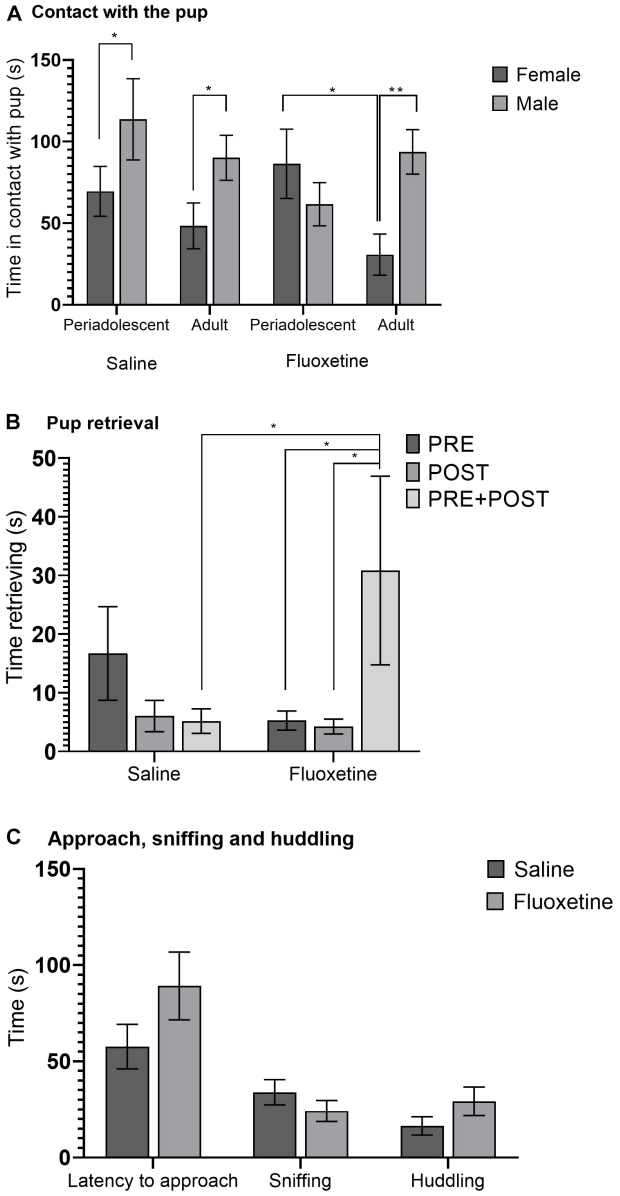


**FIGURE 3 |** Alloparental care behavior. **(A)** Mean (± SEM) duration of physical contact with the pup comparing saline and fluoxetine exposure by age and sex. **(B)** Mean (±SEM) duration of pup retrieval comparing saline and fluoxetine exposure by exposure cohort. **(C)** Mean (± SEM) latency to approach the pup, sniffing, and huddling comparing saline and fluoxetine exposure. *$p < 0.05$, **$p < 0.01$.

Duration of time spent retrieving the pup tended to be greater in males than in females ($F_{1,163} = 3.69$, $p = 0.057$). A drug condition by cohort interaction ($F_{2,163} = 3.44$, $p < 0.05$) (**Figure 3B**) indicated that in the PRE + POST cohort, FLX subjects spent more time retrieving the pup than SAL subjects ($t_{63} = 2.34$, $p < 0.05$), and that in FLX subjects, PRE + POST

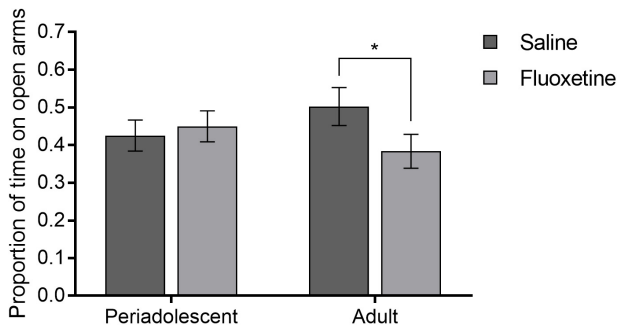


**FIGURE 4 |** Elevated plus maze. Mean (±SEM) proportion of time spent in the open arms relative to total time comparing saline and fluoxetine exposure by age. *$p < 0.05$.

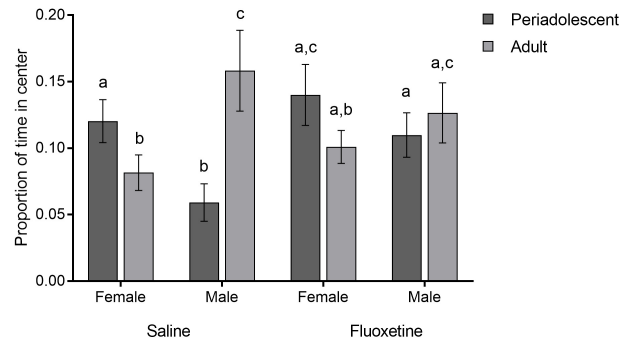


**FIGURE 5 |** Open field test. Mean (±SEM) proportion of time spent in the center relative to total time comparing saline and fluoxetine exposure by age and sex. Different letters indicate a significant difference at $p < 0.05$.

subjects spent more time retrieving than PRE ($t_{60}$ = 2.40, $p < 0.05$) and POST ($t_{58}$ = 2.47, $p < 0.05$) subjects.

Fluoxetine exposure had no effect on proximity to the pup, licking the pup, latency of approach, social investigation, or huddling (**Figure 3C**). Ratio of time spent in the same chamber of the testing arena as the pup relative to total time was not altered by drug condition, nor was latency to approach the pup, duration of sniffing, huddling, licking, or grooming of the pup. There was no indication of heightened repetitive behavior with FLX exposure, and duration of autogrooming and digging were not altered by drug condition.

### Elevated Plus Maze

Proportion of time spent in the open arms relative to total time on the maze showed an interaction of drug condition and age group ($F_{1,141}$ = 4.02, $p < 0.05$) such that FLX-exposed adults spent a lower proportion of time in the open arms compared to SAL-exposed adults ($t_{64}$ = 2.21, $p < 0.05$), while there was no such difference in periadolescent subjects (**Figure 4**). Drug condition did not alter the number of entries onto the arms of the maze, duration of freezing, or duration of autogrooming.

### Open Field Test

Proportion of time spent in the center of the open field relative to total time showed a three-way interaction of drug condition, sex, and age group ($F_{4,119}$ = 4.66, $p < 0.01$) (**Figure 5**). In SAL-exposed females, periadolescents spent more time in the center than adults ($t_{39}$ = 2.48, $p$ = 0.01), while this was not true for FLX-exposed subjects ($t_{30}$ = 1.29, $p$ = 0.20). Among SAL exposed subjects, time in the center was greater in adult males than adult females ($t_{31}$ = 3.42, $p < 0.001$), in periadolescent females than periadolescent males ($t_{44}$ = 1.94, $p$ = 0.05), and in adult males than periadolescent males ($t_{36}$ = 3.00, $p < 0.01$). There was also a trend level difference between SAL males and SAL females ($t_{76}$ = 1.91, $p$ = 0.06). There were no sex or age group differences within the FLX-exposed subjects. Duration of autogrooming and frequency of rearing were not affected by drug condition.

### Intrasexual Adult Affiliation Test

Duration of sniffing of the stimulus animal, the primary form of social investigation, did not differ by drug condition. Duration of allogrooming of the stimulus animal showed a trend level interaction of drug condition and sex ($F_{1,91}$ = 3.73, $p$ = 0.057). FLX exposed males spent more time allogrooming than SAL exposed males ($t_{49}$ = 1.77, $p$ = 0.07), and SAL females spent more time allogrooming than SAL males ($t_{48}$ = 1.91, $p$ = 0.059). Duration of time in physical contact with the stimulus animal, autogrooming, or frequency of rearing were not altered by drug condition.

Frequency of aggressive behavior was not altered by drug condition. In contrast, duration of digging showed an interaction of treatment and sex ($F_{1,73}$ = 4.62, $p < 0.05$) (**Figure 6A**). SAL males dug more than SAL females ($t_{48}$ = 2.53, $p < 0.05$), but there was no sex difference in FLX exposed subjects.

Duration of play with the stimulus animal showed an interaction of drug condition and sex ($F_{1,91}$ = 5.75, $p < 0.05$) (**Figure 6B**). FLX males played more than FLX females ($t_{45}$ = 2.23, $p < 0.05$) and SAL males ($t_{49}$ = 2.36, $p < 0.05$).

## Partner Preference Test

Difference in duration of time in the partner and stranger chambers was greater in females compared to males ($F_{1,74}$ = 12.95, $p < 0.001$) but did not differ by cohort or drug condition (**Figure 7A**). Difference in duration of time in side-by-side contact with the partner and the stranger was not altered by cohort but did show an interaction of sex and drug condition ($F_{1,73}$ = 4.01, $p < 0.05$) (**Figure 7B**). SAL females spent more time in physical contact with the partner than SAL males ($t_{40}$ = 2.62, $p < 0.01$), but there was no sex difference in the FLX condition. Within the SAL group, females formed a significant preference for the partner ($t_{24}$ = 3.44, $p$ = 0.002), while males did not ($t_{16}$ = −0.14, $p$ = 0.891). Within the FLX group, neither females ($t_{18}$ = 1.672, $p$ = 0.121) nor males ($t_{16}$ = 1.816, $p$ = 0.07) formed a significant preference.

Duration of time spent in the empty chamber in the partner preference test showed an interaction of drug condition and exposure cohort ($F_{2,70}$ = 4.17, $p < 0.05$) (**Figure 7C**). Subjects

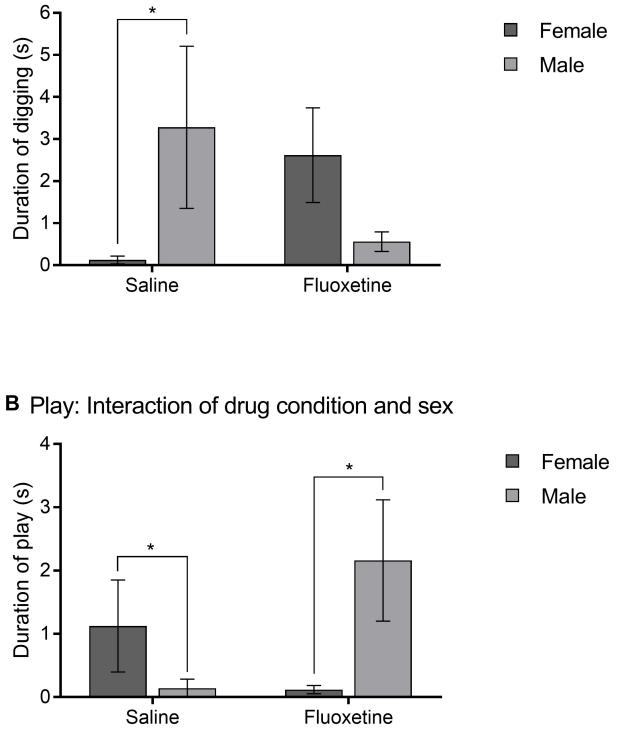


**FIGURE 6 |** Intrasexual adult affiliation. **(A)** Mean (± SEM) duration of digging comparing saline and fluoxetine exposure by sex. **(B)** Mean (±SEM) duration of play comparing saline and fluoxetine exposure by sex. *$p < 0.05$.

in the PRE cohort that were exposed to FLX spent more time in the empty chamber than those exposed to SAL ($t_{26} = 2.06$, $p < 0.05$). Time in the empty chamber was not altered by sex, nor were there differences by drug condition in the PRE + POST or POST conditions.

## Quantitative Receptor Autoradiography

### Oxytocin Receptors

Oxytocin receptors binding in the nucleus accumbens core was lower in FLX subjects compared to SAL subjects ($F_{1,43} = 3.96$, $p = 0.05$) and was greater in adult compared to periadolescent subjects ($F_{1,43} = 7.18$, $p < 0.01$). A drug condition by sex interaction ($F_{1,43} = 4.89$, $p < 0.05$) (**Figures 8A**, **9A**) indicated that FLX females had less OTR binding than SAL females ($t_{31} = 2.84$, $p < 0.01$) and FLX males ($t_{30} = 2.20$, $p < 0.05$). A drug condition by age group interaction ($F_{1,43} = 5.02$, $p < 0.05$) (**Figure 8B**) indicated that FLX adults had less OTR binding than SAL adults ($t_{28} = 2.73$, $p < 0.01$). Adults also had greater OTR binding compared to periadolescents with SAL exposure ($t_{34} = 3.50$, $p = 0.001$), but this was not the case with FLX exposure ($t_{30} = 0.31$, $p = 0.76$). OTR binding in the nucleus accumbens shell did not differ by drug condition or sex. Adult subjects had greater OTR binding in the nucleus accumbens shell than periadolescents ($F_{1,45} = 3.92$, $p = 0.05$; **Figure 8C**).

Oxytocin receptors binding in the anterior central amygdala was decreased with FLX exposure compared to SAL exposure ($F_{1,46} = 8.42$, $p < 0.01$). There was no effect of sex on OTR binding in the central amygdala. A condition by age group interaction ($F_{1,46} = 3.98$, $p = 0.05$) (**Figures 8D**, **9B**) indicated that FLX adults had lower OTR binding compared to SAL adults ($t_{66} = 3.26$, $p < 0.01$), and that SAL adults had higher OTR binding than SAL periadolescents ($t_{34} = 2.01$, $p = 0.05$), but this age difference was not found with FLX exposure. OTR binding in the lateral septum was not altered by drug condition (**Figure 8E**), sex, or age group.

Oxytocin receptors binding did not correlate with difference in contact between the partner and stranger or duration in the empty chamber in the partner preference test. There was also no correlation between OTR binding in the central amygdala and proportion of time on the open arms of the elevated plus maze.

### Vasopressin 1a Receptors

Vasopressin 1a binding in the medial amygdala was reduced by FLX exposure compared to SAL exposure ($F_{1,47} = 4.20$, $p < 0.05$) (**Figures 10A**, 9C). V1aR binding in the medial amygdala was not altered by sex or age group. V1aR binding in the lateral septum was not altered by drug condition, sex, or age group (**Figure 10B**). V1aR binding in the ventral pallidum was not altered by drug condition, sex, or age group (**Figure 10C**).

Vasopressin 1a binding density in the three ROIs quantified did not correlate with difference in contact between the partner and stranger or duration in the empty chamber in the partner preference test once adjusted to account for multiple comparisons.

### Serotonin 5-HT1a Receptors

Unexpectedly, there was no effect of FLX exposure on 5-$HT_{1A}$ receptor binding density in any ROI examined (anterior and posterior lateral septum, dorsal hippocampus, dorsal raphe, frontal cortex) nor were there any significant interactions of age group, sex, and ROI (**Figures 11A–E**).

## DISCUSSION

Understanding the etiology of the increased risk of ASD associated with developmental SSRI exposure is an area of research which can greatly benefit from animal models. Here, we used the prairie vole as a translational model in which to examine how exposure to an SSRI, FLX, affects behavior, neuropeptide receptors, and serotonin receptors in the brain.

We examined three primary behavioral domains which are associated with ASD: social behavior, repetitive behavior, and anxiety-like behavior. The first two represent the two primary diagnostic criteria for ASD, impaired social communication and stereotyped or repetitive behavior; the third represents the heightened anxiety frequently comorbid in ASD (White et al., 2009; van Steensel et al., 2011). Modeling the social communication domain of

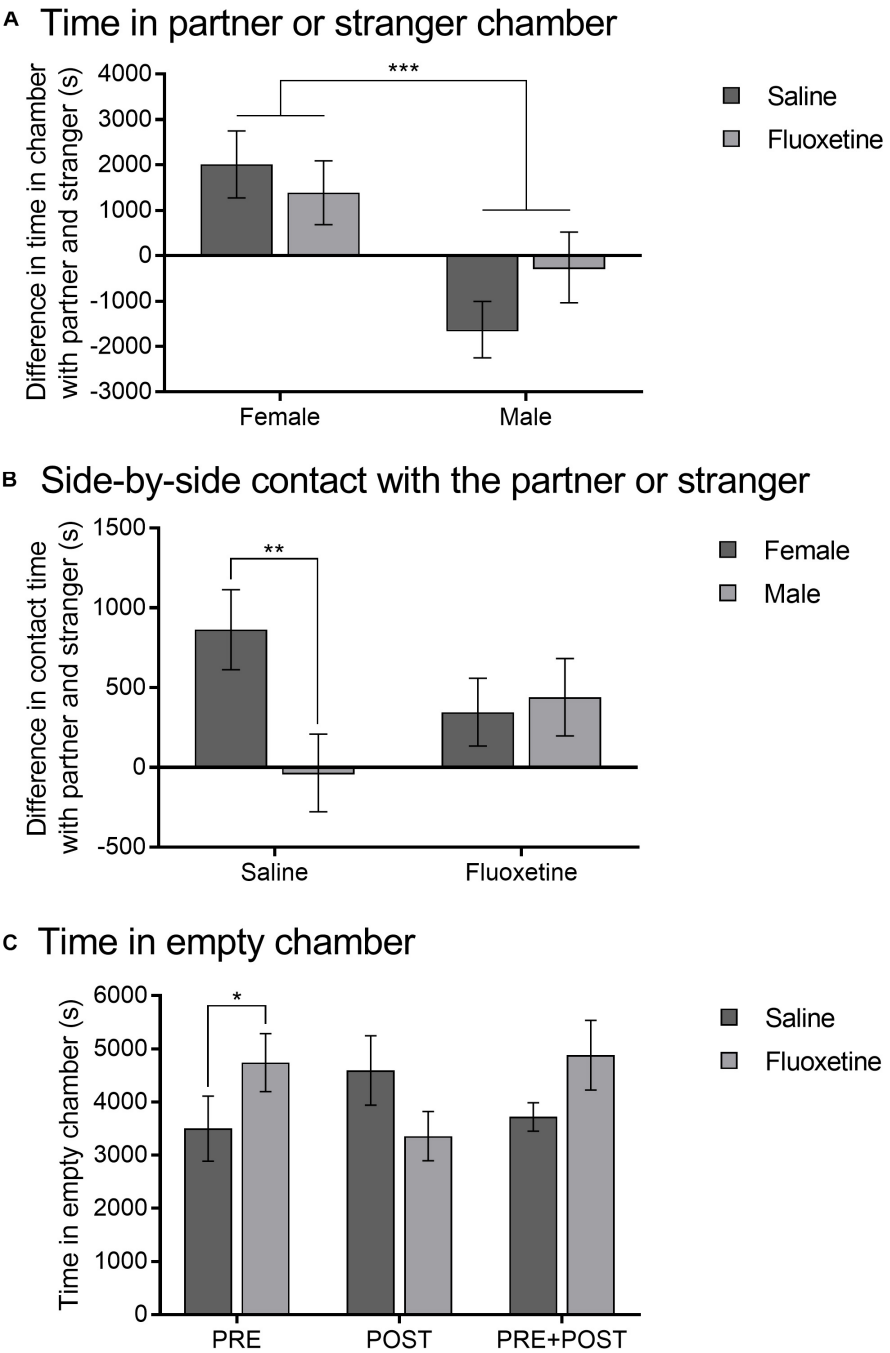


**FIGURE 7 |** Partner preference test. **(A)** Mean (±SEM) difference in duration between time spent in the partner chamber and the stranger chamber comparing saline and fluoxetine exposure by sex. **(B)** Mean (±SEM) difference in duration between time spent in side-by-side contact with the pair-mate and the stranger comparing saline and fluoxetine exposure by sex. **(C)** Mean (±SEM) duration of time in the empty chamber comparing saline and fluoxetine exposure by exposure cohort. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ASD is particularly difficult in animal models. Verbal language is uniquely human, and thus the precise deficits found in individuals with ASD cannot be modeled in any animal species.

We examined sociality by measuring species-typical behaviors involved in social interaction and looking for deficits in FLX exposed subjects. Social investigation (sniffing) was not altered by FLX with a novel social partner, be it a pup or an adult conspecific. Affiliative behavior, which is ubiquitous in prairie voles, was altered by FLX exposure (**Table 1**). We observed changes in alloparental care (**Figures 3A,B**), in play behavior with a same-sex adult (**Figure 6B**), and in time spent in the empty

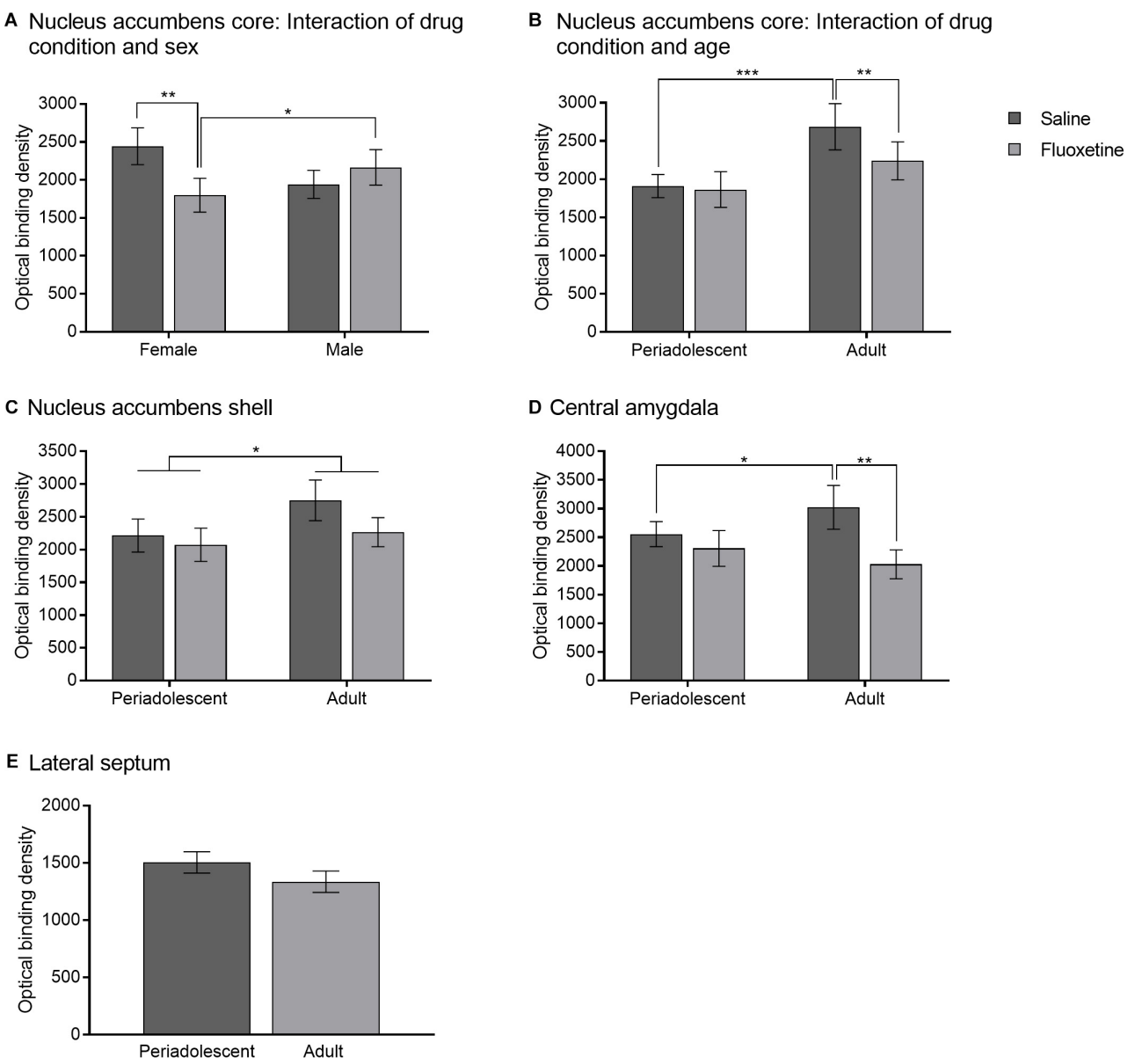


**FIGURE 8 |** Oxytocin receptor binding. **(A)** Mean (± SEM) optical binding density in the nucleus accumbens core comparing saline and fluoxetine exposure by sex. **(B)** Mean (±SEM) optical binding density in the nucleus accumbens core comparing saline and fluoxetine exposure by age. **(C)** Mean (±SEM) optical binding density in the nucleus accumbens shell comparing saline and fluoxetine exposure by age. **(D)** Mean (±SEM) optical binding density in the central amygdala comparing saline and fluoxetine exposure by age. **(E)** Mean (±SEM) optical binding density in the lateral septum comparing saline and fluoxetine exposure. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

chamber of the partner preference test (**Figure 7C**). The changes in alloparental care were primarily in retrieval behavior, with males that had been treated with both prenatal and postnatal FLX spending significantly more time retrieving (**Figure 3B**). These males were picking up the pup in their mouths and running excitedly around the test arena, in an apparently less organized manner of providing care for the pup.

During the partner preference test, prenatal FLX exposure also led subjects of both sexes to opt out of social interaction in favor of time alone in the empty cage (**Figure 7C**), indicating that FLX led to a rejection of social interaction very atypical of prairie voles. However, FLX males also spent more time in play behavior with stimulus males during the intrasexual affiliation test. Much as the research in humans suggests, prenatal SSRI exposure may increase the likelihood of asociality, or the alteration or disorganization of sociality; but it does so in subtle, non-deterministic ways.

The neurohypophyseal nonapeptides, oxytocin and vasopressin, are likely candidates to be involved in such shifts in sociality due to their developmental interaction with serotonin

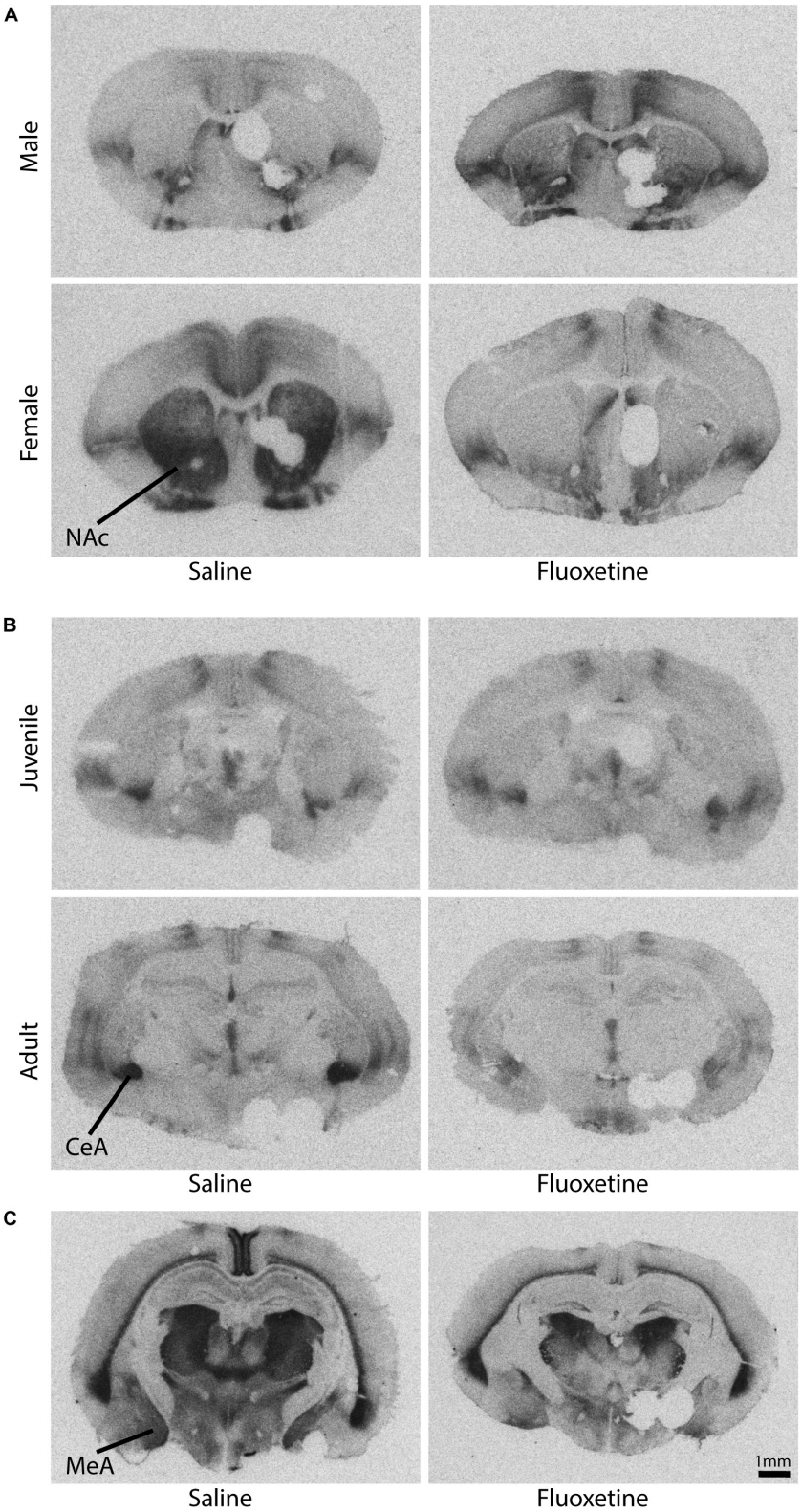


**FIGURE 9 |** Representative autoradiograms of oxytocin and vasopressin 1a receptor binding. Please note that tissue punches were taken from the left side of each brain to assess additional outcome measures not reported here. **(A)** Oxytocin receptor binding in the nucleus accumbens core shows a sex by drug condition interaction (see also **Figure 8A**). **(B)** Oxytocin receptor binding in the central amygdala shows an age by drug condition interaction (see also **Figure 8C**). **(C)** Vasopressin 1a receptor binding in the medial amygdala shows a drug condition effect (see also **Figure 9A**).

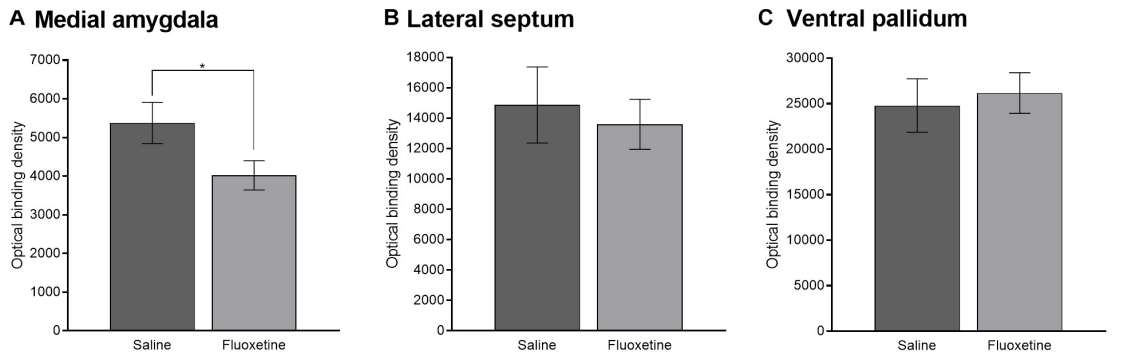


**FIGURE 10 |** Vasopressin 1a receptor binding. **(A)** Mean (±SEM) optical binding density in the medial amygdala comparing saline and fluoxetine exposure. **(B)** Mean (±SEM) optical binding density in the lateral septum comparing saline and fluoxetine exposure. **(C)** Mean (±SEM) optical binding density in the ventral pallidum comparing saline and fluoxetine exposure. $^{*}p < 0.05$.

as well as their important roles in social behavior across species (Carter and Perkeybile, 2018). We found that FLX exposure reduced the binding density of oxytocin receptors in the nucleus accumbens core and the central amygdala (**Figures 8A,B,D**), and the binding density of vasopressin 1a receptors in the medial amygdala (**Figure 9A**). While the nucleus accumbens shell has been strongly implicated in studies of prairie vole pair bonding, oxytocin receptors in the core are under-studied in the neurobiology of social behavior in voles, and may represent a new avenue of investigation.

It is likely that changes in OTR and AVPR1a underlie the differences found not only in social behavior, as described above, but also in anxiety-like behavior. Anxiety-like behavior was altered in the elevated plus maze (**Figure 4**), where adults spent less time on the open arms if developmentally exposed to FLX, regardless of the timing of exposure. This result is in line with previous research which has reported an increase in anxiety-like behavior in adults exposed to an SSRI developmentally (Ansorge et al., 2004; Boulle et al., 2016). We also found that FLX exposed subjects had lower OTR in the central amygdala during adulthood but not during periadolescence (**Figure 8D**). The amygdala is an area of the brain that is highly involved in anxiety and emotion regulation (Babaev et al., 2018). OTRs in the central amygdala are known to be involved in anxiety, as well as regulation of the hypothalamic-pituitary-adrenal axis, and can play a role in mediating the stress response (Neumann et al., 2000). Likewise, V1aR in the amygdala mediate stress and anxiety, with binding at V1aRs linked to heightened anxiety, reducing time spent in the open arms of the elevated plus maze (Hernández et al., 2016). Taken together, one potential mechanism by which developmental exposure to FLX increases anxiety in adulthood may be the reduction of OTRs and V1aRs in the amygdala.

While developmental FLX altered social and anxiety related behaviors, there was no indication of increased repetitive behaviors in FLX exposed subjects. We found no increase in stereotypies in any of the behavioral tests examined. Autogrooming and digging were not increased by FLX exposure in any of the behavioral tests in which they were measured.

Changes in offspring behavior may have been mediated by changes in the behavior of the mothers treated with FLX, although these were relatively subtle. In particular, mothers that were withdrawing from FLX spent extra time in active nursing (**Figure 2A**) and in nest-building (**Figure 2B**). The male pair mates of the FLX-withdrawing mothers also spent higher amounts of time in nest-building (although this effect was eliminated when the data were examined non-parametrically). Unfortunately, we missed the opportunity to assess the quality of the nests being produced (**Figure 2B**). Nest quality is an often-used measure of parental behavior in rodents and other species (Mann, 1993; Deacon, 2012). In three-spined sticklebacks, FLX reduced measures of male nest quality (Sebire et al., 2015); while in mice, females prenatally treated with FLX displayed lower nest quality during early days postpartum (Svirsky et al., 2016). The quality of the nest could affect various measures for the offspring including survival (Hamilton et al., 1997), thermoregulation (Gaskill et al., 2013), and even sleep (Harding et al., 2019). It is possible that the FLX-withdrawing parents put in extra time nest-building, while still producing low quality nests. A disorganized approach to nest-building would be consistent with the active nursing behavior of the mothers, which is when they locomote around the cage with the pups still attached to the nipples (prairie vole pups have milk teeth). Given that the pups are being bounced against substrate as they are dragged around, we have generally regarded this as a lower quality form of maternal behavior. Active nursing is also higher in prairie vole mothers that are broadly characterized as "low contact" mothers (Perkeybile et al., 2013). Future research on this topic should include nest quality as a variable in aiding understanding of the effects of FLX on parental behavior.

A major limitation of this study is that we did not find a partner preference in the SAL-treated males (**Figure 7B**). A possible explanation for this is that the daily injections inadvertently created a prenatal stress paradigm to which all subjects were exposed. Daily saline injections in pregnant rats have been shown to be sufficient to change several aspects of stress reactivity and the serotonin system in offspring (Peters, 1982). Prenatal stress has been shown to alter the social behavior

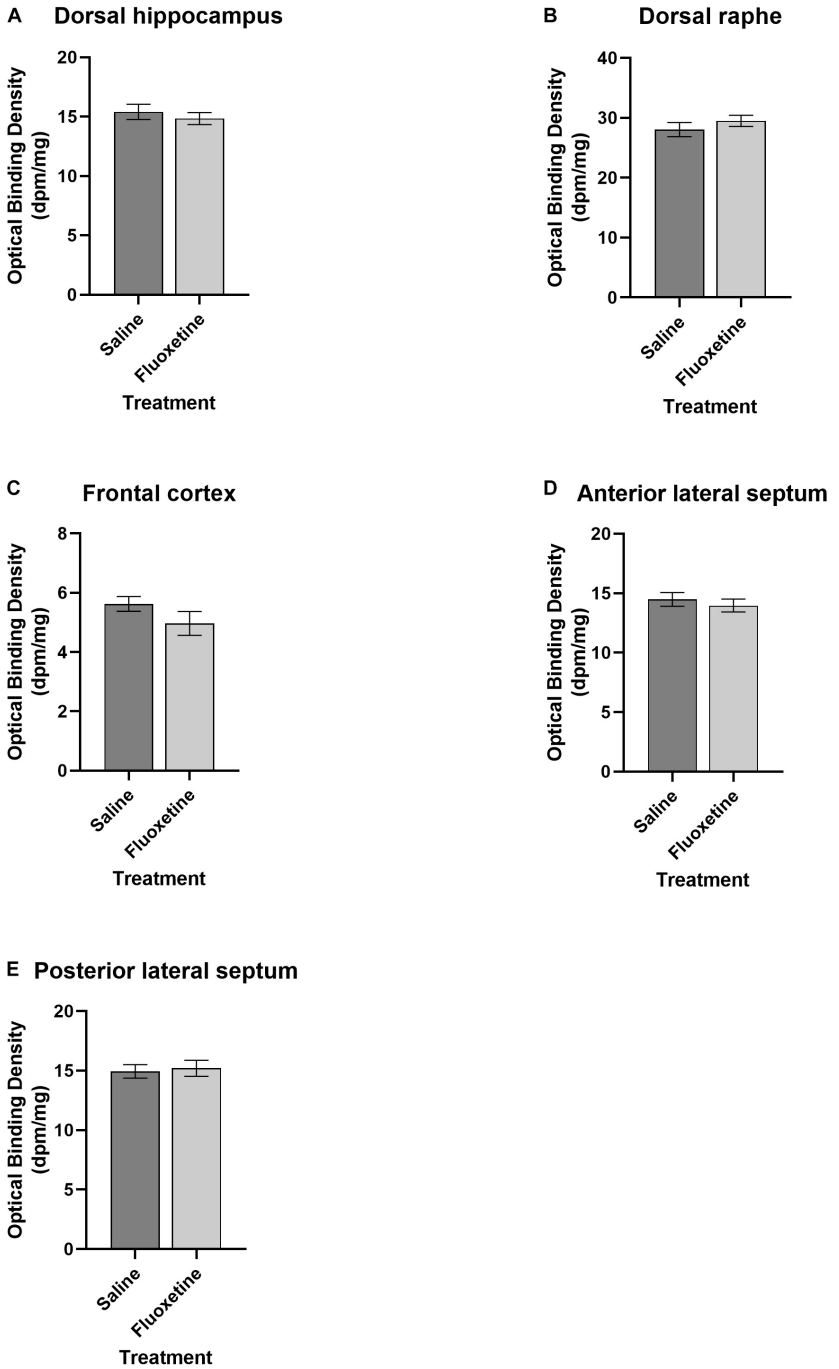


**FIGURE 11 |** Serotonin receptor 1a binding. **(A)** Mean (±SEM) optical binding density in the dorsal hippocampus comparing saline and fluoxetine exposure. **(B)** Mean (±SEM) optical binding density in the dorsal raphe comparing saline and fluoxetine exposure. **(C)** Mean (±SEM) optical binding density in the frontal cortex comparing saline and fluoxetine exposure. **(D)** Mean (±SEM) optical binding density in the anterior lateral septum comparing saline and fluoxetine exposure. **(E)** Mean (±SEM) optical binding density in the posterior lateral septum comparing saline and fluoxetine exposure.

of offspring (Weinstock, 2001; Schulz et al., 2011; Wilson and Terry, 2013) and likely prevented any of our animals from forming a preference. However, the finding that prenatally FLX exposed subjects spent more of their time alone compared to SAL treated animals suggests a change in social interest above and beyond that involved in the formation of a partner preference. Furthermore, maternal stress adds ecological validity given that in human prenatal SSRI use there is an underlying psychiatric condition for which pharmacological treatment with SSRIs has been prescribed. Chronic stress is frequently used in

**TABLE 1 |** Summary of behavioral effects of fluoxetine exposure.

| Behavioral test | Measure | Effect of fluoxetine | Interacts with | Results |
|---|---|---|---|---|
| Alloparental Care | Physical contact | Y | Sex, age group | FLX adult female < FLX peri female SAL peri female < SAL peri male |
| | Pup retrieval | Y | Exposure cohort | FLX PRE + POST > SAL PRE + POST FLX PRE + POST > FLX PRE, FLX POST |
| | Same chamber as pup | N | – | – |
| | Latency to approach | N | – | – |
| | Sniff | N | – | – |
| | Huddle | N | – | – |
| | Lick and groom | N | – | – |
| | Autogroom | N | – | – |
| | Dig | N | – | – |
| Elevated plus maze | Ratio of time on open arms | Y | Age | FLX adult < SAL adults |
| | Arm entries | N | – | – |
| | Freeze | N | – | – |
| | Autogroom | N | – | – |
| Open field test | Ratio of time in center | Y | Sex, age group | Eliminated sex and age differences seen in SAL |
| | Autogroom | N | – | – |
| | Rear | N | – | – |
| Intrasexual adult affiliation | Sniff | N | – | – |
| | Allogroom | Y | Sex | FLX male > SAL male (trend) Eliminated sex difference seen in SAL |
| | Physical contact | N | – | – |
| | Autogroom | N | – | – |
| | Rear | N | – | – |
| | Aggression | N | – | – |
| | Dig | Y | Sex | Eliminated sex difference seen in SAL |
| | Play | Y | Sex | FLX male > FLX female FLX male > SAL male |
| Partner preference test | Difference in partner and stranger chamber time | N | – | – |
| | Difference in side-by-side contact | Y | Sex | Eliminated sex difference seen in SAL |
| | Empty chamber time | Y | Exposure cohort | FLX PRE > PRE SAL |

*Y, significant effect; N, no effect; peri, periadolescent.*

the laboratory to induce a learned helplessness phenotype of depressive-like behavior to model depression (Pollak et al., 2010).

An interesting and unexpected finding was that FLX exposure eliminated sex differences across multiple behavioral tests. One example is the change in physical contact with the pup seen in the alloparental care test (**Figure 3A**). Male prairie voles are typically more alloparental than females, and here we saw that with FLX exposure, male periadolescents were not more alloparental than females, as was the case with SAL exposure. Male alloparental care is directly impacted by estrogen receptor expression, and sex-dependent changes in alloparental care with increasing age are based on changes in estrogen receptor expression (Perry et al., 2015). FLX exposure also eliminated the sex difference in partner and stranger contact in the partner preference test (**Figure 7B**). Both alloparental care and partner preference are examples of behaviors that show well-established sex differences in prairie voles. Estrogen receptor α expression has been implicated in reducing heterosexual adult contact in the partner preference test as well as male alloparental care behavior (Lei et al., 2010). FLX has estrogenic effects both *in vivo* and *in vitro* (Jacobsen et al., 2015; Pop et al., 2015; Muller et al., 2016), as does its bioactive metabolite norfluoxetine (Lupu et al., 2015). There is evidence in the literature for sex-specific effects of FLX on estrogen receptor expression (Adzic et al., 2017). FLX may have altered estrogen receptor expression, which in turn reduced affiliative behavior specifically in males, thus abolishing the sex differences seen in the SAL exposure groups. Future work should more thoroughly characterize the effects of developmental FLX on steroid receptors to further understand its behavioral effects.

Developmental timing is likely to be important in SSRI exposure. While some work has suggested that in humans, any chronic exposure in the year prior to birth results in heightened risk (Croen et al., 2011), others have found that either the first or third trimester are the periods of greatest risk (Oberlander et al., 2008; Croen et al., 2011; Harrington et al., 2014). In order to address the effects of exposure timing, we evaluated behavior in three different gross exposure cohorts spanning prenatal and postnatal development. We found few effects of FLX that were specific to an exposure cohort with the notable exception of increased duration in the empty chamber of the partner preference test in the PRE cohort. It is likely that creating shorter dosing periods which translate to specific trimesters in human pregnancy would be beneficial to more accurately determining how to best limit risk to offspring based on timing of exposure.

It is also worth pointing out that due to study design, offspring with different exposure timing were born to mothers of different parity and were potentially subject to different maternal hormone exposures. For example, pups that were part of the PRE + POST cohort were being nursed by mothers which were becoming pregnant again. To the extent that variation in maternal hormones due to parity or pregnancy may have affected hormones during the postpartum estrus or lactation (Bridges and Byrnes, 2006; Bridges, 2016), altering pup hormonal exposure *in utero* or through milk, these exposures may have varied in this study. In addition, all subjects in that cohort were litter 3 for their parents, whereas subjects in the POST cohort were all litter 2, and subjects in the PRE cohort were all litter 4; which could have also had effects on hormone exposure.

We have shown here that developmental SSRI exposure alters OTR and AVPR1a, but not 5-$HT_{1A}$, binding. Because FLX's mechanism works to increase serotonin neurotransmission by blocking reuptake of serotonin, it was surprising to find that 5-$HT_{1A}$ receptor binding was unchanged by FLX in all regions examined. Studies in mice have shown that perinatal FLX can regularize 5-$HT_{1A}$ levels that have been altered by other developmental factors (Nagano et al., 2012; Stagni et al., 2015). For the current study, it appears that the behavioral effects were mediated by OTR and V1aR without concomitant changes in the 5HT system. However, while there was no change in serotonin receptor density, actions on OTR and V1aR subsequent to FLX exposure may have been precipitated by changes in the peptides themselves, the function or location of the receptor, or other downstream cellular mechanistic pathways. Serotonin developmentally autoregulates its own innervation throughout the brain (Herlenius and Lagercrantz, 2004) and is plastic throughout development. Fetal exposure to FLX is poorly understood, yet it is clear that it leads to changes that last well into adulthood (Kiryanova et al., 2013). While SSRIs are presumed to increase extracellular serotonin in the long term, short term SSRI exposure can reduce raphe cell firing by acting on autoreceptors leading to a reduction in extracellular serotonin (Tao et al., 2000). Such activity may have neurodevelopmental consequences for offspring that have yet to be elucidated fully, but which warrant further investigation.

The serotonin system is also an extensive system with 15 different types of receptors (Carr and Lucki, 2011). We chose to examine the 1A receptor because of its autoreceptor function, but it may be the case that other exclusively post-synaptic serotonin receptors were altered while 1A was not. Further work examining other serotonin receptor populations will be important to clarify how serotonergic neurotransmission is altered by SSRI use prenatally. It is also possible that species differences between mice and voles may have altered the effects of FLX on 5-$HT_{1A}$ receptor binding.

Another area that should be considered is how exposure interacts with the maternal and early postnatal environment, as environmental moderation of SSRI effects may underlie their effects (Alboni et al., 2017). Since the prevalent and incident use of SSRI-exposed pregnancies has increased in the last two decades (Alwan et al., 2011), it is of the utmost importance that we more clearly understand the causes and consequences that prenatal SSRI exposure may have on the developing brain.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of California, Davis.

## AUTHOR CONTRIBUTIONS

RL and KB designed the research. RL, MP, CG, and SF conducted the experiments. RL, SF, and KB analyzed the data. RL wrote the first draft of the manuscript. All authors edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.584731/full#supplementary-material

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