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Long-term consequences of peri-adolescent social isolation on social preference, anxiety-like behavior, and vasopressin neural circuitry of male and female rats

Brianna L. Kinley[1]*, Robert. F. Kyne[2,3]*, Tamijah S. Lawton-Stone[1], Deena M. Walker[4], and Matthew J. Paul[2,3]

[1]Department of Biological Sciences, [2]Department of Psychology, and [3]Neuroscience Program, University at Buffalo, SUNY, Buffalo, NY, U.S.A

[4]Department of Behavioral Neuroscience, Oregon Health & Science University School of Medicine, Portland, OR, U.S.A.

*Equal contributions by authors

Address correspondence to: Matthew J. Paul (mjpaul@buffalo.edu)

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Abstract

Social isolation during the juvenile and adolescent stages (peri-adolescent social isolation) can have long-term consequences for behavioral and neural development. Most of this research, however, has relied on data from males, and very few studies have included both sexes. The present study investigated the impact of peri-adolescent social isolation on social preference, anxiety-like behavior, and vasopressin neural circuitry of male and female Long Evans rats. Rats were either housed alone for 3 weeks beginning at weaning (Isolated) or in groups (Group-housed). In adulthood, rats were tested in social preference, open field, marble burying, and light/dark box tests, and brains were processed for vasopressin immunohistochemistry. Isolated males exhibited a lower social preference score and spent more time in the light zone of the light/dark box than their group-housed counterparts. Isolated and Group-housed females did not differ in these measures. Peri-adolescent social isolation did not alter vasopressin fiber density in target areas known to influence social and anxiety-like behaviors (the lateral septum or lateral habenula), but increased fiber density in an output pathway of the circadian pacemaker (projections to the paraventricular nucleus of the thalamus); an effect detected across both sexes. A previously unreported sex difference was also detected for vasopressin fiber density in the paraventricular nucleus of the thalamus (females > males). These findings demonstrate long-term consequences of peri-adolescent social isolation on social preference, anxiety-like behavior, and the circadian vasopressin pathway and suggest that socio-affective development of males is more vulnerable to social stressors during the juvenile and adolescent stages.

Introduction

Childhood and adolescence are crucial stages for cognitive, social, and emotional development. During this period, individuals display increased sensitivity to stressful and rewarding stimuli (Eiland & Romeo, 2013; Walker *et al.*, 2017), and adverse experiences can have lasting impacts on the brain, behavior, and mental health (Gutman & Nemeroff, 2003; Lukkes, Watt, *et al.*, 2009; Walker *et al.*, 2019). Adversity increases the risk of many physical, psychological, and behavioral disorders including anxiety, depression, post-traumatic stress disorder, and substance use disorder (McFarlane *et al.*, 2005; Espejo *et al.*, 2007; Heim *et al.*, 2008; Childhood Welfare Information Gateway, 2019) all of which display stark sex differences in their emergence and presentation (Wittchen *et al.*, 1998; Merikangas *et al.*, 2010; Avenevoli *et al.*, 2015; Gogos *et al.*, 2019). Social interactions during childhood and adolescence influence the development of maladaptive behaviors in adulthood (Patterson *et al.*, 1992; Hankin *et al.*, 1998) and negative social interactions during these life stages are associated with increased rates of depression (Thapar *et al.*, 2012). Hence, understanding the impacts of the juvenile and adolescent social environment on neurobiology and behavior of both sexes is critical to our understanding of mental health disorders.

Socially isolating rodents at, or shortly after, weaning (post-weaning social isolation) has long-term consequences for neural and behavioral development and is often used as a preclinical model to study the neurobiology underlying childhood and adolescent adversity (Fone & Porkess, 2008; Burke *et al.*, 2017). Post-weaning social isolation protocols can vary markedly across studies, with animals isolated at different ages and for different durations (for a detailed review of these protocols see Lukkes, Watt, *et al.*, 2009). Broadly speaking, there are two main categories of post-weaning social isolation protocols. In most studies, isolated animals remain single-housed for the remainder of the experiment and are tested while still in isolation. This protocol has recently been referred to as isolation housing (Burke *et al.*, 2017). In other studies, animals are only isolated during the juvenile and adolescent periods then re-housed in groups for several weeks before testing. To emphasize the transient nature of the isolation period, we refer to this second protocol as peri-adolescent social isolation (PASI), meaning that isolation occurs around, but is not solely restricted to, adolescence. Distinguishing between isolation housing and PASI is important, and results can differ depending on which protocol is used. Behavior tests of isolation housing protocols can be affected by the ongoing isolation stress at the time of testing, whereas those of PASI protocols can be affected by the re-socialization period occurring after re-housing (Lukkes, Watt, *et al.*, 2009). In the present study, we used the PASI protocol, because unlike isolation housing, it can assess long-term effects of post-weaning social isolation that persist after the cessation of the social stressor.

PASI has a long-term impact on social development, but most of this research has relied on data from males. PASI has been repeatedly shown to decrease prosocial behaviors of adult male rats in the social interaction test (e.g., social approach, social contact/investigation; Hol *et al.*, 1999; Van den Berg *et al.*, 1999; Van Den Berg *et al.*, 1999; Ferdman *et al.*, 2007; Lukkes, Mokin, *et al.*, 2009; Lukkes, Vuong, *et al.*, 2009). To our knowledge, only one study has tested the impact of PASI on social behavior of female rats, and this study did not detect an effect of PASI in the social interaction test (Lukkes *et al.*, 2012). Hence, whether there are long-term consequences of PASI in female rats remains an open question.

How PASI impacts social behavior of males (and possibly females) is not understood. PASI may impact social behavior by altering preference for social stimuli. Social preference can be assessed by providing an experimental animal the opportunity to interact with a novel object and novel conspecific, either simultaneously or sequentially. The novel conspecific is typically restrained in a cage that allows limited social contact (e.g., wire-mesh cage or plastic cage with small openings) to ensure that interactions are initiated by the experimental animal. The time spent investigating the novel object and novel conspecific are compared to assess the animal's "preference" for social versus non-social stimuli. In these tests, rodents typically prefer social over non-social stimuli (e.g., Lukas *et al.*, 2011; Toth & Neumann, 2013). Effects of PASI on social preference of rats have not been reported in either sex.

Social deficits from PASI could also be the result of increased anxiety during social interactions (Lukkes, Vuong, *et al.*, 2009). Male rats subjected to PASI exhibit increased endocrine responses and fear during social or aggressive interactions (Van den Berg *et al.*, 1999; Lukkes, Mokin, *et al.*, 2009). This may reflect a more general effect on anxiety-like behavior, as male rats exposed to PASI can exhibit increased anxiety-like behavior in tests that do not include a social component (e.g., open field test and elevated plus maze; Wright *et al.*, 1991; Lukkes, Mokin, *et al.*, 2009; but see Weintraub *et al.*, 2010). Data in females are scarce, but two studies have found little to no effect of PASI on anxiety-like behavior in the open field test (tested in the dark) and the elevated plus maze (Weintraub *et al.*, 2010; Lukkes *et al.*, 2012).

The vasopressin (VP) pathway emerging from cells in the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA) is a prime candidate for mediating the effects of PASI on social behavior. This pathway regulates a variety of social and anxiety-like behaviors (Landgraf *et al.*, 1995; Liebsch *et al.*, 1996; Beiderbeck *et al.*, 2007; Veenema *et al.*, 2012, 2013; Bredewold *et al.*, 2014; Caldwell & Albers, 2016). Males have a greater number of VP cells in the BNST and MeA than females as well as more dense fiber projections to target areas such as the lateral septum (LS) and lateral habenula (LHb; reviewed in De Vries & Panzica, 2006; De Vries *et al.*, 2014). Consequently, pharmacological and genetic manipulations of this pathway often impact social and affective behaviors in a sex-specific manner (Albers, 2015; Bredewold & Veenema, 2018; Rigney *et al.*, 2019; Whylings *et al.*, 2020). The BNST/MeA VP pathway continues to develop across the juvenile and adolescent periods (De Vries *et al.*, 1981; Szot &

Dorsa, 1993), raising the possibility that adverse environments during these life stages could derail its development. Only one study has assessed the effects of post-weaning social isolation on the BNST/MeA VP pathway, which assessed vasopressin 1a receptor (V1aR) binding after isolation housing. Oliveira et al. (2019) found that isolation housing reversed the sex difference in V1aR binding in the BNST but did not alter receptor binding in the dorsal lateral septum of male or female rats. Potential long-term effects on this pathway using the PASI protocol have not been tested.

In the present experiment, we tested the hypothesis that post-weaning social isolation has long-term consequences for social preference of male and female rats. We predicted that PASI would decrease social preference in male rats. Due to the dearth of information on the effects of PASI on female social behavior, we tested whether PASI affects social preference in females, but remained agnostic to the presence or direction of the effect. We further tested the hypotheses that PASI-induced deficits in social preference, if present, would be accompanied by increased anxiety-like behavior and altered density of BNST/MeA VP fiber projections. To determine whether effects of PASI on VP-ir fibers, if present, were specific to the BNST/MeA VP pathway, we also assessed VP-ir fibers in the paraventricular nucleus of the thalamus (PVT), which originate from cells located in the suprachiasmatic nucleus of the hypothalamus (SCN; Hoorneman & Buijs, 1982; Rood *et al.*, 2013). The PVT is located at the same rostro-caudal level as the LHb, and fibers from these brain areas can be quantified from the same tissue sections within a single image. Hence, comparison of PVT and LHb vasopressin-ir staining provides a strong test of the hypothesis that group differences would manifest in one brain area, but not the other.

Methods

Animals and Housing Conditions

Fifty-nine offspring from 7 Long Evans rat breeding pairs from our colony were used as subjects. Litters were not culled or cross-fostered; offspring from each litter were distributed across groups. All rats (Group-housed and Isolated) were housed in plastic cages (44cm X 22.5cm X 20.5cm) with corn-cob bedding (Envigo, Indianapolis, IN). Room lights were set to a 12 h light/12 h dark cycle with lights off at 6:00 PM EST, and ambient temperature was maintained at 23°C. Food and water were available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University at Buffalo and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Experimental Timeline

Figure 1 illustrates the timeline of the housing manipulations, testing, and sacrifice. Rats were weaned at 21 days of age and housed either alone (Isolated; n=14 females, 11 males) or in groups of 2-3 rats/cage (Group-housed; n=19 females, 15 males). All rats were housed in the same room as our colony, and hence, Isolated rats could smell and hear other rats in the room. After 3 weeks of isolation, Isolated rats were group-housed (2-3 rats/cage) for the remainder of the experiment. Four weeks later, all rats were tested in a combined open field and social preference test (at P70-71), the marble burying test (at P72-73), and the light/dark box test (at P74-75), with 1 day between behavioral tests. Rats were sacrificed 1-2 days after the light/dark box test, and brains were removed (at P75-77). A subset of brains (n=36, 9 per group) was processed for vasopressin immunohistochemistry.

Behavioral Testing

Combined Open Field and Social Preference Test. Open field and social preference tests were conducted on the same day between zeitgeber time (ZT)5.5 and ZT10.5 under white light; lights on = ZT0 and lights off = ZT12. In the open field phase of the test, a rat was placed in an open arena (73.7cm X 73.7cm X 47.3cm) for 20 min. This phase served as an open field test, but also allowed the animal to acclimate to the testing arena for subsequent phases of the social preference test. The rat was then briefly removed while a novel empty wire-mesh cage was placed along a wall of the arena and secured to the wall with suction cups. The rat was reintroduced along the wall opposite the empty cage and allowed to explore the arena and cage for 10 min (novel object phase). The rat was again removed briefly while the empty cage was replaced with a cage containing a novel, sex-matched adult stimulus rat. The experimental rat was reintroduced again to the arena along the opposite wall of the cage and allowed to investigate the arena and the caged stimulus animal for an additional 10 min (novel animal phase). All stimulus rats were acclimated to the arena and wire-mesh cage for 10 minutes per day for 5 days before the start of the first test. Behaviors were recorded using a camera mounted above the arena. For the open field phase, time spent in the center 25% of the arena and distance travelled were assessed. For the novel object and novel animal phases, time spent in the investigation zone surrounding the novel cage or novel animal was assessed. A social preference score was calculated as (time spent investigating the novel animal – time spent investigating the novel cage) / (time spent investigating the novel animal + time spent investigating the novel cage). All measures were scored automatically using EthoVision software (Noldus Information Technology Inc., Wageningen, The Netherlands).

Marble Burying Test. Marble burying tests were conducted between ZT3.5 and ZT6 under white light. Each rat was placed in a fresh housing cage (plastic, 44cm X 22.5cm X 20.5cm) containing 5 cm of corn-cobb bedding and 12 large multi-colored marbles (diameter = 2.5cm) arranged in a 3 x 4 array on the surface of the bedding. Rats were allowed to freely move about and investigate the cage and marbles for 30 min. The number of marbles buried in the bedding by 2/3 or more was recorded at the end of the testing session.

Light/Dark Box Test. Light/dark box tests were conducted between ZT8.5 and ZT11. The rat was placed inside a dark box (38.9cm X 12.7cm X 15.2cm) with a single entrance to an illuminated open arena (40.0cm X 39.9cm X 31.2cm). The rat was allowed to explore the light and dark zones of the apparatus for 10 min. Activity in the light zone was recorded by a camera mounted above the arena. Time spent in the light zone was scored manually by a researcher unaware of group assignments using Observer software (Noldus Information Technology Inc., Wageningen, The Netherlands).

Tissue Preparation, Immunohistochemistry, and Quantification

Tissue Preparation. At sacrifice, rats were sacrificed by CO2 inhalation. Brains were removed and drop-fixed in 5% acrolein overnight, then immersed in 30% sucrose until microtome sectioning at a thickness of 40μm. Every fourth section was processed by immunohistochemistry for vasopressin.

Vasopressin Immunohistochemistry. Sections were rinsed in phosphate-buffered saline (PBS), then incubated for 30 minutes in 0.05M sodium citrate (at 70°C), 0.1M glycine (at room temperature), and 10% normal goat serum, 0.4% Triton-X, and 1% hydrogen peroxide (at room temperature), with PBS rinses between each incubation. Sections were then incubated overnight with an anti-vasopressin antibody (1:20,000 dilution, T-4563, rabbit, Bachem, Torrance, CA) at room temperature. The following day, sections were incubated in secondary antibody (60 min; biotinylated goat anti-rabbit, BA-1000, Vector Laboratories, Burlingame, CA), ABC-Elite standard kit (60 min; Vector Laboratories), and Peroxidase DAB substrate kit (30 min; Vector Laboratories, SK-4100). The proportions of solutions of the DAB kit were adjusted to prolong the DAB reaction, thereby providing more consistent results between wells: 160μl

Buffer, 320μl DAB, 2μl hydrogen peroxide, and 160μl nickel per 10ml of water. PBS rinses were conducted before and after each incubation. Sections were mounted onto gelatin-coated slides, air-dried, then coverslipped for microscope analysis.

This immunohistochemistry protocol was optimized for the detection of parvocellular vasopressin fibers. In double-label immunofluorescent pilot experiments, we found that the dilution of the primary antibody in this protocol faintly co-labels magnocellular oxytocin cells of the paraventricular and supraoptic nuclei of the hypothalamus. To be certain that measures of the present experiment reflect vasopressin staining, we restricted our analysis to brain areas where there are no oxytocin fibers (LHb and PVT) or where they are very rarely detected (LS).

Quantification of Vasopressin-ir Fibers and Cell Bodies. VP staining was visualized on a Nikon Eclipse Ni-U microscope (Nikon Instruments, Melville, NY). For most animals, 3 images were taken of the LS, LHb, and PVT – one from the rostral, mid, and caudal aspects of each brain area. The density of VP fibers was quantified by a researcher unaware of group assignments as the integrated density of staining above background using the threshold feature of ImageJ software (NIH, Bethesda, MD). For the LS and PVT, fiber density was quantified for both the left and right hemispheres within a single measure. For the LHb, fiber density was measured separately for the left and right hemispheres and then summed. Hence, rostral, mid, and caudal measures represented fiber density of both hemispheres. The integrated density across the rostral, mid, and caudal images was then averaged to provide a mean integrated density measure of each brain area for each animal. In some sections, tissue damage/folding or staining artifacts made accurate quantification impossible. Animals were included in statistical analyses as long as 2 of the 3 images could be quantified. The number of animals where 2 images could not be quantified, and therefore excluded, was: 2 for the LS, 0 for the LHb, and 0 for the PVT.

Statistical Analyses

Behavioral measures were assessed using a 2 X 2 ANOVA with housing (Isolated versus Group-housed) and sex as independent variables. When main effects or interactions were significant, post hoc comparisons were conducted using the Tukey test. Significance was assumed when $P<0.05$. Social preference was assessed in two ways. First, a social preference score was calculated (see above for equation) and differences were assessed using a 2 X 2 ANOVA with housing and sex as independent variables. We then tested whether the social preference scores of each group were statistically different from chance (i.e., no preference). Negative social preference scores indicate a preference for the novel object, whereas positive social preference scores indicate a preference for the novel animal. Equal investigation of both stimuli results in a 0 social preference score. Therefore, a One-Sample t-Test was conducted on each group to determine whether their social preference scores were statistically different from 0. All measures were assessed for outliers using the Box and Whiskers plot in SPSS that defines outliers as greater than 1.5 times the interquartile range, and outliers were removed from subsequent statistical analyses (Table 1). Potential effects of group-housing density (2/cage versus 3/cage) were assessed in Group-housed rats for each measure using a 2 X 2 ANOVA with housing density and sex as independent variables. Housing density did not alter social preference, open field, or vasopressin-ir measures, and hence, statistical analyses proceeded with both group-housing densities included as a single group for these measures. The number of marbles buried in the marble burying test and the time spent in the light zone of the light/dark box test, however, were impacted by group-housing density. Because of insufficient sample size in females housed 3/cage (there was only a single cage of 3 females), conclusions regarding effects of this housing density were considered unreliable, and rats housed at 3/cage (both male and female) were removed from marble burying and light/dark box analyses. Hence, the Group-housing variable for these tests consisted only of animals housed at 2/cage. Note that this led to a marked reduction in the sample size of Group-house males to n=6. Final group sample sizes for each analysis are denoted in the bars of each graph.

Results

Peri-adolescent social isolation decreases social preference in male rats

Isolated rats had a lower social preference score than Group-housed rats (Fig. 2A; $F_{(1,50)}$=7.19, P=0.01, η_p^2=0.13, main effect of housing, ANOVA), and there was a trend toward a significant interaction between housing and sex (P=0.078, ANOVA). Group-housed females, Group-housed males, and Isolated females each exhibited a significant preference for the novel animal over the novel cage (Group-housed females: P=0.002, Cohen's d=0.91, One-Sample t-Test; Group-housed males: P=0.042, Cohen's d=0.58, One-Sample t-Test; Isolated females: P=0.0008, Cohen's d=1.33, One-sample t-Test). Isolated males, however, did not exhibit a significant preference for either stimulus (P=0.24, One-sample t-Test). Because the One-Sample t-Test suggested that PASI only impacted male rats and the ANOVA housing X sex interaction approached significance (P=0.078), we conducted the Tukey post hoc test as a second assessment of whether the main effect of housing in the ANOVA statistical approach was significant for both sexes. In post hoc comparisons, the effect of PASI was significant in males (P=0.019, Cohen's d=0.99, Isolated males versus Group-housed males, Tukey test), but not in females (P=0.92, Isolated females versus Group-housed females, Tukey test).

PASI did not alter the time spent in the center of the open field (Fig. 2B; $F_{(1,54)}$=0.02, P=0.89 main effect of housing, ANOVA; $F_{(1,54)}$=0.19, P=0.66, housing X sex interaction, ANOVA) or the distance travelled in the arena (Fig. 2C; $F_{(1,51)}$=0.001, P=0.97, main effect of housing, ANOVA; $F_{(1,51)}$=3.29, P=0.08, housing X sex interaction, ANOVA). Females spent more time in the center ($F_{(1,54)}=9.98$, P=0.003, $\eta_p^2=0.16$, main effect of sex, ANOVA) and travelled a greater distance ($F_{(1,51)}=22.13$, P=0.00002, $\eta_p^2=0.30$, main effect of sex, ANOVA) than males.

Peri-adolescent social isolation alters anxiety-like behavior in the light/dark box test, but not the marble burying test

There was a significant interaction between housing and sex for the number of marbles buried in the marble burying test (Fig. 3; $F_{(1,42)}=5.66$, P=0.02, $\eta_p^2=0.12$, housing X sex interaction, ANOVA). Isolated males tended to bury more marbles than isolated females, but this difference fell just short of significance (P=0.05, Tukey Test); Group-housed males and females did not differ on this measure (P=0.82, Tukey Test). PASI did not significantly impact the number of marbles buried in either males (P=0.19, Tukey Test) or females (P=0.59, Tukey Test).

PASI affected the amount of time spent in the light zone of the light/dark box test (Fig. 4; $F_{(1,41)}=19.78$, P=0.00007, $\eta_p^2=0.33$, main effect of housing, ANOVA), but in a sex-dependent manner ($F_{(1,41)}=4.55$, P=0.04, $\eta_p^2=0.10$, housing X sex interaction, ANOVA). PASI increased the amount of time male rats spent in the light zone of the light/dark box (P=0.001, Cohen's d=2.39, Isolated males vs. Group-housed males, Tukey test), but did not alter this measure in females (P=0.23, Isolated females vs. Group-housed females, Tukey test). Although Group-housed females appeared to spend more time in the light zone than Group-housed males, this difference was not significant after post-hoc correction (P=0.12, Tukey Test).

Peri-adolescent social isolation impacts the circadian, but not BNST/MeA, VP pathway

Representative vasopressin staining in the LHb and PVT is illustrated in figure 5. Males had a greater density of VP-ir fibers in the LS and LHb compared to females (Fig. 6A&B; main effects of sex, ANOVA, $F_{(1,29)}=55.38$, P=0.00000003, $\eta_p^2=0.66$ for the LS; $F_{(1,31)}= 61.73$,

$P=0.000000007$, $\eta_p^2=0.67$ for the LHb). PASI, however did not alter VP-ir fiber density in either brain area ($F_{(1,29)}=0.72$, $P=0.40$, for the LS, $F_{(1,31)}=1.75$, $P=0.20$, for the LHb, main effects of housing, ANOVA; $F_{(1,29)}=0.11$, $P=0.74$, for the LS, $F_{(1,31)}=1.12$, $P=0.30$, for the LHb, housing X sex interactions, ANOVA). For the PVT, females had a greater density of VP-ir fibers than males (Fig. 6C; $F_{(1,31)}=15.11$, $P=0.0005$, $\eta_p^2=0.33$, main effect of sex, ANOVA). PASI increased fiber density in the PVT ($F_{(1,31)}=5.8$, $P=0.02$, $\eta_p^2=0.16$, main effect of housing, ANOVA); the interaction was not significant ($F_{(1,31)}=0.22$, $P=0.64$, ANOVA).

Discussion

The present study was the first to test whether social isolation of male and female rats during the juvenile and adolescent periods has long-term consequences for social preference and vasopressin neural circuitry that persist beyond the isolation period. We further assessed the impact of PASI on anxiety-like behavior in 3 behavioral tests to test the hypothesis that deficits in social preference, if present, would be accompanied by increased anxiety-like behavior. Overall, we found that PASI decreased social preference, largely in males, but this effect was not accompanied by increased anxiety-like behavior or large structural changes in vasopressin pathways emanating from the BNST and MeA. Instead, PASI increased vasopressin fiber density in the paraventricular nucleus of the thalamus in both sexes, a projection site of the SCN.

PASI decreased social preference. Although the sex X housing interaction of the ANOVA fell short of significance, the One-Sample t-Test suggested the effect was restricted to males: Group-housed males, Group-housed females, and Isolated females exhibited a significant preference for the social stimulus, whereas Isolated males did not. Hence, we conducted post-hoc tests on the ANOVA as a second assessment of whether the significant main effect of housing was due to effects in both sexes. As with the One-Sample t-Tests, Tukey post-hoc tests indicated that the effect of PASI was only significant in males: Isolated males differed from Group-housed males, whereas Isolated females did not differ from Group-housed females. Collectively, these analyses suggest that the effects of PASI on social preference are more robust in males than in females. Previous studies have reported deficits in other prosocial behaviors after PASI, including decreased social approach and social contact of male rats (Hol *et al.*, 1999; Van den Berg *et al.*, 1999; Van Den Berg *et al.*, 1999; Ferdman *et al.*, 2007; Lukkes, Mokin, *et al.*, 2009; Lukkes, Vuong, *et al.*, 2009). The present finding suggests that decreased preference for social stimuli may contribute to PASI-induced deficits in prosocial behaviors of male rats.

In contrast to the present findings, Oliveira et al. (2019) did not detect an effect of isolation housing on social preference of male rats. Other studies have also reported differing effects of PASI and isolation housing on social behavior: e.g., isolation housing increases aggression of male rats in the resident-intruder test (Tóth *et al.*, 2008; Toth *et al.*, 2011), whereas PASI does not (Potegal & Einon, 1989). It is tempting to speculate that it is the social reintegration with novel, previously isolated cage mates, rather than the period of isolation itself, that leads to deficits in social preference after PASI. Alternatively, ongoing isolation at the time of social preference testing may maintain the salience of conspecific stimuli, thereby preventing a decrease in time spent investigating the stimulus animal during testing. For example, isolation-housed rats may continue to approach and investigate the stimulus animal due to elevated aggression. Testing during the inactive phase under white light also could have affected performance in the social preference test of the present experiment; Oliveira et al. (2019) tested animals in the dark phase under dim red light. White light can decrease investigation time in social preference tests (Rein *et al.*, 2020). Importantly, rats in the present experiment spent a significant proportion of time investigating both the social and non-social stimuli, indicating that the presence of white light did not prevent exploratory behavior (mean novel object investigation time across groups = 250-311 s; mean social investigation time across groups = 285-443 s; out of a possible 600 s). Previous studies have conducted social preference tests in the dark phase under dim red light (e.g., Oliveira *et al.*, 2019) and during the light phase under white light (e.g., Lukas *et al.*, 2011; Smith *et al.*, 2015). Several other methodological differences between the two studies could also contribute to the divergent findings, including strain of rat tested (Wistar versus Long Evans), behavioral testing history (prior resident-Intruder and elevated plus maze testing versus no prior testing), habituation to the arena (30 s versus 20 min), and duration of the social preference test (4 min versus 10 min).

The absence of a significant effect of PASI on social preference of females is largely consistent with the limited number of post-weaning social isolation studies conducted to date. Isolation housing does not impact the social preference of female rats (Oliveira *et al.*, 2019), and neither PASI nor isolation housing alter prosocial behaviors of female rats in the social interaction test (Ferdman *et al.*, 2007; Lukkes *et al.*, 2012). Hermes et al. (2011) found that isolation housing coupled with early rearing decreases social interactions of female rats. Given the negative findings discussed above, it seems likely that the social deficits were due to, or at least contingent on, early weaning. Not all social behaviors of female rats are resilient to post-weaning social isolation. Isolation housing disrupts social discrimination and social recognition in female (and male) rats (Tanaka *et al.*, 2010; Oliveira *et al.*, 2019); whether similar effects are seen after PASI is not known. Future studies using a variety of social behavior assays are needed in order to fully understand the effects of isolation housing and PASI on female social behavior and to further test the hypothesis of greater vulnerability in males.

Our second prediction was that PASI-induced deficits in social preference would be accompanied by a general increase in anxiety-like behavior. Counter to our prediction, PASI increased exploratory behavior of male rats in the light/dark box test and did not affect performance on the open field and marble burying tests. These findings do not support the hypothesis that PASI-induced social deficits are due to increased anxiety. Consistent with previous PASI studies (Weintraub *et al.*, 2010; Lukkes *et al.*, 2012), we failed to detect an effect of PASI on anxiety-like behavior of females on all 3 anxiety-like behavior tests employed. Most studies using the isolation housing protocol also report limited effects on anxiety-like behavior of females in the elevated plus maze (Weiss *et al.*, 2004; Jahng *et al.*, 2012; Butler *et al.*, 2014; but see Chmelova *et al.*, 2019), but this may depend upon the behavioral test employed (Einon & Morgan, 1977; Arakawa, 2007).

Inconsistent anxiety-like behavioral responses after post-weaning social isolation are common in the literature, including across behavioral assays within individual studies (Weiss *et al.*, 2004; McCool & Chappell, 2009; Skelly *et al.*, 2015). It is important to note that factors other than anxiety can affect performance on these behavioral assays (Hascoët & Bourin, 1998; Prut & Belzung, 2003; de Brouwer *et al.*, 2019). In the present experiment, PASI increased the time males spent in the light zone of the light/dark box test, but did not alter performance in open field or marble burying tests, raising the question as to whether PASI impacted anxiety or some other internal drive/behavioral state that could affect performance on the light/dark box test. Increased time in the light zone could be due to increased general locomotor activity (Hascoët & Bourin, 1998). However, locomotor activity in the open field, as measured by distance travelled, was not affected by PASI in the present experiment. Other studies also do not report increased locomotor activity after PASI (Lukkes, Mokin, *et al.*, 2009; Lukkes, Vuong, *et al.*, 2009). The light/dark box test is also impacted by rodents' natural drive to explore novelty (Bourin & Hascoët, 2003). PASI increases contact time with novel objects in an open field (Einon & Morgan, 1977; Einon & Potegal, 1991). Hence, increased time in the light zone of the light/dark box test of isolated males could reflect increased drive to explore a novel illuminated environment. However, Isolated males did not exhibit significantly greater investigation of the novel stimulus cage during the social preference test (mean ± s.e.m. = 264.7 ± 50.6 and 296.5 ± 52.2 seconds for Group-housed and Isolated males, respectively; P=0.96, Tukey test). We did not set out to test the hypothesis that PASI impacts novelty seeking. Hence, future studies are required before definitive conclusions can be drawn.

Caution is warranted in the interpretation of the marble burying and light/dark box findings of the present experiment. Sample sizes of Group-housed rats, particularly those of Group-housed males, were markedly reduced, because preliminary analyses indicated that behavioral performance on these tests differed between animals housed 2/cage and those housed 3/cage (see Statistical Analyses section in the Methods). Hence, group-housing densities could not be analyzed as a single group in the marble burying and light/dark box tests. While this was a limitation of the present study, it raises the interesting possibility that the number of *group-housed* animals per cage can impact anxiety-like behavior. This could underlie some of the conflicting findings in the post-weaning social isolation literature, in which the number of animals housed per cage in the group-housed control often differs. Future studies are needed to directly test this hypothesis.

We failed to find support for our third prediction that PASI would alter VP-ir fiber density in the projections of the BNST/MeA VP pathway. As reported previously (De Vries *et al.*, 1981; Rood *et al.*, 2013), males had greater VP-ir fiber density than females in both the LS and LHb. PASI, however, did not alter VP-ir fiber density in either sex. Immunohistochemistry can only detect large structural changes. Hence, it remains possible that PASI impacts the microstructure or functioning of this pathway. Isolation rearing also did not alter V1aR binding in the LS but did reverse the sex difference in V1aR binding in the BNST (Oliveira *et al.*, 2019).

Other VP pathways are altered by post-weaning social isolation and could contribute to the behavioral consequences of this early life stressor. Parvocellular VP cells in the paraventricular nucleus of the hypothalamus (PVN) regulate neuroendocrine and autonomic stress responses (Swanson & Sawchenko, 1980; Palkovits, 1999; Aguilera *et al.*, 2008). Hence, changes in these cells would likely impact anxiety-like behavior and perhaps social behavior. Isolation housing decreases the number of VP-ir cells in the parvocellular division of the PVN in male, but not female rats (Tanaka *et al.*, 2010), whereas PASI increases VP mRNA in the parvocellular division of the PVN of female, but not male rats (Weintraub *et al.*, 2010). Isolation housing also decreases V1aR binding in the lateral hypothalamus and dentate gyrus of male and female rats (Oliveira *et al.*, 2019). At present, however, the functional consequences of these effects are difficult to discern.

PASI increased VP-ir fiber density in the PVT of male and female rats. These fibers originate from the suprachiasmatic nucleus of the hypothalamus (SCN) and are thought to influence circadian rhythms (Hoorneman & Buijs, 1982; De Vries & Miller, 1998; Rood *et al.*, 2013). Behavioral rhythms change during adolescence in several mammalian species, suggesting that the development of the circadian system continues across this life stage (Hagenauer & Lee, 2012). In rats, both behavioral (e.g., circadian period, phase, and chronotype) and anatomical (e.g., size of nucleus and nucleoli of cells in the SCN) changes across adolescence have been reported (Morishita *et al.*, 1974, 1978; Anderson, 1981; Hagenauer *et al.*, 2011). The present findings demonstrate that PASI disrupts the development of at least one circadian pathway and perhaps circadian regulation of behavior and/or physiology.

The present study uncovered a previously unknown sex difference – females had greater VP-ir fiber density in the PVT than males. To our knowledge, only one study has quantified VP fibers in the PVT of both sexes, but this study did not detect a sex difference in mice (Rood *et al.*, 2013). Hence, the sex difference in VP PVT may be species-dependent. Greater VP fiber density in females has been reported for other circadian outputs, including the periventricular, retrochiasmatic, and dorsomedial nuclei of the hypothalamus (Rood *et al.*, 2013). VP projections to the anteroventral periventricular nucleus of the hypothalamus regulate the circadian timing of the LH surge, and consequently ovulation, in females (Williams *et al.*, 2011; Smarr *et al.*, 2013; Bittman, 2019). The functional significance of sex differences in other VP outputs of the SCN is not known. Nevertheless, a role for VP in sex differences in behavioral timing is consistent with a recent finding that VP deficiency impacts circadian locomotor rhythms differently in male and female mice (Rohr *et al.*, 2021).

The present study found sex differences in the long-term effects of PASI on the social and anxiety-like behavior of rats, with males being more robustly affected than females. The present findings do not support the hypothesis that increased anxiety-like behavior contributes to social deficits seen after PASI, although 2 of the 3 anxiety-like behavior assays were underpowered in the present study. Contradictory outcomes across anxiety-like behavior assays in this study and in the literature suggest that other affective states should be considered. PASI likely impacts multiple facets of affective behavior, which could explain conflicting findings across behavioral tests that vary in their sensitivity to different affective behaviors, e.g., anxiety-like behavior versus exploration/novelty seeking. Nevertheless, behavioral findings of the present experiment are consistent with the hypothesis that the social, and potentially affective, development of males is more vulnerable to the effects of juvenile and adolescent social isolation. PASI effects on the macrostructure (i.e., fiber density) of VP pathways were limited to the circadian output pathway to the PVT, raising the possibility that PASI impacts circadian regulation of behavior and/or physiology. Future studies into this possibility should include both males and females as several VP output pathways of the SCN are sexually dimorphic, with greater fiber density in females. The present study adds to this list with the discovery of a sex difference in VP fiber density in the PVT. Collectively, these findings highlight the importance of considering sex as a biological variable in post-weaning social isolation studies and the far-reaching consequences of juvenile and adolescent social stress.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

MJP and DMW conceived of and designed the experiments. BLK, RFK, TSLS, and MJP conducted experiments and analyzed the data. MJP wrote the first draft of the manuscript. All authors reviewed and edited the manuscript.

Data Accessibility Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author (MJP) on request.

Abbreviations

BNST – bed nucleus of the stria terminalis
ir – immunoreactive
LHb – lateral habenula
LS – lateral septum
MeA – medial amygdala
PASI – peri-adolescent social isolation
PBS – phosphate-buffered saline
PVN – paraventricular nucleus of the hypothalamus
PVT – paraventricular nucleus of the thalamus

SCN – suprachiasmatic nucleus of the hypothalamus

V1aR – vasopressin 1a receptor

VP – vasopressin

ZT – zeitgeber time

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Figure Legends

Figure 1. Experimental Timeline. Abbreviations: OF/SP = combined open field / social preference test; MB = marble burying test; LDB = light/dark box test; Sac = sacrifice.

Figure 2. Peri-adolescent social isolation decreases social preference, but not locomotor activity of male rats. Performance on the social preference test (A) and open field test (B,C) of male and female rats group-housed or isolated from P21-P42 and tested between P70-71. (A) Mean (±s.e.) social preference score during the social preference test. (B) Mean (±s.e.) time spent in the center zone and (C) mean (±s.e.) distance travelled during an open field test. *Indicates significant difference between Group-housed and Isolated male rats ($P<0.05$, Tukey test). Females spent more time in the center and travelled a greater distance than males in the open field test ($P<0.05$, main effect of sex, ANOVA). Sample size indicated within bars.

Figure 3. Peri-adolescent social isolation does not alter anxiety-like behavior in the marble burying test. Mean (±s.e.) number of marbles buried by male and female rats, group-housed or isolated from P21-P42 and tested between P72-73. Sample size indicated within bars.

Figure 4. Peri-adolescent social isolation decreases anxiety-like behavior of male rats in the light/dark box test. Mean (±s.e.) time spent in the light zone of the light/dark box test of male and female rats, group-housed or isolated from P21-P42 and tested between P74-75. *Indicates significant difference between Group-housed and Isolated male rats ($P<0.05$, Tukey test). Sample size indicated within bars.

Figure 5. Vasopressin immunohistochemstry. Representative vasopressin staining in the lateral habenula (LHb) and paraventricular nucleus of the thalamus (PVT) in a Group-housed female (A), Group-housed male (B), Isolated female (C), and Isolated male (D).

Figure 6. Peri-adolescent social isolation increases vasopressin fiber density in the paraventriulcar nucleus of the thalamus. Mean (±s.e.) integrated density of vasopressin fibers in the lateral septum (A), lateral habenula (B), and paraventricular nucleus of the thalamus (C) of male and female rats, group-housed or isolated from P21-P42 and sacrificed between P75-77. *Indicates significant main effect of housing condition ($P<0.05$, ANOVA). Main effect of sex was also significant for all brain areas ($P<0.05$, ANOVA). Sample size indicated within bars.

Table 1. Number of outliers within each group.

Measure	Group-housed Females	Group-housed Males	Isolated Females	Isolated Males
Social Preference Score	2	0	2	1
OF Time in Center	0	0	0	1
OF Distance Travelled	1	1	2	0
MB Marbles Buried	0	0	1	0
LDB Time in Light	0	0	0	0
AVP LS	0	1	0	0
AVP LHb	0	0	0	1
AVP PVT	0	1	0	0

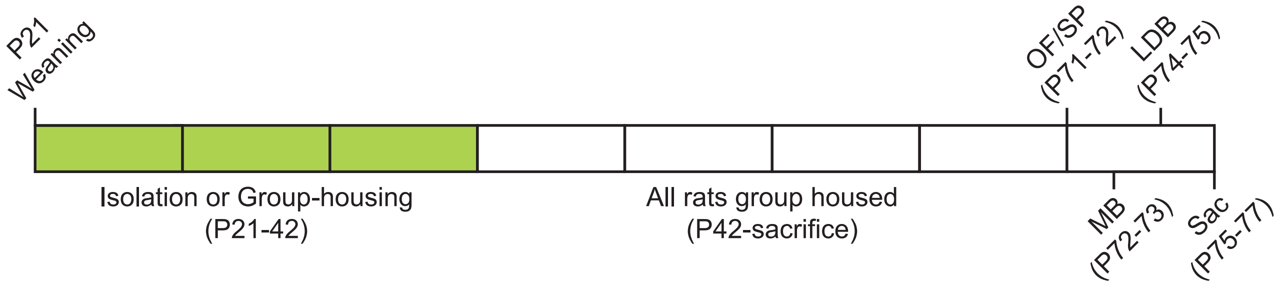
P21
Weaning
OF/SP
(P71-72)
LDB
(P74-75)
Isolation or Group-housing
(P21-42)
All rats group housed
(P42-sacrifice)
MB
(P72-73)
Sac
(P75-77)

Figure 1

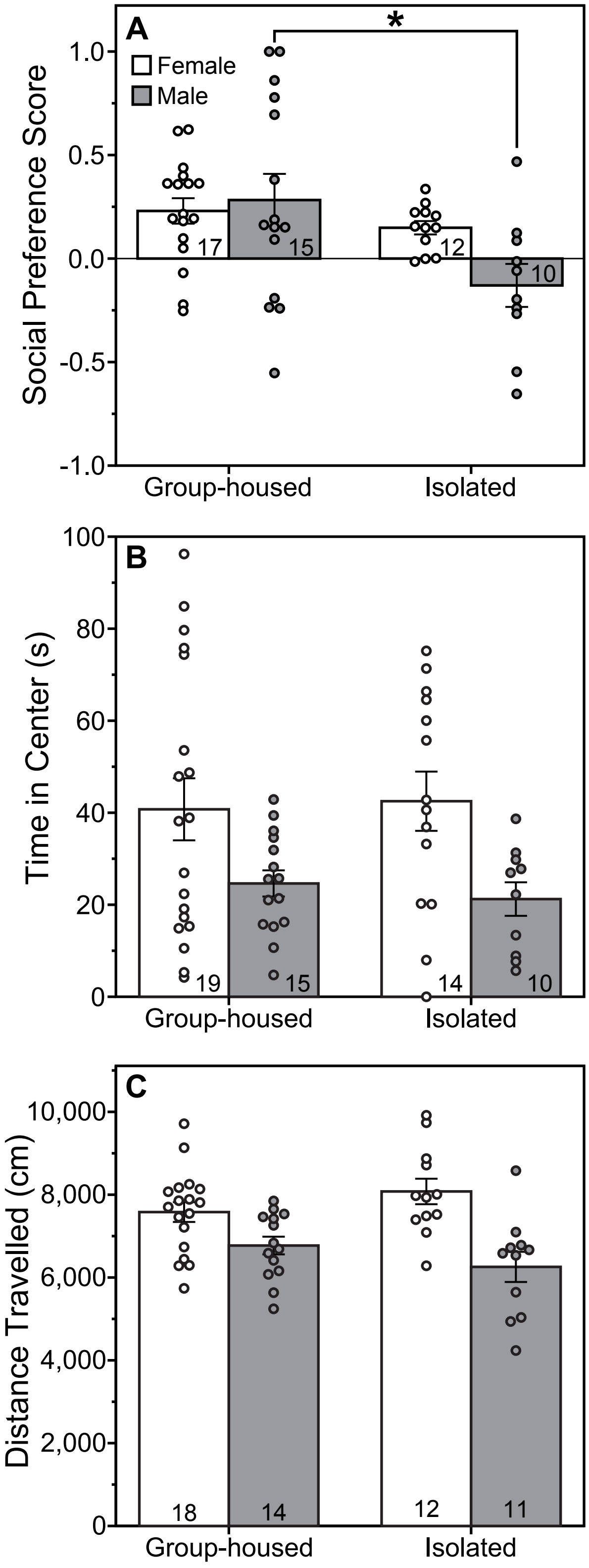
A
Female
Male
*
Social Preference Score
1.0
0.5
0.0
-0.5
-1.0
17
15
12
10
Group-housed
Isolated
B
Time in Center (s)
100
80
60
40
20
0
19
15
14
10
Group-housed
Isolated
C
Distance Travelled (cm)
10,000
8,000
6,000
4,000
2,000
0
18
14
12
11
Group-housed
Isolated

Figure 2

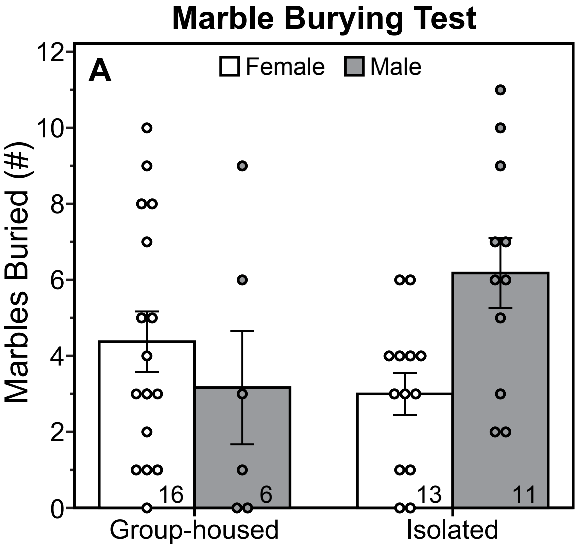
Marble Burying Test
A
Female
Male
Marbles Buried (#)
0
2
4
6
8
10
12
16
6
13
11
Group-housed
Isolated

Figure 3

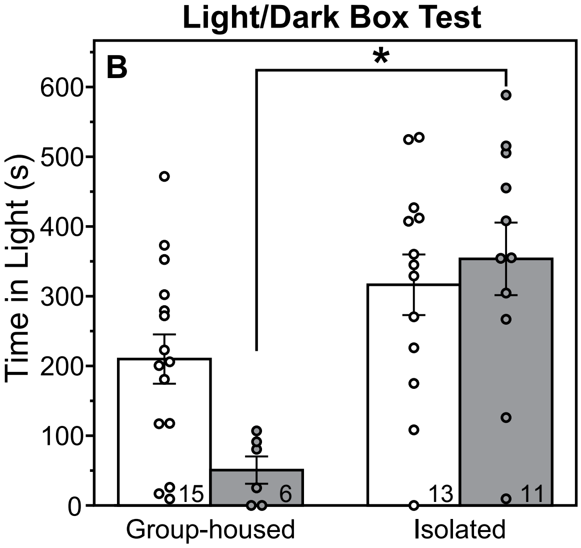
Light/Dark Box Test
B
*
Time in Light (s)
600
500
400
300
200
100
0
15
6
13
11
Group-housed
Isolated

Figure 4

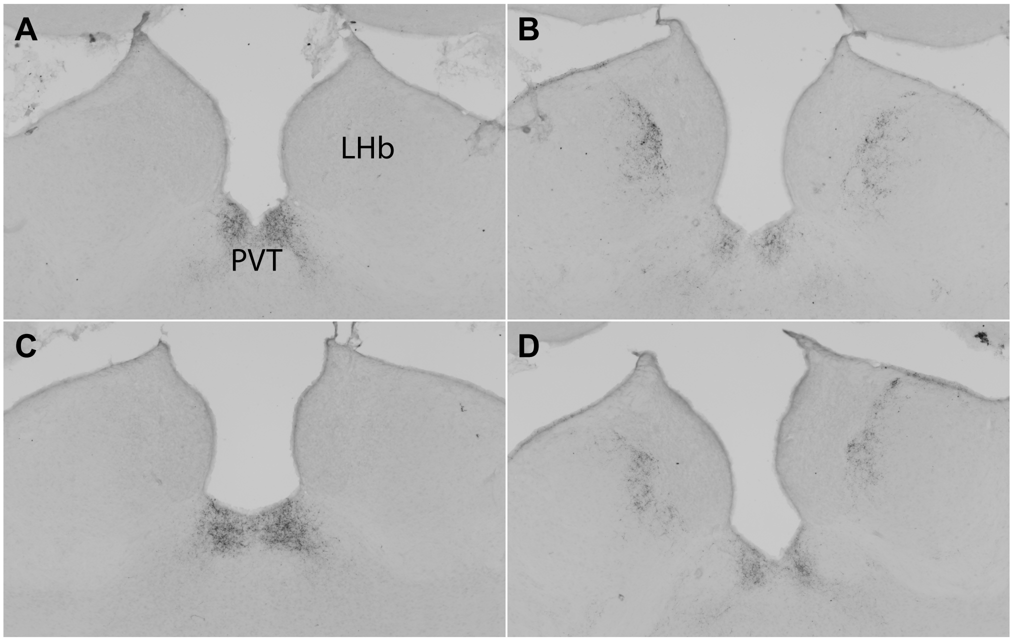

Figure 5

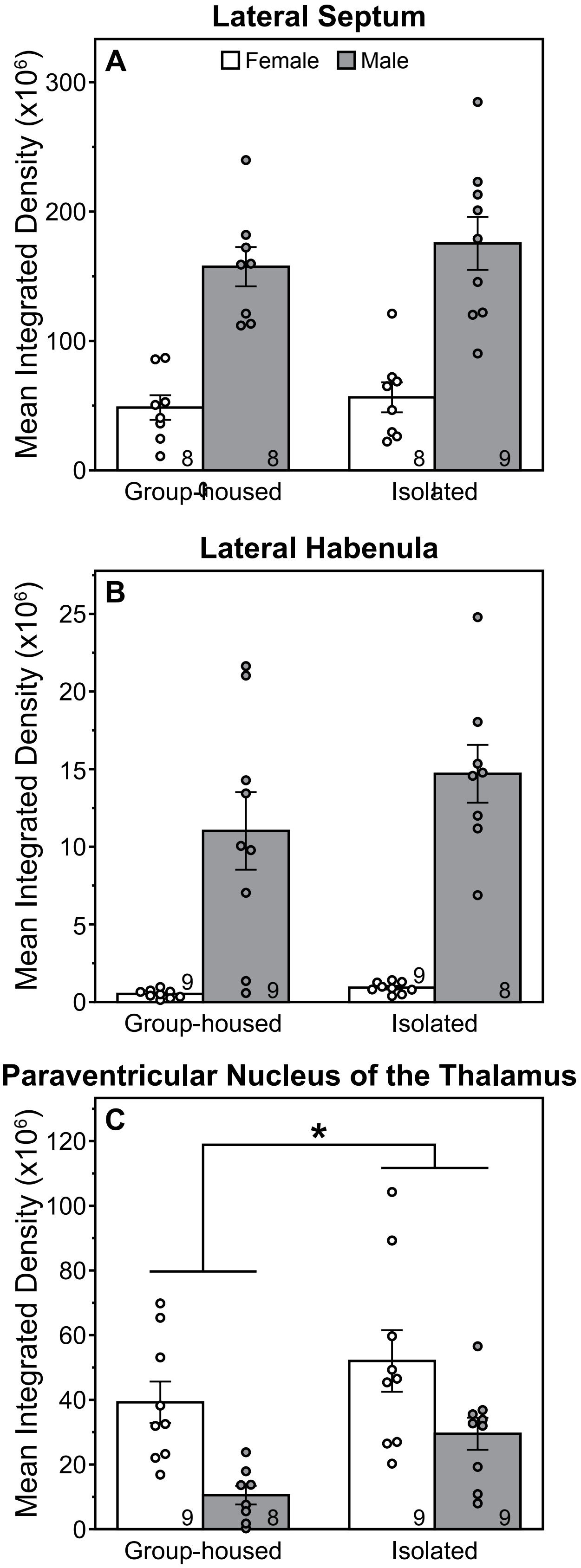
Lateral Septum
A
☐ Female ▨ Male
Mean Integrated Density (x10^6)
0
100
200
300
8
8
8
9
Group-housed
Isolated
Lateral Habenula
B
Mean Integrated Density (x10^6)
0
5
10
15
20
25
9
9
9
8
Group-housed
Isolated
Paraventricular Nucleus of the Thalamus
C
*
Mean Integrated Density (x10^6)
0
20
40
60
80
100
120
9
8
9
9
Group-housed
Isolated

Figure 6