1   **Social isolation stress in adolescence, but not adulthood, produces hypersocial behavior in**
2   **adult male and female C57BL/6J mice**
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22
23  **Abstract**
24
25  Chronic stress during the developmental period of adolescence increases susceptibility to many
26  neuropsychiatric diseases in adulthood, including anxiety, affective, and alcohol/substance use
27  disorders. Preclinical rodent models of adolescent stress have produced varying results that are
28  species, strain, sex, and laboratory-dependent. However, adolescent social isolation is a potent
29  stressor in humans that has been reliably modeled in male rats, increasing adult anxiety-like and
30  alcohol drinking behaviors, among others. In this study, we examined the generalizability and
31  sex-dependence of this model in C57BL/6J mice, the most commonly used rodent strain in
32  neuroscience research. We also performed a parallel study using social isolation in adulthood to
33  understand the impact of adult social isolation on basal behavioral phenotypes. We found that six
34  weeks of social isolation in adolescence beginning at postnatal day (PD) 28 produced a
35  hypersocial phenotype in both male and female adults in multiple assays and a female-specific
36  anxiolytic phenotype in the elevated plus maze, but it had no effects in other assays for
37  avoidance behavior, fear conditioning, alcohol drinking, reward or aversion sensitivity, novel
38  object exploration, or forced swim behavior in either sex. In contrast, social isolation in
39  adulthood beginning at PD77 produced an anxiogenic phenotype in the light/dark box but had no
40  effects on any other assays. Altogether, our results suggest that 1) adolescence is a critical period
41  for social stress in C57BL/6J mice, producing aberrant social behavior in a sex-independent
42  manner and 2) chronic individual housing in adulthood does not alter basal behavioral
43  phenotypes that may confound interpretation of behavior following other laboratory
44  manipulations.

1 **Introduction**

2  Adolescence is a critical developmental period marked by increased reward seeking and
3  impulsivity and the establishment of apposite social behaviors (Spear, 2004, Steinberg, 2004,
4  Romer, 2010, Steinberg, 2010, Leshem, 2016). In humans, adolescence is associated with
5  increased peer affiliation and separation from family (Noom et al., 1999, Keijsers et al., 2009,
6  Eichelsheim et al., 2010). In rodents and other mammals, it is marked by heightened incidence of
7  play behavior, altered social interactions, and increased exploration (Spear, 2004, Hawk et al.,
8  2009, Trentacosta and Shaw, 2009, Walker et al., 2019). The quality and quantity of social
9  interactions during adolescence have been linked to later-life behavioral outcomes in humans,
10  including rates of drug and alcohol use and the formation of healthy social relationships (Bray et
11  al., 2001, Kochenderfer-Ladd and Wardrop, 2001, Trentacosta and Shaw, 2009, Masten et al.,
12  2012, Deutsch et al., 2015, Jager et al., 2015).

13  Adolescence is also marked by increased stress sensitivity, and chronic stress exposure during
14  this period has been shown to alter brain structure and function (Paus, 2007, Eiland and Romeo,
15  2013). As peer interactions are especially important during adolescence (Steinberg, 2004, Jager
16  et al., 2015), exposure to social stress may have particularly deleterious consequences on brain
17  development and behavior (Casey et al., 2010, Platt et al., 2013, Burke et al., 2017). This
18  increased stress sensitivity may partly explain why substance use disorders and many other
19  psychiatric conditions frequently emerge during adolescence (Turner and Lloyd, 2004, Kessler et
20  al., 2005, Kessler et al., 2007, Ernst and Fudge, 2009, Casey and Jones, 2010, Blakemore and
21  Robbins, 2012). Understanding how adolescent social stress alters neurophysiology and behavior
22  may prove crucial to treating stress-related disorders in adolescence and throughout later life.

23  Adolescent social isolation in rats has emerged as preclinical model that recapitulates many of
24  the deleterious behavioral outcomes linked to chronic adolescent stress in humans (Lukkes et al.,
25  2009b, Butler et al., 2016, Walker et al., 2019). In male rats, this paradigm has been shown to
26  increase anxiety-like behavior and drug and ethanol intake and decrease fear memory extinction
27  (McCool and Chappell, 2009, Whitaker et al., 2013, Butler et al., 2014a, Karkhanis et al., 2015,
28  Skelly et al., 2015, Butler et al., 2016, Yorgason et al., 2016, Karkhanis et al., 2019), although
29  these effects were not recapitulated in female rats (Butler et al., 2014b). Isolation during
30  adolescence has also been linked to decreased social interaction in rats (Ferdman et al., 2007).
31  Less is known about the effects of protracted adolescent isolation on these behaviors in mice,
32  even though they are commonly used on neuroscience research, including studies that model
33  human psychiatric conditions such as drug self-administration that requires individual housing
34  (Becker and Ron, 2014). While some evidence suggests that isolation in adulthood is not
35  stressful for mice (Hunt and Hambly, 2006), other work presents evidence to the contrary
36  (Arakawa, 2018, Mumtaz et al., 2018, Manouze et al., 2019). The effects of isolation in
37  adolescence are even less clear. Like humans, adolescent mice demonstrate a potentiated
38  response to stress (Romeo et al., 2006). Although there are some reports that chronic social stress
39  during adolescence increases depressive- and anxiety-like behaviors and drug self-administration
40  in mice (Conrad and Winder, 2011, Lopez et al., 2011, Amiri et al., 2015), these results are
41  variable and may be strain and sex-dependent (Arakawa, 2018, Mumtaz et al., 2018, Walker et

1  al., 2019). C57BL/6J mice are commonly used in studies of alcohol self-administration (Rhodes
2  et al., 2005, Melendez et al., 2006, Lyons et al., 2008, Yoneyama et al., 2008, Hwa et al., 2011,
3  Mulligan et al., 2011) and as such are regularly singly housed for long periods of time. However,
4  the lasting behavioral effects of social isolation (either in adolescence or adulthood) on escalated
5  alcohol self-administration and anxiety-like behaviors in this strain have been variable (Lopez et
6  al., 2011, Lopez and Laber, 2015, Huang et al., 2017, Caruso et al., 2018).

7  Here we evaluated the behavioral consequences of prolonged social isolation on behavior in
8  male and female C57BL/6J mice and determined whether adolescence was a specific period of
9  stress sensitivity. Following six weeks of social isolation in adolescence or adulthood, we
10 measured anxiety, anhedonia, alcohol intake, reward and aversion sensitivity, fear memory
11 formation and social behavior in adulthood. We found that social isolation produced few
12 behavioral deficits overall, however this manipulation in adolescence led to aberrant social
13 behavior in adulthood, marked by hyper-sociability and reduced avoidance behavior. Overall,
14 these results suggest that single housing in adulthood does not robustly impact the basal
15 behavioral state of C57BL/6J mice and that adolescence is a sensitive period for the effects of
16 chronic social stress in this strain.

17 **Methods**
18 *Animals*
19
20 Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME)
21 at postnatal day (PD) 21 (for adolescent isolation experiment) or 63 (for adult isolation
22 experiment) and housed on a 12 hr:12 hour light:dark cycle with lights off at 7:30 am and *ad*
23 *libitum* access to food and water. One week after arrival, mice were randomly assigned to
24 socially isolated (SI, one mouse per cage) or maintained in group housed (GH, five mice per
25 cage) conditions for six weeks prior to behavioral testing. In the adolescent SI cohort, mice that
26 were GH through adolescence were singly housed at PD77 for the duration of the study. In the
27 adult SI cohort, GH mice remained in group-housed conditions. All experimental protocols were
28 approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine in
29 accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

30 *Behavioral Assays*

31 Assays were conducted under 250 lux lighting conditions as previously described (Pleil et al.,
32 2015, Crowley et al., 2016, Marcinkiewcz et al., 2016) and Panlab SMART 3.0 video tracking
33 software was used to track and analyze behavior, unless otherwise described. Each behavioral
34 apparatus was thoroughly cleaned with 70% ethanol prior to each trial. Timeline graphs
35 illustrating the sequence of experiments conducted in the adolescent and adult isolation cohorts
36 can be found in **Figures 1A** and **2A**, respectively.
37
38 Elevated Plus Maze

39 The elevated plus maze (EPM) test was conducted in a plexiglass maze with two open and two
40 closed arms (35 cm l × 5.5 cm w, with 15 cm h walls for closed arms) extending from a central
41 platform (5.5 cm ×5.5 cm) elevated 50 cm above the floor. At the beginning of each trial, the
42 mouse was placed in the center of the maze facing an open arm and movement was tracked

1 continuously for five minutes. The total time spent on the open and closed arms of the assay and
2 total number of open and closed arm entries (defined as placement of all four paws into the arm)
3 were quantified. Percent time spent in the open arms of the assay was calculated to measure
4 anxiety-like behavior, and closed arm entries were used as a measure of locomotion.

5 Open Field Test

6 The open field test was conducted in a plexiglass arena (50×50×34.5 cm) with a gray floor. The
7 mouse was placed in one corner of the arena and allowed to explore freely for 30 minutes. Total
8 time spent in the center of the maze (defined as having all four paws in the 25 cm x 25 cm area in
9 the center of the arena) and periphery were quantified to calculate percent center time. The total
10 distance traveled in the maze (cm) was used to measure locomotion, and percent time in the
11 center of the maze was used to assess anxiety-like behavior.

12 Light/Dark Box

13 The light/dark box assay was conducted in a rectangular box divided into two equal
14 compartments (20 cm l × 40 cm w × 34.5 cm h), one dark with a closed lid and the other with an
15 open top and illuminated by two 60 W bulbs placed 30 cm above the box. The two compartments
16 were separated by a divider with a 6 cm x 6 cm cut out passageway at floor level. At the
17 beginning of each trial, the mouse was placed in a corner of the light compartment and allowed
18 to move freely between the two compartments for 10 minutes. The number of light box entries
19 and total time spent in the light compartment as compared to the dark compartment were used to
20 assess anxiety-like behavior.

21 Social Interaction Test

22 The social interaction test was conducted in three 10-minute phases in an open plexiglass
23 arena (50 cm × 50 cm × 34.5 cm), and mice could explore freely during each phase. Between
24 each testing phase, the experimental mouse was briefly placed in a holding cage while the
25 experimenter altered the arena settings as follows: phase 1: empty arena; phase 2: two empty
26 wire mesh cups (diameter 4", height 4") located at opposite corners of the arena 10 cm from each
27 wall; phase 3: a novel age- and sex-matched mouse of the same strain was placed inside one of
28 the two cups, while the other cup remained empty. The placement of the cups and social partner
29 were pseudorandom and counterbalanced. Interaction zones for each cup were defined as
30 encompassing a 5 cm radius around the center of the cup, and the ratio of interaction time with
31 the social partner versus the empty cup during phase 3 was used to determine a social preference
32 score.

33 Novel Object Interaction

34 The novel object interaction assay was conducted under the same conditions and using the
35 same analyses as the social interaction test (see above) but using objects, in order to assess
36 whether effects observed in novel social partner preference could be generalized to a non-
37 social novel object. The objects used in this experiment included plastic cuboids with orange
38 color (3 cm × 3 cm × 6 cm) and half-sphered plastic cylinders with a blue color of the same
39 dimensions, as described in previous publications (Lueptow, 2017, Tian et al., 2019); these
40 objects were determined to be of equal interest to C57BL/6J mice in pilot testing. The objects
41 were affixed to the floor of the arena during behavioral testing, which proceeded as follows:
42 phase 1: empty arena; phase 2: two versions of the same object located at opposite corners of the

1  arena 10 cm from each wall; phase 3: a novel object replaced one of the two familiar objects in
2  the arena. The ratio of interaction time with the novel versus familiar object during phase 3 was
3  used as a novel object preference score.

### Fear Conditioning

5  Fear conditioning was performed in an operant box with a stainless-steel grid floor within a
6  sound-attenuating chamber (Colbourn Instruments, Allentown, PA). The mouse was placed in
7  the chamber at the beginning of the test, and following a five min habituation period received six
8  pairings of a 30 second, 80 dB tone (conditioned stimulus, CS) co-terminating with a 2 second,
9  0.5 mA foot shock (unconditioned stimulus, US) separated by pseudorandom intra-interval times
10 (from 31-119 seconds, with an average ITI of 75.5 seconds). Video tracking and FreezeFrame
11 software (Colbourn Instruments, Allentown, PA) were used to assess freezing behavior during
12 the 28 second period preceding the shock across tone/shock presentations.

### Home Cage Ethanol Drinking

14 We used a modified version of the standard Drinking in the Dark (DID) binge ethanol drinking
15 paradigm (mDID) to assess binge ethanol intake under limited-access conditions as well as 24-
16 hour preference for ethanol over water. Mice were singly housed for several days prior to the
17 first ethanol presentation. For each mDID cycle, the home cage water bottle was replaced with a
18 bottle containing 20% (cycles 1-4) or 30% (cycles 5-6) ethanol for two hours beginning three
19 hours into the dark cycle for three days. On day 4, two bottles (one containing ethanol solution,
20 one containing water) were placed in the cage for 24 hours (bottles were weighted after 2 hours,
21 4 hours, and 24 hours of access). Bottle weights were used to calculate ethanol and water
22 consumption daily (normalized to bodyweight) and 24 hr ethanol preference on day 4, calculated
23 as the ratio of the volume of liquid consumed from the ethanol bottle to the water bottle.

### Aversion-Resistant Ethanol Drinking

25 Consumption and preference of quinine-adulterated ethanol over water in a two-bottle choice
26 home cage assay was measured to evaluate aversion-resistant ethanol drinking behavior. Mice
27 received 4 hours of access to two bottles, one containing 20% ethanol adulterated with 100 $\mu$M
28 (Days 1 and 2) or 250 $\mu$M (Day 3) quinine hemisulfate (Sigma-Aldrich, St. Louis, MO) and the
29 other containing water. Bottle placement was pseudorandom and switched daily, and
30 consumption and preference were measured as described for mDID.

### Sucrose Preference Test

32 A similar procedure to that described above was used to evaluate consumption and preference for
33 1% (w/v) sucrose solution versus water, except that mice were given access to the sucrose and
34 water bottles for 24 hours per day. Intake and preference were measured every 24 hours for four
35 consecutive days. For all drinking experiments, empty "dummy" cages on the same rack as
36 housed behavior mice received the same ethanol, sucrose or water bottle replacement, and
37 consumption was adjusted for leak from dummy bottles and normalized to bodyweight.

### Home Cage Social Interaction

1  Home cage social interaction with a novel same-sex conspecific mouse was conducted in the
2  experimental mouse's home cage (28 cm x 18 cm x 12.5 cm). The novel mouse was placed
3  into the cage and overhead video was used to record behavior for five minutes. An experimenter
4  blind to condition hand-scored discrete behaviors performed by the experimental mouse,
5  including the number and duration of total, head-to-head, and head-to-tail social interactions, as
6  well as digging and climbing bouts.

7  *Statistical Analysis*

8  Statistical analyses were conducted using GraphPad Prism 8 software. Distributions of data
9  within group were analyzed for normality, and outliers were identified using Q-Q plots and
10  confirmed by the Rout method (Q = 0.5%); when an individual mouse's behavior was identified
11  as an outlier for at least half of the reported dependent measures for an assay, it was excluded
12  from analysis for that assay. Two-way analysis of variance (ANOVA) was used to assess the
13  effects of housing condition and sex on behavior in the elevated plus maze, open field test (adult
14  cohort), novel object test, light/dark box, and social interaction assays. Two-way repeated
15  measures ANOVA (RM ANOVA) was used to assess the effects of housing condition on home-
16  cage drinking behaviors within sex. Three-way RM ANOVA was used to assess the freezing
17  across consecutive tone/shock pairings in the fear conditioning assay and behavior in the open
18  field test across time (adolescent cohort). Equal variance across time was not assumed in RM-
19  ANOVAs with three or more repeated measures, and a Greenhouse-Geisser correction of degrees
20  of freedom was used. Significant effects in all ANOVAs were followed up with post-hoc two-
21  tailed t-tests corrected for multiple comparisons using the Holm-Sidak method, and adjusted *p*
22  values are presented. Alpha values of 0.05 were used throughout all analyses, and data are
23  presented as mean + SEM.

24  **Results**
25  *Elevated Plus Maze*

26  Following six weeks of adolescent SI or GH conditions, mice underwent testing in the EPM to
27  assess differences in anxiety-like behavior (**Figure 1B**; GH females n = 9, GH males n = 10, SI
28  females n = 10, SI males n = 9). A two-way RM ANOVA comparing the percent time spent on
29  the open arms revealed a main effect of housing condition ($F_{(1,34)} = 12.78$, $p = 0.001$) but no
30  main effect of sex ($F_{(1,34)} = 0.53$, $p = 0.472$) and a significant interaction between sex and
31  housing condition ($F_{(1,34)} = 0.41$, $p = 0.026$). Post-hoc analysis showed that this effect was driven
32  by females, as SI females spent significantly more time on the open arms than their GH
33  counterparts ($t_{(34)} = 4.17$, adjusted $p = 0.0004$), while SI males did not (adjusted $p > 0.05$). A
34  two-way RM ANOVA on the number of closed arm entries revealed no effects of housing ($F_{(1,34)}$
35  $= 0.08$, $p = 0.776$) or sex ($F_{(1,34)} = 1.41$, $p = 0.776$), nor a sex by housing condition interaction
36  ($F_{(1,34)} = 1.10$, $p = 0.301$), suggesting that the increased open arm exploration in SI females was
37  not due to a general increase in locomotion.

38  In contrast, social isolation during adulthood did not alter anxiety-like behavior on the EPM
39  (**Figure 2B**). A two-way RM ANOVA revealed a main effect of sex ($F_{(1,34)} = 6.66$, $p = 0.014$)
40  but no main effect of housing condition ($F_{(1,34)} = 0.01$, $p = 0.928$) nor a sex by housing condition
41  interaction ($F_{(1,34)} = 0.10$, $p = 0.753$). Despite this significant main effect of sex in the omnibus

1   test, post-hoc analysis did not reveal any significant differences between males and females
2   (adjusted $p > 0.05$). A two-way RM ANOVA on the number of closed arm entries revealed no
3   effects of sex ($F_{(1, 34)} = 3.17$, $p = 0.084$) or housing condition ($F_{(1, 34)} = 0.33$, $p = 0.569$), nor was
4   there a significant interaction between these factors ($F_{(1, 34)} = 1.95$, $p = 0.171$).

5   *Open Field Test*

6   To further investigate the impact of adolescent social isolation on anxiety-like and locomotor
7   behavior in early adulthood, we next compared open field exploration in GH and SI female and
8   male mice (**Figure 1C**; n = 10 per group). A three-way RM ANOVA comparing the impact of
9   sex and adolescent housing condition on the percent time spent in the center of an open field
10  across time (30 minutes total, broken into 5 minute intervals) revealed a significant main effect
11  of time ($F_{(5,180)} = 18.63$, $p < 0.0001$) but no effects of sex ($F_{(1,36)} = 3.20$, $p = 0.082$) or housing
12  condition ($F_{(1,36)} = 0.001$, $p = 0.971$). No significant interactions were identified between time
13  and sex ($F_{(5,180)} = 0.31$, $p = 0.906$), time and housing condition ($F_{(5,180)} = 0.31$, $p = 0.904$), or sex
14  and housing condition ($F_{(1,36)} = 3.35$, $p = 0.075$). While there was a significant three-way time by
15  sex by housing condition interaction ($F_{(5,180)} = 2.94$, $p = 0.014$), post-hoc analysis did not reveal
16  any significant comparisons (adjusted $ps > 0.05$). A three-way RM ANOVA comparing the total
17  distance traveled in the open field across these time points revealed a significant main effect of
18  time ($F_{(5,180)} = 57.65$, $p < 0.0001$) but no main effects of sex ($F_{(1,36)} = 0.53$, $p = 0.473$) or housing
19  condition ($F_{(1,36)} = 1.66$, $p = 0.205$). There was an interaction between time and sex ($F_{(5,180)} =$
20  $2.41$, $p = 0.038$) but no significant interaction between time and housing condition ($F_{(5,180)} =$
21  $0.85$, $p = 0.516$) or sex and housing condition ($F_{(1,36)} = 4.01$, $p = 0.052$), and no three-way
22  interaction between time, sex, and housing condition ($F_{(5,180)} = 1.57$, $p = 0.171$). Post-hoc
23  analysis did not reveal any significant differences between sexes at any time point, however
24  (adjusted $p > 0.05$).

25  In the adult SI cohort, we used a 10 min open field test (**Figure 2C**; GH females n = 8, GH males
26  n = 10, SI females n = 10, SI males n = 10). A two-way RM ANOVA comparing the percent
27  time in the center of this assay did not reveal a main effect of sex ($F_{(1,34)} = 2.29$, $p = 0.139$) or
28  housing condition ($F_{(1,34)} = 4.07$, $p = 0.051$), and the interaction between these variables also
29  failed to achieve significance ($F_{(1,34)} = 0.01$, $p = 0.931$). Interestingly, a two-way RM ANOVA
30  comparing the total distance traveled during this five minute assay did not reveal main effects of
31  sex ($F_{(1,34)} = 1.38$, $p = 0.248$) or housing condition ($F_{(1,34)} = 0.41$, $p = 0.526$) but did reveal a
32  significant interaction between these factors ($F_{(1,34)} = 18.72$, $p = 0.001$). Post-hoc comparisons
33  revealed that GH females traveled a greater distance than their SI counterparts ($t_{(34)} = 3.41$,
34  adjusted $p = 0.005$) while GH males traveled significantly less distance in this assay than SI
35  males ($t_{(34)} = 2.69$, adjusted $p = 0.021$). Furthermore, the total distance traveled was higher in GH
36  females than GH males ($t_{(34)} = 2.17$, adjusted $p = 0.037$), and higher in SI males than SI females
37  ($t_{(34)} = 4.01$, adjusted $p = 0.001$).

38  *Social Interaction Test*

39  To determine whether chronic social isolation during adolescence effects adult social behavior,
40  mice in the adolescent SI cohort underwent a social interaction test (**Figure 1D**; GH females n =

1   10, GH males n = 10, SI females n = 9, SI males n = 10). Male and female mice reared in
2   isolation, as well as GH males, demonstrated a significant preference for a social partner as
3   compared to an empty cup (one-sample *t*-tests; GH males, $t_{(9)} = 2.15$, $p = 0.004$; SI females, $t_{(8)} =$
4   2.69, $p = 0.027$; SI males, $t_{(9)} = 4.40$, $p = 0.001$); however adolescent GH females did not
5   demonstrate this social preference ($t_{(9)} = 2.15$, $p = 0.060$). Interestingly, a two-way RM ANOVA
6   analyzing preference for a social partner over a non-social object revealed a significant main
7   effect of housing condition ($F_{(1, 35)} = 5.98$, $p = 0.019$) but no main effect of sex ($F_{(1, 35)} = 2.49$, $p$
8   $= 0.123$) or interaction between these factors ($F_{(1, 35)} = 0.07$, $p = 0.787$). However, post-hoc
9   analysis did not reveal any significant differences in social preference between GH and SI
10  animals of either sex (adjusted $p > 0.05$). A two-way RM ANOVA assessing general activity in
11  this assay, as measured by combining the total time spent exploring both a social partner and a
12  non-social empty cup, revealed no significant differences between groups (main effect of sex:
13  $F_{(1, 35)} = 0.50$, $p = 0.484$; main effect of housing condition: $F_{(1, 35)} = 2.69$, $p = 0.110$; sex by
14  housing condition interaction: $F_{(1, 35)} = 0.55$, $p = 0.462$).

15  In the adult SI cohort (**Figure 2D**; GH females n = 8, GH males n = 10, SI females n = 10, SI
16  males n = 10), no group demonstrated a reliable preference for a social partner over an empty
17  cup (one-sample *t*-tests; GH females: $t_{(7)} = 2.23$, $p = 0.060$; GH males: $t_{(9)} = 1.87$, $p = 0.094$; SI
18  females: $t_{(9)} = 2.10$, $p = 0.065$; SI males: $t_{(9)} = 2.05$, $p = 0.070$). A two-way RM ANOVA did not
19  reveal significant differences in social preference between groups (main effect of sex: $F_{(1, 29)} =$
20  3.15, $p = 0.086$; main effect of housing condition: $F_{(1, 29)} = 0.02$, $p = 0.896$; sex by housing
21  condition interaction: $F_{(1, 29)} = 0.59$, $p = 0.448$). A two-way RM ANOVA comparing the total
22  combined time spent exploring both the non-social object (empty cup) and social partner
23  revealed significant main effects of sex ($F_{(1, 34)} = 10.04$, $p = 0.003$) and housing condition ($F_{(1, 34)}$
24  $= 4.32$, $p = 0.045$), but there was no interaction between these factors ($F_{(1, 34)} = 2.51$, $p = 0.122$).
25  Follow-up post-hoc analyses revealed that GH males spent more combined time exploring a
26  social partner and empty cup than GH females ($t_{(34)} = 3.27$, adjusted $p = 0.010$) and SI males ($t_{(34)}$
27  $= 2.67$, adjusted $p = 0.034$).

28  *Light/Dark Box*

29  A two-way RM ANOVA did not reveal any effects of adolescent social isolation or sex (**Figure
30  1E**; n = 10 per group) on the percent time spent in the light side of a light/dark box (main effect
31  of sex: $F_{(1, 35)} = 0.21$, $p = 0.646$; main effect of housing condition: $F_{(1, 35)} = 1.21$, $p = 0.279$; sex
32  by housing condition interaction: $F_{(1, 35)} = 0.023$, $p = 0.879$). A two-way RM ANOVA
33  comparing the effects of six weeks of adult social isolation versus group housing conditions on
34  behavior in the light/dark box in males and females (**Figure 2E**; GH females n = 8, GH males n
35  $= 10$, SI females n = 10, SI males n = 10) revealed a significant main effect of housing condition
36  ($F_{(1, 34)} = 21.78$, $p < 0.0001$), but no main effect of sex ($F_{(1, 34)} = 0.020$, $p = 0.886$) or significant
37  interaction between these variables ($F_{(1, 34)} = 0.550$, $p = 0.463$). Post-hoc analysis revealed that
38  GH animals spent significantly more time in the light compartment of the light/dark box than
39  their SI counterparts (GH males versus SI males: $t_{(34)} = 3.94$, adjusted $p = 0.0008$; GH females vs
40  SI females: $t_{(34)} = 2.70$, adjusted $p = 0.011$).

41  *Novel Object Interaction*

1 To determine whether the increased social exploration observed following adolescent social
2 isolation could be generalized to non-social contexts, we performed a novel object interaction
3 task designed similarly to the social interaction task described above (**Figure 1F**). GH females (n
4 = 9) demonstrated a preference for a novel object over a familiar object (one-sample $t$-test, $t_{(8)}$ =
5 2.71, $p = 0.026$), as did GH males (n = 10; $t_{(9)} = 4.83$, $p = 0.0009$), SI females (n = 9; $t_{(8)} = 6.02$,
6 $p = 0.0003$), and SI males (n = 9; $t_{(8)} = 3.29$, $p = 0.011$). A two-way ANOVA comparing novel
7 object preference across groups revealed a significant main effect of sex ($F_{(1, 33)} = 5.20$, $p =$
8 0.029) but no main effect of housing condition ($F_{(1, 33)} = 0.766$, $p = 0.387$) or significant
9 interaction between these factors ($F_{(1, 33)} = 1.31$, $p = 0.261$). Post-hoc analysis revealed that GH
10 males exhibited a significantly increased novel object preference as compared to GH females
11 ($t_{(33)} = 2.45$, adjusted $p = 0.039$). To assess general exploratory behavior in this assay, we
12 compared the total time that animals in each group spent exploring both the novel plus familiar
13 objects in this assay. A two-way ANOVA revealed a significant main effect of sex ($F_{(1, 33)} =$
14 17.91, $p = 0.0002$), but no main effect of housing condition ($F_{(1, 33)} = 0.54$, $p = 0.469$) or
15 interaction between these factors ($F_{(1, 33)} = 0.36$, $p = 0.553$). Post-hoc analysis revealed that GH
16 females spent significantly less time exploring the novel and familiar objects than GH males ($t_{(33)}$
17 = 3.46, adjusted $p = 0.002$). Consistent with this, SI females also spent less time exploring these
18 objects that SI males ($t_{(33)} = 2.54$, adjusted $p = 0.016$). Altogether, these results suggest that while
19 there are sex differences in the preference for and exploration of novel objects over familiar,
20 adolescent social isolation had no effect on this task. In contrast, adolescent social isolation
21 increased preference for a social partner, suggesting that its effects were specific to a social
22 context.

23 In the adult SI cohort (**Figure 2F**), GH females displayed a significant preference for the novel
24 object (n = 7; $t_{(6)} = 3.13$, $p = 0.026$), as did SI females (n = 8; $t_{(7)} = 3.07$, $p = 0.017$) and SI males
25 (n = 9; $t_{(8)} = 2.84$, $p = 0.022$), but not GH males (n = 9; $t_{(8)} = 1.99$, $p = 0.082$). A two-way
26 ANOVA comparing novel object preference across groups revealed no significant differences
27 between groups (main effect of sex: $F_{(1, 29)} = 3.15$, $p = 0.896$; main effect of housing condition:
28 $F_{(1, 29)} = 0.017$, $p = 0.896$; sex by housing condition interaction: $F_{(1, 29)} = 0.59$, $p = 0.448$). A two-
29 way ANOVA comparing the total combined time spent exploring the novel and familiar objects
30 revealed a significant main effect of sex ($F_{(1, 29)} = 10.64$, $p = 0.002$), but no main effect of
31 housing condition ($F_{(1, 29)} = 0.019$, $p = 0.890$) or sex by housing interaction ($F_{(1, 29)} = 4.13$, $p =$
32 0.051). Post-hoc tests revealed that GH males spent significantly more combined time exploring
33 a social partner and novel object than GH females ($t_{(29)} = 3.68$, adjusted $p = 0.002$)

34 *Fear Conditioning*

35 We next assessed whether adolescent social isolation impacts fear learning by measuring
36 acquisition of freezing behavior in response to a foot shock-paired tone (assessed by freezing
37 during tone presentation across six consecutive tone/shock pairings; **Figure 1G**). A three-way
38 RM ANOVA was used to measure the effects of sex and adolescent housing condition on
39 freezing behavior across time (GH females n = 5, SI females n = 4, GH males n = 5, SI males n =
40 5). This test revealed a significant main effect of time, as expected ($F_{(3,045, 45)} = 34.28$, $p <$
41 0.0001). A main effect of sex also emerged ($F_{(1, 15)} = 5.36$, $p = 0.035$) as well as a significant time

1   by sex interaction ($F_{(5, 75)} = 2.68$, $p = 0.027$). There was no significant main effect of housing

2   condition ($F_{(1, 15)} = 0.23$, $p = 0.638$), time by housing condition interaction ($F_{(5, 75)} = 0.80$, $p =$

3   0.550), sex by housing condition interaction ($F_{(1, 15)} = 0.010$, $p = 0.919$), or time by sex by

4   housing condition interaction ($F_{(5, 75)} = 0.63$, $p = 0.680$). Post-hoc comparisons did not reveal any

5   significant sex-dependent differences at any time point, however (adjusted $p > 0.05$).

6   We also assessed fear conditioning in the adult SI cohort (**Figure 2G**; GH female n = 8, GH

7   male n = 10, SI female n = 10, SI male n = 10). A three-way RM ANOVA revealed a main effect

8   of time ($F_{(3.851, 130.9)} = 78.78$, $p < 0.0001$), as well as a main effect of housing condition ($F_{(1, 34)} =$

9   4.17, $p = 0.048$) but no main effect of sex ($F_{(1, 34)} = 0.069$, $p = 0.793$). There was no interaction

10   between time and sex ($F_{(5, 170)} = 1.15$, $p = 0.336$), time and housing condition ($F_{(5, 170)} = 1.26$, $p =$

11   0.285), or sex and housing condition ($F_{(1, 34)} = 0.153$, $p = 0.697$), nor was there a significant

12   three-way interaction between these variables ($F_{(5, 170)} = 0.669$, $p = 0.646$). Post-hoc analysis did

13   not reveal any significant differences in freezing behavior across groups at any time point

14   (adjusted $p > 0.05$).

15   *Home Cage Ethanol Drinking*

16   As previous studies in rodents have demonstrated that adolescent social isolation increases home

17   cage ethanol self-administration (McCool and Chappell, 2009, Butler et al., 2014a, Skelly et al.,

18   2015, Butler et al., 2016), we next assessed whether adolescent social isolation affects binge

19   ethanol drinking in male and female C57BL/6J mice across time using a modified version of the

20   standard DID paradigm that allowed us to assess ethanol preference on day 4 of each DID cycle

21   (**Figure 3A,D**; n = 10 per group). A mixed-effects analysis was used to evaluate consumption of

22   20% ethanol across four cycles in GH and SI females (**Figure 3A**, left), revealing a main effect

23   of cycle ($F_{(6.372, 112.6)} = 4.32$, $p < 0.0001$) but no main effect of housing condition ($F_{(1, 18)} = 1.24$, $p$

24   = 0.280) or interaction between these variables ($F_{(15, 265)} = 1.43$, $p = 0.132$). To ensure that a

25   group difference was not being obscured by a ceiling effect, we next increased the concentration

26   of ethanol to 30% for two cycles, and a mixed-effects analysis revealed no effects or interactions

27   at this concentration either (main effect of cycle: $F_{(3.621, 60.52)} = 1.77$, $p = 0.153$; main effect of

28   housing condition: $F_{(1, 17)} = 0.219$, $p = 0.645$; time by condition interaction: $F_{(7, 117)} =$

29   1.72, $p = 0.111$). We also found no effect of social isolation on ethanol preference at either

30   concentration in females (**Figure 3A**, right). A mixed-effects analysis of 20% ethanol preference

31   revealed no effects (main effect of cycle: $F_{(3, 49)} = 0.097$, $p = 0.961$; main effect of housing

32   condition: $F_{(1, 18)} = 1.71$, $p = 0.207$; cycle by housing condition interaction: $F_{(3, 49)} = 2.19$, $p =$

33   0.101). Similarly, a two-way RM ANOVA assessing 30% ethanol preference revealed no effects

34   (main effect of time: $F_{(1, 17)} = 1.07$, $p = 0.316$; main effect of housing condition: $F_{(1, 17)} = 3.83$, $p$

35   = 0.252; time by housing condition interaction: $F_{(1, 17)} = 1.83$, $p = 0.194$).

36   Similar to females, social isolation did not affect ethanol consumption or preference in males

37   (**Figure 3D**, left). A mixed-effects analysis of 20% ethanol consumption (**Figure 3D**; n = 10 per

38   group) revealed a significant main effect of cycle ($F_{(7.450, 132.6)} = 4.10$, $p < 0.001$), but no main

39   effect of housing condition ($F_{(1, 18)} = 0.004$, $p = 0.947$) or interaction between these factors ($F_{(15,}$

40   $_{267)} = 0.527$, $p = 0.924$). A mixed-effects analysis of 30% ethanol intake also revealed a main

41   effect of cycle ($F_{(7, 121)} = 7.36$, $p < 0.001$), but no main effect of housing condition ($F_{(1, 18)} = 1.29$,

1  $p = 0.270$) or significant cycle by housing condition interaction ($F_{(7, 121)} = 1.63$, $p = 0.132$). A

2  mixed-effects analysis of 20% ethanol preference (**Figure 3D**, right) compared to water revealed

3  no effects (main effect of cycle: $F_{(2.357, 38.49)} = 0.325$, $p = 0.758$; main effect of housing condition:

4  $F_{(1, 18)} = 0.213$, $p = 0.649$; cycle by housing condition interaction: ($F_{(3, 49)} = 2.06$, $p = 0.117$).

5  Similarly, a mixed effects analysis assessing 30% ethanol preference did not reveal significant

6  group differences (main effect of cycle: $F_{(1, 35)} = 1.88$, $p = 0.179$; main effect of housing

7  condition: $F_{(1, 35)} = 0.151$, $p = 0.699$; cycle by housing condition interaction: $F_{(1, 35)} = 0.536$, $p =$

8  0.468).

9  *Aversion-Resistant Ethanol Drinking*

10  To assess whether adolescent social isolation alter aversion-resistant ethanol consumption, we

11  measured home cage DID intake using 20% ethanol adulterated with quinine (**Figure 3B,E**).

12  Mice were given 4 hr access to 20% ethanol containing either 100µM quinine (days 1 and 2,

13  average used for analysis) or 250µM quinine (day 3). Among female mice (ns = 9), a two-way

14  RM ANOVA for quinine-adulterated ethanol intake did not reveal any significant differences

15  (**Figure 3B**; main effect of quinine concentration: $F_{(1, 16)} = 4.21$, $p = 0.056$; main effect of

16  housing condition: $F_{(1, 16)} = 0.175$, $p = 0.681$; concentration by housing condition interaction: $F_{(1,}$

17  $_{16)} = 0.001$, $p = 0.977$). Similarly, a two-way RM ANOVA assessing quinine-adulterated ethanol

18  preference revealed no main effects of housing condition ($F_{(1, 16)} = 3.62$, $p = 0.074$) or quinine

19  concentration ($F_{(1, 16)} = 1.67$, $p = 0.214$), nor any significant interaction between these variables

20  ($F_{(1, 16)} = 0.049$, $p = 0.826$). In male mice (GH n = 10, SI n = 9), there was a significant main

21  effect of quinine concentration on ethanol intake (**Figure 3E**; $F_{(1, 17)} = 2.93$, $p = 0.105$), with the

22  higher dose of quinine suppressing ethanol consumption. However, there was no significant main

23  effect of housing condition ($F_{(1, 17)} = 2.93$, $p = 0.105$), nor a significant interaction between these

24  factors ($F_{(1, 17)} = 0.128$, $p = 0.724$). A two-way RM ANOVA comparing ethanol preference

25  across quinine concentrations did not reveal any significant differences between GH and SI male

26  mice (main effect of quinine concentration: $F_{(1, 17)} = 1.29$, $p = 0.271$; main effect of housing

27  condition: $F_{(1, 17)} = 0.108$, $p = 0.746$; concentration by housing condition interaction: $F_{(1, 17)} =$

28  0.001, $p = 0.981$).

29  *Sucrose Preference Test*

30  To determine whether social isolation during adolescence impacts general reward sensitivity, we

31  measured 1% (w/v) sucrose preference versus water across three days (**Figure 3C,F**). A two-way

32  RM ANOVA comparing adolescent GH (n = 10) and SI (n = 9) female mice revealed a

33  significant main effect of time (**Figure 3C**; $F_{(1.687, 28.68)} = 4.32$, $p = 0.028$) but no main effect of

34  housing condition ($F_{(1, 17)} = 0.342$, $p = 0.566$) or interaction between these variables ($F_{(2, 34)} =$

35  0.255, $p = 0.775$). In male mice, no differences in sucrose preference emerged (**Figure 3F**; main

36  effect of time: $F_{(1.418, 25.53)} = 2.57$, $p = 0.110$; main effect of housing condition: $F_{(1, 18)} = 0.025$, $p$

37  $= 0.874$; time by housing condition interaction: $F_{(2, 36)} = 0.331$, $p = 0.720$). Altogether, results

38  from our drinking experiments suggest that binge ethanol consumption, aversion-resistant

39  ethanol intake, and general reward sensitivity were unaltered by adolescent social isolation.

40  *Home Cage Social Interaction*

11

1    We found a robust effect of adolescent, but not adult, social isolation on increased social
2    behavior in adulthood using a social interaction paradigm in a novel environment. We further
3    probed the stability and generalizability of this phenotype using a home cage social interaction
4    test in which the experimental mouse remained in its home cage and a novel intruder conspecific
5    was placed in the cage for five min (**Figure 4**; GH females n = 9, SI females n = 9, GH males n =
6    10, SI males n = 9). Adolescent SI males and females again showed greater social interaction in
7    this paradigm. A two-way ANOVA on the total number of social interaction bouts (**Figure 4A**)
8    showed a main effect of housing condition ($F_{(1, 33)} = 19.08$, $p = 0.0001$) and no effect of sex ($F_{(1,}$
9    $_{33)} = 3.99$, $p = 0.054$) or sex by housing interaction ($F_{(1, 33)} = 1.06$, $p = 0.310$). Post-hoc t-tests
10   confirmed this effect occurred in both females ($t_{(33)} = 2.33$, adjusted $p = 0.026$) and males ($t_{(33)} =$
11   $3.87$, adjusted $p = 0.001$). This increased interaction was true for both head-to-head and head-to-
12   tail interactions. Head-to-head (**Figure 4B**): main effect of housing ($F_{(1, 33)} = 9.78$, $p = 0.004$), no
13   effect of sex ($F_{(1, 33)} = 4.13$, $p = 0.050$), and no interaction ($F_{(1, 33)} = 0.002$, $p = 0.961$); post-hoc t-
14   tests: $ps > 0,05$. Head-to-tail (**Figure 4C**): main effect of housing ($F_{(1, 33)} = 16.26$, $p = 0.0003$),
15   no effect of sex ($F_{(1, 33)} = 1.72$, $p = 0.198$), and no interaction ($F_{(1, 33)} = 2.43$, $p = 0.128$); post-hoc
16   t-tests showed the effect was driven by males: females ($t_{(33)} = 1.73$, adjusted $p = 0.094$), males
17   ($t_{(33)} = 4.01$, adjusted $p = 0.0007$). In contrast to social interactions, there was no effect of
18   adolescent SI on digging or climbing behaviors (**Figure 4D,E**). Two-way ANOVAs on the
19   number of digging bouts and the number of climbing bouts showed no effects of housing
20   condition, sex, or an interaction ($ps > 0.05$).

21   Given this distribution of behaviors during the home cage assay, adolescent SI mice spent a
22   greater proportion of time engaged in social interaction than their GH counterparts (**Figure 4F**).
23   A two-way ANOVA on the percent time spent exploring a novel social partner revealed a
24   significant main effect of housing ($F_{(1, 33)} = 7.59$, $p = 0.010$) but no main effect of sex ($F_{(1, 33)} =$
25   $0.055$, $p = 0.815$) or interaction between these variables ($F_{(1, 33)} = 2.44$, $p = 0.127$). Post-hoc
26   analysis showed that the effect of social isolation was driven by males ($t_{(33)} = 3.09$, adjusted $p =$
27   $0.008$) but did not occur in females. However, the duration of the first interaction bout was
28   longer in adolescent SI mice of both sexes (**Figure 4G**). A two-way ANOVA assessing the
29   duration of the first bout of social interaction revealed a significant main effect of housing
30   condition ($F_{(1, 33)} = 11.23$, $p = 0.002$), but no main effect of sex ($F_{(1, 33)} = 0.109$, $p = 0.742$) or
31   significant sex by housing condition interaction ($F_{(1, 33)} = 0.143$, $p = 0.707$). Post-hoc analysis
32   confirmed that both SI females and males spent more time interacting with a novel social partner
33   during this first bout than their GH counterparts (females: $t_{(33)} = 2.61$, adjusted $p = 0.027$; males:
34   $t_{(33)} = 2.13$, adjusted $p = 0.040$). Interestingly, however, SI mice had a longer latency to first
35   approach the stranger mouse, suggesting some initial inhibition of this hypersocial behavior (data
36   not shown). A two-way ANOVA revealed a main effect of housing condition ($F_{(1, 33)} = 19.00$, $p$
37   $< 0.001$), but no main effect of sex ($F_{(1, 33)} = 2.06$, $p = 0.160$) or sex by housing interaction ($F_{(1,}$
38   $_{33)} = 1.35$, $p = 0.254$). Post-hoc analysis revealed that SI males and females took significantly
39   more time to approach the novel social partner than their GH counterparts (GH females vs SI
40   females: $t_{(33)} = 2.23$, adjusted $p = 0.039$; GH males vs SI males: $t_{(33)} = 3.95$, adjusted $p = 0.001$).
41   In spite of this initial delay in interaction, the overall results support our initial findings that
42   adolescent social isolation produces an aberrant hyper-social phenotype in adulthood in
43   C57BL/6J mice.

1 **Discussion**

2 These studies were designed to assess whether the harmful and translationally-relevant
3 behavioral consequences of adolescent SI well-characterized in rats can be reliably recapitulated
4 in C57BL/6J mice, the most common laboratory mouse background strain. We further sought to
5 determine whether adolescence is a critical period for behavioral plasticity or whether a similar
6 long-term social isolation in adulthood impacts these pathology-related behaviors. Surprisingly,
7 we did not see any consistent phenotypes following adult SI, as mice displayed an anxiogenic
8 phenotype in the light/dark box assay (**Figure 2E**) but not on any other measures of anxiety-like
9 behavior. These findings indicate that singly housing mice in adulthood, as is done routinely in
10 alcohol and drug self-administration studies, among others, does not alter basal behavioral states
11 in C57BL/6J mice; thus, adult isolation is not a major confounding variable for most behavioral
12 assays including those measured herein. Similarly, we found few effects of adolescent social
13 isolation on performance in a battery of behaviors, which was surprising given the literature
14 showing the deleterious effects of stress during the adolescent period on adult behaviors.
15 However, the most robust effect of adolescent social isolation we observed was that it promoted
16 social behavior in adulthood in both sexes (**Figure 1D**; **Figure 4**), and effect remarkably similar
17 in nature to the stress imposed upon the mice.

18 Contrary to our predictions, we did not find that adolescent social isolation increases anxiety-like
19 behavior in male or female C57BL/6J mice (**Figure 1**). In fact, following adolescent isolation,
20 adult female mice spent more time in the open arms of the elevated plus maze on average, a
21 behavior which is classically interpreted as a sign of anxiolysis (**Figure 1B**). This anxiolytic
22 effect of adolescent isolation in mice has been reported elsewhere (Voikar et al., 2005, Lopez
23 and Laber, 2015). Previous studies have also found some evidence that adolescent social
24 isolation induces an anxiogenic phenotype in the light/dark box and hyperlocomotion in the open
25 field test in mice (Voikar et al., 2005, Gan et al., 2014, Amiri et al., 2015, Medendorp et al.,
26 2018), but these results have not always been reported (Koike et al., 2009). In contrast to, we
27 found no effect of adult social isolation on anxiety-like behavior in the EPM (**Figure 2B**),
28 suggesting some adolescent time period specificity for this effect. Intriguingly, we found that
29 adult social isolation increased anxiety-like behavior in the light/dark box, suggesting that if
30 anything, adult isolation produces the opposite effect of adolescent isolation. However, in both
31 cohorts, other measures of anxiety-like behavior did not recapitulate these effects, suggesting
32 there are no reliable effects of social isolation at either time point on adult anxiety-related
33 behavior in C57BL/6J mice.

34 Perhaps our most striking finding is that isolation rearing during adolescence increased social
35 exploration and interaction in adulthood. Specifically, we found that preference for a novel social
36 partner increased in both males and females following protracted adolescent isolation (**Figure
37 1D**). We extended this finding in a home cage social interaction test with a novel intruding
38 conspecific (**Figure 4**), demonstrating that this hypersocial behavior occurs in both familiar and
39 novel environments. Aberrantly high social exploration may be maladaptive in settings in which
40 social caution or defensive behavior is more appropriate, such as during exposure to an
41 unfamiliar intruder. This phenotype is similar to that observed in some developmental disorders

1    such as Williams' Syndrome, in which individuals inappropriately approach and engage with
2    strangers. However, as this behavior occurred following a longer delay before approaching the
3    stranger mouse, the social phenotype of the adolescent SI mice could be a compensatory
4    mechanism that actually promotes an adaptive social phenotype beneficial in certain contexts
5    that require social affiliation for survival. This pro-social interpretation has previously been
6    reported to occur in female mice following exposure to a developmental stressor (Koike et al.,
7    2009, Bondar et al., 2018). Interestingly, many groups have reported the exact opposite effect of
8    adolescent isolation on social behavior in mice, finding that this developmental stressor
9    decreases social interest in adulthood (Balemans et al., 2010, Medendorp et al., 2018).
10   Nonetheless, reduced social learning (Kercmar et al., 2011) and aberrant social behavior when
11   placed back into group housing in adulthood (Endo et al., 2018) have also been reported
12   following post-weaning isolation in C57BL/6J male and female mice, further supporting a
13   specific role for peri-adolescent social isolation in abnormal adult social behavior. This is
14   unsurprising given that this is a crucial developmental period for the development of prosocial
15   behaviors (Spear, 2004, Panksepp et al., 2007, Panksepp and Lahvis, 2007).

16   Interestingly, we did not identify a robust effect of isolation in adulthood on measures of social
17   interaction (**Figure 2D**), further suggesting that adolescence is a critical period for the
18   development of sensitivity to social reward. We also tested interest in a non-social novel object
19   following adolescent social isolation and found no significant effect of rearing condition on
20   novel object preference (**Figure 1F**). Again, no differences in novel object preference emerged
21   following social isolation in adulthood, although GH males spent more total time exploring the
22   social partner and novel object combined than SI males or GH females (**Figure 2F**).

23   Prolonged social isolation during adolescence or adulthood has also been reported to impact
24   aspects of fear memory formation in rats and mice (Pibiri et al., 2008, Pinna et al., 2008, Lukkes
25   et al., 2009a, Okada et al., 2015, Pinna, 2019). Here we tested the effect of sex and housing
26   condition on fear learning across six tone/footshock pairings. We did not identify any effect of
27   housing condition on fear memory formation following adolescent isolation (**Figure 1G**) but did
28   observe delayed acquisition following adult isolation, however final acquisition was similar
29   across all groups (**Figure 2G**). Together, these results suggest that singly housing C57BL/6J
30   mice during adolescence or adulthood does not reliably impact fear memory formation.

31   Adolescent isolation has been demonstrated to increase alcohol self-administration in male rats
32   and both male and female mice (Lopez et al., 2011, Butler et al., 2014b, Lopez and Laber, 2015,
33   Skelly et al., 2015, Butler et al., 2016). Here, we evaluated adolescent social isolation on binge
34   alcohol drinking using a modified DID paradigm and found no effects on 20%, 30%, and
35   quinine-adulterated 20% ethanol consumption or preference, nor on a rewarding 1% sucrose
36   solution, in either sex (**Figure 3**). Our results are inconsistent with the findings of Lopez and
37   colleagues (Lopez and Laber, 2015), who found that adolescent social isolation in C57 mice
38   produced a small but significant increase in alcohol consumption at one time point. However,
39   that study did not exam chronic home cage ethanol self-administration. Regardless, our data
40   indicate that perhaps the effects of chronic social stress in adolescence on ethanol drinking are
41   less robust than the effects reported in rats. Interestingly, adolescent social isolation has been

1    reported to produce a protracted increase in ethanol intake and preference in male C57BL/6J
2    mice given intermittent access to ethanol in their home cage, but only at a relatively low ethanol
3    concentration (5%); these differences disappeared when animals were offered a higher
4    concentration of ethanol (20%) (Advani et al., 2007). Together, these findings generally suggest
5    that adolescent isolation does not reliably produce a translationally relevant escalation of ethanol
6    self-administration in C57BL/6J mice.

7    In general, we found that C57BL/6J mice are not reliably sensitive to isolation stress. Beyond the
8    findings outlined herein, others have presented some evidence that single housing may not be
9    experienced as an adversity among C57BL/6J mice (Bartolomucci et al., 2003, Arndt et al.,
10   2009), and in fact may actually decrease social stress in males of this species (Singewald et al.,
11   2009). Others have not found evidence to support a protective effect of adolescent social
12   isolation in female C57BL/6J mice (Martin and Brown, 2010). Interestingly, the majority of
13   studies reporting a behavioral effect of adolescent isolation on anxiety-like behavior, fear
14   memory formation, or drug self-administration in have initiated isolation at the same time that
15   play behavior is typically increasing, suggesting that disruption of play behavior may be a major
16   contributor to this phenotype (Walker et al., 2019). As mice engage in less social play in
17   adolescence than rats, this may partly explain the variability in the behavioral effects of
18   adolescent isolation rearing reported here and elsewhere. Although these findings present an
19   issue for researchers interested in identifying the link between developmental stress and
20   psychopathology using mouse models on a C57BL/6J strain, the most common background for
21   genetic manipulation, it also suggests that experimentally-mandated individual housing in
22   adolescence or adulthood may not produce confounding effects on basal behavioral states that
23   experimenters prefer to avoid.

24

25   **Author contributions**

26   JKRI and MJS collected and analyzed the data. JKRI, MJS, and KEP designed the studies and
27   wrote and edited the manuscript.

28

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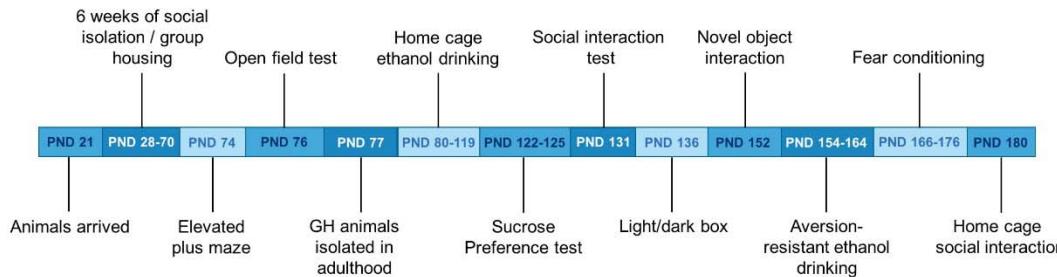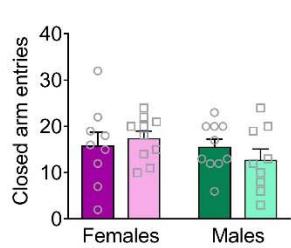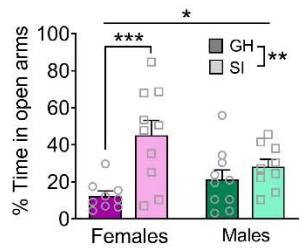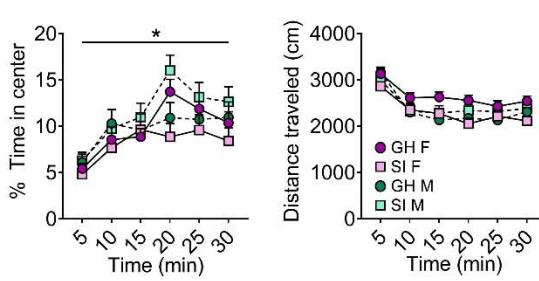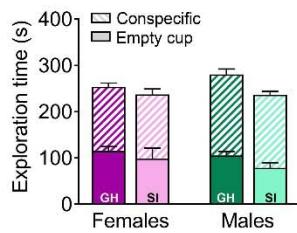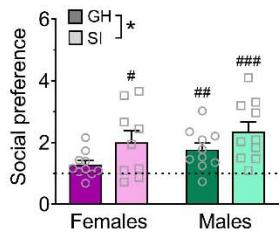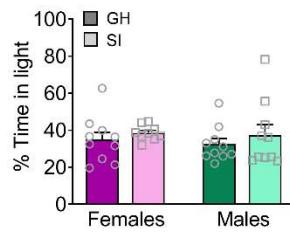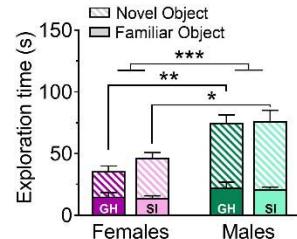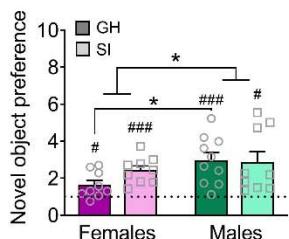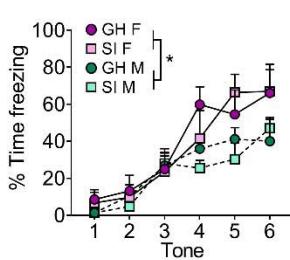
1    **Figures**



2

3    **Figure 1. Adolescent social isolation behavior battery.** (**A**) Experimental timeline. (**B**) In the
4    elevated plus maze (EPM), adolescent social isolation (SI) increases the percent time spent
5    exploring the open arms, an effect driven by females (left), without altering locomotor activity as
6    measured by closed arm entries (right). (**C**) Adolescent SI oppositely affects the percent time

1    spent exploring the center of an open field in males and females (left) but does not affect the

2    distance traveled in this assay (right). (**D**) On the social interaction test, all but GH females

3    display a significant preference for a novel social partner over an empty cup, and adolescent SI

4    increases preference (left) without impacting total time spent exploring both objects (right). (**E**)

5    Adolescent SI has no effect on anxiety-like behavior in the light/dark box. (**F**) All groups display

6    a preference for a novel object over a familiar one, and this preference was greater in males than

7    females but unaffected by adolescent SI (left). Total time spent exploring both objects is likewise

8    increased in males compared to females (right). (**G**) Females display enhanced fear conditioning

9    compared to males, but adolescent SI does not alter this measure. Data are expressed as means +

10   SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$

11   compared to null hypothesis of preference score = 1.

12
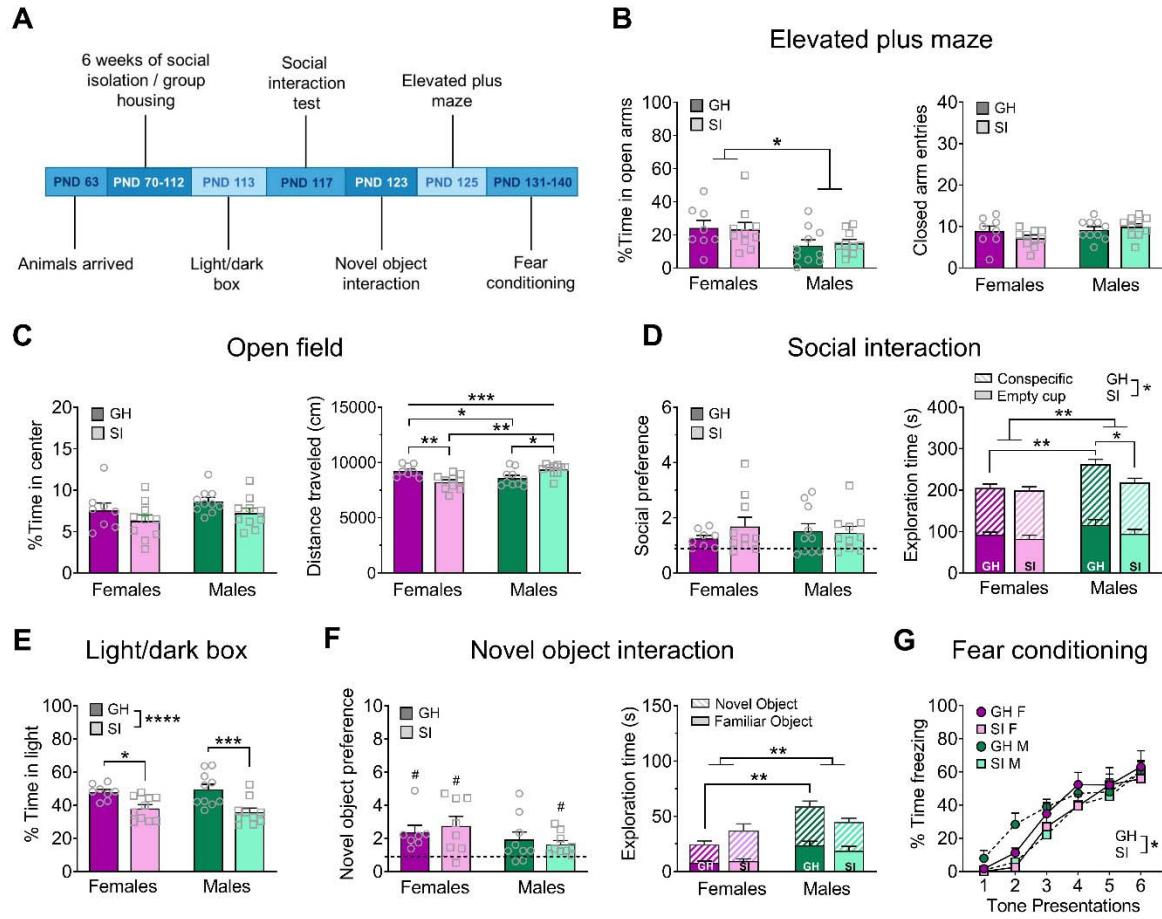
**Figure 2. Adult isolation behavior battery.** (**A**) Experimental timeline. (**B**) Females spend more time exploring the open arms of the EPM, but adult SI does not influence this measure (left); there are no difference in general locomotor behavior, measured by the number of entries into the closed arms (right). (**C**) There are no effects of sex or adult SI on the percent time spent exploring the center of the OF (left), however there is a sex-dependent effect of adult SI on the total distance traveled in the OF (right). (**D**) Adult SI does not alter preference for a novel social partner over an empty cup in the social interaction test (left) but does decrease total time spent interacting with the social partner and empty cup, an effect driven by males (right). GH males also spend more total time exploring both objects compared to GH females. (**E**) Adult SI decreases the percent time spent exploring the light side of the light/dark box in both males and females. (**F**) In the novel object interaction test, all groups except GH males display a preference for a novel vs. familiar object (left), however this is driven by greater overall interaction time with both objects in males (right). (**G**) Adult SI mice show delayed fear acquisition compared to GH mice. Data are expressed as means + SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$ between groups; [#]$p < 0.05$ compared to null hypothesis of preference score = 1.

24

## Females

**A**



## Males

**D**
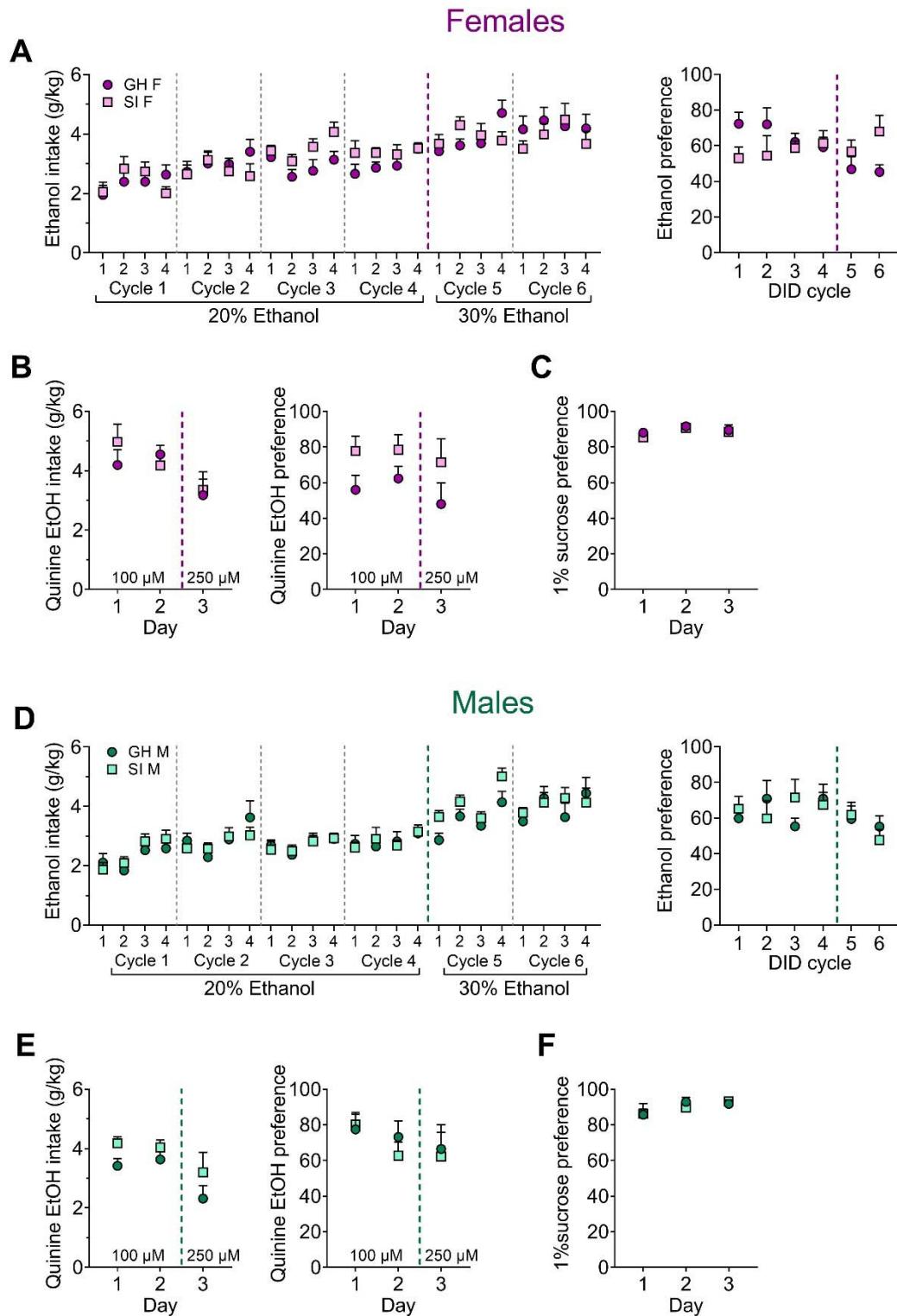


**Figure 3. Effects of adolescent social isolation on home cage ethanol drinking and reward and aversion sensitivity in adult female (A-C) and male (D-F) mice.** (**A**) There are no effects of adolescent SI on binge ethanol consumption (left) or 24-hr ethanol preference (right) across

1    six weeks of 20% and 30% ethanol in a modified EtOH DID paradigm in females. (**B**)
2    Adolescent GH and SI females display similar consumption of quinine-adulterated ethanol (left)
3    and preference for it over water (right) across multiple quinine concentrations. (**C**) Adolescent SI
4    does not alter preference for a 1% sucrose solution over water in female mice. (**D-F**) Similarly,
5    adolescent SI in males does not alter ethanol intake or preference (**D**), quinine-adulterated
6    ethanol intake or preference (**E**), or 1% sucrose preference (**F**).
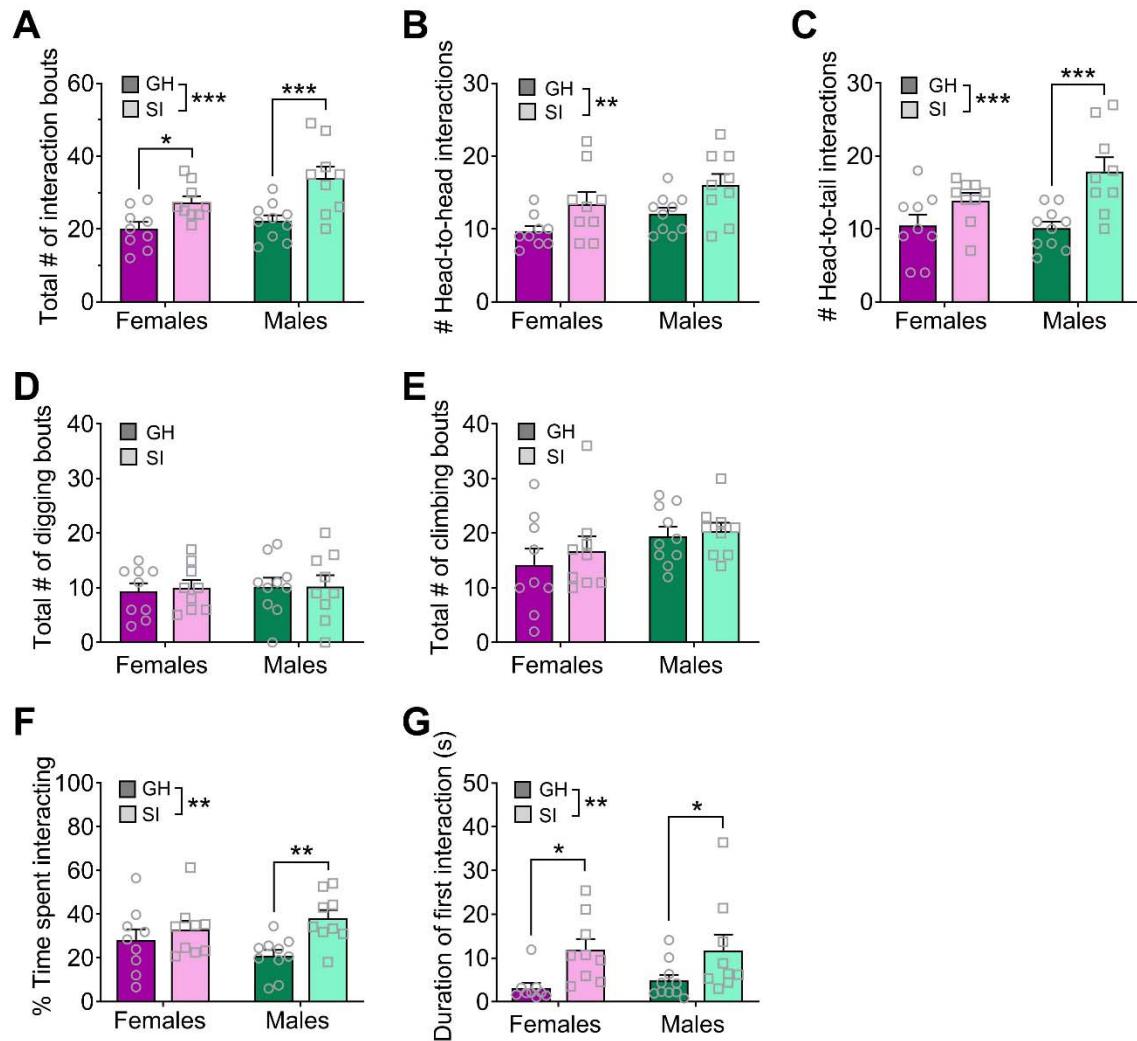


**Figure 4. Effects of adolescent social isolation on home cage social interaction in adulthood.**
(**A**) aSI mice display an increased number of social interaction bouts in both males and females.
(**B-C**) This overall phenotype is present when only head-to-head interactions (**B**) or head-to-tail
interactions (**C**) are considered. (**D-E**) In contrast, digging (**D**) and climbing (**E**) behaviors are
not altered by adolescent SI. (**F**) Adolescent SI mice spend a greater proportion of the 5 min
assay interacting with the stranger mouse than their GH counterparts, and effect driven by males.
(**G**) The duration of the first social interaction bout is longer in adolescent SI mice of both sexes.
Data are expressed as means + SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ between groups.