Prefrontal $\alpha 7$ nAChR Signaling Differentially Modulates Afferent Drive and Trace Fear Conditioning Behavior in Adolescent and Adult Rats

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Increased level of kynurenic acid is thought to contribute to the development of cognitive deficits in schizophrenia through an $\alpha 7$ nAChR-mediated mechanism in the prefrontal cortex (PFC). However, it remains unclear to what extent disruption of PFC $\alpha 7$ nAChR signaling impacts afferent transmission and its modulation of behavior. Using male rats, we found that PFC infusion of methyllycaconitine (MLA; $\alpha 7$ nAChR antagonist) shifts ventral hippocampal-induced local field potential (LFP) suppression to LFP facilitation, an effect only observed in adults. Hippocampal stimulation can also elicit a GluN2B-mediated LFP potentiation (when PFC GABA_AR is blocked) that is insensitive to MLA. Conversely, PFC infusion of MLA diminished the gain of amygdalar transmission, which is already enabled by postnatal day (P)30. Behaviorally, the impact of prefrontal MLA on trace fear-conditioning and extinction was also age related. While freezing behavior during conditioning was reduced by MLA only in adults, it elicited opposite effects in adolescent and adult rats during extinction as revealed by the level of reduced and increased freezing response, respectively. We next asked whether the late-adolescent onset of $\alpha 7$ nAChR modulation of hippocampal inputs contributes to the age-dependent effect of MLA during extinction. Data revealed that the increased freezing behavior elicited by MLA in adult rats could be driven by a dysregulation of the GluN2B transmission in the PFC. Collectively, these results indicate that distinct neural circuits are recruited during the extinction of trace fear memory in adolescents and adults, likely because of the late-adolescent maturation of the ventral hippocampal-PFC functional connectivity and its modulation by $\alpha 7$ nAChR signaling.

Key words: adolescence; $\alpha 7$ nAChR; amygdala; fear conditioning; prefrontal cortex; ventral hippocampus

Significance Statement

Abnormal elevation of the astrocyte-derived metabolite kynurenic acid in the prefrontal cortex (PFC) is thought to impair cognitive functions in schizophrenia through an $\alpha 7$ nAChR-mediated mechanism. Here, we found that prefrontal $\alpha 7$ nAChR signaling is recruited to control the gain of hippocampal and amygdalar afferent transmission in an input-specific, age-related manner during the adolescent transition to adulthood. Behaviorally, prefrontal $\alpha 7$ nAChR modulation of trace fear memory was also age-related, likely because of the late-adolescent maturation of the ventral hippocampal pathway and its recruitment of PFC GABAergic transmission enabled by local $\alpha 7$ nAChR signaling. Collectively, these results reveal that distinct $\alpha 7$ nAChR-sensitive neural circuits contribute to regulate behavior responses in adolescents and adults, particularly those requiring proper integration of hippocampal and amygdalar inputs by the PFC.

Introduction

Several neural processes contributing to prefrontal cortex (PFC) maturation undergo major remodeling during adolescence (Caballero et al., 2016; Caballero and Tseng, 2016) to enable the acquisition of adult cognitive abilities (Casey et al., 2000; Best and Miller, 2010). As PFC processing and integration of inputs matures through adolescence (Caballero et al., 2016), any disruption that compromises the protracted trajectory of prefrontal development is expected to confer vulnerability to the onset of mental disorders (Caballero and Tseng, 2016) that display cognitive deficits and associated dysregulation of affect (Paus et al., 2008; Gogtay et al., 2011; Volk and Lewis, 2014). Thus,

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elucidating which signaling mechanisms are recruited to strengthen the functional connectivity of PFC afferent transmission is key to reveal how cognitive impairments could emerge in psychiatric disorders when such recruitment fails to occur (Caballero et al., 2016; Caballero and Tseng, 2016).

Of particular interest is the increased level of kynurenic acid in the PFC and its potential link to the onset of cognitive deficits in schizophrenia (Erhardt et al., 2007; Wonodi and Schwarcz, 2010; Myint, 2012). In addition to disrupting NMDAR function (Kessler et al., 1989; Parsons et al., 1997), nanomolar concentrations of kynurenic acid can elicit a state of excitatory-inhibitory imbalance in the PFC through a presynaptic $\alpha 7$ nAChR-mediated mechanism (Flores-Barrera et al., 2017). Certainly, prefrontal regulation of cognitive behavior, such as working memory, behavioral flexibility and attention, requires proper levels of $\alpha 7$ nAChR and NMDAR function (Alexander et al., 2012, 2013; Phenix et al., 2020) and integration of hippocampal and amygdalar inputs by the PFC (Floresco et al., 1997; Ishikawa and Nakamura, 2003; Tse et al., 2015). Since $\alpha 7$ nAChRs are well-positioned to regulate glutamate release in the PFC (Bortz et al., 2016), any disruption of local $\alpha 7$ nAChR signaling is expected to impact the gain of afferent glutamatergic transmission and the recruitment of postsynaptic NMDAR-mediated plasticity by hippocampal and amygdalar inputs (Flores-Barrera et al., 2014).

The aim of the present study is to determine how disruption of $\alpha 7$ nAChR signaling in the PFC impacts afferent information processing and its control of behavioral responses in adolescent and adult rats. To this end, local field potential (LFP) recordings were combined with PFC infusions of antagonists to reveal whether $\alpha 7$ nAChR modulation of ventral hippocampal and basolateral amygdalar transmission is input-specific and age-regulated. Similar pharmacological manipulations were implemented to assess the contribution of prefrontal $\alpha 7$ nAChR signaling in modulating behavior using a trace fear conditioning paradigm. Such a behavioral construct was preferred because proper processing of hippocampal and amygdalar afferent information by the PFC is needed for the learning and extinction of conditioned fear memories (Ishikawa and Nakamura, 2003; Sierra-Mercado et al., 2011; Gilmartin et al., 2012, 2014; Sotres-Bayon et al., 2012).

Materials and Methods

All experimental procedures were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee and met the National Institutes of Health guidelines for care and use of laboratory animals. Male Sprague Dawley rats were purchased from Envigo. Upon arrival, rats were allowed to habituate for at least 7 d before being subjected to any surgical procedures. They were group housed (two to three rats per cage), maintained under constant temperature (21–23°C) and light/dark cycle (14/10 h) with food and water available *ad libitum*. All chemicals were obtained from Sigma, except for methyllycaconitine (MLA) and indiplon that were obtained from Tocris.

In vivo recordings of LFP responses in the PFC

All recordings and PFC infusions procedures were conducted as previously described (Cass et al., 2013; Thomases et al., 2013, 2014; Caballero et al., 2014b). Briefly, rats were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic frame, and maintained at 37–38°C with a steady supplement of 300–400 μ l/h of 8% chloral hydrate. After exposing the skull, two burr holes were drilled to place the recording electrode in the medial PFC and the stimulating electrode within the ventral hippocampus or the basolateral amygdala. All LFP recordings were obtained using a concentric bipolar electrode attached to a 28G cannula (PlasticsOne), amplified (Cygnus Technology), filtered (1- to

100-Hz bandwidth), and digitized (Digidata 1440A, Molecular Devices) at a sampling rate of 10 kHz. The intensity of stimulation was chosen from the 0.2- to 0.8-mA range (mean intensity: $\sim$ 0.6 mA) using 300- μ s duration square pulses delivered every 15 s through a computer-controlled pulse generator (Master 8, A.M.P.I.). Typically, a 10-min LFP baseline recording was collected prior PFC infusions of 0.8 μ l (0.1 μ l/min) artificial CSF (aCSF)-containing vehicle, picrotoxin (50 μ M in 0.1% DMSO), MLA (300 nM), or picrotoxin+MLA. The dose of MLA was chosen because it blocks $\alpha 7$ nAChR function and disrupts PFC synaptic transmission *in vivo* and *ex vivo* (Flores-Barrera et al., 2017). A protocol of high-frequency stimulation (four trains of 50 pulses each at 100 Hz every 15 s) was then delivered into the ventral hippocampus or basolateral amygdala $\sim$ 30 min post-PFC infusion, and changes in the slope of LFP responses (from the onset to peak amplitude) were determined. The 30-min postinfusion period was included to monitor the stability of the evoked LFP response. Only recordings with a reliable 10- to 12-min baseline of LFP responses ($<15\%$ variability in slope using a bin size of 2 min) before the delivery of the high-frequency stimulation protocol were included.

Assessing the effects of PFC infusion of MLA on trace fear conditioning and extinction

Rats underwent survival surgery for bilateral cannula placement targeting the medial PFC region at least 8 d (range: 8–13 d) before behavioral testing. All PFC infusions were performed 20 min before the start of behavioral testing and consisted of simultaneous delivery of 0.8 μ l aCSF alone or in combination with MLA (300 nM), MLA+ifenprodil (10 μ M), or MLA+indiplon (10 μ M/0.04% DMSO), using a 33G infusion cannula protruding 0.5 mm beyond the tip of the guide cannula. The doses of MLA, ifenprodil and indiplon were chosen from previous studies showing their preferential effects on $\alpha 7$ nAChR, GluN2B, and GABAR transmission, respectively (Flores-Barrera et al., 2014, 2017).

Survival surgery

Rats were deeply anesthetized in a chamber saturated with 5% isoflurane (Somnosuite Unit, Kent Scientific). The level of anesthesia was monitored by assessing absence of the withdrawal reflex (hindlimb compression reflex). Before mounting the rats in the stereotaxic apparatus using non-rupture ear bars (Kopf), the head was shaved and the skin overlying the skull was then infiltrated with 2% lidocaine hydrochloride. Throughout the surgical procedure, isoflurane anesthesia (3%–5%) was maintained using a Somnosuite Unit with the body temperature kept within 37–38°C (TCAT-2LV heating pad, Physitemp). Burr holes were drilled in the skull to enable placement of a 26G guide cannula (Plastics One) targeting the dorsal border of the medial PFC bilaterally at a 25° angle (2.7–3.2 mm anterior to bregma; 3.2 mm lateral; 3.5–4.0 mm below the brain surface). At least 2 skull screws (Plastics One) were used to anchor the acrylic cement head assembly. After securing the guide cannula with acrylic cement (Stoelting), a 33G dummy cannula was screwed into the guide cannula to prevent clogging. All dummy cannulas were replaced by those protruding 0.5 mm beyond the tip of the guide cannula 24 h before testing the impact of PFC infusions on behavior.

Behavioral testing

We adapted a fear conditioning paradigm used by Zhang and Rosenkranz (2013). Briefly, all testing chambers (Ugo Basile) were housed in sound attenuating cabinets with white noise (60–70 dB; Scientific Design). The conditioning phase begins with a 120-s habituation period followed by the presentation of five trials of 220 s each using a pseudorandom inter-trial interval of 240–280 s. In each trial, a neutral tone (10 s, 1500 Hz, 85 dB) was paired with a footshock (1 s, 0.4 mA) at a delay of 20 s from the end of the tone (ANY-Maze, Stoelting). The extinction phase begins 24 h later in a visually and tactilely distinct chamber. Following 120 s of habituation, rats were tested with 14 trials of 60 s each from which the conditioned tone was presented for 20 s without footshock (ANY-Maze, Stoelting). The acquisition of fear extinction is typically revealed by the degree of conditioned freezing to the tone that diminishes over repeated trials. All behavioral changes were recorded by an infrared camera connected to a computer, and the time spent freezing (lack of non-respiratory

Figure 1. PFC infusion of MLA shifts ventral hippocampal-induced LFP suppression to LFP facilitation. **A**, Summary diagram and coronal sections (inset images) showing the anatomic location (mm relative to bregma) of LFP recordings within the medial PFC and the stimulation sites in the ventral hippocampus shown in **B**. **B**, Ventral hippocampal high frequency stimulation (HFS) typically elicits a pattern of sustained LFP suppression in the PFC of adult rats (P60–P90) that remained unaltered following aCSF infusion ($n = 6$). However, a potentiation of LFP responses emerged in the PFC following local infusion of MLA ($n = 8$). Bar graph summarizing the mean normalized LFP response obtained from the last 10 min post-HFS ($***p < 0.0001$, unpaired t test). Inset traces are examples of hippocampal-evoked LFP taken from 5 min pre-HFS (–5) and 35 min post-HFS (+35) illustrating the effect of MLA (calibration: 2 mV/20 ms). **C**, No apparent changes in prefrontal LFP responses were observed following hippocampal HFS in P30–P44 rats. Relative to aCSF ($n = 5$), PFC infusion of MLA ($n = 7$) failed to disrupt the pattern of hippocampal-evoked LFP.

Figure 2. PFC infusion of MLA does not disrupt the LFP facilitation elicited from the ventral hippocampus. **A**, Summary of the recording and stimulating electrodes placement (mm relative to bregma). **B**, Ventral hippocampal HFS typically elicits a pattern of sustained LFP potentiation in the PFC of adult rats (P60–P90) when local GABA_A receptors are blocked with picrotoxin ($n = 6$). Infusion of MLA along with picrotoxin ($n = 7$) failed to disrupt this facilitation. Inset bar graph summarizing the mean LFP response obtained from the last 10 min post-HFS. Examples traces of hippocampal-evoked LFP taken from 5 min pre-HFS (–5) and 35 min post-HFS (+35) illustrating the lack of effect of MLA (calibration: 2 mV/20 ms).

movement >0.5 s) per trial (% freezing) was determined offline from trial to trial as previously described (Caballero et al., 2020; Flores-Barrera et al., 2020).

Finally, a separate cohort of rats was included to assess the effect of MLA on contextual fear memory. Briefly, rats were trained using the same trace fear conditioning protocol described above. However, the shock intensity was increased to 0.5 mA to obtain sufficient levels of freezing response during the contextual testing phase. The context retention test begins 24 h later in the same training chamber in the absence of tone and shock. After 120 s of habituation, changes in freezing behavior were recorded for 16 min, and the time spent freezing per epoch of 4 min (% freezing) was determined.

Histology

At the end of the experiments (electrophysiology and behavior), rats were euthanized, and their brains quickly removed. Brains were then

blocked, fixed in 10% formalin overnight, and stored in 30% sucrose before sectioning as previously described (Cass et al., 2013; Thomases et al., 2013). The exact location of all recording, stimulating and infusion sites were determined by Nissl staining.

Statistical analysis

Data were summarized as mean $\pm$ SEM and differences among experimental conditions were considered statistically significant at $p < 0.05$. More specifically, all electrophysiological changes resulting from PFC infusion of MLA were compared with aCSF controls by Student's t test because they involve a single continuous dependent variable. On the other hand, changes in the level of freezing behavior across treatment conditions or age groups were assessed by two-way and three-way ANOVA (treatment or age $\times$ trials or epochs) for testing comparisons along three or more dependent variables.

Figure 3. PFC infusion of MLA attenuates basolateral amygdalar-induced facilitation of LFP. **A**, Summary diagram and coronal sections (inset images) showing the anatomic location (mm relative to bregma) of LFP recordings within the medial PFC and the stimulation sites in the basolateral amygdala shown in **B**. **B**, Relative to aCSF ($n = 10$), PFC infusion of MLA in adult rats (P60–P90, $n = 7$) markedly diminished the amplitude of basolateral amygdalar-induced LFP facilitation as revealed by the mean normalized LFP response obtained from the last 10 min post-HFS (bar graph, $**p < 0.001$, unpaired t test). Inset traces are examples of amygdalar-evoked LFP taken from 5 min pre-HFS (–5) and 35 min post-HFS (+35) illustrating the effect of MLA observed in adult rats (calibration: 2 mV/20 ms). **C**, PFC infusion of MLA ($n = 5$) also reduced the amplitude of amygdalar-induced LFP facilitation observed in the PFC of P30–P44 rats (aCSF, $n = 5$). Bar graph summarizing the mean normalized LFP response obtained from the last 10 min post-HFS ($**p < 0.001$, unpaired t test). Inset traces are examples of amygdalar-evoked LFP taken from 5 min pre-HFS (–5) and 35 min post-HFS (+35) illustrating the effect of MLA (calibration: 2 mV/20 ms).

Results

We first examined how blocking $\alpha 7$ nAChR signaling in the PFC impacts afferent drive originated from the ventral hippocampus by means of local field potential (LFP) recordings *in vivo*. To this end, the $\alpha 7$ nAChR antagonist MLA (300 nM) was locally infused into the PFC prior to high frequency stimulation of the ventral hippocampus (four trains of 50 pulses each delivered at 100 Hz every 15 s). This stimulation protocol typically elicits a sustained suppression of LFP in the PFC that emerges after postnatal day (P)45 (Caballero et al., 2014b; Fig. 1) through the recruitment of a developmentally regulated prefrontal GABAergic transmission (Caballero et al., 2014a, 2020). Relative to aCSF controls ($n = 6$), PFC infusion of MLA ($n = 8$) shifted hippocampal-induced LFP suppression to LFP facilitation (Fig. 1A,B), an effect that was not present in adolescent P30–P44 rats ($n = 5$ aCSF, $n = 7$ MLA; Fig. 1C). Interestingly, such an effect was no longer apparent in the presence of picrotoxin ($n = 6$ picrotoxin, $n = 7$ picrotoxin + MLA; Fig. 2) indicating that MLA is disrupting the GABAergic component of the PFC response. In addition, these results further revealed that the NMDAR-GluN2B component contributing to the potentiation of LFP in the PFC when local GABA_A receptors are blocked (Caballero et al., 2014a; Flores-Barrera et al., 2014) is insensitive to MLA (Fig. 2). Together, these findings point to an age-dependent recruitment of $\alpha 7$ nAChR signaling by ventral hippocampal inputs that preferentially impacts the gain of prefrontal GABAergic function.

We next asked whether PFC inputs originated from the basolateral amygdala are also modulated by $\alpha 7$ nAChR signaling. Contrary to the impact of hippocampal stimulation, basolateral amygdala high frequency stimulation elicits a pattern of sustained LFP facilitation in the PFC that is already enabled

Figure 4. Adolescent and adult rats exhibited different levels of freezing response during trace fear conditioning and extinction. **A**, A progressive increase in freezing behavior was observed in both adult (P70–P90; $n = 16$) and adolescent (P38–P44; $n = 15$) rats during conditioning (main effect of trials, $F_{(4,145)} = 30.0$, $p < 0.0001$; two-way ANOVA). However, the adolescent group showed an overall lower freezing pattern than adults (main effect of age, $F_{(1,145)} = 46.3$, $***p < 0.0001$; two-way ANOVA). **B**, Similarly, both age group of rats display comparable patterns of conditioned freezing to the tone that diminishes over repeated trials during extinction testing 24 h later (main effect of trial $F_{(13,406)} = 27.1$, $p < 0.0001$; two-way ANOVA). The two-way ANOVA also revealed a significant main effect of age ($F_{(1,406)} = 7.6$, $*p < 0.01$) as a result of an overall lower freezing response in the adolescent group.

by P30 (Caballero et al., 2014b; Fig. 3). Accordingly, the amplitude of the potentiated amygdalar LFP response was markedly attenuated by PFC infusion of MLA in both P30–P44 ($n = 5$ aCSF, $n = 5$ MLA) and P60–P90 ($n = 10$ aCSF, $n = 7$ MLA) age groups (Fig. 3). Thus, PFC $\alpha 7$ nAChR signaling is recruited as early as P30 to facilitate afferent transmission of basolateral amygdala inputs.

At the behavioral level, we implemented a trace-fear conditioning paradigm paired with local infusion of MLA to determine how PFC disruption of $\alpha 7$ nAChR signaling impacts behavior in adolescent (P38–P44) and adult (P70–P90) rats. Although the age ranges in the behavioral cohort are more narrowed than that included in the electrophysiology groups because of the recovery period from the survival surgical procedure for cannula placement (see Materials and Methods), they are still within P30–P44 and P60–P90. Data obtained from all aCSF groups revealed that adolescent rats ($n = 15$) show lower freezing than adults ($n = 16$) during acquisition (Fig. 4A), while both age groups display similar patterns of extinction behavior (Fig. 4B). Of note, the impact of MLA during acquisition was also age related, such that it reduced the freezing response in adults ($n = 6$ aCSF, $n = 8$ MLA; Fig. 5A–C) without disrupting the pattern of freezing behavior in adolescents ($n = 6$ aCSF, $n = 8$ MLA; Fig. 5D–F). Conversely, PFC infusion of MLA elicited opposite effects in adolescent and adult rats during extinction (Fig. 6). Relative to aCSF controls, MLA increased the level of freezing response to the conditioned tone in adults ($n = 6$ aCSF, $n = 8$ MLA; Fig. 6A–C), while it reduced freezing in adolescents ($n = 7$ aCSF, $n = 8$ MLA; Fig. 6D–F). These results suggest that adolescent and adult rats recruit distinct neural circuits during the extinction of trace fear memory, a behavioral response known to require proper integration of ventral hippocampal and amygdalar inputs by the PFC (Sierra-Mercado et al., 2011). Thus, it is possible that the delayed maturation of the ventral hippocampal pathway (Caballero et al., 2014b) contributes to the opposite, age-dependent effect of MLA as $\alpha 7$ nAChR signaling in the PFC emerges after P45 to enable the GABA_A component of the hippocampal-evoked response (Fig. 1).

If the late-adolescent onset of PFC $\alpha 7$ nAChR modulation of hippocampal inputs (Fig. 1) sets the age-dependent effect of MLA during extinction (Fig. 6), the increased freezing behavior observed in adult rats could result from two concurrent events triggered by a disruption of the GABA_A-mediated LFP suppression (Fig. 1), while the GluN2B-mediated LFP potentiation component remains intact (Fig. 2). To test this hypothesis, ifenprodil (10 μ M) was co-delivered with MLA into the PFC to block the GluN2B-mediated potentiation of the hippocampal transmission (Flores-Barrera et al., 2014). Data revealed that the inclusion of ifenprodil was sufficient to mitigate the enhanced freezing response elicited by MLA in adult rats during extinction ($n = 5$ aCSF, $n = 5$ MLA+ifenprodil; Fig. 7A,B). However, this was not the case when the GABA_A receptor-positive allosteric modulator indiplon (10 μ M; Flores-Barrera et al., 2017) was delivered along with MLA to partially compensate for the diminished GABAergic function ($n = 6$ aCSF, $n = 6$ MLA+indi-plon; Fig. 7C). Together, the results indicate that an imbalanced potentiation of PFC GluN2B transmission

Figure 5. PFC infusion of MLA reduces the level of freezing response during the acquisition of trace fear memory only in adult rats. **A**, Summary of aCSF and MLA infusion sites within the PFC of adult rats. **B**, Relative to aCSF ($n = 6$), infusion of MLA ($n = 8$) into the PFC of adult rats (P70–P90) markedly diminished the freezing response during conditioning (main effect of treatment, $F_{(1,60)} = 67.9$, $***p < 0.0001$; main effect of trials, $F_{(4,60)} = 8.7$, $p < 0.0001$; two-way ANOVA). **C**, Twenty-four hours later, MLA-treated rats showed a lower level of conditioned freezing response during extinction testing (main effect of treatment, $F_{(1,168)} = 23.5$, $***p < 0.0001$; main effect of trials, $F_{(13,168)} = 10.3$, $p < 0.0001$; two-way ANOVA). **D**, Summary of aCSF and MLA infusion sites within the PFC of adolescent rats. **E**, Relative to aCSF ($n = 8$), PFC infusion of MLA ($n = 9$) did not alter the pattern of freezing response in P38–P44 rats during conditioning. **F**, Both aCSF-treated and MLA-treated rats showed similar levels of conditioned freezing behavior during extinction testing 24 h later.

underlies the increased level of freezing response during extinction when prefrontal disruption of $\alpha 7$ nAChR signaling occurs in adults.

Finally, it is possible that $\alpha 7$ nAChR signaling in the PFC is also recruited during contextual fear association in trace conditioning (Gilmartin and Helmstetter, 2010; Orsini et al., 2011; Kim and Cho, 2017; Twining et al., 2020). Thus, another cohort of adolescent and adult rats was generated to examine the impact of MLA during the context retention test 24 h later. Here, the shock intensity during conditioning was increased to obtain sufficient levels of freezing behavior during the contextual testing phase. As a result, the age effect obtained during conditioning with a lower shock intensity (Fig. 4A) is no longer apparent (Fig. 8A). Remarkably, adolescent rats continue to show a pattern of lower freezing than adults during re-exposure to the training context (Fig. 8B). However, PFC infusion of MLA did not alter the distinct levels of freezing response observed in adolescent (P38–P44: $n = 6$ aCSF, $n = 7$ MLA) and adult rats (P70–P90: $n = 6$ aCSF, $n = 7$ MLA; Fig. 8B). Together, these results show that the level of contextual freezing response is developmentally regulated but independent of prefrontal $\alpha 7$ nAChR signaling.

Discussion

The present study reveals that prefrontal $\alpha 7$ nAChR signaling is recruited by ventral hippocampal and basolateral amygdalar inputs to modulate the gain of afferent transmission in an age-related manner. While amygdalar inputs in the PFC are already enabled by $\alpha 7$ nAChR signaling at P30, its modulation of ventral

Figure 6. PFC infusion of MLA enhances the level of freezing response during extinction testing only in adult rats. **A**, Summary of aCSF and MLA infusion sites within the PFC of adult rats. **B**, All adult rats (P70–P90) assigned to receive either aCSF or MLA during extinction testing (day 2) showed similar level of freezing response during conditioning (day 1). **C**, Relative of aCSF ($n = 6$), PFC infusion of MLA ($n = 8$) markedly increased the level of conditioned freezing response during extinction testing (main effect of treatment, $F_{(1,140)} = 81.7$, $***p < 0.0001$; main effect of trials, $F_{(13,140)} = 13.4$, $p < 0.0001$; two-way ANOVA). **D**, Summary of aCSF and MLA infusion sites within the PFC of adolescent rats. **E**, All P38–P44 rats assigned to receive either aCSF or MLA during extinction testing (day 2) showed similar level of freezing response during conditioning (day 1). **F**, Relative of aCSF ($n = 7$), PFC infusion of MLA ($n = 8$) diminished the overall level of freezing response in P38–P44 rats during extinction (main effect of treatment, $F_{(1,182)} = 12.4$, $**p < 0.001$; main effect of trials, $F_{(13,182)} = 5.6$, $p < 0.0001$; two-way ANOVA).

hippocampal transmission does not emerge until late adolescence when the GABA_A component of the prefrontal response becomes online. Remarkably, a similar age-related modulation of trace fear behavior by PFC $\alpha 7$ nAChR was observed in tandem with the delayed maturation of the hippocampal pathway. Thus, it is conceivable that distinct $\alpha 7$ nAChR-sensitive neural circuits contribute to regulate behavioral responses in adolescents and adults, particularly when proper integration of hippocampal and amygdalar inputs by the PFC is required.

Despite its widespread expression, our data indicate that the recruitment $\alpha 7$ nAChR signaling by PFC afferent transmission is input and synapse specific. While the GluN2B-mediated potentiation of hippocampal inputs (Flores-Barrera et al., 2014) is insensitive to MLA, PFC $\alpha 7$ nAChR modulation of the amygdalar pathway is already enabled by P30, likely through a mechanism that facilitates glutamate release (Konradsson-Geuken et al., 2009; Bortz et al., 2016; Yarur et al., 2020). GABAergic synapses in the PFC are also regulated by $\alpha 7$ nAChR (Couey et al., 2007; Aracri et al., 2010; Flores-Barrera et al., 2017) in a manner that enables ventral hippocampal inputs to enhance PFC inhibitory control of afferent drive after P45 (Caballero et al., 2014b). In fact, the pattern of LFP potentiation observed in the PFC following MLA infusion (Fig. 1) resembles those elicited by picrotoxin (Cass et al., 2013; Caballero et al., 2014b; Thomases et al., 2014), which point to a GABAergic mechanism underlying the $\alpha 7$ nAChR control of hippocampal inputs. Thus, any disruption that compromises $\alpha 7$ nAChR function in the PFC is expected to limit its optimal computational capacity and the control of input selectivity by local inhibition (Lew and Tseng, 2014).

Parallel to the gain of GABA function in the PFC during adolescence are the increased level of parvalbumin (PV) expression and glutamatergic transmission onto PV-positive fast-spiking interneurons (FSI; Caballero et al., 2014a). Although not all FSI express $\alpha 7$ nAChR-sensitive currents (Porter et al., 1999; Couey et al., 2007; Poorthuis et al., 2013), nAChR stimulation facilitates the transmission of excitatory inputs onto these interneurons in the PFC, likely through a presynaptic mechanism (Couey et al., 2007). Therefore, $\alpha 7$ nAChR signaling could strengthen PFC inhibitory control of afferent drive by increasing the gain of excitatory synapses onto PV-positive FSI during adolescence. In fact, genetic deletion of $\alpha 7$ nAChR markedly reduced the level of cortical PV and markers of GABA function (Lin et al., 2014), resembling the GABAergic deficit observed in the PFC when local recruitment of PV-positive FSI during adolescence is limited (Cass et al., 2013; Caballero et al., 2020; Flores-Barrera et al., 2020). Collectively, these results suggest an $\alpha 7$ nAChR mechanism underlying the maturation of PV-positive FSI in the PFC. A deficient recruitment of prefrontal FSI function by $\alpha 7$ nAChR in adulthood will likely disrupt the inhibitory control of afferent drive and its impact on behavior, as seen when PV expression in the PFC fails to reach adult levels (Caballero et al., 2020).

Prefrontal control of behavior requiring proper integration of hippocampal and amygdalar inputs also undergoes developmental changes during adolescence. Of particular interest is the regulation of conditioned fear memories by PFC nAChRs (Raybuck and Gould, 2010; Kutlu et al., 2018) and the underlying glutamatergic mechanism driving freezing behavior (Gilmartin and Helmstetter, 2010; Gilmartin et al., 2012, 2013a,b). In this regard, the age-dependent facilitation of freezing responses observed during trace fear conditioning could result from the gain of PFC GluN2B function to strengthen hippocampal inputs after P45 (Flores-Barrera et al., 2014). However, it is unlikely that inhibition of such GluN2B transmission is driving the reduced level of freezing observed following PFC $\alpha 7$ nAChR blockade in adults since this glutamatergic input is insensitive to nanomolar infusion of MLA. Instead, the effect of MLA during conditioning is likely due to a disruption of basolateral amygdala transmission to the PFC as similar behavioral deficit was observed following functional disconnection of the amygdalar-prefrontal pathway (Gilmartin et al., 2012). Our data also revealed that $\alpha 7$ nAChR signaling is not recruited during the acquisition of trace fear conditioning in adolescent rats, further indicating that different PFC-dependent processes regulate this behavior in an age-related manner. In addition to ventral hippocampal and amygdalar inputs, thalamic afferents also continue to develop into early adulthood, which are likely to impact PFC maturation and its control of behavior (Ferguson and Gao, 2014; Parnaudeau et al., 2018).

Associated with the strengthening of glutamatergic transmission is the functional maturation of prefrontal GABAergic circuits during adolescence that enables ventral hippocampal inputs to enhance PFC inhibitory control of afferent drive in adulthood (Caballero and Tseng, 2016; Caballero et al., 2016). Such gain of GABAergic function is critical to support the extinction of a trace fear memory that requires intact hippocampal-PFC connectivity (Caballero et al., 2020; Flores-Barrera et al., 2020). Remarkably, the enhanced freezing response elicited by MLA in adult rats during extinction resembles the behavioral deficit observed following a developmental disruption that renders the PFC disinhibited (Caballero et al., 2020; Flores-Barrera et al., 2020). While these results are consistent with the view that PFC interneurons are recruited to facilitate the extinction of learned behaviors (Sotres-Bayon et al., 2012; Courtin et al., 2014; Sparta et al., 2014), they also imply a disinhibitory mechanism underlying the effect of MLA in adults. By limiting the gain of inhibitory synapses (Flores-Barrera et al., 2017), the resulting impact of MLA during extinction becomes shifted because of an imbalanced facilitation of the PFC GluN2B transmission (Flores-Barrera et al., 2014). Accordingly, the enhanced freezing response elicited by MLA during extinction was mitigated by the GluN2B antagonist ifenprodil, which also blunted the potentiation of hippocampal-driven LFP response in the PFC (Flores-Barrera et al., 2014). Thus, it is possible that coordinated feedforward inhibitory control of glutamatergic inputs by $\alpha 7$ nAChR in the PFC dictates the level of conditioned freezing that diminishes over repeated trials during trace fear extinction. The opposite freezing response elicited by MLA in adolescent rats further reveals that distinct PFC mechanisms are recruited to regulate trace fear extinction behavior when the functional connectivity of the hippocampal-prefrontal pathway is not fully matured.

The level of contextual freezing behavior is also developmentally regulated, but insensitive to PFC infusion of MLA. Relative to adolescents, adult rats displayed an enhanced freezing response during re-exposure to the training context. While several mechanisms (Gilmartin and Helmstetter, 2010; Gilmartin et al., 2012, 2013a,b) could contribute to driving such age-dependent freezing response, recent work has highlighted the role of ventral hippocampal inputs to the PFC in modulating contextual fear association in trace conditioning

Figure 7. PFC infusion of ifenprodil prevents the enhanced freezing response elicited by MLA in adult rats during extinction. **A**, All adult rats (P70–P90) assigned to receive PFC infusions exhibited similar patterns of increased freezing response during conditioning. **B**, Summary of aCSF, MLA+Ifenprodil, and MLA+Indiplon infusion sites within the PFC of adult rats. **C**, Relative to aCSF ($n=5$), the typical heightened freezing behavior observed with MLA alone (Fig. 6C) is no longer apparent when the GluN2B antagonist ifenprodil ($10 \mu\text{M}$, $n=5$) was co-administered into the PFC (main effect of treatment, $p=0.15$; main effect of trials, $F_{(13,112)}=14.5$, $p<0.0001$; two-way ANOVA). **D**, In contrast, PFC co-infusion of the GABA $_A$ $\alpha 1$ -positive allosteric modulator indiplon ($10 \mu\text{M}$, $n=6$) along with MLA failed to reduce the enhanced freezing response elicited by MLA alone (main effect of treatment, $F_{(1,126)}=19.1$, $***p<0.0001$; main effect of trials, $F_{(13,126)}=15.4$, $p<0.0001$; two-way ANOVA).

Figure 8. PFC infusion of MLA does not disrupt the pattern of freezing behavior during re-exposure to the training context. **A**, All adults (P70–P90) and adolescents (P38–P44) rats assigned to receive either aCSF or MLA during the context retention test (day 2) showed similar level of freezing response during conditioning (day 1). **B**, Relative to aCSF ($n=6$, P70–P90; $n=6$, P38–P44), PFC infusion of MLA in adult ($n=7$, P70–P90) and adolescent ($n=7$, P38–P44) rats failed to disrupt the level conditioned freezing response that diminishes over time (4 min/epoch) during context retention testing (main effect of epochs, $F_{(3,88)}=8.1$, $***p<0.0001$; three-way ANOVA). Notably, adolescents displayed an overall lower freezing pattern than adults (main effect of age, $F_{(1,88)}=11.2$, $**p<0.002$; three-way ANOVA).

(Twining et al., 2020). In this regard, the enhanced contextual freezing response observed in adults could result from the gain of PFC GluN2B transmission that potentiates hippocampal inputs after P45 (Flores-Barrera et al., 2014) that is

also insensitive to MLA. Although the gain of hippocampal inputs after P45 also strengthens PFC inhibitory control of afferent drive (Caballero et al., 2014b), it is unlikely that this component is recruited during the contextual retention testing because of its sensitivity to MLA, whereas the behavioral response is not. Collectively, our data suggest that contextual and cue-mediated association in trace fear conditioning can be dissociated at the level of circuitry and PFC $\alpha 7$ nAChR signaling. Whether the contextual control of extinction observed in adolescents and adults involve hippocampal-PFC connections directly or indirectly via midline thalamic inputs (Orsini et al., 2011; Xu and Südhof, 2013; Kim and Cho, 2017) remains to be determined.

In sum, any disruption of prefrontal $\alpha 7$ nAChR function during development (e.g., by elevation of brain kynurenic acid levels) is expected to limit the gain of afferent transmission and negatively impact the functional connectivity between the PFC, ventral hippocampus and basolateral amygdala in an age-dependent manner. Our data also reveal that behavioral responses requiring PFC integration of hippocampal and amygdalar inputs mature through adolescence, which can be compromised by a deficient prefrontal $\alpha 7$ nAChR function as seen in psychiatric disorders exhibiting deficits in cognitive and affective domains (Young and Geyer, 2013; Parikh et al., 2016; Notarangelo and Pocivavsek, 2017). Future studies are warranted to identify the cholinergic origin underlying the input-specific, age-related recruitment of $\alpha 7$ nAChR transmission in the PFC, and its contribution to the maturation of prefrontal cognitive functions during adolescence.

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