

Developmental Shifts in Amygdala Activity during a High Social Drive State

 Nicole C. Ferrara,^{1,2} Sydney Trask,³ Brittany Avonts,^{1,2}  Maxine K. Loh,^{1,2} Mallika Padival,^{1,2} and J. Amiel Rosenkranz^{1,2}

¹Department of Foundational Sciences and Humanities, Discipline of Cellular and Molecular Pharmacology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064, ²Center for Neurobiology of Stress Resilience and Psychiatric Disorders, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064, and ³Department of Psychological Sciences, Purdue University, West Lafayette, Indiana, 47907

Amygdala abnormalities characterize several psychiatric disorders with prominent social deficits and often emerge during adolescence. The basolateral amygdala (BLA) bidirectionally modulates social behavior and has increased sensitivity during adolescence. We tested how an environmentally-driven social state is regulated by the BLA in adults and adolescent male rats. We found that a high social drive state caused by brief social isolation increases age-specific social behaviors and increased BLA neuronal activity. Chemogenetic inactivation of BLA decreased the effect of high social drive on social engagement. High social drive preferentially enhanced BLA activity during social engagement; however, the effect of social opportunity on BLA activity was greater during adolescence. While this identifies a substrate underlying age differences in social drive, we then determined that high social drive increased BLA NMDA GluN2B expression and sensitivity to antagonism increased with age. Further, the effect of a high social drive state on BLA activity during social engagement was diminished by GluN2B blockade in an age-dependent manner. These results demonstrate the necessity of the BLA for environmentally driven social behavior, its sensitivity to social opportunity, and uncover a maturing role for BLA and its GluN2B receptors in social engagement.

Key words: amygdala; development; GluN2B; social

Significance Statement

Social engagement during adolescence is a key component of healthy development. Social drive provides the impetus for social engagement and abnormalities underlie social symptoms of depression and anxiety. While adolescence is characterized by transitions in social drive and social environment sensitivity, little is known about the neural basis for these changes. We found that amygdala activity is uniquely sensitive to social environment during adolescence compared with adulthood, and is required for expression of heightened social drive. In addition, the neural substrates shift toward NMDA dependence in adulthood. These results are the first to demonstrate a unique neural signature of higher social drive and begin to uncover the underlying factors that heighten social engagement during adolescence.

Introduction

Social engagement during development is essential to gain social and cognitive skills needed during adulthood (Hol et al., 1999;

Van Den Berg et al., 1999; Allemand et al., 2015; Powell et al., 2015). Adolescence is a developmental period characterized by increased sensitivity to the social environment and interaction (Varlinskaya et al., 1999; Douglas et al., 2004; Pietropaolo et al., 2004; Varlinskaya and Spear, 2008). Numerous psychiatric disorders with prominent social deficits emerge during adolescence and can be exacerbated by low quality social environment (Moy et al., 2004; Schneider et al., 2006; MacFabe et al., 2011; Lee et al., 2014; Favre et al., 2015; Burke et al., 2017; Meyer and Lee, 2019). In rodents, the duration and diversity of social interaction and sensitivity to the social environment declines from adolescence to adulthood. Specifically, adult conspecifics shift from social play toward a greater proportion of time spent engaging in cautious investigation (e.g., sniffing), defining two clusters of age-specific behaviors (Douglas et al., 2004; Varlinskaya and Spear, 2008).

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Correspondence should be addressed to Nicole C. Ferrara at nicole.ferrara@rosalindfranklin.edu.

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Isolation can help understand the influence of the social environment on age-specific social behaviors (Hol et al., 1999; Van Den Berg et al., 1999; Varlinskaya and Spear, 2008). Brief isolation (<7 d) increases the propensity to socially interact in a manner akin to states of increased motivated drive (Varlinskaya and Spear, 2008). Adolescents are sensitive to brief isolation and show robust increases in social engagement compared with adults (Varlinskaya and Spear, 2008), but it is unclear how maturing neural circuitry contributes to changes in social drive across ages.

Developmental shifts in social behavior have been linked to brain maturation, and abnormal social environments impact the maturation of neural circuits during adolescence (Silva-Gómez et al., 2003; Pellis et al., 2010; Miyazaki et al., 2012; Wang et al., 2012; Hinton et al., 2019; Ferrara et al., 2021). While several brain regions are sensitive social circumstances and mature during adolescence, the amygdala is sensitive to changes in the social environment and has been implicated in psychiatric disorders characterized by changes in social behavior. The adult basolateral amygdala (BLA) has an important role in social recognition, social interaction, and social learning (Truitt et al., 2007; Wellman et al., 2016; Zinn et al., 2016; Paine et al., 2017; Twining et al., 2017; Allsop et al., 2018; Rosenberger et al., 2019; Fustiñana et al., 2021). However, little is known about the environmental influence on BLA function, and BLA regulation of behavior produced by brief changes to the social environment.

The BLA undergoes synaptic and structural changes from adolescence to adulthood that occur alongside refinement of social behavior (Bessières et al., 2019; Ferrara et al., 2021). NMDA receptor expression increases following isolation periods with facilitated interaction in adults, while suppression of social behavior by NMDA antagonists and regulation of BLA activity increases with age (Monyer et al., 1994; Sajdyk and Shekhar, 1997; Delaney et al., 2013; Zhang et al., 2013; Gan et al., 2014; Morales and Spear, 2014; Jacobs and Tsien, 2017; Bessières et al., 2019). Changes in the regulation of social behavior by NMDA antagonism may be because of opposing changes in NMDA subunit expression in the developing BLA (De Armentia and Sah, 2003; Bessières et al., 2019). The GluN2B NMDA receptor subunit plays a critical role in social recognition (Jacobs et al., 2015) and social behavior during adverse social experiences (Day et al., 2011), with GluN2B-sensitive social interaction increasing with age (Morales and Spear, 2014). This suggests a role for GluN2B in the BLA in experience-dependent plasticity, including social experiences, which may shift over the course of development.

Here, we used a brief isolation (2 h) to measure BLA sensitivity to an acute social change that increases social drive, and the role of the BLA in isolation-driven facilitation of social behaviors. We then tested the contribution of GluN2B-containing NMDA receptors to brief social isolation on BLA neuronal activity and social engagement.

Materials and Methods

Experiments were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science.

Subjects

Subjects were male Sprague Dawley rats purchased from Envigo ($n = 207$) and housed two to three per cage in the Rosalind Franklin University animal facility. Rats had free access to food and water at all times and were maintained on a reverse light cycle (12/12 h light/dark). Adolescent rats arrived to the animal facility at postnatal day (PND) 20–PND21, and adults arrived at PND64–PND69. At the time of recording

and behavior, adolescents were between the ages of PND28–PND38, and adults were PND71–PND120.

Virus surgery

For DREADD experiments, DREADD-Gi (AAV5-CaMKII-HM4DG-mcherry) generously gifted by Bryan Roth to AddGene (Addgene viral prep #50477; RRID:Addgene_50477) or control virus (AAV5-CaMKII-mcherry) generously gifted by Karl Deisseroth to AddGene (Addgene viral prep #114469-AAV5; RRID:Addgene_114469) were bilaterally infused into the BLA. Rats were first anesthetized with 4% isoflurane and oxygen until deeply anesthetized, and maintained at 2–2.5% for the remainder of the surgery. A 10- μ l Hamilton syringe with a 24-gauge needle (World Precision Instruments) containing the virus was mounted onto a stereotaxic infusion pump (World Precision Instruments). All groups received bilateral infusions of virus (0.5 μ l/side; 50 nl/min) into the BLA (adolescent: P: 2.8, ML: 4.6; V: 7.9; adult: P: 3.1, ML: 5.2, V: 8.2) from bregma (Paxinos and Watson, 2007). The syringe was left in place for an additional 10 min following virus infusion to allow for diffusion. Approximately two weeks later, animals were tested for social interaction.

For fiber photometry experiments, pAAV.Syn.GCaMP6s.WPRE.SV40 (Chen et al., 2013) was a gift to Addgene from Douglas Kim & GENIE Project (Addgene viral prep #100843-AAV5; RRID: Addgene_100843). Viral infusion parameters were identical except that GCaMP6s was unilaterally infused. The infusion hemisphere was counterbalanced. Immediately after virus infusion, optical fibers were implanted into the BLA (adolescent: P: 2.8, ML: 4.8, V: 7.2; adult: P: 3.1, ML: 5.4, V: 7.4; 400 μ m, Doric Lens). Skull screws were implanted surrounding the fiber and metabond glue (Parkell Inc.) was layered onto the skull. Acrylic cement (Dentsply Sirona) was then layered on top of the glue. Rats were given a minimum 10 d of recovery before handling, and recordings took place 14 d following surgery. To confirm viral placement, BLA images were captured on a Nikon Eclipse E600 microscope and virus spread was mapped onto coronal sections (40 μ m) according to the rat brain atlas (Paxinos and Watson, 2007).

Anesthetized in vivo extracellular recording

Isolated and control rats were anesthetized with urethane (1.5 g/kg in 0.9% saline, i.p.). Rats were then mounted onto a stereotaxic device (Kopf Instruments) and body temperatures were maintained at 36–37°C using a heating pad (Model TC-1000; CWE). Coordinates for surgery were chosen based on the rat brain atlas (Paxinos and Watson, 2007). Adult BLA coordinates were A/P –3.1 mm, M/L +5.0 mm, and D/V –6.5 to –7.2 mm. Adolescent BLA coordinates were A/P –2.9 mm, M/L +4.8 mm, and D/V –6.5 to –7.0 mm. For ifenprodil experiments, dH2O or ifenprodil [2 μ g/ μ l (Jarome et al., 2011), 0.5 μ l; Sigma] were infused into the BLA (adult: A/P –3.0 mm, M/L 5.1 mm, and D/V –7.0 mm; adolescent: A/P –2.8 mm, M/L 4.9 mm, and D/V –6.8). Drugs were loaded into a 10 μ l Hamilton syringe with a 24-gauge needle (World Precision Instruments) mounted onto a stereotaxic infusion pump (World Precision Instruments). Drug was infused at a rate of 100 nl/min and the needle was left in place for 2 min to allow for drug diffusion.

Single-barrel glass recording electrodes were pulled (PE-2; Narishige) and broken under a microscope for a 1–2 μ m in diameter tip. The electrode was then filled with Pontamine (2% Chicago Sky Blue 6B; Sigma-Aldrich) in 2 M NaCl. The electrode was mounted onto the stereotaxic device and slowly lowered into the brain, targeting the BLA, with a hydraulic microdrive (SKU 50-12-9-02 and SKU 50-12-1C; Frederick Haer & Co). Signals were amplified via a headstage preamplifier and filtered (Model 1800; AM Systems) at low cut off frequency of 100 Hz and a high cut off frequency of 10 kHz. Signals were transmitted to an audio monitor (Model AM7, Grass Instruments, Astro-Med Inc). Following filtering from the amplifier, digitized outputs were recorded and monitored on a personal computer (Mac Pro; Apple) using Axograph X software and stored for later analysis. Placements were verified with Pontamine injection, confirmed under a microscope, and mapped onto coronal sections, as seen in Selleck et al. (2018).

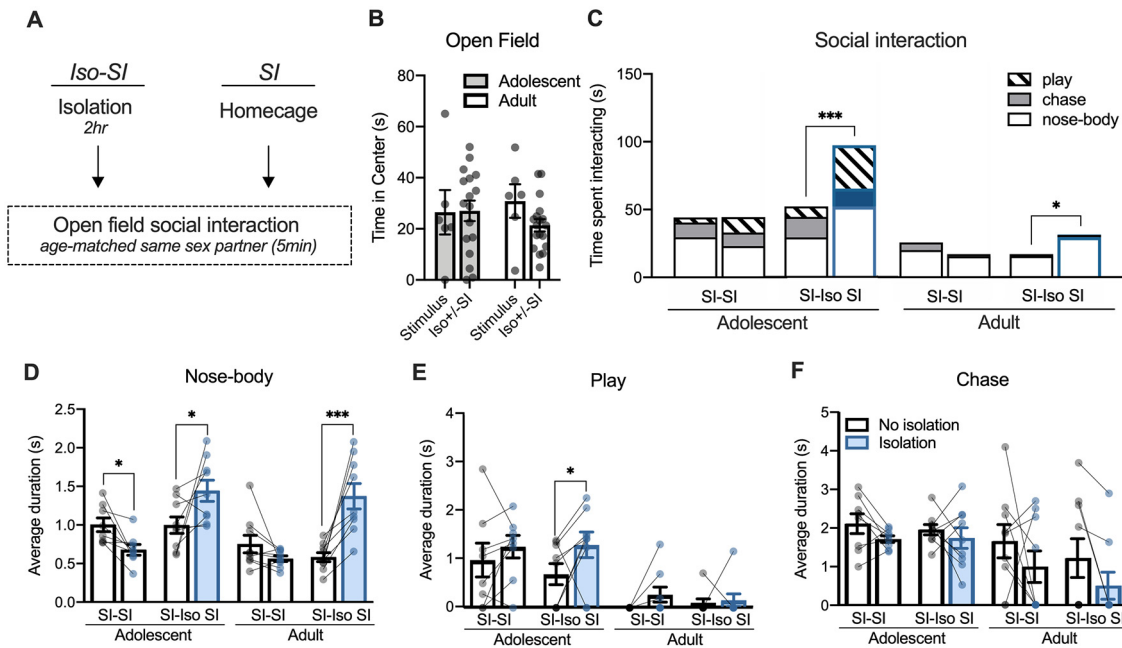


Figure 1. Brief isolation facilitates age-specific social behaviors. Adults and adolescents were divided into two groups and were exposed to two social interaction (SI) sessions. One group received two SI exposures (SI-SI), where graphed results are displayed as day 1 and day 2 (left to right, adolescent $n = 8$, adult $n = 9$). The other group received two SI exposures, where either the first or second interaction was preceded by a brief isolation in a counterbalanced manner (Iso-SI, adolescent $n = 9$, adult $n = 9$; **A**). Groups were preexposed to the open field apparatus the day before SI, and there was not a significant time in center difference between groups in SI-SI or Iso-SI groups (isolation condition) or stimulus rats (**B**). Regardless of age, SI-SI groups did not spend a significantly different amount of time socially interacting with a novel partner on day 1 or day 2, while isolation increased SI (**C**). Social behaviors were broken down and the average duration of each behavior was quantified. Isolation increased the average duration of nose-body contact in adults and adolescents (**D**). Isolation increased play behavior in adolescents (**E**). Isolation did not impact chase (**F**). Error bars represent the SEM; * $p < 0.05$, *** $p < 0.0005$, *post hoc* after two-way RM-ANOVA.

Open field social interaction test

All rats were individually placed into a black opaque Plexiglas open field apparatus (100×100 cm) for acclimation the day before social interaction and/or isolation manipulations for 5 min. Open field behavior and social interaction were measured in a dimly lit room (10–15 lux white light and dim red light). During the following 2 d, rats were placed into the apparatus with a novel same-sex age-matched Sprague Dawley stimulus rat and were allowed to freely interact for 5 min. Groups that were isolated were placed into a transparent Plexiglas transport cage (length: 28.58 cm, width: 17.78 cm, height: 20.32 cm) containing bedding and were at least 12 inches from all other cages in the animal colony during the dark cycle for 2 h. Social interactions with and without isolation were counterbalanced. For DREADD experiments, groups received an intraperitoneal infusion of saline 2 h and 40 min before social interaction or CNO (1 mg/kg of body weight) 40 min before isolation. All interactions were video recorded and captured with AnyMaze software (Stoelting). Videos were then uploaded into CowLog software (3.0.2; Hänninen and Pastell, 2009) and scored for nose-body contact, play, and chase behaviors by a rater blind to condition. Data were exported in a CSV file and then transferred to an excel file where the sum and average duration of each interaction were calculated.

Social preference apparatus and test

For fiber photometry experiments, ifenprodil dose was chosen based on prior work demonstrating reductions in social interaction in adults but not adolescents to study the emergence of developmental differences with GluN2B antagonism (Morales and Spear, 2014). The social preference apparatus was 62.23×31.75 cm with opaque black Plexiglas flooring and outer walls, and clear transparent internal walls in a dimly lit room (10–15 lux white light and dim red light). Apparatus acclimation and counterbalancing of isolation-interaction or interaction were identical to open field procedures. Exceptions were that the social preference test lasted 15 min and novel partners and objects were placed under a wire cage (27.94×8.89 cm) at either side of the apparatus. The side in which the partner or object was placed was counterbalanced between

rats and between days. AnyMaze software was used to track the head of the rat throughout the entire session and segments of the social preference apparatus were divided into an investigation region where rats were allowed to directly interact through a cage, and a proximity region (4.5 inches from the cage) for each partner or object side (Fig. 8A). The time spent with the head of the rat at the social or non-social cage was considered investigation. Bins (1 s, 900 s total) of the data were exported into a CSV file for later MATLAB analysis.

Fiber photometry recordings and analysis

To capture calcium transients during social interaction test, data analysis and procedures were similar to Lerner et al. (2015). Here, a 465-nm (sinusoidal modulation at 211 Hz) and 405-nm LED (sinusoidal modulation at 531 Hz) were emitted through a fiber optic patch cord (Doric Lenses). The patch cord was then secured to the fiber optic implant on the rat skull. Synapse software (Tucker Davis Technologies), was used to control LEDs during behavior. Simultaneously, AnyMaze software was used to record rat behavior. Fiber photometry data were collected (RZ10X system amplifier and processor, Tucker Davis Technologies) and exported into MATLAB for analysis. Similar to Lerner et al. (2015), a least-squares linear fit was used to align 405- to 465-nm signals. A $\Delta F/F$ signal was calculated using the fitted 405-nm signal and normalizing this to the 465-nm signal. $\Delta F/F$ values were then converted to a z score using the mean and standard deviation (SD) from the entire session each day (bin $\Delta F/F$ value subtracted from the mean $\Delta F/F$ divided by the $\Delta F/F$ SD; Cai et al., 2020; Sathyanesan et al., 2021). The time of each $\Delta F/F$ z score value was matched to the time in which the rat head was at the cage (investigation) and in the proximity of the cage and were then averaged. A calcium transient event was defined as a change at least three SDs above the $\Delta F/F$ z score mean of the entire behavioral session using the find peaks function in MATLAB. The proportion of events in investigation and proximal regions was then quantified.

Synaptosomal fractionation

Animals were deeply anesthetized with isoflurane immediately following isolation or control. Brains were immediately removed, flash frozen with

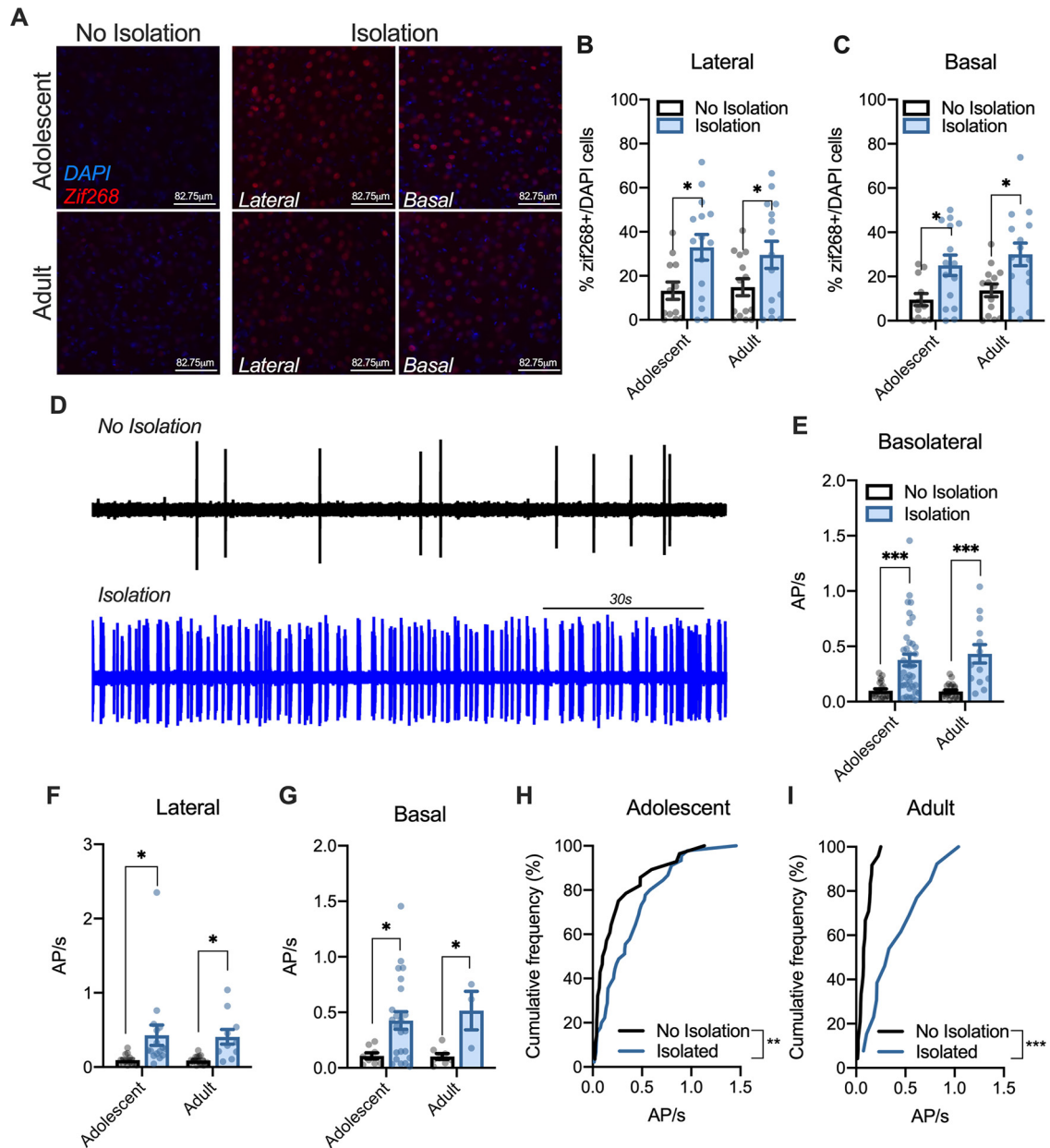


Figure 2. Brief isolation increases BLA activity in adults and adolescents. Zif268 expression was quantified and normalized as a proportion of total DAPI staining in the sampled region in non-isolated adults (subject $n = 5$, slice $n = 14$) and adolescents (subject $n = 4$, slice $n = 12$) and were compared with isolated adults (subject $n = 5$, slice $n = 15$) and adolescents (subject $n = 4$, slice $n = 15$). Left, zif268 and DAPI expression in an example basal amygdala image of no isolation adolescent (top) and adult (bottom) groups. Right, Expression in isolated adolescent (top) and adult (bottom) groups (**A**). Zif268 expression was increased following isolation in adolescents and adults in the lateral and basal amygdala (**B**, **C**). *In vivo* recording of adolescent BLA action potentials in no isolation (top) and isolation (bottom) groups (**D**). Isolation increased firing frequency in adolescents (no isolation: subject $n = 6$, neuron $n = 28$; isolation: subject $n = 11$, neuron $n = 44$) and adults (no isolation: subject $n = 9$, neuron $n = 24$; isolation: subject $n = 6$, neuron $n = 13$; **E**). When divided into lateral and basal amygdala divisions, isolation significantly increased firing frequency in the lateral (no isolation: adolescent neuron $n = 14$, adult neuron $n = 16$; isolation adolescent neuron $n = 16$, adult neuron $n = 10$; **F**) and basal (no isolation: adolescent neuron $n = 8$, adult neuron $n = 8$; isolation adolescent neuron $n = 23$, adult neuron $n = 3$; **G**). The BLA distribution of responses was different (F test) between no isolation and isolation adolescent (**H**) and adult (**I**) groups. Error bars represent the SEM; * $p < 0.05$, ** $p < 0.003$, *** $p < 0.0001$, *post hoc* after two-way ANOVA, unless otherwise indicated.

dry ice, and stored at -80°C until dissected. Crude synaptosomal fractions were obtained as previously described (Jarome et al., 2011; Ferrara et al., 2017). Amygdalae were dissected out and homogenized in TEVP buffer with 320 mM sucrose and then centrifuged at $1000 \times g$ for 10 min. The supernatant was removed and centrifuged at $10,000 \times g$ for 10 min, and the remaining pellet was denatured in lysis buffer (all in 100-ml DDH₂O; 0.605 g Tris-HCl, 0.25 g sodium deoxycholate, 0.876 g NaCl, 1 $\mu\text{g/ml}$ PMSF, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, and 10 ml 10% SDS). Protein levels were measured with a Bradford-based protein assay (catalog #500-0006, Bio-Rad Laboratories).

Western blotting

Groups were killed immediately after a 2-h isolation or from their home-cage. After tissue fractionation, protein levels were normalized and loaded onto an SDS-PAGE gel and then transferred to a membrane using a transfer apparatus (Bio-Rad). Membranes were incubated in blocking buffer for 1 h before overnight incubation in primary solution at 4°C that contained antibodies for GluN2A (1:500, #4205, Cell Signaling Technology), GluN2B (Cell Signaling Technology, 1:1000, #D8E10), and β -actin (Cell Signaling Technology, 1:1000, #8H10D10). Membranes were then incubated in the appropriate secondary antibody

(goat anti-rabbit #sc-2004, goat anti-mouse #sc-2005; 1:20,000; Santa Cruz Biotechnology) for 1 h and prepped in a chemiluminescence (West Dura, Thermo Scientific) solution for 3 min. Protein bands were detected on film (Kodak) and densitometry was performed using ImageJ software (NIH). Full-length membranes can be found in Extended Data Fig. 6-1.

Immunofluorescence

Groups were deeply anesthetized following isolation manipulation or home cage and perfused with 0.1 M PBS followed by 4% PFA. Brains were sliced on a vibratome in 40- μ m sections and mounted onto gelatinized slides. Slides were rehydrated in wash buffer (PBS + 0.05% Tween 20), endogenous peroxidase activity was blocked (PBS + 0.3% H₂O₂), and slices were permeabilized (PBS + 0.03% Triton X-100). Slices were then incubated in blocking solution for 1 h (PBS + 0.7% NGS), and then anti-EGR1 (Cell Signaling, 1:500, # 15F7) overnight at 4°C. Slices were then incubated in a secondary solution (1:500, Alexa Fluor 594, Invitrogen) for 2 h, rinsed with wash buffer, and coverslipped with a DAPI counterstain.

Microscopy

Amygdala regions were identified based on the rat brain atlas (Paxinos and Watson, 2007). Lateral and basal amygdala regions were captured on a Nikon Eclipse E600 microscope using a 20 $\times$ objective lens. Three square sections in each region were captured and analyzed bilaterally by an individual blind to condition. Images were then exported as TIFF files and particles were quantified using ImageJ software (NIH). Particles were counted by using the subtract background function and were made binary. The watershed function was then used to separate overlapping particles and particles were counted using the “analyze particles” function with a circularity between 0.04 and 1.00 (Ferrara et al., 2019a,b).

Experimental design and statistical analyses

Statistical analyses and graphs were made using Prism 8 software (GraphPad), with the exception of effect size which was calculated in IBM SPSS. α Was set to $p = 0.05$. A two-way ANOVA with age (adult, adolescent) or isolation experience (isolation, no isolation) and drug manipulation (CNO, saline; ifenprodil, dH2O) as factors was used for analyses and is described in detail below. Fisher's LSD *post hoc* tests were used to follow-up on main effects or interactions. For electrophysiology data, outliers were detected using the ROUT method ($Q = 1\%$) and removed from all subsequent analyses. Graphed data are presented as mean with SEM.

For behavioral experiments, adult and adolescent rats underwent two social interaction tests. For Figure 1, eight adolescent rats and nine adult rats were exposed to two social interaction sessions separated by 24 h with an age-matched novel partner. To assess the impact of isolation, nine adolescents and adults were exposed to an isolation-interaction and a social interaction with an age-matched novel partner separated by 24 h (counterbalanced). Data from this experiment were analyzed using a 2 $\times$ 2 repeated-measures (RM)-ANOVA with session (interaction-interaction, isolation-interaction) and age (adolescent, adult) as factors. Chemogenetic inactivation of the BLA was used to determine if BLA activity was required for isolation-driven increases in interaction using a 2 $\times$ 2 RM-ANOVA with condition (no isolation, isolation) and virus (mCherry, Gi) as factors. Similar to our initial behavioral results, isolation increased social interaction in mCherry adults ($n = 9$) and adolescents ($n = 12$). BLA inactivation before isolation reduced this effect in both adults ($n = 11$) and adolescents ($n = 9$). In our final behavioral experiment, we used a social preference apparatus to measure the contribution of GluN2B-containing NMDA receptors to BLA activity during social investigation. Data were analyzed using a 2 $\times$ 2 RM-ANOVA with condition (no isolation, isolation) and age (adolescent, adult) as factors. Isolation increased social investigation in adolescent ($n = 6$) and modestly increased interaction in adult ($n = 6$) vehicle conditions, while GluN2B antagonism with ifenprodil reduced this effect at both ages (adolescent: $n = 7$; adult: $n = 8$).

To assess changes in BLA activity following isolation, zif268 expression was quantified in homecage (adult: subject $n = 5$, slice $n = 14$; adolescent: subject $n = 4$, slice $n = 12$) and isolation conditions (adult: subject $n = 5$, slice $n = 15$; adolescent: subject $n = 4$, slice $n = 15$) and with *in vivo* recordings in adolescents (no isolation: subject $n = 6$, neuron $n = 28$;

isolation: subject $n = 11$, neuron $n = 44$) and adults (no isolation: subject $n = 9$, neuron $n = 24$; isolation: subject $n = 6$, neuron $n = 13$). These were measured with a 2 $\times$ 2 ANOVA with age (adolescent, adult) and condition (isolation, no isolation) as factors (Fig. 2). We found that isolation increased BLA activity in both adults and adolescents. A second order polynomial best fit line was applied to cumulative histograms, and F tests were used to compare the quadratic, linear, and constant components of the polynomial model in Figure 2H,I.

Changes in GluN2B-containing NMDA receptor synaptic expression were quantified with Western blotting following isolation with a 2 $\times$ 2 ANOVA with age (adolescent, adult) and condition (isolation, no isolation) as factors. Isolation did not change adolescent BLA GluN2B synaptic expression (no isolation $n = 10$, isolation $n = 8$), but did increase expression in adults (no isolation $n = 12$, isolation $n = 11$). GluN2B antagonism with ifenprodil following isolation was then measured with a 2 $\times$ 2 RM-ANOVA with drug (vehicle, ifenprodil) and age (adolescent, adult) as factors and showed increased sensitivity to antagonism in adults relative to adolescents. A two-tailed t test was used to compare difference score values between adult and adolescent ifenprodil groups. A second order polynomial best fit line was applied to cumulative histograms, and F -tests were used to compare the quadratic, linear, and constant components of the polynomial model.

We next used fiber photometry to measure BLA activity and sensitivity to GluN2B antagonism during social investigation. This was analyzed using a 2 $\times$ 2 RM-ANOVA with condition (no isolation, isolation) and target (object, partner) as factors for adults (vehicle $n = 6$, ifenprodil $n = 8$) and adolescents (vehicle $n = 6$, ifenprodil $n = 7$). We found that isolation increased BLA activity during partner investigation in adults and adolescents, and this was reduced with ifenprodil infusion more robustly in adults relative to adolescents.

Results

Brief isolation facilitates age-specific social behaviors

Groups were first preexposed to the open field apparatus, and the time in center was analyzed with a 2 (age: adolescent, adult) $\times$ 2 (condition: stimulus rat or social interaction testing) ANOVA. There were no differences between groups (largest F value: interaction $F_{(1,43)} = 0.9969$, $p = 0.3236$; Fig. 1B). Similar to prior work (Varlinskaya et al., 1999; Varlinskaya and Spear, 2008; Pellis et al., 2010), nose-body contact, play, and chase were the most frequent social interactions and were quantified to capture age-specific social changes after brief isolation. To account for potential differences in social behavior across sessions, groups were exposed to two social interactions with no preceding manipulation (SI-SI group; adolescent $n = 8$, adult $n = 9$; Fig. 1A) or with a 2-h isolation preceding one of the social interaction sessions (Iso-SI, counterbalanced; adolescent $n = 9$, adult $n = 9$). Social interaction was analyzed with a 2 (session: day 1, day 2) $\times$ 2 (age: adolescent, adult) RM-ANOVA. Within adolescent and adult cohorts, isolation increased the time spent socially interacting (Fig. 1C; age $\times$ session two-way RM-ANOVA, interaction $F_{(3,31)} = 8.00$, $p = 0.0004$, $\eta_p^2 = 0.436$; main effect of session $F_{(3,31)} = 4.82$, $p = 0.036$, $\eta_p^2 = 0.318$; *post hoc* adolescent: $p = 0.0005$; *post hoc* adult: $p > 0.999$). In contrast, repeated social interaction sessions alone did not significantly impact time engaged in social interaction in adults or adolescents (Fig. 1B; *post hoc* adolescent: $p > 0.999$; *post hoc* adult: $p = 0.298$).

Although repeated social interaction sessions did not change overall time engaged in social behavior, when type of social behavior was examined, there was a decrease in the average duration of nose-body investigation across days in adolescents but not adults (Fig. 1D; age $\times$ session two-way RM-ANOVA, interaction $F_{(3,31)} = 12.20$, $p < 0.0001$, $\eta_p^2 = 0.541$; main effect of session $F_{(3,31)} = 5.716$, $p = 0.023$, $\eta_p^2 = 0.356$; SI-SI sessions *post hoc*: adolescent: $p = 0.047$; SI-SI sessions *post hoc* adult: $p = 0.21$). In contrast, brief isolation increased the average duration of nose-

body contact in adolescents (Fig. 1D; Iso-SI sessions *post hoc*, $p=0.0052$) and adults (Fig. 1D; Iso-SI sessions *post hoc*, $p<0.0001$). Similarly, brief social isolation increased play behavior in adolescents (Fig. 1E; age $\times$ session two-way RM-ANOVA; main effect of session $F_{(3,31)} = 6.57$, $p=0.02$, $\eta_p^2 = 0.389$; no interaction $F_{(3,31)} = 1.033$, $p=0.392$; Iso-SI sessions *post hoc* $p=0.012$) and was stable across sessions in adolescents without isolation (SI-SI *post hoc*; $p=0.258$). There was little play behavior observed in adults, nor any change in play behavior in either session in adults (SI-SI *post hoc* adult: $p=0.286$; Iso-SI *post hoc* $p=0.826$). Although chase behavior differed between adults and adolescents (age $\times$ session two-way RM-ANOVA, main effect of age $F_{(3,31)} = 3.47$, $p=0.028$, $\eta_p^2 = 0.251$; main effect of session $F_{(3,31)} = 5.47$, $p=0.026$, $\eta_p^2 = 0.346$; no interaction: $F_{(3,31)} = 0.299$, $p=0.83$), it was relatively similar across sessions in adolescents whether or not rats were exposed to brief isolation (Fig. 1F; SI-SI *post hoc* $p=0.3727$; Iso-SI *post hoc*; $p=0.605$). Chase behavior was similarly insensitive to brief isolation in adults (Fig. 1F; SI-SI *post hoc* $p=0.127$; Iso-SI *post hoc* $p=0.099$). These results demonstrate that brief social isolation facilitates social behaviors, and identifies increased investigative behaviors (e.g., nose-body contact) in adults and increased play and investigative behaviors in adolescents as measures that can reflect increased social drive.

Brief isolation increases BLA activity in adults and adolescents

To determine if a brief change to the social environment that increases social drive also impacts amygdala activity, we measured effects of brief isolation on immediate early gene *zif268* expression to assess overall cellular activity (adult $n=5$, adolescent $n=4$; three bilateral slices/group; Fig. 2A). A 2 (condition: no isolation, isolation) $\times$ 2 (age: adolescent, adult) ANOVA was used to assess changes in *zif268* expression following isolation. Brief isolation increased *zif268* expression in the lateral amygdala (Fig. 2B; age $\times$ condition two-way ANOVA, main effect of condition $F_{(1,52)} = 10.68$, $p=0.002$, $\eta_p^2 = 0.170$; no interaction $F_{(1,52)} = 0.227$, $p=0.636$) in adults (*post hoc* $p=0.049$) and adolescents (*post hoc* $p=0.012$). Similarly, isolation increased *zif268* expression in the basal amygdala (Fig. 2C; age $\times$ condition two-way ANOVA, main effect of condition $F_{(1,52)} = 14.63$, $p=0.0004$, $\eta_p^2 = 0.220$; no interaction $F_{(1,52)} = 0.009$, $p=0.92$) in adults (*post hoc* $p=0.007$) and adolescents (*post hoc* $p=0.013$). While there are likely a variety of brain regions impacted by brief isolation, our results indicate that BLA activity is increased during states promoting social behavior in adults and adolescents.

We next used extracellular electrophysiological recordings to quantify increases in BLA neuronal activity *in vivo* (Fig. 2D for example adolescent traces). A 2 (condition: no isolation, isolation) $\times$ 2 (age: adolescent, adult) ANOVA was used to assess changes in firing rate. Brief isolation increased firing frequency in the BLA in adults and adolescents (Fig. 2E; age $\times$ condition two-way ANOVA, condition main effect $F_{(1,92)} = 35.27$, $p<0.0001$, $\eta_p^2 = 0.277$; no interaction $F_{(1,92)} = 0.351$, $p=0.555$; *post hoc*: adult: $p<0.0001$, adolescent: $p<0.0001$). This increase of firing rate was seen in lateral (Fig. 2F; age $\times$ condition two-way ANOVA, condition main effect $F_{(1,51)} = 13.11$, $\eta_p^2 = 0.205$; no interaction $F_{(1,51)} = 0.007$, $p=0.932$; *post hoc*: adult: $p=0.039$; adolescent: $p=0.0174$) and basal nuclei (Fig. 2G; age $\times$ condition two-way ANOVA, condition main effect $F_{(1,38)} = 9.505$, $\eta_p^2 = 0.200$; no interaction $F_{(1,38)} = 0.157$, $p=0.694$; *post hoc*: adult: $p=0.0498$; adolescent: $p=0.0135$). To quantify and measure differences in the distribution of neuronal firing rates, a cumulative

histogram was created and groups were compared. The distribution of BLA firing rates between non-isolated and isolated cohorts was significantly different in adolescents (Fig. 2H; *F* test of quadratic component of a second order polynomial best-fit line; $F_{(1,56)} = 15.53$, $p=0.002$; linear component: $F_{(1,56)} = 8.273$, $p=0.0057$; constant: $F_{(1,56)} = 11.54$, $p=0.0013$) with a similar pattern in adults (Fig. 2I; *F* test, $F_{(1,30)} = 128.4$, $p<0.001$; linear component: $F_{(1,30)} = 281.1$, $p<0.0001$; constant: $F_{(1,30)} = 2.799$; $p=0.1047$). These results demonstrate that brief isolation shifts the distribution of neuronal firing rates and increases BLA neuronal activity in both adults and adolescents. Histologic verification of neurons recorded within the BLA can be found in Figure 3.

Effects of brief isolation on social behaviors require BLA activity

Because we found that brief isolation facilitated both social interaction and BLA activity, we next wanted to determine if this increase in neuronal activity was necessary for changes in social behavior. Isolation-driven changes in BLA activity were inhibited using an inhibitory DREADD (AAV5-CaMKII-HM4DGi-mCherry; adolescent $n=9$, adult $n=11$), and within-subject changes in BLA inhibition were assessed relative to a control DREADD condition (AAV5-CaMKII-mCherry; adolescent $n=12$, adult $n=9$; Fig. 4A). Similar to above, groups were first preexposed to the open field apparatus and time in center was analyzed with a one-way ANOVA. There were no differences in adolescent groups ($F_{(2,27)} = 0.3849$, $p=0.6842$; Fig. 4B) or adult groups ($F_{(2,26)} = 0.6781$, $p=0.5163$; Fig. 4F). The next day, groups were exposed to a baseline social interaction and a brief isolation before another social interaction (counterbalanced, 20–28 h apart). All groups were given CNO (1 mg/kg) before the isolation-social interaction and vehicle before a baseline social interaction.

A RM 2 (condition: no isolation, isolation) $\times$ 2 (DREADD: AAV-mCherry, AAV-DREADD-Gi) ANOVA was used to assess effects of social isolation on social interaction if BLA inactivation occurred before isolation. As above, brief isolation increased nose-body contact in adolescents within the mCherry condition (Fig. 4C; condition $\times$ DREADD two-way RM-ANOVA, main effect of condition $F_{(1,19)} = 11.37$, $p=0.003$, $\eta_p^2 = 0.374$; no interaction $F_{(1,19)} = 0.775$, $p=0.380$; AAV-mCherry *post hoc* $p=0.0042$). Relative to their own baseline social interaction, there were no isolation-driven increases in nose-body contact with BLA inhibition (Fig. 4C; DREADD-Gi *post hoc* $p=0.116$). As above, brief isolation also increased play behavior in adolescents (Fig. 4D; condition $\times$ DREADD two-way RM-ANOVA, main effect of condition $F_{(1,19)} = 4.679$, $p=0.0435$, $\eta_p^2 = 0.198$; no interaction $F_{(1,19)} = 3.164$, $p=0.091$; AAV-mCherry *post hoc*: $p=0.014$) when compared within the mCherry group. In the BLA inactivation group, changes in play behavior were not different between no isolation or isolation conditions (Fig. 4D; AAV-DREADD-Gi *post hoc* $p>0.999$). Chase behavior was not significantly impacted by brief isolation in adolescents (Fig. 4E; no main effect of condition: $F_{(1,19)} = 2.751$, $p=0.114$ and no interaction $F_{(1,19)} = 3.343$; $p=0.083$). In adults with mCherry virus, brief isolation increased nose-body contact (Fig. 4G; condition $\times$ DREADD two-way RM-ANOVA, main effect of condition $F_{(1,18)} = 11.92$, $p=0.003$, $\eta_p^2 = 0.398$; interaction $F_{(1,18)} = 4.549$, $p=0.047$, $\eta_p^2 = 0.202$; AAV-mCherry *post hoc*: $p=0.0028$), but not within the Gi BLA inactivation group (AAV-DREADD-Gi *post hoc* $p=0.677$). As above, play behavior was rare in adults and was not influenced by brief isolation when isolation driven

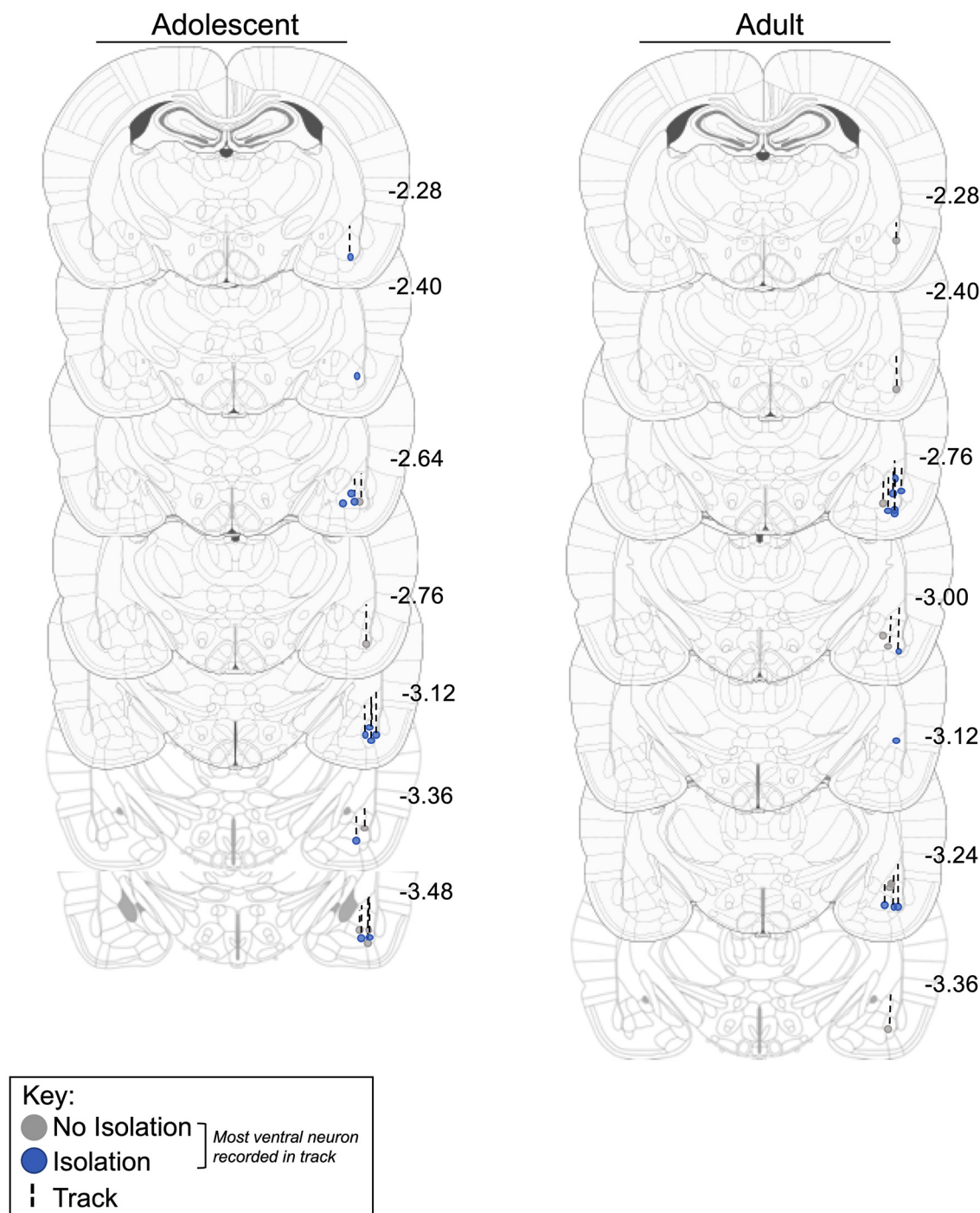


Figure 3. Histologic verification of amygdala placements for *in vivo* spontaneous activity recordings. Spontaneous activity recording placements for adults and adolescents seen in Figure 2. Circles indicate the final neuron recorded in each track.

changes in behavior were compared within the mCherry or Gi group (Fig. 4H; condition $\times$ DREADD two-way RM-ANOVA no main effect of condition: $F_{(1,18)} = 0.45$, $p = 0.511$, and no interaction $F_{(1,18)} = 0.475$, $p = 0.499$), or chase behavior in adults (Fig. 4I; condition $\times$ DREADD two-way RM-ANOVA no main effect of condition: $F_{(1,18)} = 0.117$, $p = 0.736$; and no interaction $F_{(1,18)} = 0.322$, $p = 0.577$). These results show a necessity for BLA activity in the enhancement of social interaction following brief social isolation in adults and adolescents. Histologic verification of BLA viral expression can be found in Figure 5.

GluN2B-containing NMDA receptors are required for isolation-dependent increases in BLA neuronal activity

GluN2-containing NMDA receptors play an essential role in BLA plasticity, and as such could contribute to increased BLA neuronal activity caused by brief isolation. To determine if brief isolation alters GluN2 receptors, changes in the synaptic expression of GluN2-containing NMDA receptors as a result of isolation were measured in adults and adolescents using a 2 (condition: no isolation, isolation) $\times$ 2 (age: adolescent, adult; $n = 8$ –12) ANOVA (Fig. 6A). Brief isolation did not alter the synaptic expression of GluN2A receptor subunit (Fig. 6B; age $\times$

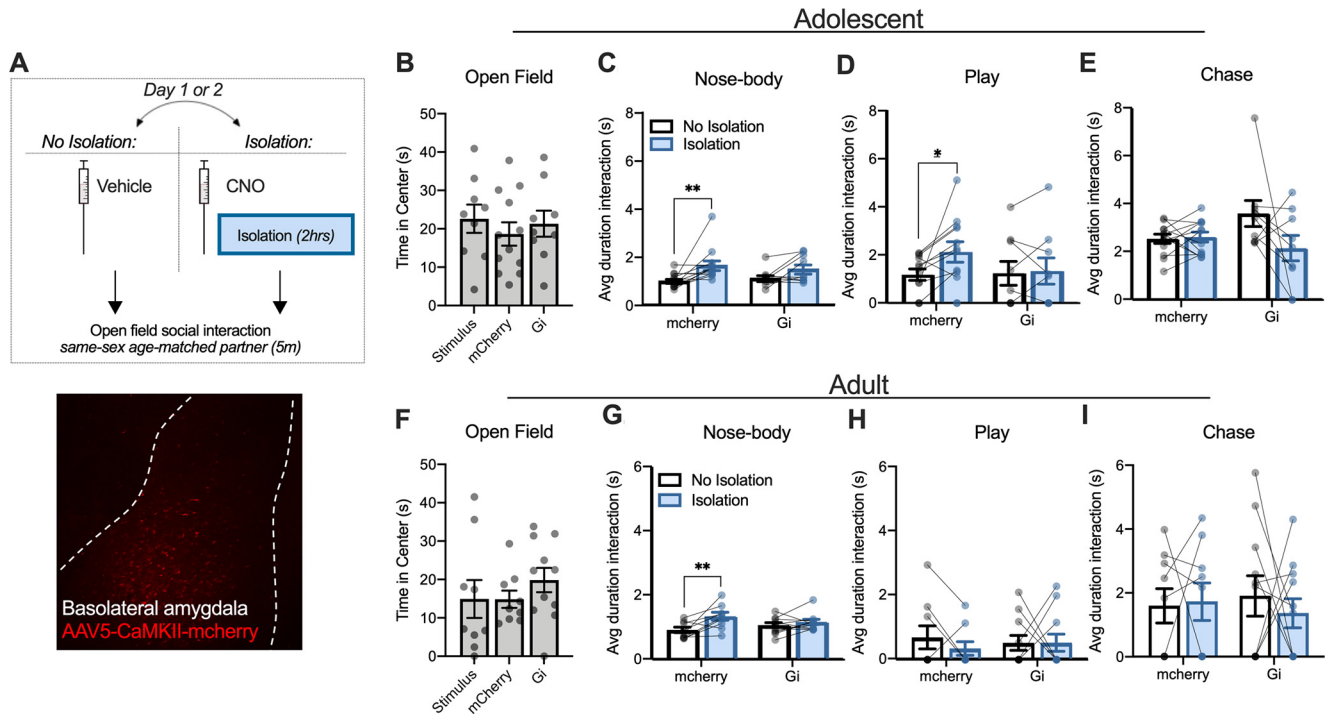


Figure 4. Effects of brief isolation on social behaviors require BLA activity. Adolescents and adults were infused with AAV5-CamKII-HM4DGI-mCherry (Gi, adolescent $n = 9$, adult $n = 11$) or AAV5-CamKII-mCherry (mCherry, adolescent $n = 12$, adult $n = 9$) into the BLA. All groups were administered CNO (1 mg/kg, i.p.) before an isolation-interaction and vehicle before a baseline social interaction, order was counterbalanced (**A**). There were no differences for the time in center between adolescent groups (**B**). Isolated adolescents engaged in longer durations of nose-body (**C**) and play (**D**) behavior, which was reduced in Gi groups. Isolation had no effect on chase (**E**). There were no differences for time in center for adult groups (**F**). In adults, isolation increased nose-body contact which was reduced in the Gi group (**G**). Play (**H**) and chase (**I**) were not impacted by isolation. Error bars represent the SEM; * $p < 0.05$, ** $p < 0.01$, *post hoc* after two-way RM-ANOVA.

condition two-way ANOVA, largest F value: no interaction $F_{(1,33)} = 0.691$, $p = 0.412$). However, brief isolation increased synaptic expression of GluN2B in adults (Fig. 6C; age $\times$ condition two-way ANOVA, main effect of condition $F_{(1,40)} = 5.605$, $p = 0.029$, $\eta_p^2 = 0.123$; no interaction $F_{(1,40)} = 0.602$, $p = 0.442$; adults *post hoc* $p = 0.028$; no isolation $n = 12$; isolation $n = 11$) but not adolescents (*post hoc* $p = 0.278$; no isolation $n = 10$; isolation $n = 11$). There were no differences in actin expression in adults or adolescents (Fig. 6D; age $\times$ condition two-way ANOVA, largest F value: no main effect of age $F_{(1,40)} = 0.4622$, $p = 0.501$). While this does not rule out alterations between adults and adolescents, it links changes in GluN2B synaptic expression to isolation within age groups.

To test whether GluN2B-containing NMDA receptors contribute to isolation-driven increase in BLA neuronal activity, adults and adolescents received an intra-BLA infusion of the GluN2B antagonist ifenprodil or vehicle into contralateral hemispheres (counterbalanced; adolescent $n = 6$, adult $n = 8$; Fig. 6E). A RM 2 (age: adolescent, adult) $\times$ 2 (drug: vehicle, ifenprodil) ANOVA was used to measure changes in firing rate in vehicle and ifenprodil conditions between age groups (example traces in Fig. 6F). Ifenprodil infusions following brief isolation reduced BLA neuronal activity in adults (Fig. 6G; age $\times$ drug two-way RM-ANOVA, main effect of drug $F_{(1,12)} = 11.55$, $p = 0.0053$, $\eta_p^2 = 0.490$; no interaction $F_{(1,12)} = 1.0$, $p = 0.337$; adult *post hoc*: $p = 0.0113$) but not adolescents. The degree of inhibition among neurons recorded following ifenprodil infusion was greater in adults than adolescents ($t_{(30)} = 2.618$, $p = 0.0137$; Fig. 6H). Ifenprodil did not significantly change the distribution of BLA neuronal firing rates in adolescents (Fig. 6I; F test of best-fit to quadratic component of second order polynomial, $F_{(1,6)} =$

0.6288, $p = 0.4587$; linear: $F_{(1,6)} = 1.8$, $p = 0.2282$; constant: $F_{(1,6)} = 0.4065$, $p = 0.5473$) but did shift the distribution in adults (Fig. 6J; F test, quadratic: $F_{(1,10)} = 44.12$, $p < 0.0001$; linear: $F_{(1,10)} = 34.34$, $p = 0.0002$; constant: $F_{(1,10)} = 0.1844$, $p = 0.6767$). Together, these results indicate that brief isolation increases GluN2B synaptic expression between no isolation and isolation adult groups. This change between adult groups may contribute to increased BLA neuronal activity and ifenprodil sensitivity along with increased social drive when compared within the adult cohort. Histologic verification of neurons recorded in the BLA can be found in Figure 7.

Adolescent BLA activity is increased during social investigation following isolation

After identifying the BLA as a critical site for increased social drive caused by brief isolation, we sought to determine the relationship between BLA activity and social motivation during development. To achieve this goal, BLA neuronal calcium transients (pAAV.Syn.GCaMP6s.WPRE.SV40) were recorded in adults and adolescents during a social preference task to directly compare social and non-social exploration, and changes produced by brief social isolation. Here, rats could freely interact with a novel object or partner under a wire cage. Social interaction was considered the time spent investigating a partner through a cage, while social preference was considered the relative time exploring novel object or partner as well as in proximal areas to cages (Fig. 8A; Moy et al., 2004). All groups were exposed to two counterbalanced social interaction tests, one control and the other preceded by 2-h isolation, to test the effects of brief isolation on BLA activity during social behavior. Brief isolation increased social preference in adolescents and modestly in

adults (Fig. 8A; main effect of condition $F_{(1,10)} = 12.83$, $p = 0.005$, $\eta_p^2 = 0.562$; no interaction $F_{(1,10)} = 0.222$, $p = 0.648$; adolescent *post hoc*: $p = 0.0168$; adult *post hoc*: $p = 0.052$), confirming the usefulness of this assay to measure sensitivity to brief social isolation. Isolated groups were given vehicle (adolescent $N = 6$, adult $n = 6$) or ifenprodil (adolescent $n = 7$, adult $n = 8$; 3 mg/kg, i.p.) to understand how GluN2B-containing NMDA receptors contribute to age differences in the effects of social isolation on BLA activity and social behavior. A RM 2 (target: object, partner) $\times$ 2 (condition: no isolation, isolation) ANOVA was used to quantify changes in investigation of and time proximal to a social partner or an object. Brief isolation increased the social investigation in adolescents (Fig. 8D; target $\times$ condition two-way RM-ANOVA, main effect of target $F_{(1,5)} = 19.89$, $p = 0.007$, $\eta_p^2 = 0.799$; no interaction $F_{(1,5)} = 6.329$, $p = 0.0535$; isolation *post hoc* $p = 0.0045$) and adults (Fig. 8B; main effect of target: $F_{(1,5)} = 24.99$, $p = 0.0041$, $\eta_p^2 = 0.833$; interaction $F_{(1,5)} = 20.43$, $p = 0.0063$, $\eta_p^2 = 0.803$; isolation *post hoc* $p = 0.0006$). The impact of isolation on social interaction was modestly reduced with ifenprodil infusion in adults (Fig. 8C; target $\times$ condition two-way RM-ANOVA, main effect of target $F_{(1,7)} = 5.955$, $p = 0.0447$, $\eta_p^2 = 0.460$; no interaction $F_{(1,7)} = 3.619$, $p = 0.099$; partner *post hoc*: $p = 0.011$) and was reduced in adolescents (Fig. 8E; main effect of target $F_{(1,6)} = 7.159$, $p = 0.0367$, $\eta_p^2 = 0.544$; no interaction $F_{(1,6)} = 0.274$, $p = 0.620$; partner *post hoc*: $p = 0.4788$).

To understand how BLA neuronal activity changes as a result of isolation during a social preference task, the average $\Delta F/F$ of BLA neuronal Ca^{2+} transients and the number of events were quantified as an indicator of neuronal activity during investigation and when in proximity to cages containing a novel object or partner using fiber photometry (Fig. 9A for example trace and Fig. 9H for viral expression). Sensitivity to brief isolation could be reflected in increased neuronal activity during social investigation and/or in areas proximal to social interaction sites. A RM 2 (condition: no isolation, isolation) $\times$ 2 (target: object, partner) ANOVA was used to measure changes in BLA activity during the social preference task. In adolescents, baseline BLA neuronal activity was similar during object or partner interactions (vehicle control, Fig. 9C; target $\times$ condition two-way RM-ANOVA, interaction $F_{(1,5)} = 8.279$, $p = 0.0347$, $\eta_p^2 = 0.624$; no main effect of target $F_{(1,5)} = 0.501$, $p = 0.511$; target *post hoc*: $p = 0.413$; $\Delta F/F$ while in center for no isolation: $M = 0.066$). However, brief isolation increased BLA neuronal activity during partner relative to object interactions (Fig. 9C; target *post hoc*: $p = 0.029$; $\Delta F/F$ while in center for isolation: $M = 0.084$). Likewise, BLA neuronal activity at baseline was similar when proximal to object or partner (Fig. 9D; target $\times$ condition two-way RM-

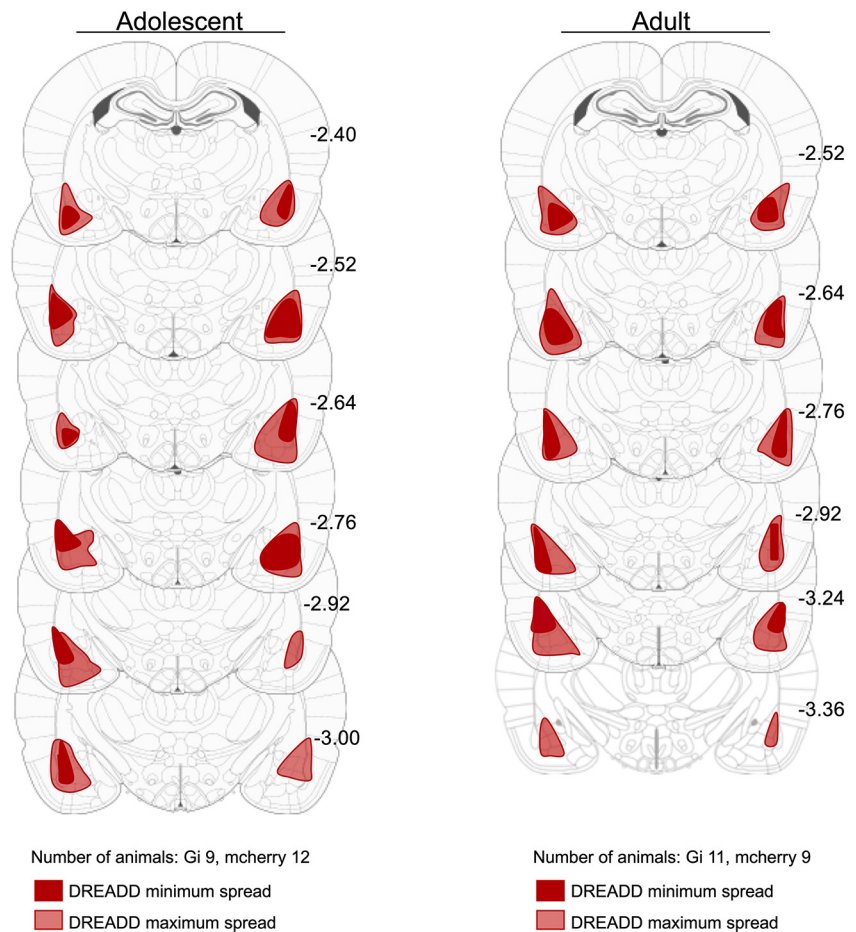


Figure 5. Viral placement and spread for BLA DREADD manipulations. Spread of DREADD virus in adult and adolescent groups for behavior seen in Figure 3. Numbers listed indicate number of animals in each group. Gi = AAV5-CAMKII-DREADD-Gi-mCherry; mCherry = AAV5-CAMKII-mCherry.

ANOVA, target main effect $F_{(1,5)} = 17.44$, $p = 0.0087$, $\eta_p^2 = 0.777$; no interaction $F_{(1,5)} = 5.502$, $p = 0.066$; target *post hoc*: $p = 0.382$), but brief isolation increased BLA activity when proximal to partner relative to object (Fig. 9D; target *post hoc*: $p = 0.0196$). To understand the contribution of the GluN2B subunit in the regulation of isolation-dependent changes in BLA neuronal activity during social behavior, a separate cohort of adolescents were treated identically as above with the exception that this group received ifenprodil instead of vehicle before behavior. Similar to vehicle control, adolescents show similar BLA neuronal activity whether interacting with object or partner (Fig. 9F; investigation target $\times$ condition two-way RM-ANOVA, target main effect $F_{(1,6)} = 6.546$, $p = 0.043$, $\eta_p^2 = 0.523$; no interaction $F_{(1,6)} = 0.786$, $p = 0.410$; target *post hoc*: $p = 0.25$; $\Delta F/F$ while in Center for no isolation: $M = 0.055$). Furthermore, even in the presence of ifenprodil, brief isolation still increased BLA neuronal activity during investigation of a partner relative to object (Fig. 9F; investigation, target *post hoc*: $p = 0.02$; $\Delta F/F$ while in center for isolation: $M = -0.259$). However, in the presence of ifenprodil, there were no BLA activity differences in proximal exploration between partner and object (Fig. 9G; target $\times$ condition two-way RM-ANOVA, largest F value: interaction $F_{(1,6)} = 5.401$, $p = 0.059$). This indicates that increased social drive state can increase BLA sensitivity preferentially for social engagement in adolescents, and although this BLA sensitivity during social investigation is not mediated by GluN2B, subtle increases in BLA sensitivity to social proximity is sensitive to GluN2B antagonism.

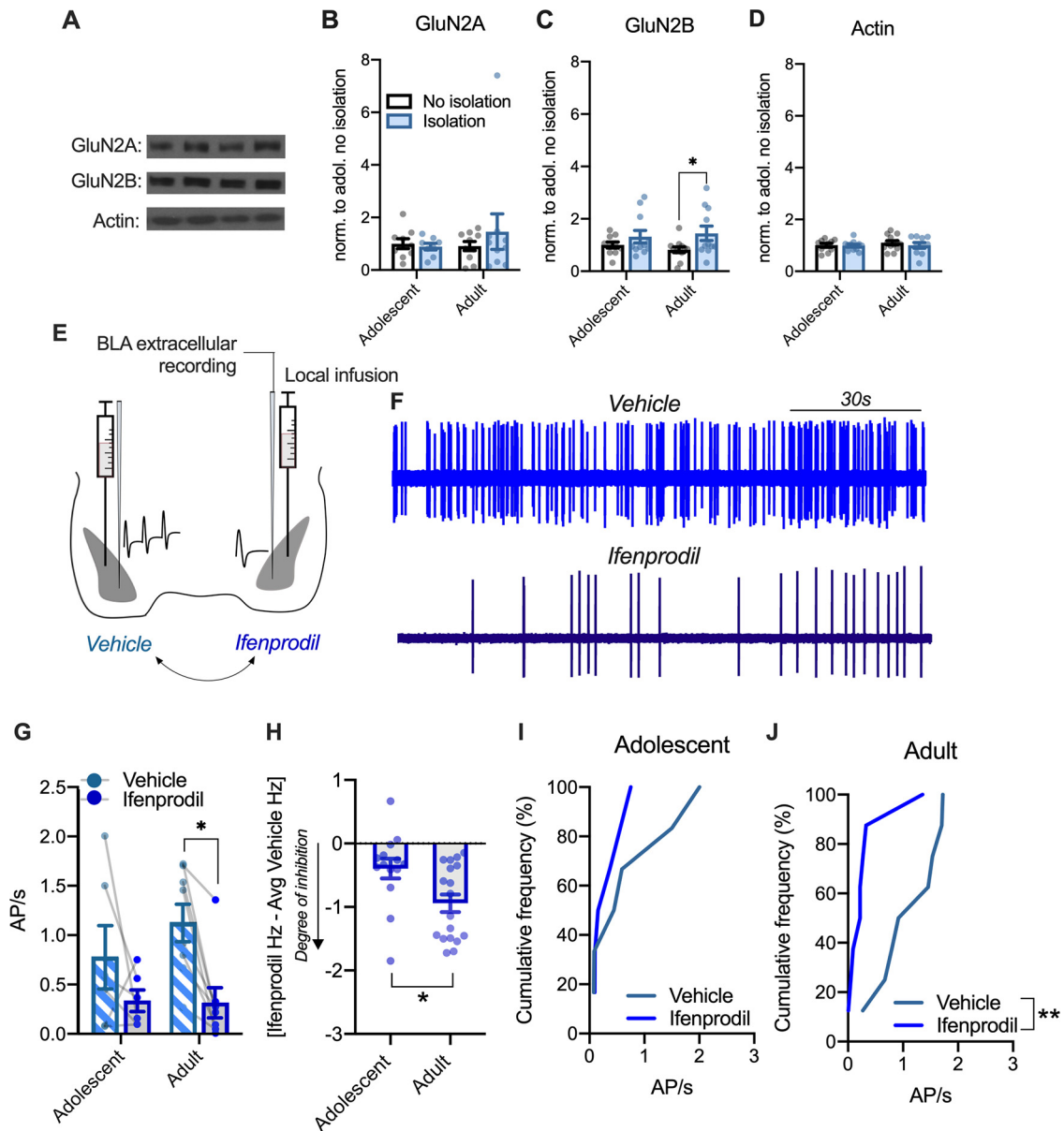


Figure 6. GluN2B-containing NMDA receptors are required for isolation-dependent increases in BLA neuronal activity. Amygdalae from adults and adolescents were dissected in isolated and non-isolated groups. Expression of NMDA receptor subunits GluN2A (no isolation: adolescent $n = 9$, adult $n = 10$; isolation: adolescent $n = 8$, adult $n = 10$) and GluN2B (no isolation: adolescent $n = 10$, adult $n = 12$; isolation: adolescent $n = 11$, adult $n = 11$) were quantified in crude synaptosomal fractions and normalized to actin (**A**; full-length membranes can be found in Extended Data Figure 6-1). These normalized NMDA subunit receptor values were then normalized to look at the degree of change from the adolescent no isolation condition. GluN2A expression did not change in adults or adolescents as a result of isolation (**B**). Isolation increased GluN2B expression in adults (**C**). There were no differences in actin expression (**D**). Isolated groups were infused with vehicle into one hemisphere and ifenprodil in the opposing hemisphere (adolescent $n = 6$, adult $n = 8$; counterbalanced) and spontaneously firing BLA neurons were recorded (**E**). Example trace of a BLA neuron recorded following vehicle infusion (top) and ifenprodil infusion (bottom; **F**). Ifenprodil infusions reduced firing frequency in isolated adults and adolescents (averaged for each rat shown here; two-way RM-ANOVA; **G**). Using a change score, the degree of inhibition was greater in adults compared with adolescents following ifenprodil infusion (adolescent cells $n = 14$, adult cells $n = 18$; unpaired t test; **H**). The distribution of responses following ifenprodil infusion was similar in isolated adolescents (**I**) but was different in adults (**J**; F test). Error bars represent the SEM; * $p < 0.05$, ** $p < 0.0001$, *post hoc* after two-way ANOVA unless otherwise noted.

To directly compare sensitivity to ifenprodil between groups, we examined the difference score [partner $\Delta F/F$ – object $\Delta F/F$] in investigation and proximity regions as a measure of BLA activity preference for social or object targets. BLA activity increased during social investigation after social isolation (Fig. 9*I*; drug $\times$ condition two-way RM-ANOVA, main effect of condition $F_{(1,11)} = 4.839$, $p = 0.0501$, $\eta_p^2 = 0.306$; no interaction $F_{(1,11)} = 1.989$, $p = 0.186$), but this was similar across drug and vehicle groups (no main effect of drug or *post hoc* differences). However, BLA activity in social proximity increased after social isolation, and this was sensitive to ifenprodil (Fig. 9*J*; drug $\times$ condition two-way RM-ANOVA, main

effect of drug $F_{(1,11)} = 5.308$, $p = 0.042$, $\eta_p^2 = 0.326$; no interaction $F_{(1,11)} = 0.128$, $p = 0.728$), although effects of ifenprodil were not significantly different in groups that were not isolated (drug *post hoc*: $p = 0.354$) or that were isolated (drug *post hoc*: $p = 0.136$). This suggests that minor GluN2B sensitivity is a feature of adolescent BLA activity when in proximity to a conspecific after isolation, but not when actively socially interacting.

In addition to an averaged $\Delta F/F$, we also quantified individual Ca^{2+} transients (events >3 SD from average $\Delta F/F$) during social interaction (Gunaydin et al., 2014). The percentage of events in proximal and investigation areas of the object and partner targets

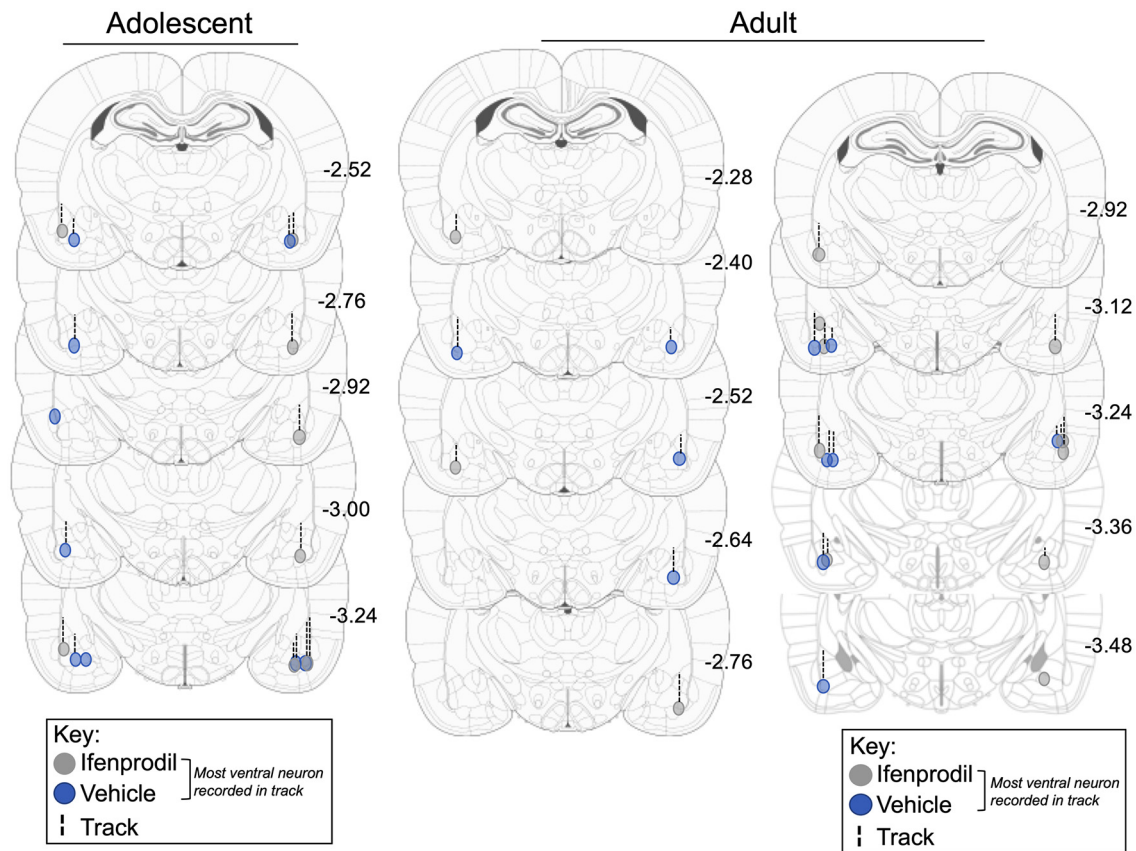


Figure 7. Histologic verification of amygdala placements following ifenprodil infusion and *in vivo* recordings. Spontaneous activity recording placements for adults and adolescents after ifenprodil (gray) or vehicle (blue) infusion seen in Figure 4. Circles indicate the final neuron recorded in each track.

relative to the total number of events for each animal during the behavioral session was compared across groups. This was compared during the social preference session using a RM 2 (location: proximity, investigation) $\times$ 2 (target: object, partner) ANOVA. In the vehicle baseline condition, there were no differences between object and partner events (Fig. 9B, left; target $\times$ location two-way RM-ANOVA, largest F value: location $F_{(1,5)} = 3.500$, $p = 0.120$), but an increase following brief isolation (Fig. 9B, right; target $\times$ location two-way RM-ANOVA, interaction $F_{(1,5)} = 15.34$, $p = 0.0112$, $\eta_p^2 = 0.754$; main effect of target $F_{(1,5)} = 56.63$, $p = 0.007$, $\eta_p^2 = 0.919$; main effect of location $F_{(1,5)} = 10.94$, $p = 0.021$, $\eta_p^2 = 0.686$) in proximal partner (target *post hoc*: $p = 0.012$) and investigation areas (target *post hoc*: $p = 0.002$) relative to object areas. In the absence of isolation in the ifenprodil condition, there were baseline differences in object and partner events (Fig. 9E, left; target $\times$ location two-way RM-ANOVA, main effect or target $F_{(1,6)} = 6.638$, $p = 0.042$, $\eta_p^2 = 0.525$; no interaction $F_{(1,6)} = 3.121$, $p = 0.128$) in investigation areas (target *post hoc*: $p = 0.019$) but not proximal areas (target *post hoc*: $p = 0.185$). In the presence of ifenprodil, there was still a greater proportion of events after isolation during social investigation (Fig. 9E, right; target $\times$ location two-way RM-ANOVA, interaction $F_{(1,6)} = 10.55$, $p = 0.018$, $\eta_p^2 = 0.638$; main effect of target $F_{(1,6)} = 10.04$, $p = 0.019$, $\eta_p^2 = 0.626$; main effect of location $F_{(1,6)} = 13.25$, $p = 0.011$, $\eta_p^2 = 0.688$; target *post hoc*: $p = 0.0121$) but not when in proximal regions (target *post hoc*: $p = 0.12$). Together, these results demonstrate that social isolation increases adolescent BLA activity during social investigation, and when near social investigation sites, and systemic ifenprodil does not interfere with this effect, but may subtly reduce BLA activity when near, but not engaged with a social partner.

GluN2B-containing NMDA receptors mediate increased BLA activity during social investigation in adults

Using the same approach described in adolescents, adult BLA activity was measured during a social preference task. A RM 2 (condition: no isolation, isolation) $\times$ 2 (target: object, partner) ANOVA was used to measure changes in BLA activity. In the vehicle group, BLA neuronal activity was different between object and partner investigation (Fig. 10A; investigation target $\times$ condition two-way RM-ANOVA, interaction $F_{(1,5)} = 8.531$, $p = 0.033$, $\eta_p^2 = 0.631$; main effect of target $F_{(1,5)} = 20.09$, $p = 0.007$, $\eta_p^2 = 0.801$; $\Delta F/F$ while in center for no isolation: $M = -0.286$), where BLA activity was elevated for partner compared with object (target *post hoc*: $p = 0.008$). Similarly, after isolation, BLA activity was increased during partner investigation compared with object (Fig. 10A; target *post hoc*: $p = 0.004$; $\Delta F/F$ while in center for no isolation: $M = 0.041$). Unlike adolescents, there were no significant differences in BLA activity in the proximal regions of an object or partner in the vehicle group (Fig. 10B; target $\times$ condition two-way RM-ANOVA, largest F value: main effect target $F_{(1,5)} = 3.014$, $p = 0.143$). Baseline BLA activity during partner interaction was replicated without ifenprodil (Fig. 10C; target $\times$ condition two-way RM-ANOVA, interaction $F_{(1,7)} = 5.819$, $p = 0.046$, $\eta_p^2 = 0.454$; no main effect target $F_{(1,7)} = 5.353$, $p = 0.054$; target *post hoc*: $p = 0.008$; $\Delta F/F$ while in center for no isolation: $M = -0.153$). However, ifenprodil reduced the effect of brief isolation on BLA activity during partner interaction (Fig. 10C; *post hoc*: $p = 0.629$; $\Delta F/F$ while in center for isolation: $M = 0.215$). While there was a main effect of condition in proximal regions ($F_{(1,7)} = 16.24$, $p = 0.005$, $\eta_p^2 = 0.699$; no interaction $F_{(1,7)} = 1.666$, $p = 0.238$; Fig. 10D), there were no differences in

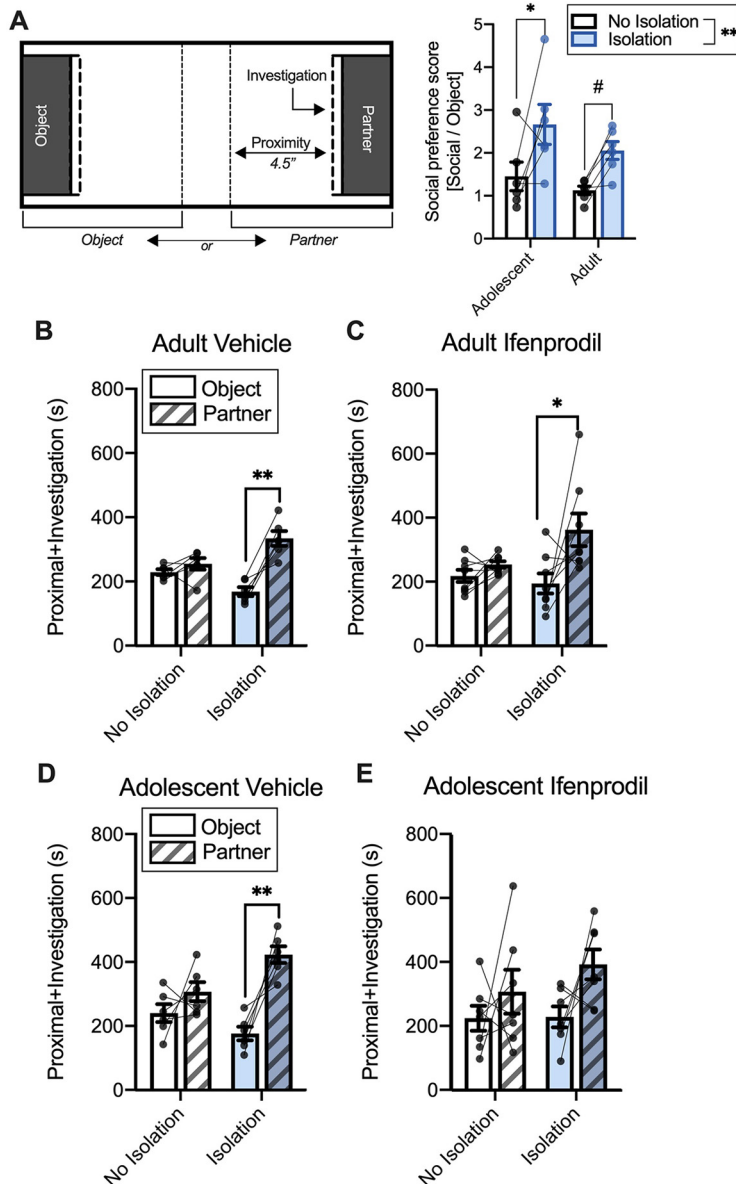


Figure 8. Brief isolation increases partner preference and is reduced with ifenprodil infusion. For fiber photometry experiments, behavior during the 15-min social preference test was measured. Time spent investigating and in the proximal region for an object or partner (indicated with O and P, respectively) was quantified and a change score was created (time spent in social/object area). Isolation significantly increased social preference in adolescents and modestly in adults (A). Adults spent more time in a social area following isolation when infused with vehicle (B) or ifenprodil (C). Adolescents in the vehicle condition spend more time in a partner area after isolation (D), and this was reduced with ifenprodil infusion (E). Error bars represent the SEM; ** $p < 0.01$, * $p < 0.05$, # $p = 0.052$.

BLA activity in the absence of isolation (target *post hoc*: $p = 0.103$) or following isolation (target *post hoc*: $p = 0.960$). Further, vehicle and ifenprodil groups were significantly different when investigating using a difference score ([partner $\Delta F/F$ – object $\Delta F/F$]; Fig. 10G; drug $\times$ condition two-way RM-ANOVA, interaction $F_{(1,12)} = 12.04$, $p = 0.0046$, $\eta_p^2 = 0.501$, largest main effect F : drug $F_{(1,12)} = 0.770$, $p = 0.398$), where BLA activity was significantly lower in the ifenprodil group after isolation (Fig. 10G; $p = 0.015$), indicating that ifenprodil infusion reduces socially driven BLA activity. At proximal regions, there were no differences between groups using a difference score (Fig. 10H; largest F value: no interaction drug $\times$ condition two-way RM-ANOVA $F_{(1,12)} = 3.856$, $p = 0.073$).

The effects of brief isolation on the distribution of Ca^{2+} transients during social and non-social exploration was compared using a RM 2 (location: proximity, investigation) $\times$ 2 (target: object, partner) ANOVA. There was no difference at baseline for the relative distribution of Ca^{2+} transients between object and partner proximal areas (vehicle, Fig. 10E, left; target $\times$ location two-way RM-ANOVA, largest F value: no main effect of location $F_{(1,5)} = 0.176$, $p = 0.692$). However, brief isolation shifted the distribution of events (vehicle, Fig. 10E, right; target $\times$ location two-way RM-ANOVA, main effect of target $F_{(1,5)} = 11.12$, $p = 0.021$, $\eta_p^2 = 0.690$; main effect of location $F_{(1,5)} = 7.662$, $p = 0.040$, $\eta_p^2 = 0.605$; no interaction $F_{(1,5)} = 1.408$, $p = 0.289$), with preferential occurrence of Ca^{2+} transients during partner exploration and when in proximity compared with object following isolation (Fig. 10E, right; target *post hoc* investigation: $p = 0.036$, target *post hoc* proximity: $p = 0.04$). When ifenprodil was administered, there was no baseline difference between the proportion of events in social or nonsocial proximal areas (Fig. 10F, left; target $\times$ location two-way RM-ANOVA, largest F value: main effect of target $F_{(1,7)} = 4.244$, $p = 0.078$). However, ifenprodil blocked the effects of brief isolation, and isolation no longer caused a shift toward preferential BLA activity when investigating or near a partner (Fig. 10F, left; target $\times$ location two-way RM-ANOVA, largest F value: main effect of target $F_{(1,7)} = 2.504$, $p = 0.158$). This indicates that increased social drive state enhances adult BLA sensitivity to social investigation in a manner that requires GluN2B. Viral spread for both adults and adolescent cohorts can be found in Figure 11.

Discussion

We investigated developmental differences in social behavior and BLA activity, and its sensitivity to a brief isolation that increases social drive. We found that brief isolation facilitated age-specific social interaction as well as BLA neuronal activity. There was an increased ability for GluN2B antagonism to dampen isolation-driven BLA neuronal activity in adults relative to adolescents, associated with increased synaptic expression of GluN2B between adult isolation and no isolation conditions. Further, adult BLA activity during a social preference task was increased during partner investigation, and this was reduced with GluN2B antagonism following isolation. In contrast, ifenprodil had little effect after brief isolation on adolescent BLA activity during social investigation. These results demonstrate that the BLA is sensitive to and critical for enhancing social drive, but the role of GluN2B in social behavior changes from adolescence to adulthood.

Long-term social isolation (more than four weeks) can increase social aggression, and when isolated during adolescence,

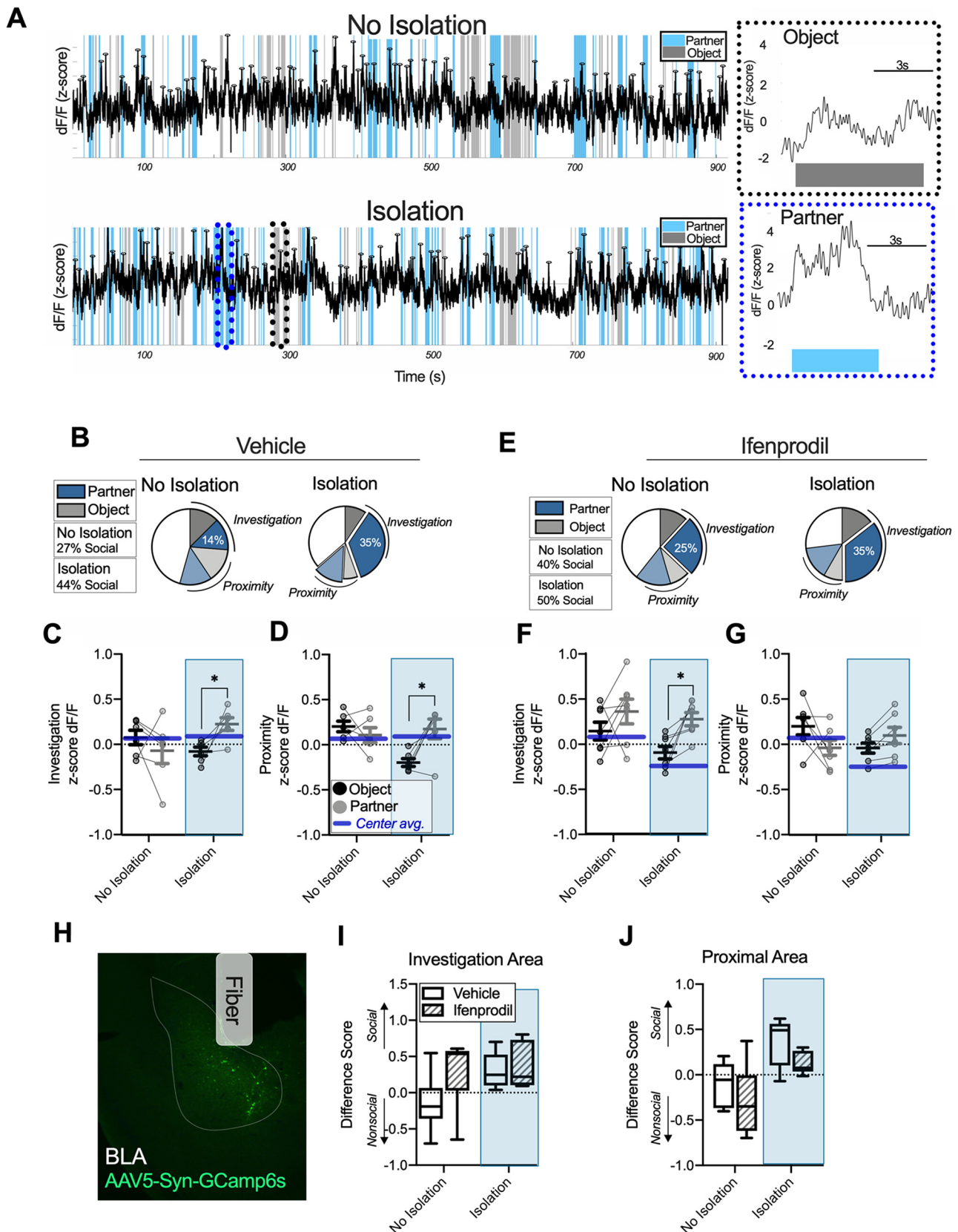


Figure 9. Adolescent BLA activity is increased during social investigation following isolation. All adolescents and adults were randomly assigned to a vehicle or ifenprodil group and were exposed to two social preference tests. In one test, groups explored a novel object or partner after vehicle infusion. The other test was preceded by a 2-h isolation where groups received a vehicle or ifenprodil administration (3 mg/kg, i.p.). Test order was counterbalanced. Example fiber photometry traces (z-scored dF/F) during a social preference task in an animal that was not isolated (top) and isolated (bottom), where blue marks indicate partner investigation and gray indicates object investigation (A). Expanded timeframe of dF/F during object or partner investigation measured after brief isolation. In vehicle infused groups ($n = 6$), there were no differences in the proportion of events (peaks three SDs above the mean) that occurred during investigation or proximal regions; however, the proportion of events in social compartments (investigation and proximal regions) significantly increased following isolation when compared

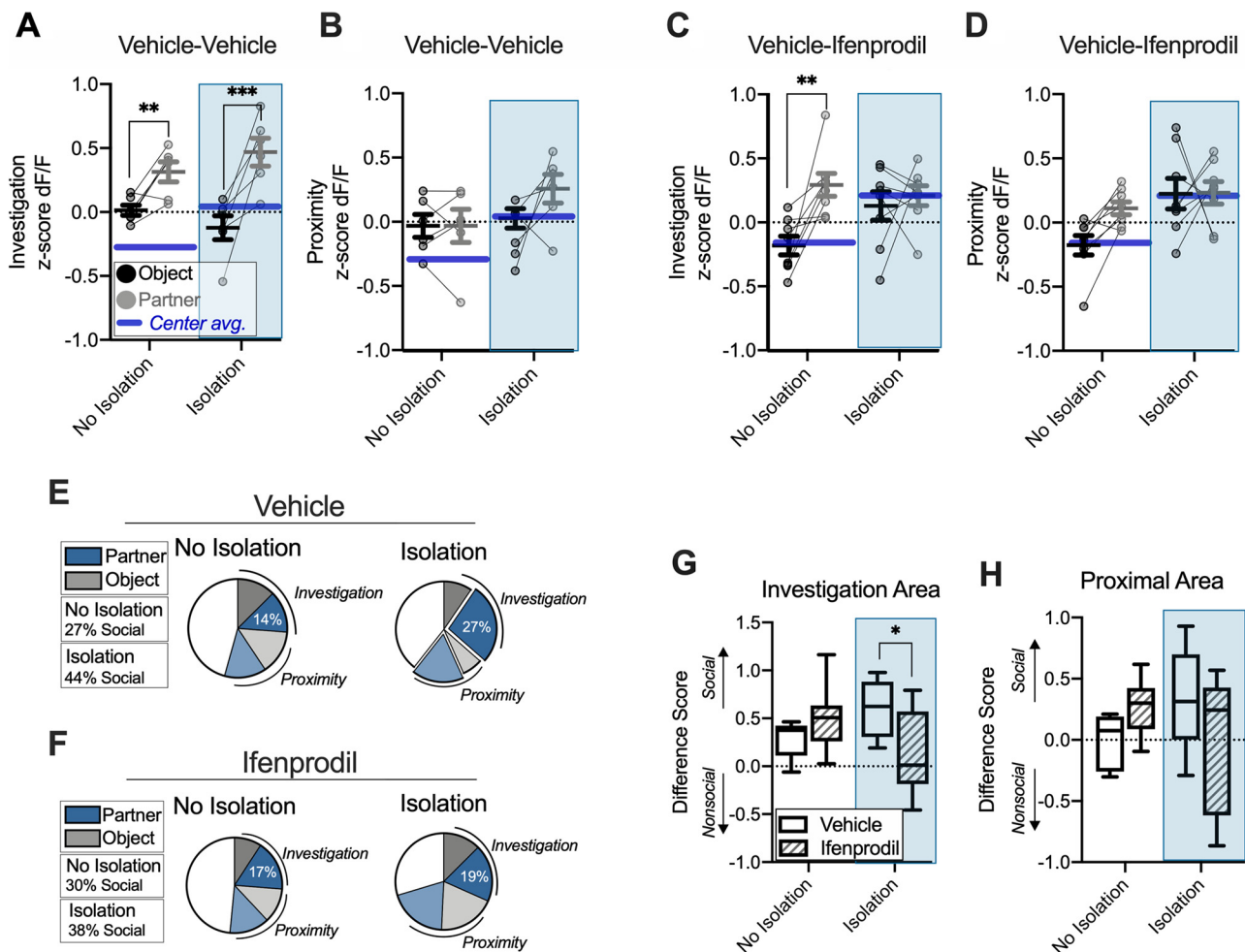


Figure 10. GluN2B-containing NMDA receptors mediate increased BLA activity during social investigation in adults. In isolated and non-isolated conditions for adults, BLA activity was increased in partner investigation regions compared with object (A), but there was no difference in proximal regions (B). In the ifenprodil group ($n = 8$), BLA activity was higher in partner investigation sites compared with an object, but the effect of isolation on BLA activity during social investigation was mitigated (C). Consistent with the vehicle group, there were no differences in BLA activity in proximal regions (D). In the vehicle adult group ($n = 6$), there were no differences in events (peaks three SDs above the mean) that occurred in investigation or proximal regions, while isolation increased the proportion of events in partner investigation and proximal regions when compared with object (E). In the ifenprodil group, there were no differences in events in proximity or investigation areas and isolation no longer had an effect (F). While ifenprodil had no significant effect on BLA activity preference for social investigation, ifenprodil decreased the effect of isolation on this BLA activity preference (G) but had no effect on BLA activity when in proximal areas (H). Error bars represent the SEM. Horizontal blue lines indicate the average dF/F while in the center; ** $p < 0.01$, *** $p < 0.001$, *post hoc* two-way RM-ANOVA.

can impair cognitive flexibility and social learning (Matsumoto et al., 2005; Yusufshaq and Rosenkranz, 2013; Amitai et al., 2014). This long-term isolation during a critical developmental period has been linked to symptomology of neurodevelopmental disorders (Amitai et al., 2014; Matsumoto et al., 2019). Brief isolation can increase social behavior, believed to be regulated

through a drive or homeostatic mechanism (Hol et al., 1999; Matthews and Tye, 2019). Although the same manipulation at different durations results in an opposing behavioral effect, the neural circuitry impacted may be similar and provide insight for neurodevelopmental disorder symptomology that emerges during adolescence. In this case, brief or extended isolation impacting neural circuitry critical for social function (including but not limited to several amygdala subregions, the prefrontal cortex, and the nucleus accumbens) may strengthen and/or weaken a social neural network promoting (with brief isolation) or impairing (with long-term isolation) social behavior (Manduca et al., 2016; Franklin et al., 2017; Selimbeyoglu et al., 2017; Twining et al., 2017; Kopec et al., 2018; Fustiñana et al., 2021). Social isolation may impact discrete BLA neural ensembles connected to brain regions implicated in social avoidance or approach to promote these behaviors (Rogers-Carter et al., 2018; Vasconcelos et al., 2019; Fustiñana et al., 2021). Many stressors are known to engage BLA activity but few facilitate social investigation, indicating that isolation-driven facilitation of social interaction may be attributed to the shift in

with object (B). Similarly, BLA dF/F was not different between an object and partner during investigation or when in proximal regions but did increase toward a partner when compared with an object following isolation (C) and when in proximity to partner compared with object (D). In the ifenprodil group ($n = 7$), the proportion of events was significantly higher in partner investigation sites compared with object in the presence and absence of isolation (E). After ifenprodil, BLA dF/F activity was similar to vehicle during investigation, and similarly increased by isolation (F). However, effects of isolation on BLA dF/F activity while in proximal areas was no longer seen after ifenprodil (G). Example image of GCamp6s virus expression in the BLA (H). A difference score was assessed [partner z score – object z score] in investigation and proximal areas. There was a main effect of isolation on BLA dF/F, but no differences between ifenprodil or vehicle groups at investigation areas (I) or proximal areas (J). Error bars represent the SEM. Horizontal blue lines indicate the average dF/F while in the center; * $p < 0.05$. Whiskers for box-and-whisker plots represent maximum and minimum values.

the social environment perhaps in combination with engagement of a larger social neural circuit (Varlinskaya et al., 2010; Green et al., 2013; Munshi et al., 2020).

While maturing circuits play a role in social engagement, the level of engagement in age-specific social behaviors in turn is also an important factor for behavioral and neural maturation (Varlinskaya et al., 1999; Han et al., 2011; van Kerkhof et al., 2013; Domínguez et al., 2019). We found that isolation increases social interaction in a manner consistent with increased social drive (Latane et al., 1972; Varlinskaya et al., 1999) and is sensitive to BLA manipulations, consistent with BLA sensitivity to social stressors (Patel et al., 2018; Munshi et al., 2020) and regulation of learned and innate social behaviors (Jasnow et al., 2005; Twining et al., 2017; Allsop et al., 2018; Patel et al., 2018). We found that BLA activation as a result of isolation was required for increased social drive, showing a common substrate through which high social motivation states are translated. While we found similar increases in lateral and basal divisions, distinct neuronal populations within BLA differentially contribute to social behavior (Fustiñana et al., 2021) and sensitivity to the social environment may be distinct between age groups perhaps based on function and maturation of distinct inputs (Kwapis et al., 2017; Selleck et al., 2018; Polepalli et al., 2020). In line with this, distinct morphologic changes are found in brain regions that project to and from the BLA following prolonged social isolation (Wang et al., 2012). While there are clear differences in BLA activity during novel partner or object investigation, the center averages included in photometry figures showed very little difference between isolation and no isolation manipulations in adolescents infused with vehicle, but adults showed a general increase in BLA activity while in the center with isolation. Despite increases in adult center averages, BLA activity still was highest during partner investigation. Changes in center data between isolation conditions could be a result orientation and/or trajectory of the rat while in the center, toward the social stimulus rat (Fustiñana et al., 2021).

Our findings indicating isolation-driven increases in BLA activity during social investigation are in line with BLA necessity for isolation-driven changes in social interaction across ages. Prior work has shown that sensitivity to the social environment gradually declines with age (Douglas et al., 2004; Varlinskaya and Spear, 2008) and activity of brain regions sensitive to changes in social circumstances, like the BLA, may also be differentially impacted during social experiences (Fustiñana et al., 2021). Sociability is often measured by time in social compartments and areas (Moy et al., 2004). In this instance, the impact of the social environment on engagement of BLA activity can be measured as a change in the degree of neural activity at social versus nonsocial investigation or at regions near these interaction sites (e.g., proximal areas). While brief isolation increased BLA activity during social interaction in both ages, BLA activity increased in proximal

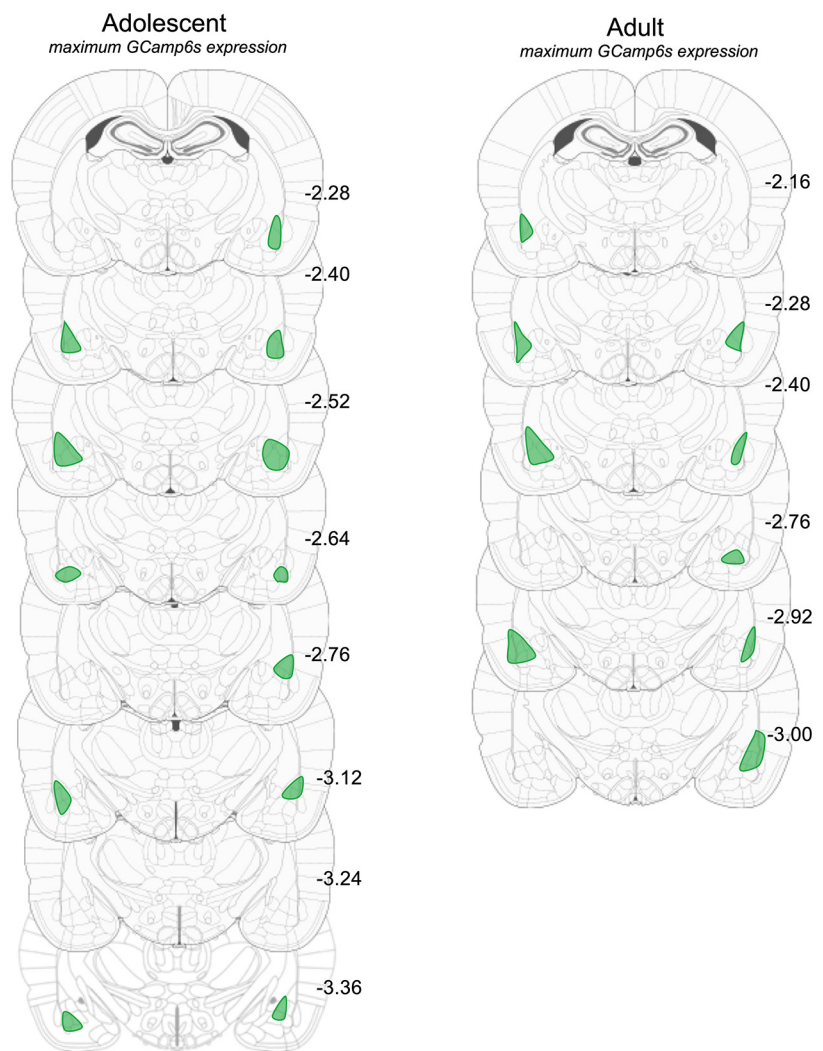


Figure 11. GCamp6s viral verification and spread adult and adolescent fiber photometry experiments. Spread of GCamp6s virus for fiber photometry data seen in Figures 9, 10.

partner regions only in adolescents, which may be related to adolescent social sensitivity relative to adults. Our results indicate that the BLA activity is engaged during social interaction across ages, but BLA activity is more sensitive to changes in the social environment in adolescents.

During development, NMDA subunit composition and sensitivity to antagonism changes in the BLA and for BLA-mediated behaviors (De Armentia and Sah, 2003; Delaney et al., 2013; Baker and Richardson, 2017; Bisby et al., 2018), and the ability for NMDA antagonism to regulate social behavior increases with age (Morales and Spear, 2014). Similarly, we found that GluN2B-containing NMDAR antagonism decreased adult BLA activity to a greater extent than adolescents. Isolation-driven enhancement of BLA activity during social interaction was less sensitive to GluN2B antagonism in adolescents, which may be attributed to ongoing cortical-BLA maturation. Prefrontal cortical inputs to the BLA mature during adolescence, and a greater number of NMDA receptors are postsynaptic to cortical inputs in the BLA compared with thalamic inputs (Farb and LeDoux, 1997; Arruda-Carvalho et al., 2017; Selleck et al., 2018). While antagonism with ifenprodil was not completely without effect in adolescents, the results suggest that glutamatergic inputs may contribute to a heightened social drive state in adults via GluN2B receptors. Additionally, we did not test the impact of isolation combined with social interaction on BLA

activity. Based on our work and previous results demonstrating BLA engagement during social interaction, it is possible that the combination of a brief change to the social environment with interaction (rather than isolation alone) would uncover a different role for NMDA-mediated amygdala activity between age groups that should be addressed in future work.

Prior work has demonstrated that animal shipment is a stressor that can impact behavior (Sachs and Lumia, 1981; Sanchez et al., 2021). In the current study, rats were shipped within 10 d of their target postnatal day and underwent isolation and subsequent behavioral and molecular assessments shortly after. This shipment stressor could therefore emphasize isolation effects between age groups and should be considered for future work, as this could impact social cognition. Additionally, CNO can have off-target DREADD effects masked by stress that is differentially impacted during aging (Manvich et al., 2018; Martinez et al., 2019). It is possible that the combination of shipment stress and CNO impacted our DREADD results such that isolation effects were exacerbated and not detected in our preexposure open field data. While we included control conditions in an attempt to rule out off-target effects, future work should investigate the importance of shipment stress in combination with isolation on social behavior, particularly when using CNO for DREADD manipulations.

Together, the current results may provide a clearer understanding of the neural mechanisms sensitive to changes in social circumstances during a developmental period in which there is increased diagnosis of neuropsychiatric disorders (Lee et al., 2014; Burke et al., 2017; Meyer and Lee, 2019). This increased risk of disorder diagnosis may in part be because of disruptions in the maturation of social behaviors and/or BLA activity during adolescence, making a clear understanding of social engagement and BLA activity essential for understanding core behavioral symptoms underlying neuropsychiatric disorders (Seffer et al., 2015; Banerjee et al., 2016; Barrett et al., 2017; Ferrara et al., 2021). While we found overlapping mechanisms regulating social drive in both age groups, the way in which BLA activity is regulated is different, where adult increases in activity are dependent on GluN2B receptor activity to a greater extent than adolescents. This provides a framework and supports literature identifying a critical role for the BLA in the modulation of age-specific behaviors as a result of subtle changes to the social environment that become dependent on GluN2B receptors with age.

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