# Effects of Post-Weaning Social Isolation and Oxytocin on Adult Anxiety and Sociability in Female Rats

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Positive social interactions during childhood and adolescence are essential for human neurobehavioral development. All social animals show persistent physiological and behavioral deficits after extended periods of social isolation. Our goal was to determine whether post-weaning social isolation negatively impacts adult anxiety and sociability in female Long Evans rats. Additionally, we tested whether administration of the pro-social hormone, oxytocin, could prevent behavioral deficits induced by isolation. On post-natal day (PND) 21, subjects were randomly assigned to social isolation (n = 30) or group housing (n = 30). Half of the subjects in each housing condition received 1 mg/kg oxytocin every three days for thirty days. The other half received saline-vehicle control. On PND 51-52, anxiety was assessed in the elevated-plus-maze (EPM). Subjects were then group housed ("resocialization") in novel triplets. Due to the potential influence of ovarian hormones on social motivation, daily vaginal cytology was initiated to track subjects' estrous cycles. On PND 66-67, subjects completed a second EPM test. On PND 70-72, subjects' social motivation was assessed using a three-chamber sociability apparatus. Isolated subjects exhibited higher anxiety in the first EPM test (immediately following isolation) compared to group housed subjects ($p < 0.01$). In the second EPM test, however, there were no group differences in anxiety, suggesting that the detrimental effects of post-weaning social isolation on anxiety had been redressed by resocialization. Also as predicted, previously isolated subjects exhibited reduced social motivation compared to group-housed subjects ($p < 0.05$). These findings suggest that post-weaning isolation leads to persistent social deficits that cannot be explained by an increase in generalized anxiety. Lastly, oxytocin treatment did not prevent the detrimental behavioral effects of post-weaning social isolation, which suggests that the developmental consequences of adolescent social deprivation may not be caused by reduced oxytocin output. Overall, this study substantiates the theory that early social isolation has detrimental effects on adult emotionality and behavior. Future animal research should continue to explore the potential clinical utility of oxytocin in treating social deficits.

Abbreviations: PND – post-natal day; EPM – elevated plus maze; OT – oxytocin; VEH – vehicle; GH – group housed; SI – socially isolated; OVX – ovariectomized

Keywords: social isolation; development; adolescence; oxytocin; anxiety; sociability

## Introduction

Mammals suffer profound physiological, behavioral, and psychological complications when deprived of developmentally appropriate social stimulation (Fone and Porkess, 2008; Harlow et al., 1965; McKinney, 1974). In humans, early-life isolation and other negative social experiences increase vulnerability to neuropsychiatric disorders, such as depression, schizophrenia, and substance abuse (Bos et al., 2011; Humphreys and Zeanah, 2015). Animal models designed to assess the negative developmental consequences of social deprivation can help clarify the link between early life experiences and adult

psychopathology in humans**.** They allow researchers to control and manipulate the early social environment of test subjects, and also test the value of prospective treatments. While the adverse impact of post-natal maternal deprivation on neurobehavioral development has long been known (Bowlby, 1969; Marco et al., 2015), the effects of adolescent social isolation are less defined. Adolescence serves as a critical psychological and physiological transition between childhood and adulthood, and peer-to-peer social interactions are particularly salient during this time (Spear, 2000; Tzanoulinou and Sandi, 2016). Several rodent studies have shown that social isolation during pre-adolescence/adolescence induces behavioral deficits and abnormalities that can persist into adulthood (Burke et al., 2017; Butler et al., 2016; Fone and Porkess, 2008; Hall, 1998; Lukkes et al., 2009). Our laboratory is particularly interested in how social experiences during late childhood and adolescence shape the emergence of normal, or abnormal, adult social behavior. In the current study, we hypothesized that a period of post-weaning social isolation in female rats would negatively impact their sociability as adults. Furthermore, we hypothesized that administration of the peptide hormone, oxytocin, during the period of isolation would attenuate these negative developmental consequences.

Animal research on the social effects of post-weaning isolation has produced surprisingly inconsistent findings. Male rats given two weeks of post-weaning isolation (PND 22-35) expressed decreased anogenital investigation and social approach in adulthood, but no changes in general locomotor activity or anxiety (Hol et al., 1999; van den Berg 1999 a,b). In contrast, several studies have reported *increased* social activity in male and female rats when they are tested immediately following periods of social isolation (Tanaś et al., 2015; Varlinskaya and Spear, 2008). However, such testing procedures have focused primarily on the short-term, often reversible consequences of social deprivation. Lukkes et al. (2012) demonstrated that a resocialization period between isolation and behavioral testing is necessary if one wishes to assess the persistent neurobehavioral effects of isolation. Using this paradigm, Lukkes et al. (2009) reported that male rats isolated from PND 21-42 and then resocialized until testing in early adulthood (PND 59) expressed deficits in a social interaction test. In contrast, Meng et al. (2010) assessed the effects of four weeks of post-weaning isolation (PND 21- 48) followed by four weeks of social housing on subsequent social behavior. When isolated subjects were assessed in the social interaction test immediately following isolation, they exhibited significantly higher levels of both aggression and social behavior (e.g., approaching, sniffing, anogenital investigation) compared to group-housed controls. However, after the resocialization period, these differences disappeared; there were no permanent effects of post-weaning social isolation on adult sociability.

Due to the inconsistent findings across social isolation literature, it is difficult to determine whether pre-adolescent/adolescent social isolation increases the expression of adult social behavior, diminishes it, or has no developmental effect. One factor complicating the comparison of social isolation studies is the substantial variance in procedures, especially with regards to: duration of isolation, inclusion of a resocialization period, and timing of behavioral testing. Furthermore, there are far more social isolation studies that use male subjects over female subjects (Lukkes et al., 2009). It is possible that females have differing sensitivity to the detrimental effects of post-weaning isolation or the pro-social effects of oxytocin compared to males. Given the relative dearth of studies on female rats, a primary goal of the current experiment was to address that gap in the literature. We utilized naturally cycling (non-ovariectomized) female rats as subjects and included estrous status as a subject variable. Our laboratory has previously shown that estrous female rats may, under some circumstances, express increased social motivation to approach a female conspecific (Lopez et al., 2007). As such, we tracked estrous status through vaginal cytology throughout sociability testing.

In the current study, we explored the long-term, developmental consequences of prolonged post-weaning social deprivation.

Female subjects were isolated for over four weeks starting at PND 21, encompassing all of pre-adolescence and the majority of adolescence (Lukkes et al., 2009; Renard et al., 2014; Sengupta, 2013). Subjects were then given two weeks of resocialization prior to behavioral testing. Our primary outcome variable was social motivation, as assessed by approach behavior in the three-chambered sociability apparatus (Crawley, 2004). This apparatus and model was specifically developed to assess deficits in social motivation induced by various experimental procedures, including genetic manipulation. The advantage of this model over standard social interaction tests is that subjects can approach and investigate target conspecifics who are constrained within cages and thus cannot engage in physical interaction. This allows one to assess social motivation that is not influenced by a concurrent physical, social experience (either positive or negative). While this model was originally designed for mice, it has since been validated in rats (Poirier et al., 2014; Reilly et al., 2015; Stefanik et al., 2015; Wu et al., 2016; Zhang et al., 2015).

An additional goal of the current experiment was to assess the effects of chronic oxytocin on rats subjected to prolonged post-weaning social deprivation. Oxytocin is a peptide hormone, synthesized in the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus. There is substantial evidence that oxytocin can decrease aggression and increase sociability in rodents (Bowen et al., 2011; Calcagnoli et al., 2014; Carter et al., 2008; Lukas et al., 2011; Suraev et al., 2014). Furthermore, chronic oxytocin administered during adolescence may have persistent pro-social effects. Bowen et al. (2011) administered 1mg/kg oxytocin intraperitoneally (IP) to male rats for ten consecutive days starting at PND 33. Approximately two weeks after the final injection, animals were placed in a social interaction test. Oxytocin-treated subjects showed higher levels of social interaction compared to controls. Similarly, Suraev et al. (2014) administered 0.5-1.0 mg/kg of oxytocin (IP) or an oxytocin receptor agonist (TGOT) to male rats once every three days starting at PND 28 for a total of ten injections. Oxytocin-treated subjects expressed greater social behavior with other oxytocin-treated subjects in social interaction tests conducted at PND 70 (2 weeks after the final injections). However, there were no significant effects of treatment on social preference for a novel conspecific versus a toy rat. Subjects treated with oxytocin during adolescence also showed significantly higher plasma oxytocin concentrations as adults, compared to controls.

While both Bowen et al. (2011) and Suraev et al. (2014) demonstrated a persistent increase of sociability in rats treated with chronic oxytocin, neither examined sociability in previously isolated subjects. Over the past decade, there has been burgeoning clinical interest in oxytocin's potential to alleviate social deficits associated with autism, social anxiety disorder, depression, and numerous other psychiatric disorders (Kirsch, 2015; Zik and Roberts, 2015). Preclinical work, primarily in mice, has indicated that oxytocin may be able to rescue social deficits induced by direct genetic manipulation (e.g., MAGEL2), inbreeding (e.g., BALB/cByJ), or early environmental insult (e.g., in utero exposure to valproic acid; reviewed in Peñagarikano, 2017). However, no laboratory has specifically assessed oxytocin's ability to rescue neurobehavioral deficits induced by post-weaning social isolation. Post-weaning isolation arguably represents a more accessible model for researchers and one that focuses upon critical developmental processes occurring during late childhood and adolescence. Tanaka et al. (2010) reported that female rats given two weeks of post-weaning isolation expressed fewer oxytocin-immunoreactive cells within their hypothalamus and expressed abnormal social recognition, compared to group-housed controls. This suggests that social experiences, including play behavior, during pre-adolescence/ adolescence serve as critical input in the development of neuropeptide systems that regulate the emergence of "normal" adult social behavior.

In the current study, female subjects were either socially isolated or group-housed immediately following weaning. In addition, half of the subjects within each of the housing conditions received 1 mg/kg oxytocin every three days for 30 days. Subjects were assessed

for anxiety in the elevated plus maze (EPM) before being group housed in novel triplets ("resocialization") for two weeks. A second EPM trial followed this resocialization period, as well as sociability assessment in the three-chambered apparatus. We hypothesized that previously isolated subjects would display reduced social motivation as adults, but not elevated general anxiety. Furthermore, we predicted that oxytocin treatment would prevent the development of this social deficit; isolated subjects that received oxytocin would show higher social motivation compared to isolated controls. We did not have specific predictions regarding the effect of oxytocin on anxiety and sociability in group-housed subjects, although previous findings indicate that chronic oxytocin can enhance baseline sociability in rats (Bowen et al., 2011; Calcagnoli et al., 2014; Suraev et al., 2014).

## Material and Methods

*Subjects*

Sixty female Long-Evans rats (Charles River, Wilmington, MA) were used as subjects. Four female ovariectomized (OVX) rats (Charles River, Wilmington, MA) were used as "targets" in the sociability tests. Naturally cycling female rats emit pheromones and vary their behavior across the estrous cycle in ways that may or may not subtly influence the behavior of female conspecifics. As such, to eliminate this potential confound, we used OVX females as social targets.

Subjects arrived at our laboratory on PND 21; we confirmed with the vendor (Charles River) that subjects were weaned immediately prior to shipping. Sociability targets were 60 days old on arrival.

All animals were kept on a 12:12 reverse light-dark schedule (lights off from 11:00-22:00), with behavioral testing occurring during the active portion of the subjects' cycles. Food and water were provided *ad libitum*. The vivarium was maintained at a stable temperature of 23˚C, with approximately 30% humidity. All experimental protocols were approved by the campus Institutional Animal Care and Use Committee in compliance with the National Institute of Health's Guide for the Case and Use of Laboratory animals.

*Elevated Plus Maze*

The elevated plus maze (EPM) was constructed of wood and painted black. The maze, mounted on a central post, was elevated 66.5 cm off of the ground. The maze consisted of four arms (63 cm L x 19 cm W), organized in a plus shape around a central platform (19 cm x 19 cm). Closed arms had walls (50 cm H), while open arms did not. Like arms were positioned opposite each other (e.g., one closed arm was across from the other closed arm). A digital camera (SONY HDR-AS200 Action Camera) was mounted above the maze and used to record subject trials.

The EPM is widely used to assess acute, unconditioned anxiety in laboratory rodents (Pellow et al., 1985). Subjects placed into an EPM for the first time typically spend the vast majority of their time within the closed arms, where they feel safer and less vulnerable. Experimental manipulations (such as drug treatments) that increase time spent within open arms are deemed anxiolytic, while those that decrease time spent in open arms are anxiogenic.

*Three-chambered Sociability Apparatus*

Two identical three-chamber sociability apparatuses were used (see Figure 1). Each apparatus was constructed from an aluminum frame (80/20$^{TM}$), transparent Plexiglas walls, and an opaque black, Masonite floor. The apparatus was divided into three equally sized chambers, individually measuring 33 cm L x 38 cm W x 35 cm H. Chambers were separated from each other by Plexiglas walls with a single 20 cm x 20 cm opening at the base, large enough for adult rats to easily pass through. In addition, opaque Plexiglas barriers without openings could be inserted between chambers to hold subjects within the center one until a trial was initiated.

A removable cage was located within each of the side chambers of the apparatus. These cages were used to hold the respective "targets" during sociability trials. Cages were made of a circular white ABS plastic base and

top (printed with a 3D printer) and 20 0.64 cm-diameter stainless steel rods spaced evenly around the perimeter. Each cage measured 18 cm diameter x 14 cm tall and was large enough to hold a single adult male or female rat comfortably. ]

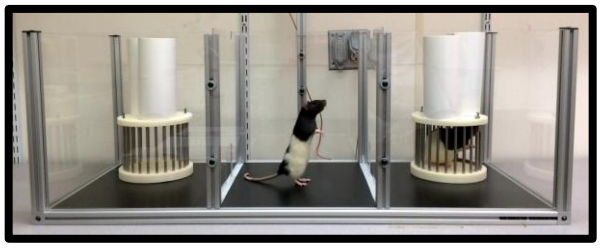

**Figure 1.** Image of the three-chambered sociability apparatus. In this example, the female subject is located in the central chamber. The chamber on the left contains an empty cage ("empty target"). The chamber on the right contains a female conspecific with the cage ("female target"). Note that during the actual trials, overhead red lights were used for illumination.

The three-chamber sociability apparatus has been cited as a valid and reliable apparatus for measuring social motivation in both mice and rats (Crawley, 2004; Kaidanovich-Beilin et al., 2011; Nadler et al., 2004; Poirier et al., 2014; Stefanik et al., 2015; Yang et al., 2011; Zhang et al., 2015).

Subjects typically express normal social motivation by: 1) making more entries into the "social chamber" (the one that contains a same-sex, conspecific target) vs. an empty chamber, 2) spending more time in the social chamber, and 3) spending more time investigating the social target.

### *Housing Manipulation and Oxytocin Administration*

Figure 2 displays a timeline of the various experimental procedures. Upon arrival, subjects were randomly assigned to one of two housing conditions (n = 30 each): socially isolated (SI) and group-housed (GH). GH subjects were housed in groups of three. All subjects were housed in the same room and exposed to an identical laboratory environment. Previous research has shown that extensive interaction between the test animals and care staff may attenuate the experimental effects of social isolation (Rosa et al., 2005). Thus, throughout this phase of the experiment, subject handling by animal care staff and experimenters was kept to the minimum necessary (e.g., as necessitated by administering injections, or changing subjects' cages on a weekly basis). This housing manipulation was maintained for 31 days, between PND 21-51. This period captures the majority of time between weaning and adulthood in rats, including all of pre-adolescence and most of adolescence (Lukkes et al., 2009; Renard et al., 2014).

Starting on PND 22, each subject was administered either oxytocin (1 mg/kg, intraperitoneal, approximate injection volume of 0.1 ml) or saline vehicle once every 3 days throughout this housing manipulation phase. As such, each animal received a total of 10 injections. Oxytocin was purchased from Bachem (Torrance, CA). Animals were injected approximately 1-2 hrs after waking up. This dosage and administration route is based on prior research in which oxytocin was administered to rats of approximately the same age (Bowen et al., 2011; Ferris et al., 2015; Suraev et al., 2014). Most pertinently, Suraev et al. (2014) gave male Long-Evans rats a dose of 1 mg/kg oxytocin every 3 days between PND 28-55; this regimen induced lasting changes in social behavior, as well as an enduring increase in plasma oxytocin.

### *Pre-Resocialization Behavioral Testing*

On PND 50 and 51, subjects were put through an EPM task to assess their level of general anxiety. This test occurred at the end of the housing manipulation and thus assessed the short-term effects of social isolation on general anxiety.

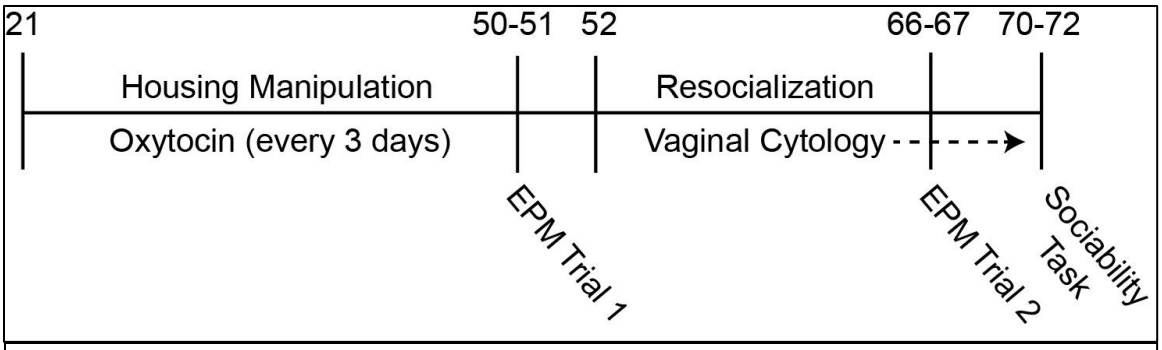


**Figure 2.** Timeline of experimental procedures. Age of subjects (post-natal day) is indicated above timeline.

The testing room was lit with dim white light. Subjects were placed in the center of the maze and then allowed to explore the apparatus for five minutes. Trials were recorded with a digital camera mounted above the maze. The apparatus was cleaned with 70% ethanol between trials. Raters blind to the group status of the subjects subsequently coded the following dependent variables: total arm entries, open arm entries, and time spent (s) in open arms. An arm-entry was operationally defined as having all four limbs within the arm. Total arm entries are often used as a metric of locomotor function. Open arm entries and time spent in open arms are typically converted into percentages (% open-arm entries % time spent in open-arms, respectively) and are reflective of a subject's anxiety level; higher numbers indicate less anxiety.

### *Resocialization*

On PND 52, all subjects were group-housed (in groups of three) with novel same-sex conspecifics. GH subjects were rehoused with novel cage-mates (who were also previously group-housed), while SI subjects were rehoused with other SI subjects (Lukkes et al., 2012). This resocialization period lasted 14 days.

### *Post-Resocialization Behavioral Testing*

On PND 66 - 67 subjects were again tested for general anxiety using the EPM. The procedure was identical to that used during the pre-resocialization test.

On PND 70 - 72 subjects were tested for social motivation using the three-chambered sociability apparatus. Approximately one-third of the subjects were tested each day; an equal number of subjects from each group were tested each day. Sociability testing was done under red light. A single trial consisted of the following: the female subject was placed within the central chamber and allowed to explore the entire apparatus for 10 min. After this brief "habituation" period, opaque Plexiglas dividers were inserted to confine the subject to the center chamber. While the subject was confined, the side chambers were prepared. An OVX female rat was placed within the cage of one side chamber ("female target"), while the cage in the opposite chamber was kept empty ("empty target"). The location of the empty target vs. female target was counterbalanced between experimental groups. Female targets had been previously habituated by placing them within the cages in the apparatus for 10 min on two consecutive days prior to testing. After the targets were positioned, the dividers were removed and the subject was free to roam throughout the apparatus for 10 min. The trial was recorded using overhead-mounted digital camera. The apparatus was cleaned with 70% ethanol between trials.

Subsequently, a researcher blind to the experimental condition of the subjects coded the following dependent variables using the video files: number of entries into each side-chamber, time spent in each side-chamber, and time spent investigating each cage. A chamber entry was defined as all four limbs being within the chamber. Investigation was operationally

defined as any kind of physical contact with a cage (including, but not limited to, touching and sniffing the cage with the head and nose, climbing upon the cage with the front paws, and rubbing the cage with the side of the body). While different laboratories have coded investigation time in subtly different ways, a common feature of this dependent variable is to focus on the time the subject spends in close proximity (typically 1-3 cm) to the targets. Experimental manipulations that reduce social motivation should reduce 1) the percentage of entries into the "social chamber" (where the conspecific target is located), 2) reduce the percentage of time spent in the social chamber, and 3) reduce the investigation time of the social target.

### *Vaginal Cytology*

Subjects in this experiment were intact, naturally cycling female rats. Social motivation may be influenced by ovarian hormones (e.g., Lopez et al., 2007). Additionally, estradiol may affect oxytocin regulation in the brain (Choleris et al., 2003). We therefore tracked the estrous status of our subjects throughout the latter portion of this experiment so that the number of estrous and non-estrous subjects could be counterbalanced across groups in sociability testing.

Starting at the beginning of the resocialization period (PND 52) through the end of behavioral testing (PND 72), experimenters obtained daily vaginal smears from all female subjects. A Q-tip moistened with physiological saline was inserted into the vagina, rotated slightly, and removed. The Q-tip was then smeared onto a blank microscope slide. The smears were inspected using a compound light microscope to determine the current stage of vaginal estrous for each female. This was accomplished by examining the relative proportions of nucleated cells, cornified cells or leukocytes (Cora et al., 2015; Goldman et al., 2007). Proestrous smears were defined by clusters of nucleated cells with some cornified epithelial cells and no leukocytes. Estrous smears were defined by a majority of cornified cells, some nucleated cells and the absence of leukocytes. Diestrous smears were defined by the presence of leukocytes.

Using nearly three weeks of vaginal estrous data, we attempted to 1) predict the status of each subject on their day of sociability testing and 2) schedule subjects such that estrous status was counterbalanced across experimental conditions (Cora et al., 2015). Within 60 minutes of each subject completing their sociability trial, a final vaginal smear was collected and used to confirm the subject's status. The four vaginal estrous stages (proestrous-estrous-metestrus-diestrus) were simplified into two behavioral categories: estrous and non-estrous. Subjects were considered to be "estrous" if their cytology indicated either a proestrous or estrus vaginal stage (or a transitional stage between them). Estrous female rats typically show enhanced receptivity, proceptivity, attractiveness, and sexual motivation when paired with male rats (Beach, 1976), and they may exhibit enhanced social motivation for conspecific females (Lopez et al., 2007). Subjects were considered to be "non-estrous" if they were either in vaginal metestrus or diestrus.

## Results

### *Weight*

Table 1 presents the mean (± SEM) weight of the four treatment groups at the following three ages: PND 21, PND 49 and PND 63. On PND 21, subjects arrived. On PND 49, subjects had previously received nine injections of oxytocin/saline and were nearly finished with the housing manipulation. On PND 63, subjects were nearing the end of their resocialization period. A 3 (age) x 2 (housing) x 2 (oxytocin) analysis of variance (ANOVA) was performed on these weight data. There was an overall main effect of age ($F(1,112 = 453.6$, $p < 0.01$). Three post-hoc, paired sample t-tests (using a Bonferroni correction to give an alpha of 0.016) revealed that the subjects' weight was significantly higher at PND 49 than at PND 21 ($t(59) = 19.6$, $p < 0.001$), significantly higher at PND 63 than at PND 21 ($t(59) = 88.3$, $p < 0.01$), and significantly higher at PND 63 than PND 49

($t(59) = 3.6$, $p < 0.01$). There were no other significant main effects or interactions. These results indicate that subjects gained weight as they aged and that weight was not significantly affected by the subjects' housing condition or whether they received chronic oxytocin.

| | PND 21 | PND49 | PND63 |
|---|---|---|---|
| SI-VEH | 0.033 ± .002 | 0.175 ± .005 | 0.21 ± .006 |
| SI-OT | 0.037 ± .001 | 0.173 ± .003 | 0.204 ± .003 |
| GH-VEH | 0.035 ± .001 | 0.200 ± .030 | 0.204 ± .004 |
| GH-OT | 0.037 ± .001 | 0.182 ± .004 | 0.219 ± .005 |

**Table 1.** Mean (±SEM) weights (kg) of subjects in the four experimental groups at PND 21, PND 49 and PND 63. SI = socially isolated, GH= group housed, VEH = saline vehicle, OT = oxytocin.

## *Pre-Resocialization EPM*

To assess the effects of housing condition and oxytocin on anxiety (prior to resocialization), total arm entries, percent open arm entries, and percent open arm time were compared. Percent open arm entries was calculated as the proportion of open arm entries to total arm entries. Percent open arm time was calculated as the proportion of time spent in open arms relative to total trial time (300 seconds). A 2 (housing) x 2 (oxytocin) ANOVA was used to analyze each of these dependent variables.

For total arm entries, there was no significant effect of housing ($F(1,56) = 2.37$, $p = 0.13$), oxytocin treatment ($F(1,56) = 0.13$, $p = 0.72$), or interaction of housing and oxytocin treatment ($F(1,56) = 0.34$, $p = 0.56$). Figure 3 displays the mean (± SEM) percent open arm entries displayed by the four experimental groups. A 2 x 2 ANOVA revealed a significant main effect of housing ($F(1,56) = 8.42$, $p < 0.01$) on this dependent variable. Socially isolated subjects expressed a significantly lower percentage of open arm entries (estimated marginal mean = 10.9%) compared to group housed subjects (estimated marginal mean = 19.4%). There was no main effect of oxytocin ($F(1,56) = 0.09$, $p = 0.77$) or interaction of housing and oxytocin treatment ($F(1,56) = 2.49$, $p = 0.62$). A 2 x 2 ANOVA on mean (± SEM) percent time spent in open arms revealed no significant effect of housing ($F(1,56) = 3.78$, $p = 0.06$), oxytocin ($F(1,56) = 0.13$, $p = 0.72$), or interaction of housing and oxytocin treatment ($F(1,56) = 0.03$, $p = 0.86$).

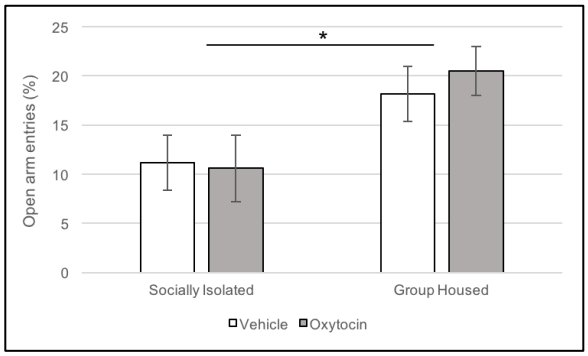


**Figure 3.** Mean (±SEM) percentage of open arm entries in the pre-resocialization EPM task. * indicates main effect of housing ($F(1,56) = 8.42$, $p < 0.01$).

## *Post-Resocialization EPM*

One trial was excluded from data analysis due to camera failure during the EPM trial. The subject dropped was from the GH, vehicle-treated condition.

2 x 2 ANOVA's revealed no significant experimental effects on any of the dependent variables. For total arm entries, there was no effect of housing ($F(1,55) = 0.92$, $p = 0.34$), oxytocin ($F(1,55) = 2.60$, $p = 0.11$), or interaction of housing and oxytocin treatment ($F(1,55) = 0.07$, $p = 0.80$). For percent open arm entries, there was no effect of housing ($F(1,55) = 0.13$, $p = 0.72$), oxytocin ($F(1,55) = 0.10$, $p = 0.75$), or interaction of housing and oxytocin treatment ($F(1,55) = 0.11$, $p = 0.74$). For percent time in open arms, there was no effect of housing ($F(1,55) = 0.10$, $p = 0.75$), oxytocin ($F(1,55) = 0.49$, $p = 0.49$), or interaction of housing and oxytocin treatment ($F(1,55) = 0.01$, $p = 0.92$). Figure 4 displays mean (± SEM) percent open arm entries shown by the four experimental groups in the post-resocialization EPM test.

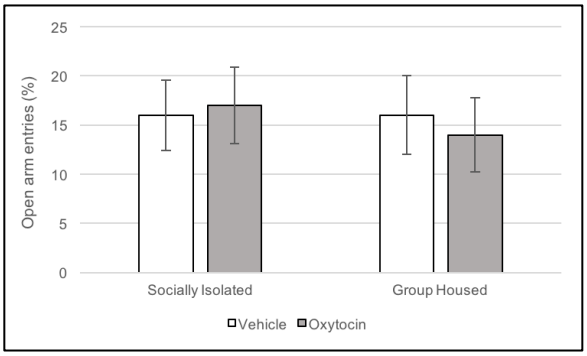


**Figure 4.** Mean (±SEM) percentage of open arm entries in the post-resocialization EPM task.

*Sociability*

One subject was excluded due to abnormal behavior during her sociability trial, which resulted in her being a statistical outlier. On average, subjects spent approximately 70% of their time during the sociability trial in one of the two side-chambers. The subject that was excluded spent only 13% of the trial in either side-chamber (this datum was more than 2 standard deviations from the mean). This subject was from the GH, oxytocin-treated condition.

We first converted each of the sociability dependent variables into percentages. Percent female chamber entries was calculated as the proportion of entries to the chamber containing the female target relative to total chamber entries. Percent female chamber time was calculated as the proportion of time spent in the chamber containing the female target relative to the total trial time (600 s). Percent female investigation time was calculated as the proportion of time spent investigating the female target relative to the total trial time.

To determine whether estrous status had an effect on the social motivation of the subjects, we conducted an initial analysis incorporating estrous status as a subject variable. A 2 (estrous status) x 2 (housing) x 2 (oxytocin) ANOVA was run on each sociability dependent variable. Estrous status had no significant effect on: percent female chamber entries ($F(1,51) = 0.25$, $p = 0.62$), percent female chamber time ($F(1,51) = 0.20$, $p = 0.65$), and percent female investigation time ($F(1,51) = 0.23$, $p = 0.63$). Based on these results, we removed estrous status from the remainder of analyses and conducted 2 (housing) x 2 (oxytocin) ANOVA's on each sociability dependent variable.

A 2 x 2 ANOVA on percent female chamber entries revealed no significant effect of housing ($F(1,55)=0.00$, $p = 0.99$), oxytocin ($F(1,55)=1.08$, $p = 0.30$), or interaction of housing and oxytocin ($F(1,55)=0.55$, $p = 0.46$). A 2 x 2 ANOVA on percent female chamber time also revealed no significant effect of housing ($F(1,55)=0.80$, $p = 0.38$), oxytocin ($F(1,55)=2.94$, $p = 0.09$), or interaction of housing and oxytocin ($F(1,55)=0.06$, $p = 0.81$).

Figure 5 displays the mean (± SEM) percent time spent investigating the female target. A 2 x 2 ANOVA on these data revealed a significant main effect of housing status ($F(1,55) = 4.11$, $p < 0.05$). Group housed subjects spent a greater percentage of the trial investigating the female target (estimated marginal mean = 31.8%) compared to socially isolated subjects (estimated marginal mean = 27.0%). There was no significant main effect of oxytocin ($F(1,55)=2.49$, $p = 0.12$), or interaction between housing and oxytocin treatment ($F(1,55)=0.01$, $p = 0.91$).

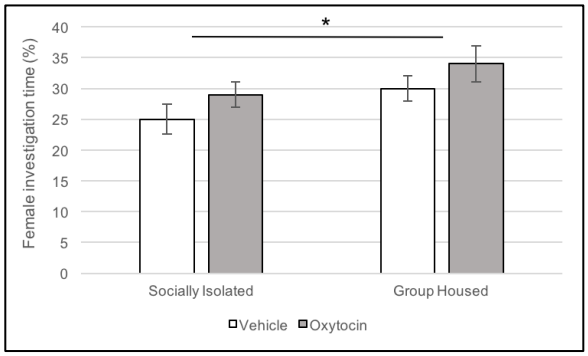


**Figure 5.** Mean (±SEM) percent time spent investigating the female target during the sociability task. Percent female investigation time was calculated as the proportion of time spent investigating the female target relative to the total trial time (600 s). * indicates main effect of housing ($F(1,55) = 4.11$, $p = 0.048$).

To further explore this main effect of housing on female investigation time, we conducted a series of follow-up analyses. Previous experiments have shown that rodents show preference for a conspecific target versus an empty target in the three-chambered task (e.g., Moy et al., 2004; Wu et al., 2016). This

can be assessed by specifically comparing time spent investigating the conspecific target versus time spent investigating the empty target.

Figure 6 displays the mean (± SEM) percent time investigating the female vs. empty targets for each of the four groups. A 2 (housing) x 2 (oxytocin) x 2 (target) ANOVA revealed a main effect of target ($F(1,55) = 9.89$, $p < 0.01$), with subjects overall spending a significantly greater percentage of time investigating the female target (estimated marginal mean = 29.4%) vs. the empty target (estimated marginal mean = 23.5%). There was also a main effect of oxytocin ($F(1,55) = 4.789$, $p = 0.03$), with oxytocin-treated subjects spending a greater percentage of time investigating either target (estimated marginal mean = 27.8%) compared to vehicle-treated subjects (estimated marginal mean = 25.1%). There were no significant interactions of housing and oxytocin treatment.

We then proceeded with a series of planned comparisons. Each planned comparison was a paired-sample *t*-test (one-tailed, because

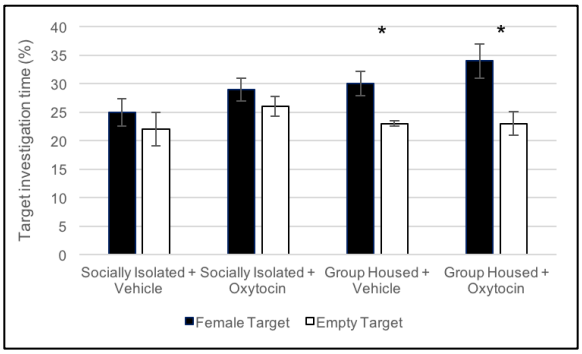


**Figure 6.** Mean (±SEM) percent investigation time of female and empty targets during the sociability task for the four experimental groups. * indicates a significant difference between female target and empty target investigation time (paired-sample *t*-test, $p < 0.05$)

of our directional hypotheses), comparing mean time spent investigating the female target vs. the empty target within each group. Vehicle-treated GH subjects spent a significantly greater percentage of the trial investigating the female target (30.1% ± 2.1) than the empty target (22.9% ± 1.5; $t(14) = 2.691$, $p = 0.01$). Oxytocin-treated GH subjects also spent a significantly greater percentage of the trial investigating the female target (33.6% ± 2.9) than the empty target (23.1% ± 2.1; $t(13) = 2.209$, $p = 0.02$). In contrast, neither vehicle-treated SI subjects ($t(14) = 0.59$, $p = 0.28$) nor oxytocin-treated SI subjects ($t(14) = 1.12$, $p = 0.14$) showed a significant target preference.

## Discussion

Social isolation during the pre-adolescent/adolescent developmental period induces long-lasting neurobehavioral deficits in rodents (Fone and Porkess, 2008; Hall, 1998; Lukkes et al., 2009). The current experiment expanded upon this literature by: 1) testing naturally-cycling females, 2) specifically assessing the effects of isolation on social motivation using the three-chambered sociability task, and 3) testing whether concurrent oxytocin administration during isolation could "rescue" affected behavior. The results of the experiment indicate that a substantial period (31 days) of post-weaning social isolation does significantly disrupt adult sociability – even when isolated subjects were given a period of resocialization prior to behavioral testing. Specifically, previously isolated subjects expressed lower motivation to investigate a novel female conspecific compared to group-housed subjects. This was reflected in a significantly lower percentage of female investigation time (Figure 4) and an absence of preference for the female target over the empty target (Figure 5). In comparison, group-housed subjects expressed a significant preference for a novel female over an empty cage. Such a preference is indicative of "normal" social motivation (Crawley, 2004; Moy et al., 2004; Yang et al., 2011). Due to the decrease of social motivation in the socially isolated, but not group-housed subjects, we can conclude that post-weaning isolation causes abnormal development of sociability in female rats.

This reduction in social motivation cannot be explained as a mere byproduct of increased general anxiety. Socially isolated subjects did express increased anxiety in the EPM immediately following their period of isolation (Figure 2), replicating previous

findings (Einon and Morgan, 1977; Hellemans et al., 2004; Leussis and Andersen, 2008, Lukkes et al., 2012; Skelly et al., 2015). However, a 14-day resocialization period abolished this effect. In the post-resocialization EPM test, there were no significant differences between the previously isolated and group-housed subjects on any anxiety measure. Yet, these groups did exhibit differences in the sociability test. Thus, the reduced motivation to approach novel females displayed by the isolated subjects was likely not a manifestation of enhanced generalized anxiety. This behavioral dissociation also presents a strong argument against using indices of sociability as measures of "anxiety behavior" (Lukkes et al., 2009). While state or trait anxiety may very well influence the expression of social motivation, social motivation is an independent construct generated by distinct biological systems.

The change in EPM results from pre- to post-resocialization also indicates that resocialization may be able to ameliorate the detrimental effects of social isolation on anxiety. This is a disputed topic (Lukkes et al., 2009; Lukkes et al., 2012). For instance, Wright et al. (1991) showed that 30 days of resocialization after a 30-day period of post-weaning social isolation did not rescue enhanced anxiety in male rats. However, other laboratories have shown beneficial effects of resocialization. van den Berg et al. (1999a) reported that male rats socially isolated for 13 days from PND 21, resocialized for three weeks, and then tested in the EPM showed no differences in anxiety compared to group-housed controls. Weintraub et al. (2010) tested both female and male rats, isolated from PND 30 – PND 50 and subsequently resocialized for 20 days. These subjects behaved no differently from group-housed controls in the EPM. Similarly, female rats isolated between PND 21 – PND 42 and then resocialized for two weeks did not show substantially heightened anxiety in a novel open field test (Lukkes et al., 2012). Our findings corroborate these latter experiments, and present a particularly strong case because subjects were tested in the EPM both before and after resocialization (a methodology uncommon in this literature). Furthermore, our data support the conclusion that resocialization can reverse the detrimental impact of isolation rearing on anxiety, but not sociability, in female rats.

Oxytocin administration during the period of social isolation did not, contrary to our hypotheses, prevent the emergence of abnormal social behavior. There was no significant difference between oxytocin-treated subjects and vehicle-treated controls, regardless of prior housing condition, on female investigation time or target preference. The only effect of oxytocin observed was that oxytocin-treated subjects spent more time investigating either target (female or empty) compared to vehicle-treated subjects. This cannot be easily explained as an enduring anxiolytic effect (e.g., Cohen et al., 2010), since there was no effect of oxytocin treatment in the EPM.

Other studies have shown that chronic administration of oxytocin to adolescent rats enhances their sociability as adults (Bowen et al., 2011; Suraev et al., 2014). However, the current study differs from these previous ones in several important ways, including: 1) our subjects were socially isolated during pre-adolescence/adolescence, 2) our subjects were female. Regarding this latter factor, there is substantial evidence for sex differences in oxytocin function within rodents (Dumais and Veenema, 2016). Perhaps post-weaning oxytocin administration is capable of enhancing adult social behavior in male rats but not females. One additional factor that may have influenced results was the specific sociability test utilized. Suraev et al. (2014) evaluated the long-term effects of adolescent oxytocin treatment on adult social behavior, assessed by both a social interaction test (in which subjects were allowed to physically interact with one another) and a social preference test (preference for a novel rat vs. a toy rat located in cages to prevent significant physical interaction). Interestingly, oxytocin treatment enhanced sociability in the social interaction test but not the social preference test. One speculative possibility is that chronic oxytocin treatment during adolescence does not directly impact social motivation, *per se*, but rather enhances the reward value of physical social contact. To borrow Berridge's terminology, oxytocin treatment may enhance social liking but not

social wanting (Berridge et al., 2009; Chevallier et al., 2012).

The absence of an anxiolytic effect of oxytocin in the EPM, especially in the pre-resocialization task, was somewhat surprising. Several studies have reported an anxiolytic, anti-stress effect of both acute and chronic oxytocin administration in rodents (for a review, see Neumann and Slattery, 2016). However, results have not been entirely consistent, and oxytocin's effects on the expression of stress-related behaviors appear to be influenced by a variety of factors, including subject species and sex, as well as trait anxiety. Only one other study has specifically examined the effects of chronic oxytocin on anxiety behavior in the EPM in female rats (Windle et al., 1997). Female Sprague-Dawley rats were ovariectomized and implanted with silastic capsules containing estradiol benzoate. Oxytocin-treated animals received intracerebroventricular infusions (100 ng/h) for 5 days prior to testing in the EPM. EPM trials were 15 minutes in length. Interestingly, there were no effects of oxytocin treatment on anxiety-behavior in the EPM if the testing took place in a familiar environment. However, when subjects were tested in an unfamiliar environment, and experienced higher levels of anxiety, oxytocin did have a significant anxiolytic effect. There are numerous methodological differences between this study and our own so it is difficult to resolve the discrepancy in findings. Perhaps our oxytocin dose was not high enough to affect anxiety, or our subjects did not experience enough anxiety for oxytocin to have an impact. Further work may clarify the conditions under which oxytocin might be utilized as an effective treatment for stress and anxiety-related disorders (Neumann and Slattery, 2016).

The biochemical mechanisms by which post-weaning isolation negatively impacts the development of social motivation remain unclear (Fone and Porkess, 2008; Lukkes et al., 2009). There is evidence that isolation can affect the development of serotonergic, dopaminergic, and neuropeptide pathways in the brain that are implicated in the regulation of emotive behaviors (Lukkes et al., 2009). One intriguing possibility is that post-weaning isolation suppresses the development of the oxytocin system itself. In support of this, Tanaka et al. (2010) reported that female rats isolated for at least 2 weeks following weaning expressed fewer oxytocin-immunoreactive cells in their hypothalamus compared to group-housed controls. Furthermore, the isolated females exhibited impaired social recognition, but not enhanced anxiety, in behavioral testing (Tanaka et al., 2010). The authors argue that a lack of social stimuli during the pre-adolescent/adolescent period caused a decrease in oxytocin release in females. In our experiment, we did not find that exogenous oxytocin administration during post-weaning isolation prevented the development of abnormal sociability. However, a major limitation of our study was a lack of any direct measurement of the oxytocin system (plasma levels, cell counts, receptor densities, etc.) within our subjects. As such, we do not know for certain if the social deficits observed were in any way mediated by dysfunctional oxytocin pathways.

In conclusion, we found that post-weaning isolation in naturally cycling female rats 1) increased general anxiety in the short term, and 2) reduced social motivation in the long term. A period of group-housing between isolation and adult behavioral assessment revealed that resocialization could redress anxiety but not sociability. As such, adolescent social isolation may differentially impact emotional systems in females, having a more persistent effect on social motivation than on anxiety. Chronic oxytocin treatment during the period of isolation had no short or long-term effects on anxiety or sociability in any of the subjects. It is possible that a different dosage regimen of oxytocin, or an alternate timing of oxytocin administration (immediately prior to sociability testing, for example) might have had a positive effect. Given the enormous potential in the use of oxytocin for the treatment of social deficits across a spectrum of human developmental and psychiatric conditions, continued preclinical work in this area is vital. In particular, the specific conditions under which oxytocin does and does not have behavioral effects must be clarified.

## Acknowledgements

The authors would like to thank Eric Egan for his time and expertise in constructing the three-chambered sociability apparatuses. This project was supported by a grant from the Student Opportunity Funds of Skidmore College.

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