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ARTICLE

GluN2B inhibition confers resilience against long-term cocaine-induced neurocognitive sequelae

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Cocaine self-administration can disrupt the capacity of humans and rodents to flexibly modify familiar behavioral routines, even when they become maladaptive or unbeneficial. However, mechanistic factors, particularly those driving long-term behavioral changes, are still being determined. Here, we capitalized on individual differences in oral cocaine self-administration patterns in adolescent mice and revealed that the post-synaptic protein PSD-95 was reduced in the orbitofrontal cortex (OFC) of escalating, but not stable, responders, which corresponded with later deficits in flexible decision-making behavior. Meanwhile, NMDA receptor GluN2B subunit content was lower in the OFC of mice that were *resilient* to escalatory oral cocaine seeking. This discovery led us to next co-administer the GluN2B-selective antagonist ifenprodil with cocaine, blocking the later emergence of cocaine-induced decision-making abnormalities. GluN2B inhibition also prevented cocaine-induced dysregulation of neuronal structure and function in the OFC, preserving mature, mushroom-shaped dendritic spine densities on deep-layer pyramidal neurons, which were otherwise lower with cocaine, and safeguarding functional BLA→OFC connections necessary for action flexibility. We posit that cocaine potentiates GluN2B-dependent signaling, which triggers a series of durable adaptations that result in the dysregulation of post-synaptic neuronal structure in the OFC and disruption of BLA→OFC connections, ultimately weakening the capacity for flexible choice. And thus, *inhibiting* GluN2B-NMDARs promotes resilience to long-term cocaine-related sequelae.

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INTRODUCTION

Behavioral flexibility, meaning, the capacity to deviate from routine behavioral sequences, is necessary for navigating dynamic environments. It is important for planning actions and making choices both large and small, and deficits in flexible action are thought to be core features of multiple neuropsychiatric diseases, including cocaine use disorder (CUD) [1].

The orbitofrontal cortex (OFC) performs myriad functions in support of flexible behavior [2–5]. A prevailing model posits that the OFC encodes a cognitive map of task space [6, 7], a schematic representation of task-relevant variables and associations within a particular environment [8]. Cognitive maps allow for the efficient integration of new learning into existing knowledge, enabling adaptive modification of action strategies when familiar expectancies are violated [9]. These OFC-dependent task space representations are destabilized by cocaine, which may be a mechanism by which cocaine results in rigid behavioral patterns that are resistant to change [10]. Neurobiological mechanisms remain unclear.

While there is debate regarding the precise nature of a potential *etiological* role for aberrant decision-making processes in drug misuse [1, 11, 12], it is clear that impairments in behavioral flexibility represent a durable consequence of cocaine in both humans and model organisms [13, 14], persisting despite abstinence [14, 15], and often in parallel with cocaine craving [16, 17]. Progressive alterations in decision-making processes may be particularly insidious when coinciding with sensitive developmental periods [14, 18, 19]. For instance, adolescent-onset, *versus* adult-onset, cocaine use decreases the likelihood that individuals will seek treatment across the lifespan [20, 21].

Here we aimed to understand how cocaine, particularly during adolescence, affects OFC-dependent action flexibility. Cognizant that the transition from recreational cocaine use to CUD is highly heterogeneous between individuals [21], and drug self-administration patterns can differ greatly between mice [22], we hypothesized that individual differences in cocaine-seeking behavior may help to reveal neurobiological factors by which cocaine disrupts action flexibility, or that confer resilience to the drug. We found that adolescent mice apparently resilient to cocaine had low GluN2B content in the OFC. This observation led to the discovery that GluN2B blockade obstructs the capacity of cocaine to degrade OFC neural structure and decision-making capacity, including its capacity for functional interaction with the basolateral amygdala (BLA), later in life. Thus, GluN2B levels or activity in adolescence have long-term ramifications for the structure and function of mature excitatory OFC neurons.

MATERIALS AND METHODS

Animals

Experiments used C57BL/6J mice (Jackson Laboratory, #000664), except for dendritic spine imaging experiments, which used B6.Cg-Tg(*Thy1*-YFP)HJrs/J

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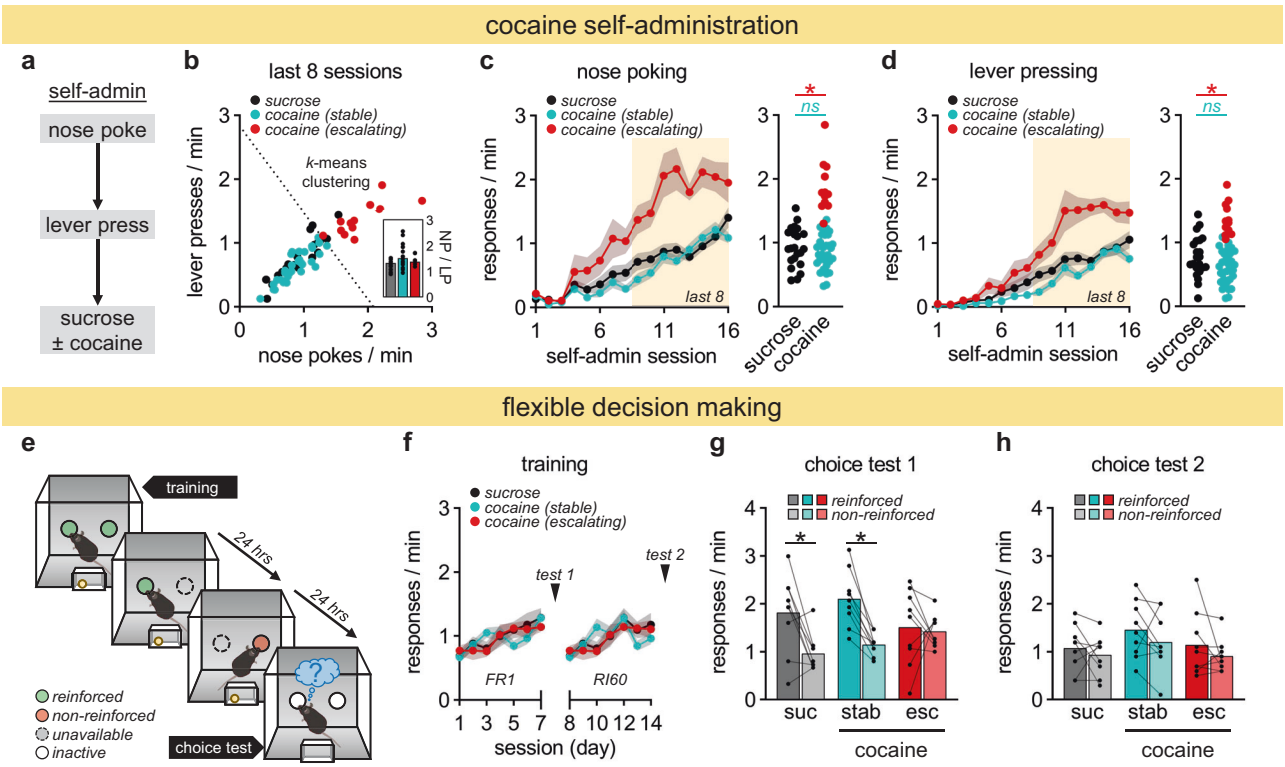


Fig. 1 Cocaine persistently disrupts flexible decision-making behavior. a Response sequencing during oral cocaine self-administration sessions, conducted in adolescence. **b** Average nose poke (NP) and lever press (LP) responses across last 8 sessions. Stable and escalatory response groups defined by *k*-means clustering. *Inset*. Ratio of NP to LP responses. **c**, **d** *Left panels*. NP and LP responses per session. *Right panels*. Average NP and LP responses across last 8 sessions. **e** Behavioral procedure assessing flexible decision making later in life. **f** Responses across training. **g**, **h** Choice test responses after one action was unexpectedly not reinforced, first following FR1 training (test 1; impaired in escalating cocaine responders) and then RI training (test 2). Data presented as individual points or mean ± S.E.M. $*p < 0.05$ (post-hoc). *See* Table S2 *for complete statistics and group sizes*.

mice (#003782). Male mice were used due to previously reported sex differences in the long-term effects of oral cocaine self-administration on the behaviors examined here [22]. Mice were provided food and water *ad libitum*, except during food-reinforced testing, when they were food-restricted until body weights were ~93% of baseline. Procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Emory University IACUC.

Oral cocaine self-administration

Operant conditioning chambers were equipped with two nose-poke apertures, a retractable lever, and a separate magazine for liquid dipper access or food pellet delivery (Med Associates). Mice were trained using an instrumental response sequence whereby two nose-poke apertures (active *vs*. inactive) were initially available. A nose-poke response in the active aperture extended the lever, pressing on which provided brief (10 s) access to 100 µL of a 10% w/v sucrose solution with or without 75 µg/mL cocaine (Sigma-Aldrich) (Fig. 1a) [22]. Mice underwent 16 daily self-administration sessions starting at postnatal day (P) 31. Sessions lasted 60 min or until 30 dipper access bouts were earned.

In order to group mice according to response patterns, we performed an unbiased two-dimensional *k*-means clustering analysis, using average response rates across the final eight self-administration sessions, ultimately categorizing cocaine-exposed mice into stable and escalating responder groups (Fig. 1b–d; Fig. S1). Escalating responders displayed both heightened seeking and taking responses compared to "stable" or control mice (Fig. 1c, d), resulting in a ~50% increase in the estimated effective dose of cocaine for escalating *vs*. stable responders across all sessions (Fig. S2). Mice were then left undisturbed and tested in a food-reinforced task 3 weeks later.

Food-reinforced response training

Starting between P60-70, mice were trained to nose poke for 20 mg grain pellets (Bio-Serv) in operant conditioning chambers (Med Associates). Initial training used a fixed ratio (FR1) reinforcement schedule whereby each nose poke delivered one food pellet reinforcer. For experiments when mice underwent multiple training conditions, we next utilized 30- or 60-second random interval (RI30 or RI60, respectively) reinforcement schedules whereby each reinforcer delivery was followed by a random interval of time in which nose-poke apertures were inactive. Mice underwent daily training sessions lasting either 70 min or until mice earned 30 reinforcers from responding on each aperture (60 total).

Test for behavioral flexibility (Fig. 1e)

Following food-reinforced training, we assessed the capacity of mice to modify response strategies based on reinforcer availability. Mice underwent two, 25-minute sessions occurring on consecutive days. On the first day, one nose-poke aperture was occluded while responding on the available aperture remained reinforced. On the second day, the opposite aperture was occluded, but responding on the available aperture was no longer reinforced. Instead, pellets were delivered into the magazine at a rate equal to what each mouse earned during the previous day's reinforced session. On a third day, mice performed a 10-minute **choice test**, conducted in extinction, when both apertures were again available. For experiments when mice underwent multiple-choice tests, the aperture that was to be non-reinforced was reversed so that mice could not utilize information from prior tests to inform subsequent responding.

Instrumental reversal learning

Following the test immediately above, some mice were returned to the operant conditioning chambers with access to both nose-poke apertures and a newly available lever. Both apertures were inactive, while lever-pressing was reinforced on an FR1 schedule with the same food pellet as above. Sessions lasted 25 min. For each session, a reversal rate was computed as reinforced responses (lever presses) divided by total responses (nose pokes+lever presses).

Protein quantification

Mice were anesthetized with isoflurane and decapitated. Brains were extracted, flash frozen, and sectioned into 1 mm coronal slices and dissected using a 1 mm tissue core. Tissue was homogenized. Protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Fisher). 15 µg of total protein was separated by SDS-PAGE on 4–20% gradient Tris-glycine gels (Bio-Rad), transferred to a PVDF membrane (Bio-Rad), blocked with 5% non-fat dry milk, and incubated overnight at 4 °C in primary antibody solution (Table S1). Membranes were incubated in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactivity was assessed using Pierce ECL chemiluminescence substrate (Thermo Fisher) and measured using a ChemiDoc XRS + Imaging System (Bio-Rad). Densitometry values were normalized to each sample's respective loading control (HSP-70). Protein levels were expressed as a fold change compared to control sample means from each membrane replicate.

Experimenter-administered cocaine and ifenprodil

Cocaine (10 mg/kg; Sigma-Aldrich) and ifenprodil (10 mg/kg; Tocris) in saline were administered *i.p.* daily from P31-35, with ifenprodil administered 30 min prior to cocaine.

Dendritic spine imaging and reconstruction

Immediately following the choice test of the behavioral flexibility assay, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and trans-cardially perfused and fixed in 4% paraformaldehyde. Brains were sectioned into 50 µm coronal sections using a freezing microtome. Basal dendritic segments located 50–150 µm from the soma on YFP-expressing neurons were imaged with a spinning disk confocal microscope (VisiTech International). Z-stack series (0.1 µm steps) were collected using a 100×1.4 NA oil-emersion objective. Three-dimensional dendritic morphology reconstructions were performed using the FilamentTracer module in Imaris (Oxford Instruments). Dendritic spines were classified using established parameters for pyramidal prefrontal cortex neurons [23]. Eight cells were analyzed/mouse and one dendrite was analyzed/cell.

Projection-selective chemogenetic manipulations

Intracranial surgeries were performed two weeks prior to behavioral testing. Mice were anesthetized with ketamine (80 mg/kg) and dexmedetomidine (0.5 mg/kg). Viral vectors were infused over 5 min using a microliter syringe (Hamilton). Infusion coordinates, relative to bregma (mm): OFC (ML:±1.40, AP:+2.50, DV: −2.80), BLA (ML:±3.00, AP: −1.50, DV: −4.90). A retrogradely transported Cre-recombinase construct (AAVrg-hSyn-HI-eGFP-Cre-WPRE-SV40; Addgene, #105540) was infused into the OFC alongside an anterogradely transported Cre-dependent, inhibitory chemogenetic receptor construct in the BLA (AAV5-hSyn-DIO-hM4D(Gi)-mCherry; Addgene, #44362). Clozapine-N-oxide (CNO; 0.1 or 1.0 mg/kg; 2% DMSO and saline; RTI International) was administered *i.p.* immediately following non-reinforced sessions.

Experiment overview (outlined Fig. S3)

Experiment 1 identified individual differences in oral cocaine self-administration and measured response flexibility and protein content in the OFC following drug washout (Figs. 1 and 2).

Experiment 2 utilized experimenter-administered cocaine and ifenprodil and measured response flexibility and dendritic spine morphology following drug washout (Figs. 3 and 4).

Experiment 3 utilized experimenter-administered cocaine and ifenprodil and chemogenetically manipulated BLA-OFC interactions (Fig. 5).

Data analysis

Statistical analysis was performed using SPSS v.27 (IBM). Tests were two-tailed with $\alpha < 0.05$. Response rates were compared using ANOVA, with repeated measures when appropriate. For dendritic spine analysis, hierarchical data structure was controlled using linear mixed-effect modeling (LMM) [24]. Intra-class correlation coefficients (ICC) were computed using random-intercept models to assess correlated error from subject-wise clustering. We then performed a 2-factor (cocaine × ifenprodil) LMM with an interaction term to test differences in dendritic spine parameters. *Post-hoc* analysis following interaction effects was performed for pair-wise comparisons as a 1-factor LMM.

RESULTS

Individual differences in cocaine-seeking behavior

We first examined cocaine self-administration in adolescent mice using a self-paced response sequence task [nose-poke (NP)→lever-press (LP)], which provided mice brief access to a sucrose solution with or without cocaine (Fig. 1a). NP and LP patterns revealed a broad spectrum of both seeking (NP) and taking (LP) behaviors among cocaine-exposed mice, including individual response rates that were similar to, or even lower than, those of control mice that received only sucrose. Mice were thus divided into "low" and "escalating" responders, which significantly differed (Fig. 1b: main effects comparing the last 8 sessions, used to classify mice: NP: $F_{1,43} = 97.2$, $p < 0.001$; LP: $F_{1,43} = 75.7$, $p < 0.001$; NP/LP: $F_{2,64} = 1.63$, $p = 0.204$). Figure 1c depicts NPs across training (session × group: $F_{30,960} = 3.64$, $p < 0.001$). Figure 1d depicts LPs across training (session × group: $F_{30,960} = 3.63$, $p < 0.001$). Average NP and LP rates also differed (Fig. 1c: $F_{2,64} = 51.4$, $p < 0.001$; Fig. 1d: $F_{2,64} = 32.4$, $p < 0.001$). Throughout this report, key ANOVA terms are reported in the main text, and complete statistics can be found in Tables S2–S6.

Mice were left undisturbed for 3 weeks, into adulthood, then we assessed flexible choice behavior, first following training using an FR reinforcement schedule, which generates response patterns that are sensitive to changes in outcome expectancies [25] (Fig. 1e). Prior cocaine did not impact initial training (Fig. 1f; Fig. S4: session × group: $F_{13,312} = 1.10$, $p = 0.338$). Mice were then given isolated access to one aperture, and responding remained reinforced. The next day, they had access to the opposite aperture, and responding was not reinforced; instead, pellets were delivered non-contingently. Prior cocaine did not alter responding when nose-poking was unexpectedly non-reinforced (Fig. S5), but cocaine-exposed mice categorized as escalating responders failed to later prefer the reinforced *vs.* non-reinforced behavior in a choice test–indicating an inability to update action strategies (Fig. 1g: test 1: reinforcement × group: $F_{1,24} = 3.47$, $p = 0.048$).

We next trained mice using an RI schedule, which over time weakens behavioral sensitivity to changes in outcome expectancies, causing organisms to persist in established behavioral sequence, even if not explicitly reinforced [25, 26]. Here, all mice displayed inflexible choice behavior, as expected (Fig. 1h: reinforcement × group: $F_{1,24} < 1$), thus confirming that our procedure is capable of detecting failures in flexible action due to drugs or reinforcement conditions alike.

Cocaine alters post-synaptic protein levels in the OFC

We next dissected OFC tissue from these same mice, focusing on the ventrolateral OFC, which is sensitive to changes in outcome expectancy [4, 27], and quantified several synaptic and plasticity-related proteins (Fig. 2a). PSD-95, a post-synaptic marker of excitatory synapses, was reduced in escalating, but not stable, responders (Fig. 2b: $F_{2,24} = 8.31$, $p = 0.002$), a presumed dose-dependent effect of cocaine mirroring the pattern of behavioral inflexibility. Meanwhile, the pre-synaptic marker synaptophysin was unchanged (Fig. 2c: $F_{2,24} = 2.32$, $p = 0.120$). Given that PSD-95 can regulate synaptic plasticity via interaction with glutamate receptors [28], we next quantified glutamate receptor subunit levels. Levels of the obligate GluN1 or the GluN2A subunit of NMDARs did not differ (Fig. 2d, e: GluN1: $F_{2,24} < 1$; GluN2A: $F_{2,24} < 1$). However, GluN2B was reduced in stable responders, which cannot be obviously explained by cocaine dose, given that GluN2B levels in escalating responders were similar to controls (Fig. 2f: $F_{2,24} = 4.81$, $p = 0.017$). This also did not reflect general alterations in glutamate receptor density, as AMPAR subunit GluA1 also did not differ (Fig. 2g: $F_{2,24} < 1$). Further, several other structural and functional synaptic regulatory proteins did not differ (Fig. S6). Meanwhile, PSD-95 and GluN2B correlated with individual choice behavior, with increased PSD-95 and decreased

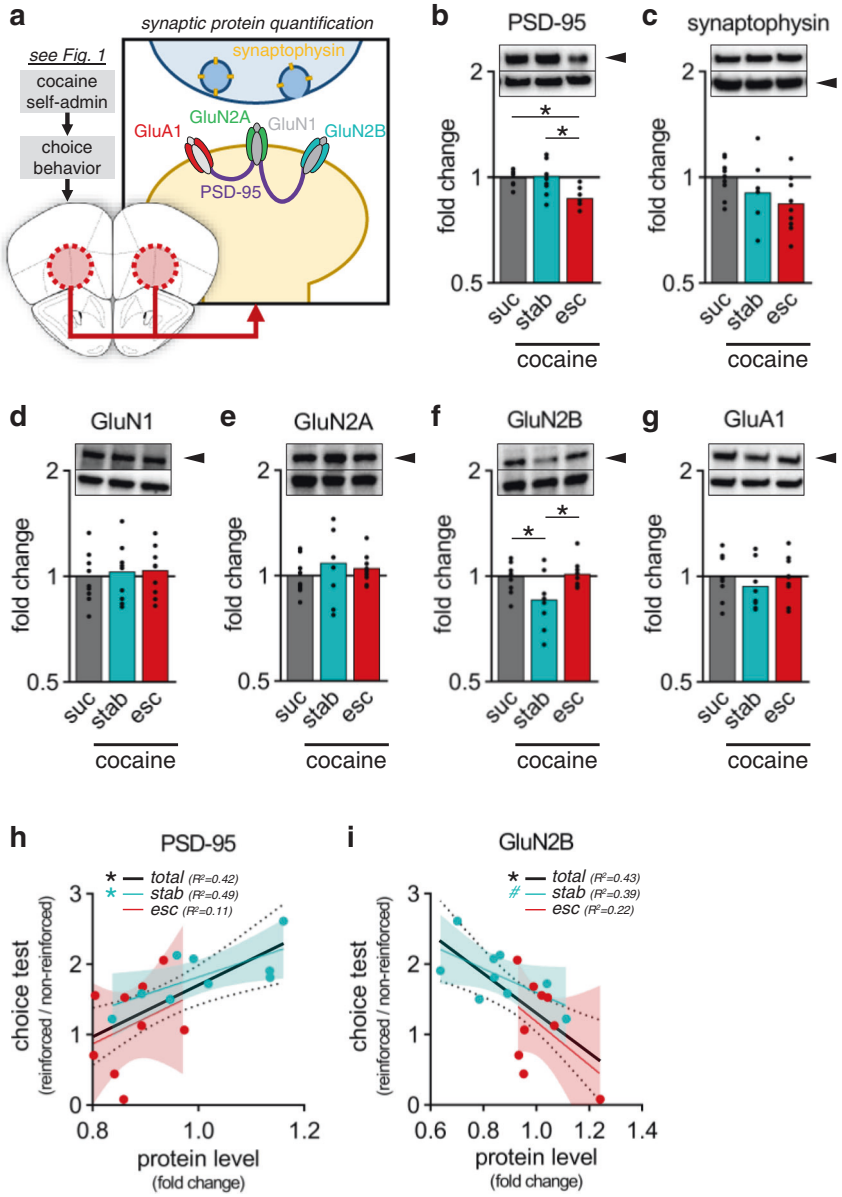


Fig. 2 Quantification of synaptic and plasticity-related protein levels in the OFC following oral cocaine self-administration. a Synaptic localization of quantified proteins in the OFC. **b**, **c** Quantification of synaptic marker protein levels in the OFC: PSD-95 (lower among escalating responders) and synaptophysin. **d–g** Quantification of glutamate receptor subunit levels in the OFC: GluN1, GluN2A, GluN2B (lower among stable responders), and GluA1. Values normalized to loading controls (HSP-70) and expressed as fold change from sucrose controls. Representative blots show target protein (black arrow) and HSP-70 loading controls (no arrow). **h**, **i** Correlation between choice test preference (*from* Fig. 1g) and PSD-95 or GluN2B levels. 95% confidence intervals (shading). Data presented as individual points. $^{*}p < 0.05$ (post-hoc), $^{\#}p = 0.07$. *See* Table S3 *for complete statistics and group sizes*.

GluN2B associating with flexible action updating (Fig. 2h, i: PSD-95: $F_{1,16} = 11.7$, $p = 0.004$; GluN2B: $F_{1,16} = 12.0$, $p = 0.003$).

GluN2B inhibition preserves behavioral flexibility following cocaine

These discoveries led us to test whether attenuating GluN2B-mediated signaling would confer resilience to cocaine-induced impairments in flexible choice (Fig. 3a). We administered the GluN2B-selective antagonist ifenprodil shortly before *i.p.* cocaine injections. We opted for experimenter-administered cocaine to ensure that mice received a dose that would reliably induce action inflexibility, in order to then attempt to combat it. Neither cocaine nor ifenprodil affected response training (Fig. 3b: session × cocaine × ifenprodil: $F_{6,198} = 1.68$, $p = 0.127$). As with cocaine self-administration, experimenter-administered cocaine disrupted flexible choice behavior, and this phenomenon was blocked by ifenprodil (Fig. 3c: reinforcement × cocaine × ifenprodil: $F_{1,33} = 5.85$, $p = 0.021$). To further solidify this evidence that GluN2B blockade buoys OFC function, we also tested these mice in an instrumental reversal task, another OFC-dependent task [29], in which pressing a previously unavailable lever was reinforced, while nose-poking was not. Here, ifenprodil also mitigated cocaine-induced deficits in reversal learning (Fig. 3d: session × cocaine × ifenprodil: $F_{3,99} = 3.71$, $p = 0.014$). Specifically, while cocaine had no effects on the acquisition of a new response (Fig. 3e: session × cocaine × ifenprodil: $F_{3,99} = 1.13$, $p = 0.295$), it perpetuated nose-poking, which was blocked by ifenprodil (Fig. 3f: session × cocaine × ifenprodil: $F_{3,99} = 2.78$, $p = 0.045$).

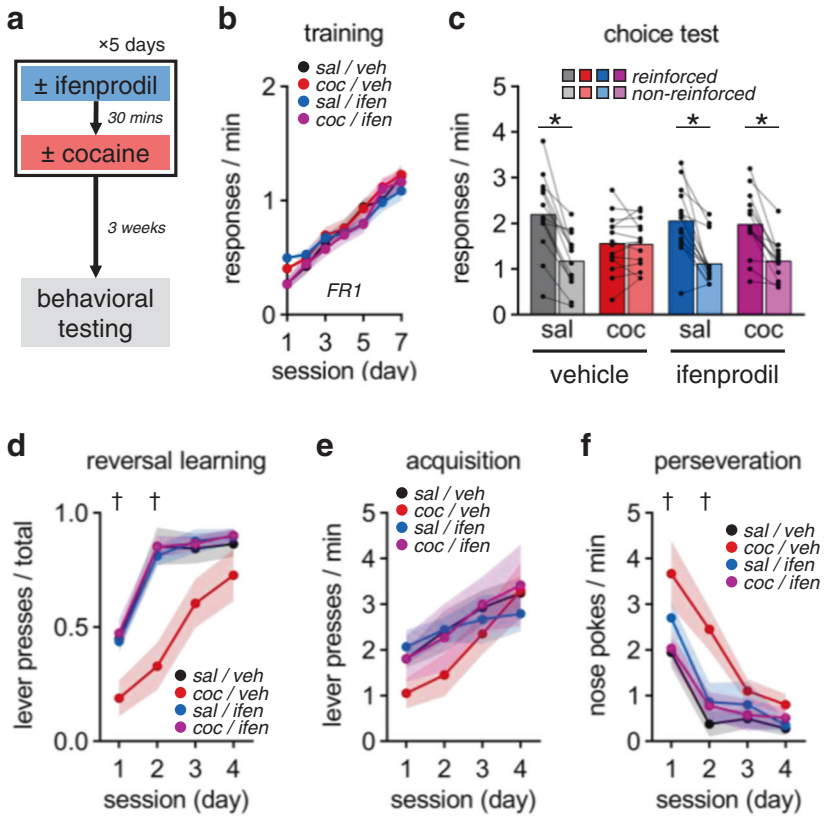


Fig. 3 GluN2B inhibition by ifenprodil blocks cocaine-induced decision-making deficits. a Ifenprodil and cocaine administration. **b** Responses across training. (**c**) Choice test responses. Cocaine impaired action flexibility, which was protected by ifenprodil. **d–f** Similar patterns were detected in instrumental reversal learning responses: reversal rate, response acquisition, and perseverative responding. Data presented as individual points or mean ± S.E.M. *$p < 0.05$ (post-hoc). †$p < 0.05$ (2-factor ANOVA, cocaine × ifenprodil). *See* Table S4 *for complete statistics and group sizes*.

GluN2B inhibition blocks cocaine-induced dendritic spine dysregulation in the OFC

We next examined in the same mice the density and structure of dendritic spines, the principal sites of excitatory neurotransmission in the brain [30], on ventrolateral OFC pyramidal neurons (Fig. 4a, b; Fig. S7). We visualized deep-layer neurons [31], which send and receive the bulk of long-range cortical projections [32] and appear to be especially vulnerable to cocaine compared to other prefrontal cortical cell types [33]. Cocaine reduced overall spine density (as with self-administered cocaine [34]), while ifenprodil prevented spine loss (Fig. 4c: cocaine × ifenprodil: $F_{1,16} = 7.35$, $p = 0.015$).

Notably, spine densities correlated with choice behavior, with a greater abundance of dendritic spines associated with response flexibility (Fig. 4d: $F_{1,18} = 4.68$, $p = 0.044$). Stratification of dendritic spines by morphologic subtypes revealed that cocaine-induced spine loss was driven by reduction of mushroom-shaped spines (Fig. 4e: cocaine × ifenprodil: $F_{1,16} = 6.96$, $p = 0.009$), while thin- and stubby-shaped spines were unaffected (Fig. 4f: thin: cocaine: $F_{1,16} < 1$; ifenprodil: $F_{1,16} < 1$; cocaine × ifenprodil: $F_{1,16} = 1.12$, $p = 0.306$; Fig. 4g: stubby: cocaine: $F_{1,16} < 1$; ifenprodil: $F_{1,16} < 1$; cocaine × ifenprodil: $F_{1,16} < 1$). This pattern of spine loss resulted in a reduction in the relative abundance of mushroom-shaped spines (Fig. 4h: cocaine × ifenprodil: $F_{1,16} = 5.20$, $p = 0.033$), containing more mature, stable synaptic connections [35]. This pattern is significant because the presence of these mature spines, and the cellular factors that stabilize them, in the ventrolateral OFC are necessary for action flexibility [14, 27, 36–38].

GluN2B-mediated resilience to cocaine requires BLA→OFC projections

Our findings lead to the question: Might GluN2B blockade confer behavior resilience to cocaine by preserving the ability of OFC neurons to receive inputs necessary for action flexibility? A prominent source of input arises from the BLA [39, 40]. BLA lesions disrupt reward-related activity of OFC neurons [41, 42], and BLA→OFC projections are required for goal-seeking action [43]. Thus, we expressed inhibitory (Gi-coupled) chemogenetic receptors on BLA→OFC projection neurons (Fig. 5a–d). We administered the chemogenetic ligand CNO immediately following the non-reinforced session, when familiar reinforcement conditions were violated (Fig. 5e), and excitatory OFC neuron plasticity is necessary for the consolidation of adaptive action strategies [44]. We tested multiple CNO doses and training conditions to identify a behaviorally sub-threshold CNO dose that *weakens*, but does not completely abolish, the capacity for flexible learning and memory. Groups did not differ during response acquisition (Fig. 5f: session × CNO condition: $F_{20,270} < 1$). A 0.1 mg/kg CNO dose did not disrupt flexible choice following FR training but *did* exhibit an additive effect with brief RI training, while 1.0 mg/kg induced inflexible choice throughout (Fig. 5g: test 1: reinforcement × CNO: $F_{2,27} = 4.92$, $p = 0.015$; Fig. 5h: test 2: main effect of reinforcement: $F_{1,27} = 15.8$, $p < 0.001$; reinforcement × CNO: $F_{2,27} = 2.39$, $p = 0.111$, followed by planned comparisons).

Next, we used this 0.1 mg/kg dose to attenuate BLA→OFC projection activity in cocaine-exposed mice that had been treated with ifenprodil (Fig. 5i). Groups again did not differ during training (Fig. 5j: session × ifenprodil × CNO: $F_{10,290} < 1$). Ifenprodil again

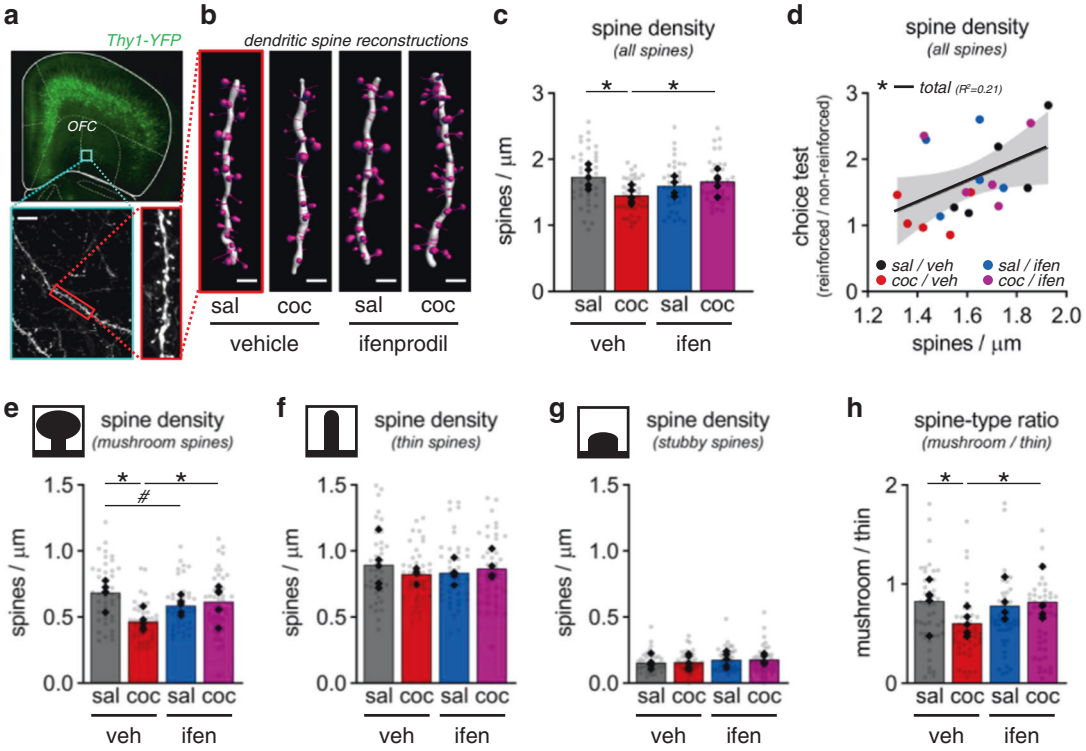


Fig. 4 GluN2B inhibition blocks cocaine-induced dendritic spine loss in the OFC. a Dendritic spine imaging from the OFC. Scale bar = 100 µm. **b** Representative three-dimensional dendritic spine reconstructions. Scale bar = 2 µm. **c** Dendritic spine density for all spines, lower following cocaine, and protected by ifenprodil. **d** Correlation between individual choice behavior and dendritic spine density across all groups. 95% confidence interval (grey shading). **e–g** Dendritic spine density stratified by mushroom-, thin-, or stubby-type spines. **h** Mushroom-to-thin spine-type ratio. Data presented as individual points (solid = per animal; transparent = per dendrite). $^{*}p < 0.05$, $^{\#}p = 0.0.038$ (post-hoc). *See* Table S5 *for complete statistics and group sizes*.

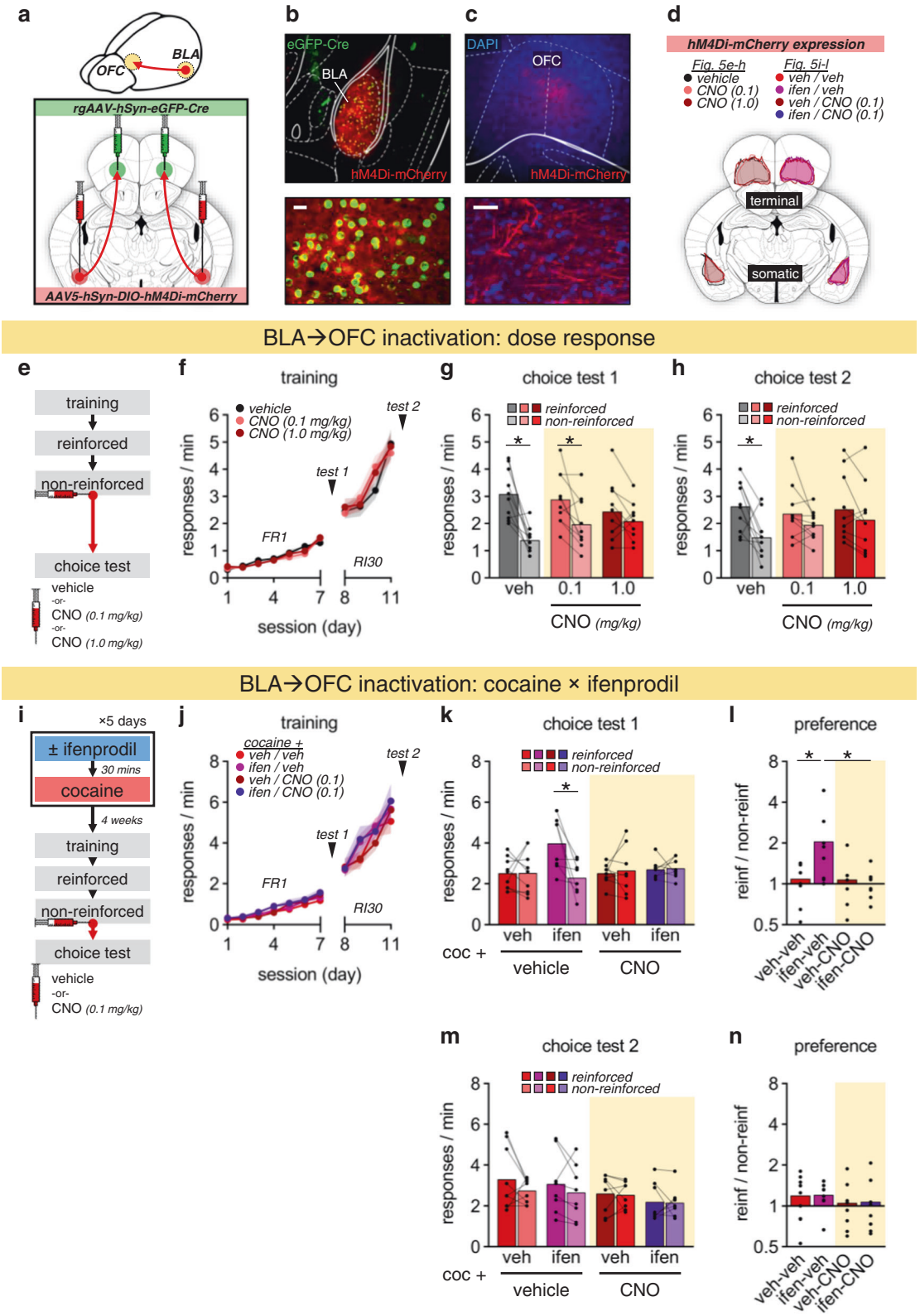
preserved flexible choice behavior in cocaine-exposed mice, while dampening BLA→OFC projections abolished this protective effect (Fig. 5k: reinforcement × ifenprodil × CNO: $F_{1,29} = 4.38$, $p = 0.045$). The same data can be reported as preference ratios (simply, responses in the reinforced/non-reinforced condition, such that scores >1 reflect action flexibility). Again, an interaction effect was detected, with post-hoc comparisons indicating that ifenprodil preserved action flexibility (Fig. 5l: ifenprodil × CNO: $F_{1,29} = 4.79$, $p = 0.037$). And again, we confirmed that all groups developed response inflexibility with extended training, as expected (Fig. 5m, n: reinforcement × ifenprodil × CNO: $F_{1,29} < 1$; preference ratio: ifenprodil × CNO: $F_{1,29} < 1$). Thus, the mechanism by which ifenprodil confers long-term resilience to cocaine-induced behavioral inflexibility appears to involve the preservation of functional BLA→OFC connections.

DISCUSSION

We demonstrate that both self- and experimenter-administered cocaine during adolescence disrupt the capacity of mice to later flexibly modify familiar behaviors when reinforcement conditions unexpectedly change. We capitalized on individual differences in cocaine self-administration patterns and revealed that the post-synaptic protein PSD-95 was lower in the OFC of mice that displayed escalating cocaine-seeking behavior, which corresponded with long-term deficits in flexible decision-making behavior. Meanwhile, GluN2B content was lower in the OFC of mice that were apparently *resilient* to escalatory cocaine-seeking behaviors. This discovery led us to pharmacologically inhibit GluN2B, attempting to confer resilience to cocaine. GluN2B blockade indeed obstructed the long-term behavioral and neuronal structural effects of cocaine, maintaining mature, mushroom-shaped dendritic spine densities on deep-layer pyramidal neurons in the OFC and preserving functional BLA→OFC connections. Cocaine can rapidly induce the insertion of GluN2B-containing NMDARs into post-synaptic membranes, resulting in long-lasting synaptic changes that persist even following prolonged abstinence [45, 46]. Because GluN2B inhibition coincident with cocaine administration here prevented the emergence of behavioral inflexibilities, we posit that inhibiting GluN2B-mediated signaling promotes resilience to long-term cocaine-related sequelae by interrupting the acute, early-stage synaptic response to cocaine. GluN2B inhibition in this context thus blocks cascading cocaine-induced morphological changes among deep-layer OFC neurons, which weaken the capacity for flexible choice *before* these deficits emerge and become entrenched.

Individual differences in cocaine seeking reveal biological-behavioral correlates

We utilized an oral cocaine self-administration procedure to resolve individual differences in drug-seeking behavior in mice. Orally-ingested cocaine readily penetrates the brain [47], and has reinforcing properties in both mice [22] and rats [48]. One advantage is that it allows for a non-drug control group that robustly responds for reinforcement (as opposed to animals receiving *i.v.* saline, which largely do not respond). We replicate existing evidence that some mice respond for a cocaine + sucrose solution comparably to mice receiving sucrose alone; meanwhile, other cocaine-reinforced mice escalate in responding [22]. Only these escalating responders later displayed deficits in an OFC-dependent decision-making task, concurrent with PSD-95 loss in the OFC. Meanwhile, stable responders had lower GluN2B content in the OFC, which could not be explained by intrinsic differences in motivation or reward processing per se, given that these mice responded equivalently to



sucrose-reinforced mice by the end of the training period, and the chained response was comparably efficient between groups, meaning, the number of nose pokes generated for access to the reinforced lever was indistinguishable. This discovery would have evaded us were it not for attention to individual differences, since these distinctions would not have been detected.

This unexpected outcome—a potential role for reduced GluN2B-mediated signaling in conferring resilience to cocaine – led us to administer the GluN2B-selective antagonist ifenprodil coincident with cocaine to attempt to block long-term deleterious cocaine-induced sequelae. We opted here for experimenter-administered cocaine to ensure that mice uniformly received a

Fig. 5 Preservation of flexible decision making following cocaine by GluN2B inhibition requires functionally intact BLA→OFC projections. a Combinatorial viral targeting of BLA→OFC projections. **b–d** eGFP-Cre driving hM4Di-mCherry expression in cell bodies in the BLA and axon terminals in the OFC. Scale bar = 25 µm. **e** Timing of CNO administration (0.1 or 1.0 mg/kg) occurs following a session when responding is unexpectedly not reinforced, this experimental design allowing us to selectively manipulate projection activity during a period of new memory encoding. **f** Responses across training. **g**, **h** Choice test responses following FR and moderate RI training, which has additive impact with 0.1 mg/kg CNO in obstructing action flexibility. **i** Timing of ifenprodil, cocaine, and CNO administration. **j** Responses across training. **k** Choice test responses following FR training, with effects of ifenprodil obstructed by subthreshold CNO. **l** Choice test response preference ratios revealing the same pattern. **m** Choice test responses following moderate RI training. **n** Choice test response preference ratios. Data presented as individual points or mean ± S.E.M. *$p < 0.05$ (post-hoc or planned comparison). *See* Table S6 *for complete statistics and group sizes*.

"high" dose that would reliably induce action inflexibility. Ifenprodil meanwhile *preserved* action flexibility, potentially attributable to the preservation of dendritic spine densities on deep-layer OFC neurons. NMDA receptors are ionotropic glutamate receptors composed of two GluN1 subunits and two GluN2 or GluN3 subunits. Ifenprodil can preferentially inactivate extra-synaptic dimers and block LTD [49]. Reducing extra-synaptic NMDA receptor activity is also thought to improve signal-to-noise ratio of synaptic NMDA receptor signaling [50]. Both of these mechanisms would be associated with the anchoring and maintenance of mature dendritic spine subtypes [30], though it is worth noting that while low GluN2B apparently aids, it is not *necessary*, for action flexibility, given that sucrose self-administering mice were able to update action strategies without the 'benefit' of low GluN2B. It is also likely one of multiple outcomes associated with drug resilience, even potentially compensating for other neurobiological sequelae.

Resilience to cocaine requires BLA→OFC projections

Dendritic spines house the post-synaptic component of a vast majority of synaptic connections in the mammalian brain [30], leading to the question: Which functional inputs to the OFC are preserved by GluN2B blockade? BLA lesions alter reward-related activity of OFC neurons [41, 42], and BLA-OFC interactions are involved in many facets of reinforcement learning [51, 52], leading us to investigate functional BLA→OFC connections, the goal being to test whether the behavioral resilience conferred by ifenprodil required functionally intact BLA→OFC connections. A challenge in designing this experiment is that inactivating BLA→OFC projections in typical, healthy mice obstructs response flexibility in the same task [43], which would make it difficult to draw conclusions regarding the protective actions of GluN2B inhibition with previously established procedures. To circumvent this issue, we established a behaviorally sub-threshold dose of the chemogenetic ligand CNO – meaning, a dose that was not sufficient to disrupt flexible action in cocaine-naïve mice expressing the same chemogenetic receptors. Here, sub-threshold silencing obscured the protective effects of ifenprodil, suggesting that GluN2B blockade preserves functional BLA→OFC connections rendered vulnerable by cocaine. Notably, other inputs to the OFC, such as the ventral hippocampus, support aspects of response flexibility in this task [53] and play major roles OFC-dependent representations of task variables [54–56]. The BLA is thought to broadcast a salience signal regarding task features most relevant to reward acquisition; thus, BLA→OFC inputs might organize multiplexed inputs by biasing the allocation of certain OFC neurons into the memory traces that subserve later adaptive responding.

CONCLUSIONS

Here, we administered cocaine during adolescence, given that adolescent-onset drug use is more likely to be associated with relapse across the lifespan [57]. During this late developmental epoch, cortical GluN2B levels decline, in favor of GluN2A receptor subunits, a process thought to optimize synapse and spine stabilization and refinement [58–60]. Meanwhile, cocaine self-administration can increase cortical GluN2B content [61], and developmental cocaine alters dendritic spine developmental trajectories on layer V OFC neurons [14]. Our findings suggest that GluN2B blockade is sufficient to protect connections necessary for flexible goal-seeking behavior, conceivably safeguarding neurodevelopmental processes otherwise disrupted by cocaine. Our investigation is complementary to others in which pharmacologic GluN2B antagonism occluded cocaine- and heroin-seeking behavior [14, 62], cocaine-induced locomotor sensitization [63, 64], and drug-induced habit [14, 65]. In these cases, though, GluN2B inhibitors were largely administered *following* some degree of drug exposure, and/or in mature organisms. Here, we reveal a novel, prophylactic effect of developmental treatment. A key next question is whether GluN2B blockade preserves *learning*-related structural plasticity in the OFC [27], and following drug self-administration; resolving this issue would more fully reveal ontogenetic factors that impact OFC function later in life.

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AUTHOR CONTRIBUTIONS

Conceptualization: DCL, EGP, SLG. Investigation: DCL, EGP, NMD. Formal analysis: DCL. Writing: DCL, SLG. Funding acquisition: DCL, SLG. Supervision: SLG.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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