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Late-adolescent onset of prefrontal endocannabinoid control of hippocampal and amygdalar inputs and its impact on trace-fear conditioning behavior

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Prefrontal cortex (PFC) maturation during adolescence is characterized by structural and functional changes, which involve the remodeling of GABA and glutamatergic synapses, as well as changes in the endocannabinoid system. Yet, the way PFC endocannabinoid signaling interacts with local GABA and glutamatergic function to impact its processing of afferent transmission during the adolescent transition to adulthood remains unknown. Here we combined PFC local field potential recordings with local manipulations of 2-AG and anandamide levels to assess how PFC endocannabinoid signaling is recruited to modulate ventral hippocampal and basolateral amygdalar inputs in vivo in adolescent and adult male rats. We found that the PFC endocannabinoid signaling does not fully emerge until late-adolescence/young adulthood. Once present, both 2-AG and anandamide can be recruited in the PFC to limit the impact of hippocampal drive through a CB1R-mediated mechanism whereas basolateral amygdalar inputs are only inhibited by 2-AG. Similarly, the behavioral effects of increasing 2-AG and anandamide in the PFC do not emerge until late-adolescence/young adulthood. Using a trace fear conditioning paradigm, we found that elevating PFC 2-AG levels preferentially reduced freezing behavior during acquisition without affecting its extinction. In contrast, increasing anandamide levels in the PFC selectively disrupted the extinction of trace fear memory without affecting its acquisition. Collectively, these results indicate a protracted recruitment of PFC endocannabinoid signaling, which becomes online in late adolescence/young adulthood as revealed by its impact on hippocampal and amygdalar-evoked local field potential responses and trace fear memory behavior.

INTRODUCTION

Major structural and functional changes occurring in the prefrontal cortex (PFC) during adolescence are thought to facilitate the maturation of adult cognitive abilities [1–4]. These developmental changes involve the remodeling of excitatory and inhibitory synapses in the PFC, which are somewhat dependent upon afferent drive from major limbic areas [5, 6]. Of particular interest are inputs originating from the basolateral amygdala and the ventral hippocampus, two limbic structures that relay emotionally salient and contextual information to the PFC, respectively [7–10].

We recently found that afferent drive arising from the ventral hippocampus and basolateral amygdala elicit distinct forms of PFC local field potential (LFP) responses in an age-dependent and input-specific manner [11, 12]. While the functional connectivity of amygdalar inputs to the PFC is fully established by postnatal day (P) 30, afferent evoked plasticity derived from hippocampal inputs does not fully emerge until P50 [11]. The latter is due in part to a protracted recruitment of PFC GABAergic and GluN2B transmission in the PFC by ventral hippocampal inputs after P45 [11–14]. Collectively, these events have been proposed to enable PFC maturation and its regulation of behavior [6, 15].

Among the different modulators of synaptic activity in the PFC, the endocannabinoid system is of relevance as its key signaling components (2-arachidonoyl glycerol—2-AG—and anandamide) also undergo major developmental changes during the adolescent transition to adulthood [16–19]. Through the cannabinoid receptor type 1 (CB1R), both 2-AG and anandamide provide a powerful presynaptic inhibitory regulation of cortical excitatory and inhibitory transmission [20]. Of note, PFC levels of anandamide steadily increase by almost 3-fold from early to late adolescence, while the trajectory of 2-AG shows an overall reduction [16, 18]. Parallel to these distinct changes in 2-AG and anandamide are the downregulation of CB1R expression in the PFC and its inhibitory control of excitatory synaptic transmission during adolescence [17]. Thus, it is conceivable that the impact of these developmental events within the endocannabinoid system are linked, which are expected to emerge during the adolescent transition to adulthood and modulate key afferent processes underlying PFC maturation [21, 22].

The goal of the present study is to determine the extent to which local endocannabinoid signaling in the PFC regulates afferent transmission in vivo, and whether such modulation changes over the course of the adolescent transition to adulthood. To this end, we conducted LFP recordings and behavioral testing combined with pharmacological manipulations of PFC 2-AG and anandamide levels to reveal how local endocannabinoid signaling impacts ventral hippocampal and basolateral amygdalar inputs

and their control of trace fear conditioning behavior. This behavioral construct was chosen because it requires the integration of amygdala and hippocampal inputs [23–27] and of its sensitivity to changes in synaptic activity associated to PFC maturation [28–30].

METHODS AND MATERIALS

All experimental procedures were approved by the University of Illinois at Chicago and the Rosalind Franklin University Institutional Animal Care Committee in accordance with NIH guidelines. Upon arrival, male Sprague Dawley rats (Envigo, Indianapolis, IN) were group-housed (3 per cage), kept under constant temperature (21–23 °C), humidity, and light/dark cycle with food and water available *ad libitum*, and allowed to habituate for at least 7 days before receiving any experimental manipulation. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for JZL184 and URB597, which were obtained from Tocris Bioscience (Ellisville, MO).

In vivo local field potential (LFP) recordings in the PFC

All procedures were conducted as previously described [11]. Briefly, rats were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic apparatus (ASI Instruments, MI), and maintained at 37–38 °C using a Physitemp TCAT-2LV Controller (Physitemp Instruments, NJ). Supplemental 8% choral hydrate (400 µl/h, i.p.) was delivered for the duration of the recording session through a cannula attached to a minipump (BASi, West Lafayette, IN).

All LFP recordings were conducted in the medial PFC (mainly within the prelimbic region) using a concentric bipolar electrode (SNE-100 × 50 mm; Rhodes Medical Instruments Inc., Summerland, CA) attached to a 28G cannula to enable local delivery of artificial cerebrospinal fluid (aCSF) alone or aCSF containing the CB1R agonist WIN-55,212-2, the monoacylglycerol lipase (MAGL) inhibitor JZL184 or the fatty acid amide hydrolase (FAAH) inhibitor URB597. In some recordings, the GABA-AR antagonist picrotoxin was included to isolate the glutamatergic component of the hippocampal transmission whereas the CB1R inverse agonist AM251 was used to determine whether the effects resulting from the enzyme inhibitors are CB1R mediated. All drugs were dissolved in 0.02–0.09% DMSO.

Another concentric bipolar electrode (NE-100 × 50 mm; Rhodes Medical Instruments Inc., Summerland, CA) was lowered into the basolateral amygdala or ventral hippocampus to elicit single LFP every 15 s through a computer-controlled pulse generator (Master-8 AMPI, Jerusalem, Israel). After a period of stable recording following PFC infusions (0.8–1.0 µL, 0.1 µL/min) of aCSF alone or in combination with one of the cannabinoid modulators, a protocol of high-frequency stimulation (HFS) (4 trains of 50 pulses each at 100 Hz) was delivered into the ventral hippocampus or basolateral amygdala to elicit changes in the slope of the evoked LFP (measured from the onset to the peak amplitude of the LFP response) and normalized to baseline values as previously described [11]. The timing used for the PFC infusion of the CB1R agonist WIN was around 15 min whereas for the enzyme inhibitors (JZL184 and URB597) and related aCSF controls was 50 min prior to the delivery of the HFS. Each data point before (baseline) and after the HFS was computed by averaging the slope value of eight evoked LFP responses (i.e., 2 min window). Thus, all time-course plots shown in the figures were generated using a bin size window of 2 min (i.e., mean slope value from 8 LFP responses per data point) normalized to baseline values (i.e., 10 min prior to the delivery of the HFS protocol).

Trace fear conditioning and extinction following PFC infusions of endocannabinoid enzyme inhibitors

Ten days prior to behavioral testing, rats were anesthetized with 5% isoflurane mixed with oxygen (Somnosuite Unit, Kent Scientific, CT), placed in a stereotaxic apparatus (Kopf, Tujunga, CA) and the skull exposed for placement of infusion cannulas targeting the prelimbic PFC at a 25° angle, as previously described [29]. Constant level of isoflurane (2–3%) was delivered during the surgical procedure using a Somnosuite Unit with the body temperature kept within the 37–38 °C range (TCAT-2LV controller heating pad, Physitemp). After securing the guide cannulas with acrylic cement (Stoelting Co., IL), a 33G dummy cannula was screwed into the guide cannulas until the day prior to fear conditioning testing, when the dummy cannulas were replaced by those protruding 0.5 mm beyond the tip of the guide cannulas. Thus, all PFC infusions (50 min prior to behavioral testing) were delivered using a 33G cannula protruding 0.5 mm beyond the tip of the guide cannula as previously described [29].

We implemented a trace fear conditioning protocol [28–30] from which all testing chambers (Ugo Basile) were housed in sound-attenuating cabinets with white noise (60–70 dB; Scientific Design). Briefly, the conditioning phase consists of a 120 s habituation period followed by five trials of pairing a neutral tone (10 s, 1500 Hz, 85 dB) with a footshock (1 s, 0.4 mA) delivered at a delay of 20 s from the end of the tone using a pseudorandom inter-trial interval of 240–280 s (ANY-Maze, Stoelting). The extinction phase begins 24 h later in a visually and tactilely distinct chamber. Following 120 s of habituation, rats were exposed to 14 trials of 60 s each from which the conditioned tone was presented for 20 s without footshock. An infrared camera connected to a computer was used to record the behavioral changes and the time spent freezing (lack of non-respiratory movement >0.5 s) from trial to trial (% freezing) was measured offline as previously described [28–30].

Histology

All animals were euthanized at the end of the experiments and the exact location of all recording, stimulating, and infusion sites were determined by Nissl-stained sections as previously described [28–30].

Statistical analysis

Data were shown as mean ± SEM and differences among experimental conditions were considered significant when $p < 0.05$. Student t test was used to reveal group effects involving a single continuous variable whereas 2-way repeated-measures ANOVA was applied to determine significant effects along three or more variables (StatSoft, Tulsa, OK).

RESULTS

We first examined the effects of the CB1R agonist WIN on prefrontal cortex (PFC; prelimbic region) LFP responses elicited from the ventral hippocampus using a protocol of HFS that is sensitive to reveal age-related changes in PFC plasticity [11, 12, 30]. Typically, the impact of ventral hippocampal HFS on prefrontal LFP begins to emerge after P45 as assessed by changes in the slope of the evoked response from the onset to peak amplitude as previously described [11]. Relative to aCSF controls, PFC infusion of WIN (3 µM) failed to disrupt the pattern of LFP in P30–44 rats (Fig. 1A, B). However, the characteristic LFP suppression observed in the P65–95 age group was no longer apparent following PFC infusion of WIN (Fig. 1C). Such inhibitory effect of WIN begins to emerge around P50 (Supplementary Fig. 1A, B). In addition to LFP suppression, ventral hippocampal HFS can also elicit a form of LFP potentiation in the adult PFC when local GABA-AR is blocked [11, 31]. In the presence of picrotoxin (GABA-AR blocker), a developmentally regulated GluN2B-mediated LFP potentiation becomes apparent in the PFC after P50 [12]. Interestingly, this sustained LFP facilitation is also sensitive to PFC infusion of WIN such that the amplitude/slope of the ventral hippocampal-evoked response is gradually reduced to 50% of the initial potentiation (Fig. 1D). Of note, this inhibitory effect of WIN also begins to appear around P50 (Supplementary Fig. 1C, D). Similarly, prefrontal LFP responses elicited from the basolateral amygdala are also sensitive to CB1R stimulation. As shown before [11], basolateral amygdalar HFS induces a pattern of sustained LFP facilitation in the PFC that is already enabled by P30 (Fig. 1E–G). Accordingly, PFC infusion of WIN (3 µM) diminished the level of LFP potentiation in both P30–44 (Fig. 1F) and P65–95 (Fig. 1G) age groups. Together, these results indicate that activation of CB1R in the PFC can change the gain of ventral hippocampal and amygdalar inputs by limiting the impact of HFS-induced suppression and facilitation of LFP transmission.

We next asked which endocannabinoids are recruited following hippocampal and amygdalar HFS stimulation in adult rats by comparing the impact of PFC infusions of JZL184 (2 µM) vs. URB597 (2 µM), two enzyme inhibitors of MAGL and FAAH which are capable of hydrolyzing 2-AG and anandamide, respectively. Typically, HFS of the ventral hippocampus elicits a pattern of sustained LFP suppression in the PFC (as seen in aCSF controls) that was no
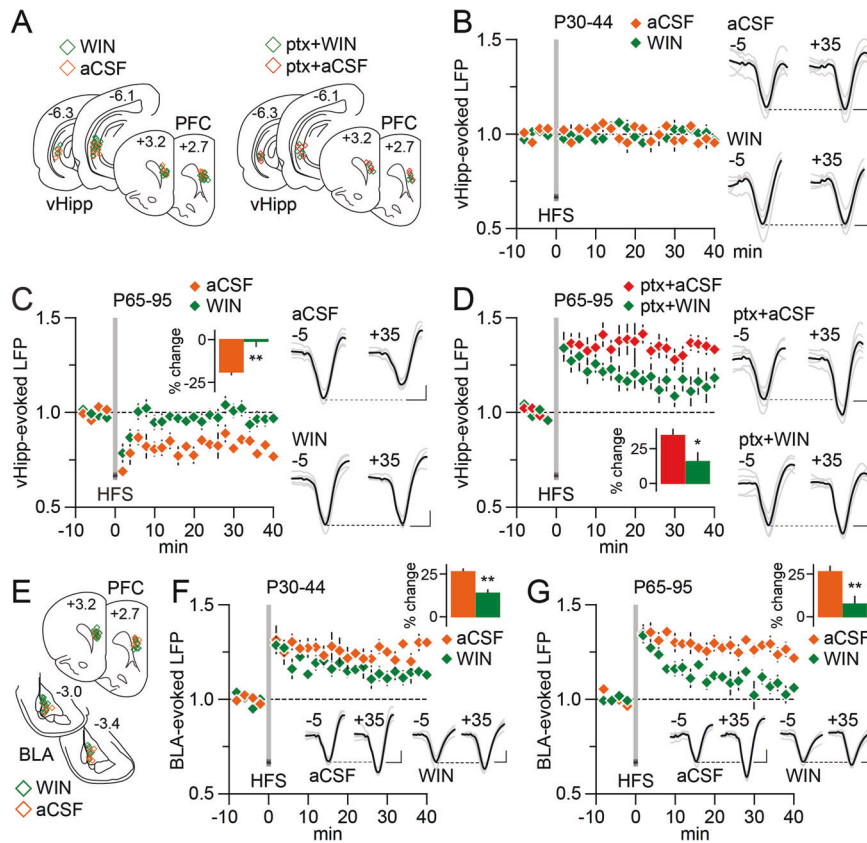
Fig. 1 PFC infusion of the CB1R agonist WIN limits the impact of ventral hippocampal and basolateral amygdalar-evoked LFP responses.
A Ventral hippocampal (vHipp) stimulation and PFC recording sites. Green: WIN and picrotoxin (ptx) + WIN; Orange: aCSF; Red: ptx + aCSF. **B** PFC infusion of WIN (3 μM; n = 8) does not alter the pattern of LFP response observed in P30–44 rats (aCSF, n = 9) following high-frequency stimulation (HFS) of the ventral hippocampus. **C** In adult rats (P65–95; aCSF, n = 6), ventral hippocampal HFS typically elicits a sustained pattern of GABA-AR-mediated LFP suppression in the PFC that is no longer observed following infusion of WIN (n = 7). Inset: mean LFP response from the last 10 min post-HFS (**$p < 0.005$, unpaired t test). **D** Ventral hippocampal HFS can elicit a pattern of sustained LFP potentiation in the PFC of adult rats (P65–95) when local GABA$_A$R are blocked (ptx + aCSF, n = 8). This potentiation is mediated by a developmentally regulated GluN2B transmission in the PFC [11] that also attenuated following prefrontal infusion of WIN (ptx + WIN, n = 7). Inset: mean LFP response from the last 10 min post-HFS (*$p < 0.05$, unpaired t test). **E** Basolateral amygdalar (BLA) stimulation and PFC recording sites. Green: WIN; Orange: aCSF. **F** Relative to aCSF controls (n = 8), PFC infusion of WIN (n = 8) in P30–40 rats reduced the amplitude of LFP potentiation elicited by basolateral amygdalar HFS. Inset: mean LFP response from the last 10 min post-HFS (**$p < 0.005$, unpaired t tests). **G** Similarly, PFC infusion of WIN in adult rats (n = 6) markedly attenuated the potentiated amygdalar-evoked LFP (aCSF, n = 5). Inset: mean LFP response from the last 10 min post-HFS (**$p < 0.005$, unpaired t tests). NOTE: All example LFP traces shown are from 5 min pre-HFS (−5′) and 35 min post-HFS (+35′). Calibration: 3 mV/25 ms.

longer observed following PFC delivery of the MAGL inhibitor JZL184 (Fig. 2A, B). PFC Co-infusion of JZL184 with the CB1R antagonist AM251 at a concentration that did not elicit any disruption when administered alone (green line in Fig. 2C; see Supplementary Fig. 2A) effectively re-established the normal pattern of sustained LFP suppression, indicating that the effect of 2-AG is mediated by CB1R (Fig. 2C). Similarly, PFC elevation of anandamide by local delivery of the FAAH inhibitor URB597 also abolished the hippocampal-induced LFP suppression, a response that was reinstated following PFC co-infusion of AM251 (Fig. 2D–F). On the other hand, the GluN2B-mediated LFP facilitation elicited by hippocampal HFS (Fig. 1D; see [12]) was only sensitive to PFC infusion of JZL184 (Fig. 3A, B). This effect was also reinstated when AM251 (which did not elicit any disruption when administered alone; green line in Fig. 3C; see Supplementary Fig. 2B) was co-infused into the PFC (Fig. 3C). Of note, PFC elevation of anandamide with URB597 failed to disrupt the pattern of hippocampal-induced LFP facilitation (Fig. 3D–F). Together, these results show that activation of CB1R is required to enable the inhibitory action of 2-AG and anandamide in the PFC. The data also reveal that PFC 2-AG signaling is preferentially recruited by the glutamatergic

component of the LFP potentiation whereas the GABA component of the PFC response is sensitive to both 2-AG and anandamide.

We next examined whether a similar endocannabinoid regulation of LFP emerges in the PFC following HFS of the basolateral amygdala. To test this, the extent of amygdalar-evoked LFP was assessed following PFC infusions of JZL184 (2 μM) or URB597 (2 μM). Relative to aCSF controls, PFC delivery of JZL184 markedly attenuated the level of amygdalar-evoked LFP facilitation (Fig. 4A, B). This inhibitory action of JZL184 was no longer observed when the CB1R antagonist AM251 (which did not elicit any disruption when administered alone; see Supplementary Fig. 2C and green line in Fig. 4C) was co-infused into the PFC (Fig. 4C). Interestingly, PFC infusion of URB597 did not disrupt the pattern of basolateral amygdalar-evoked LFP potentiation (Fig. 4D–F). These results indicate that 2-AG signaling is preferentially recruited by basolateral amygdalar inputs, which in turn limit the gain of amygdalar-PFC transmission through a CB1R-dependent mechanism.

At the behavioral level, we implemented a trace-fear conditioning paradigm in adolescent and adult rats paired with local infusions of JZL184 and URB597 to determine how PFC elevations
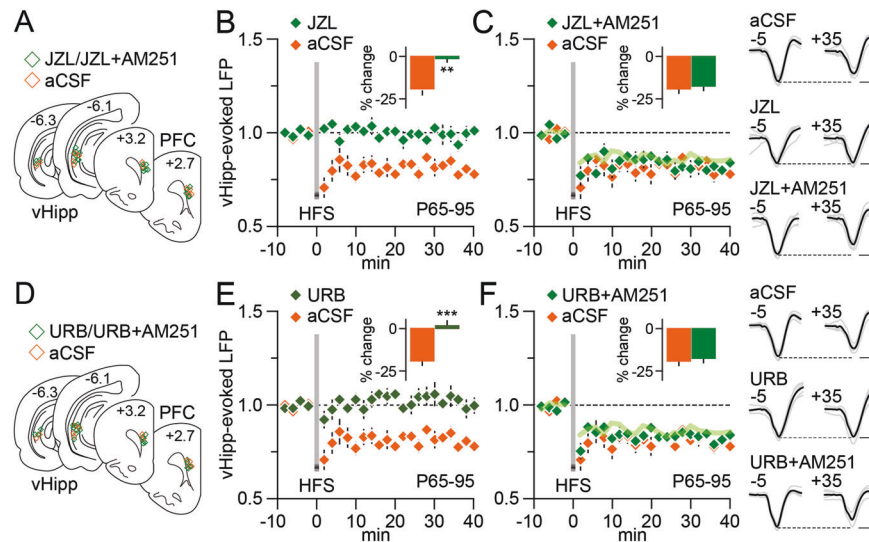
Fig. 2 PFC infusion of JZL184 and URB597 blocks the LFP suppression elicited from the ventral hippocampus. A Ventral hippocampal (vHipp) stimulation and PFC recording sites. Green: JZL and JZL + AM251; Orange: aCSF. **B** Ventral hippocampal high-frequency stimulation (HFS) elicits a pattern of LFP suppression in the PFC of adult rats (P65–95; aCSF, n = 6) that is not apparent following PFC infusion of JZL (2 µM, n = 5). Inset: mean LFP response from the last 10 min post-HFS (**$p < 0.005$, unpaired t test). **C** A normal pattern of LFP suppression re-emerged in the PFC when AM251 (2 µM) was co-infused with JZL (JZL + AM251, n = 6). PFC infusion of AM251 alone (green line) does not alter the normal pattern of LFP suppression (see Supplementary Fig. 2A). Inset: mean LFP response from the last 10 min post-HFS. **D** Ventral hippocampus (vHipp) stimulation and PFC recording sites. Green: URB and URB + AM251; Orange: aCSF. **E** The distinctive pattern of ventral hippocampus-evoked LFP suppression (aCSF, n = 6) is no longer observed following PFC infusion of URB (2 µM, n = 5). Inset: mean LFP response from the last 10 min post-HFS (***$p < 0.0005$, unpaired t test). **F** PFC inclusion of URB along with AM251 (URB + AM251, n = 7) restored the normal pattern of LFP observed following ventral hippocampus HFS. Data from PFC infusion of AM251 alone (green line, same as in (**C**)) was included for comparison (see Supplementary Fig. 2A). Inset: mean LFP response from the last 10 min post-HFS. NOTE: All example LFP traces shown are from 5 min pre-HFS ($-5'$) and 35 min post-HFS ($+35'$). Calibration: 3 mV/25 ms.
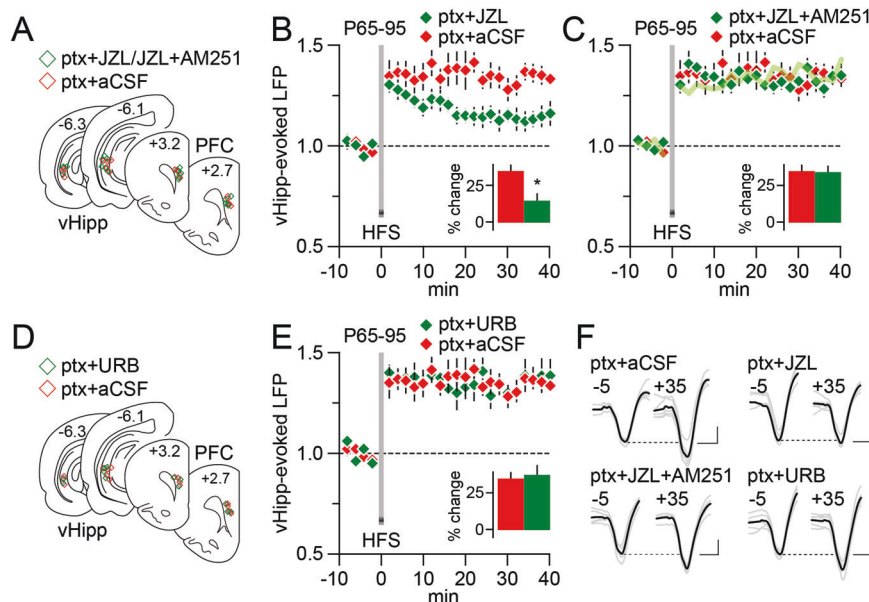

Fig. 3 PFC infusion of JZL184 (but not URB597) diminishes the LFP potentiation elicited from the ventral hippocampus. A Ventral hippocampal (vHipp) stimulation and PFC recording sites. Green: picrotoxin (ptx) + JZL and ptx + JZL + AM251; Red: ptx + aCSF. **B** PFC infusion of JZL along with picrotoxin (ptx + JZL, n = 6) diminished the amplitude of LFP potentiation (ptx-aCSF, n = 8) elicited by ventral hippocampal high-frequency stimulation (HFS). Inset: mean LFP response from the last 10 min post-HFS (*$p < 0.01$, unpaired t test). **C** This inhibitory effect was blocked when AM251 was co-infused with JZL into the PFC (ptx + AM + JZL, n = 6). Data from PFC infusion of ptx + AM251 alone (n = 5, *green line*; see Supplementary Fig. 2B) was included for comparison. Inset: mean LFP response from the last 10 min post-HFS. **D** Ventral hippocampal (vHipp) stimulation and PFC recordings sites. Green: picrotoxin (ptx) + URB; Red: ptx + aCSF. **E** Infusion of URB along with picrotoxin into the PFC (ptx + URB, n = 5) did not disrupt the normal pattern of hippocampal-induced LFP potentiation (ptx + aCSF, n = 8). Inset: mean LFP response from the last 10 min post-HFS. **F** Example traces of LFP taken from 5 min pre-HFS ($-5'$) and 35 min post-HFS ($+35'$). Calibration: 3 mV/25 ms.
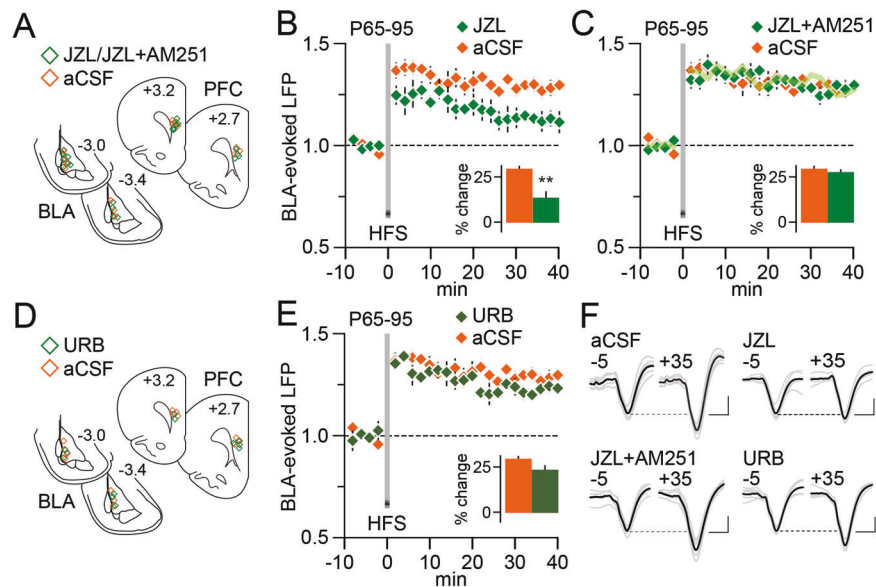
Fig. 4 PFC infusion of JZL184 (but not URB597) diminishes the LFP response elicited from the basolateral amygdala. A Basolateral amygdala (BLA) stimulation and PFC recording sites. Green: JZL and JZL + AM251; Orange: aCSF. **B** PFC infusion of JZL (n = 7) reduced the amplitude of LFP potentiation (aCSF, n = 7) elicited following high-frequency stimulation (HFS) of the basolateral amygdala. Inset: mean LFP response from the last 10 min post-HFS (**$p < 0.005$, unpaired t test). **C** This inhibitory effect was not observed when AM251 was co-infused with JZL (JZL + AM251, n = 6). Data from PFC infusion of AM251 alone was included for comparison (n = 5, *green line;* see Supplementary Fig. 2C). Inset: mean LFP response from the last 10 min post-HFS. **D** Basolateral amygdala (BLA) stimulation and PFC recording sites. Green: URB; Orange: aCSF. **E** PFC infusion of URB (n = 6) does not disrupt the amplitude of amygdalar-induced LFP potentiation (aCSF group, n = 7). Inset: mean LFP response from the last 10 min post-HFS. **F** Example traces of LFP from 5 min pre-HFS (−5′) and 35 min post-HFS (+35′). Calibration: 3 mV/25 ms.

of 2-AG and anandamide impact freezing behavior during the acquisition and extinction of conditioned fear memories. As observed before [30], adolescent rats (P37–44) typically exhibit lower freezing response than adults (P75–95) during the acquisition of trace fear conditioning (main effect of age from all aCSF groups, ***$p < 0.0001$; two-way ANOVA). Note that due to the recovery period needed from the cannula placement procedure (Materials & Methods) the age ranges included in the behavioral cohorts are more narrowed than those from the LFP recordings, which are still within P30–P44 and P65–P95 for adolescent and adult rats, respectively.

Data obtained from P37–44 rats (Supplementary Fig. 3A) revealed that PFC infusions of JZL184 (2 μM) or URB597 (2 μM) did not disrupt the level of freezing responses during the acquisition of trace fear conditioning or the pattern of cue-induced extinction on day 2 (Supplementary Fig. 3B, C). In contrast, PFC infusion of JZL184 in adult rats (Fig. 5A) markedly reduced the level of conditioned-freezing behavior (Fig. 5B). Twenty-four hours later, both aCSF- and JZL184-treated rats exhibited similar patterns of cue-induced extinction (Fig. 5C). On the other hand, the level of freezing response during the acquisition phase in adult rats is insensitive to PFC infusion of URB597 (Fig. 5D). Accordingly, aCSF- and URB597-treated rats showed similar patterns of cue-induced freezing behavior during extinction test on day 2 (Fig. 5E). Together, these results indicate that PFC 2-AG signaling can be recruited to modulate the acquisition of trace fear memory in adults.

We also examined the impact of PFC infusions of JZL184 (2 μM) and URB597 (2 μM) during the extinction of trace fear memory. Therefore, another cohort of adolescent and adult rats exhibiting similar levels of freezing behavior during fear conditioning were assigned to receive either aCSF, JZL184, or URB597 prior to extinction testing on day 2. Data obtained from P37–44 rats (Supplementary Fig. 3D) indicate that the level of freezing behavior during extinction testing remains unaltered following PFC infusions of JZL184 or URB597 (Supplementary Fig. 3E, F).

Similarly, PFC infusion of JZL184 in adult rats (Fig. 5F) failed to disrupt the typical pattern of freezing behavior on day 2 (Fig. 5G, H). However, a higher freezing response over the course of extinction trials was observed following PFC infusion of URB597 relative to the aCSF group in adults (Fig. 5I, J). Collectively, these results show that anandamide signaling in the PFC can be recruited to modulate freezing behavior during the extinction of cue-induced trace fear memory in adults.

DISCUSSION
The present study was conducted using male rats and it remains to be determined whether a similar age-related modulation of PFC inputs (within the prelimbic region) and associated behavior responses by local endocannabinoids occur in females. Nevertheless, our data obtained from male rats indicates a protracted recruitment of the PFC endocannabinoid signaling after P45 that becomes fully online by P65 to control PFC function in vivo. Once present, local PFC 2-AG and anandamide can limit the impact of ventral hippocampal drive whereas basolateral amygdalar inputs are preferentially inhibited by 2-AG. Behaviorally, PFC elevation of 2-AG reduced the level of freezing during the acquisition of trace fear conditioning without impacting its extinction. Conversely, enhancing anandamide in the PFC did not disrupt the acquisition of trace fear memory but increased the level of freezing behavior during its extinction. Collectively, it is conceivable that the recruitment of PFC endocannabinoid signaling begins after P45 to modulate the strength of ventral hippocampal and amygdalar inputs and associated behavioral responses. Future studies are needed to identify the precise late-adolescent period (P50–60) during which the PFC endocannabinoid system reaches its full modulatory potential in vivo.

PFC infusion of WIN revealed an age-dependent inhibitory control of ventral hippocampal inputs by CB1R that coincides with the recruitment of GABA and GluN2B transmission to sustain the characteristic pattern of LFP suppression and facilitation observed
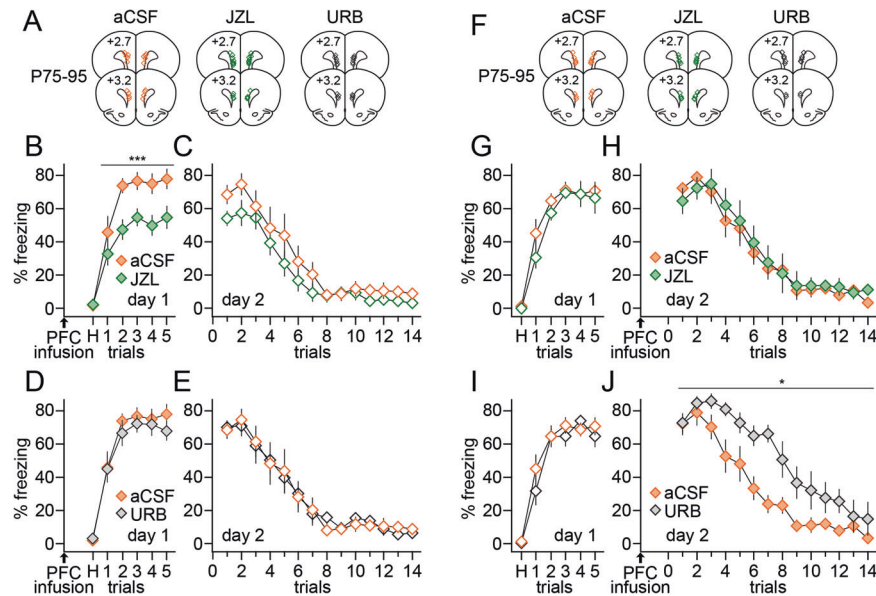
Fig. 5 Differential impact of PFC infusion of JZL184 and URB on the level of freezing response during the acquisition and extinction of trace fear memory in adult rats (P75-95). A Summary of the infusion sites within the PFC prior to the acquisition of trace fear conditioning. **B** Relative to aCSF controls (n = 9), PFC infusion of JZL (2 μM, n = 11) markedly diminished the freezing behavior during the acquisition of trace fear conditioning (main effect of treatment, $F_{1,18} = 10.6$, ***$p = 0.004$; main effect of trials, $F_{5,90} = 54.8$, $p < 0.001$; 2-way RM-ANOVA). **C** Rats that received JZL on day 1 did not exhibit a significantly lower level of freezing on day 2 (main effect of treatment, $F_{1,18} = 1.8$, $p = 0.2$, 2-way RM-ANOVA). **D** PFC infusion of URB (2 μM, n = 11) did not disrupt the normal pattern of freezing behavior observed in aCSF controls (n = 9) during the acquisition of trace fear conditioning. **E** Both aCSF- and URB-treated rats on day 1 showed similar patterns of freezing response to the conditioned tone during extinction testing on day 2. **F** Summary of the infusion sites within the PFC prior to the extinction of trace fear memory. **G** Freezing response during the acquisition of trace fear conditioning from adult rats assigned to receive aCSF or JZL on day 2. **H** PFC infusion of JZL (2 μM, n = 8) fails to disrupt the level of freezing behavior during extinction trials on day 2. **I** Freezing response during the acquisition of trace fear conditioning from adult rats assigned to receive aCSF or URB on day 2. **J** Relative to aCSF controls (n = 8), PFC infusion of URB (2 μM, n = 7) elicited a higher level of freezing response to the conditioned tone during extinction trials on day 2 (main effect of treatment, $F_{1,13} = 7.7$, *$p < 0.02$; main effect of trial, $F_{13,169} = 49.9$, $p < 0.001$; treatment x trial interaction, $F_{13,169} = 2.5$, $p < 0.005$; 2-way RM-ANOVA). In fact, a lower rate of extinction becomes apparent as the curve of freezing behavior was shifted to the right.

in the adult PFC [11, 12]. CB1R activation also reduced the strength of amygdalar inputs in the PFC, an inhibitory effect that is already enabled by P30 and becomes more robust after P65 as the NMDAR-mediated potentiation of the evoked LFP reaches its maximal level [11, 12]. This suggests that proper fine-tuning of CB1R signaling in the PFC is critical for the functional strengthening of key GABAergic and glutamatergic synapses that typically begin during adolescence [6, 15] and are needed for optimal control of input selectivity by the PFC [31, 32].

Despite the widespread expression of CB1R in cortical circuits [17, 33], our data indicate that the recruitment of 2-AG and anandamide signaling by afferent transmission in the PFC could be input and synapse specific via a CB1R-dependent mechanism. The effect of 2-AG on ventral hippocampal-driven LFP is likely due to inhibition of PFC GABA and GluN2B transmission, which emerge after P45 to sustain the HFS-induced LFP suppression and potentiation, respectively [11, 12]. In contrast, the inhibitory action of anandamide on ventral hippocampal transmission is limited to the GABA component of the PFC response as revealed by the preferential effect of the FAAH inhibitor URB597 on LFP suppression (Fig. 2) while the LFP potentiation remains intact (Fig. 3). PFC elevation of 2-AG, but not anandamide reduced the amplitude of amygdalar-evoked LFP potentiation (Fig. 4), which is also dependent on prefrontal NMDAR transmission [12]. Together, these results show that the impact of 2-AG (directly or indirectly) is not limited to excitatory synapses whereas anandamide's inhibitory action in the PFC becomes apparent only when local GABAergic transmission is recruited by afferents.

Previous studies have reported the presence of 2-AG synthesizing machinery at excitatory synapses and the ability of 2-AG to diminish PFC glutamatergic transmission [34, 35]. Similarly, the

2-AG degrading enzyme MAGL is also located at the presynaptic compartment of excitatory synapses [36, 37]. Therefore, the inhibitory action on LFP observed following PFC infusion of the MAGL inhibitor JZL184 could result from local suppression of glutamatergic transmission onto pyramidal neurons and GABAergic interneurons. In fact, PFC NMDAR transmission is needed to sustain the LFP facilitation elicited from ventral hippocampal and basolateral amygdalar stimulation [11, 12]. Furthermore, a presynaptic inhibition of glutamatergic inputs by 2-AG is expected to limit the recruitment of GABAergic activity in the PFC [22], which is developmentally regulated and required for sustaining the characteristic ventral hippocampal-evoked LFP suppression [11, 28, 29, 31]. Future studies are warranted to reveal the precise synaptic compartment underlying the inhibitory action of 2-AG in the PFC and whether a developmental shift in 2-AG levels is sufficient to disrupt the gain of PFC NMDAR and GABAergic transmission through adolescence.

Contrary to the impact of 2-AG on glutamatergic inputs, the inhibitory action of anandamide in the PFC is limited to the recruitment of local GABAergic activity without disrupting the NMDAR component of the LFP potentiation. Another distinction is the fact that the anandamide degrading enzyme FAAH is located at the postsynaptic compartment across different brain regions [38] including cortical pyramidal cells [39] and GABA interneurons [40]. It is therefore conceivable that the inhibitory effect of anandamide in the PFC occurs at local GABA synapses onto pyramidal output neurons or by limiting the strength of long-range excitatory inputs (i.e., ventral hippocampus) driving the activity of interneurons. While the proposed mechanisms are not mutually exclusive, the expression of FAAH within GABAergic interneurons in the PFC has yet to be determined. Finally, our

findings also imply that developmental changes in cortical anandamide levels could disrupt the trajectory of PFC maturation by preventing the gain of local GABA transmission that typically begins after P45 [15, 22].

At a behavioral level, the endocannabinoid system has been implicated in the regulation of conditioned fear memories [41–44]. Particularly, its role in freezing responses during the acquisition and extinction of trace fear conditioning is of interest due to the age-dependent nature of this behavioral paradigm [30]. Our study demonstrates that enhancing 2-AG in the PFC reduces the level of freezing behavior during the acquisition of trace fear conditioning only in adults (Fig. 5B). Such age-dependent modulation by 2-AG in the PFC could result from the inhibition of the NMDAR component of both ventral hippocampal- and amygdalar-evoked LFP potentiation as the underlying mechanism driving freezing behavior includes NMDAR signaling [45, 46]. Further supporting the view that PFC NMDAR plasticity is required for the acquisition of trace fear memory is the fact that freezing behavior during conditioning remained unaltered following PFC elevation of anandamide, which spared the NMDAR component of the LFP potentiation.

The behavioral impact of anandamide in the PFC becomes apparent in adult rats only during the extinction phase of trace fear conditioning as revealed by the enhanced level of freezing to the conditioned tone on day 2 of testing (Fig. 5J). Similar heightened freezing behavior can be found when PFC GABA function is compromised while the recruitment of NMDAR transmission by afferent drive is spared [28–31]. Accordingly, PFC elevation of anandamide preferentially disrupts the GABA component of ventral hippocampal-evoked LFP without disrupting the NMDAR-mediated potentiation of the response. These findings imply that any NMDA/GABA imbalance in the PFC could disrupt the normal pattern of freezing behavior during extinction of trace fear memory (see [30]). Further supporting a disinhibitory mechanism underlying the behavioral impact of anandamide are studies showing that PFC interneurons are recruited to facilitate the extinction of learned behaviors [27, 47, 48]. By limiting the gain or recruitment of GABA function in the PFC, the influence of anandamide during extinction leads to an imbalanced facilitation of NMDAR/glutamatergic transmission, a potential mechanism that could contribute to the anxiogenic effects of anandamide [49, 50]. This highlights the significance of maintaining proper levels of anandamide to enable the gain of GABA activity in the PFC, as it has the potential to dictate the trajectory of PFC maturation and its control of behavioral responses [3, 15].

While the mechanisms underlying the age-related changes within the brain endocannabinoid system remain unclear, it is conceivable that the distinct trajectories of CB1R, 2-AG, and anandamide levels in the PFC [16–18] are coordinated to impact the development of key afferent processes contributing to PFC maturation [21, 22, 51]. In this regard, a shift in PFC 2-AG levels is likely to exert a broader effect on excitatory synapses. On the other hand, PFC perturbations of anandamide levels during development are expected to impact the recruitment of GABA activity, which is known to undergo maturational changes [3, 15] and is key for regulating freezing behavior during the extinction of trace fear memory [28–30]. Future studies involving disruption of PFC anandamide and 2-AG levels during critical periods of brain development in male and female rodents are needed to assess whether the observed effects are linked to the maturation of the endocannabinoid system and sex specific and whether the underlying mechanisms by which homeostatic adaptations of synaptic activity are susceptible to endocannabinoids.

DATA AVAILABILITY
All data supporting the conclusions of this manuscript will be made available to any qualified researcher without undue reservation.

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AUTHOR CONTRIBUTIONS

Hanna M. Molla and Kuei Y. Tseng designed the study, wrote the manuscript, and prepared the figures. Hanna M. Molla performed all electrophysiological and behavioral experiments and data analyses under the supervision of Kuei Y. Tseng. Anabel M. M. Miguelez Fernández assisted Hanna M. Molla with the execution of the behavioral experiments.

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