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Experience-dependent plasticity of Locus Coeruleus glutamatergic synapses during the adolescence to adulthood transition

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Abstract

The prefrontal cortex (PFC) integrates emotion and cognitive control upon novel salient experiences, and it provides one of the main cortical glutamatergic inputs to the neuromodulatory nucleus Locus Coeruleus (LC), the major source of Norepinephrine (NE) for the entire forebrain. The LC is promptly recruited by salient and arousing stimuli, and through NE release, modulates the animals' internal states and behavior. While the role of LC-mediated NE release in target brain regions has been extensively studied, it remains to be established how LC activity is shaped by impinging PFC inputs. Adaptive changes at the PFC→LC synapse may occur upon exposure to salient novel experience, or during critical developmental periods for PFC maturation, like adolescence. During adolescence, the maturation of the PFC is protracted, and emotional responses are mainly driven by subcortical brain regions, such as the Central Nucleus of the Amygdala (CeA). Notably, CeA sends strong Corticotropin-releasing factor (CRF)-containing innervation to the LC; the contribution of this neuropeptidergic input to the modulation of PFC afferents across different post-natal developmental stages is unknown.

We combined neurophysiological, optogenetic, and behavioral approaches to probe the PFC→LC synapses at two different age-periods. Our data show that PFC→LC synapses undergo developmental changes in synaptic strength and long-term synaptic plasticity mechanisms. This is accompanied by differences in valence assignment to novel stimuli and divergent dynamic modulation of the Endocannabinoid signaling (eCB) in adolescents compared to adult mice. Finally, we provide evidence of developmentally regulated functional interaction between the CRF and the eCB signaling in the LC.

In summary, our results reveal a new mechanism of experience-dependent neuromodulated plasticity at PFC→LC synapses and uncover diverse molecular players involved in shaping these synapses during the transition from adolescence to adulthood. Our findings contribute to the understanding of how LC activity is regulated by cortical and subcortical inputs during different neurodevelopmental stages.

The Locus Coeruleus: a multifaceted nucleus in the brain stem

The Locus Coeruleus (LC) is the largest cluster of norepinephrine (NE) containing neurons located bilaterally in the brainstem (Poe et al., 2020) **(Figure 1)**. The LC belongs to a central NE system along with other smaller NE nuclei (A1, A2, A5, A7, A-for adrenaline) scattered throughout the pons and medulla (Fuxe, 1965).

Although comprising only a tiny population of cells (around 1600 unilaterally in the rodent brain (Swanson, 1976)), the LC provides NE to virtually the entire central nervous system (CNS). Massively arborized ascending projections expand across the whole forebrain, as well as descending fibers ramify throughout the brainstem and the spinal cord (Grzanna et al., 1977; Swanson and Hartman, 1975) **(Figure 1)**. Given its diffused projections, it was no surprise, that this tiny nucleus in the brainstem contributes to myriad brain functions. The LC activation has been linked to sensory gating (Devilbiss and Waterhouse, 2004), sleep-waking cycle (Aston-Jones and Bloom, 1981; Hayat et al., 2019), attention shifting (Aston-Jones et al., 1994), orienting reflex (Bouret and Sara, 2005), analgesia (Hirschberg et al., 2017), affective response to novelty (Gompf et al., 2010; Lustberg et al., 2020a), stress and anxiety (Borodovitsyna et al., 2018b; McCall et al., 2017; Reyes et al., 2015), fear conditioning and extinction (Giustino et al., 2019; Mueller et al., 2008; Uematsu et al., 2017).

Despite the wide palette of LC-NE dependent, sometimes even opposing (fear conditioning vs fear extinction (Uematsu et al., 2017)) functions, several observations lead to the consensus opinion that the LC is a homogenous structure, and NE transmission occurs uniformly across CNS neural networks. Considerable physiological evidence from intracellular membrane potential recordings, extracellular local field potential measures, and single-unit recordings proposed that LC-NE neurons exert synchronous firing patterns and globally broadcast NE across functionally diverse brain regions (Aston-Jones et al., 1997, 1986; Aston-Jones and Bloom, 1981; Berridge and Waterhouse, 2003; Chen and Sara, 2007; Finlayson and Marshall, 1988; Loughlin et al., 1982). In support of the homogeneous population synchrony of the LC nucleus, the functional diversity of LC-NE neurons was accounted for different compositions of postsynaptic NE receptors and spatiotemporal dynamics of NE release (Agster et al., 2013; Giustino and Maren, 2018). From a technical perspective, the small LC size has been an impeding factor to selectively target only LC nucleus without affecting nearby structures. Extensively branched LC-NE projections throughout the brain did not permit to isolate the NE effect on a single brain region (Schwarz and Luo, 2015). Finally, the lack of conventional synaptic contacts between the LC axon terminals and postsynaptic neurons suggested that NE release occurs via "volume" transmission", characterized by a non-synaptic or a paracrine neurotransmitter's diffusion within the extracellular fluid by varicosities along axons (Agnati et al., 1995). This knowledge lead to

the assumption of a non-specific release of NE within LC terminal fields. Only a decade later it was demonstrated, that NE release can also occur via synaptic release (Agster et al., 2013). Together, the aforementioned obstacles have made it challenging to establish whether diverse LC functions stem from anatomically and functionally diverse NE cells.

Even with these limitations, more comprehensive views emerged along with advanced experimental techniques uncovering the heterogeneity of the LC-NE system. The goal of the following chapter is to summarize the key features of the organization and function of the LC-NE nucleus, highlighting the diversity of this nucleus that extend beyond the outdated view of a homogeneous unit.

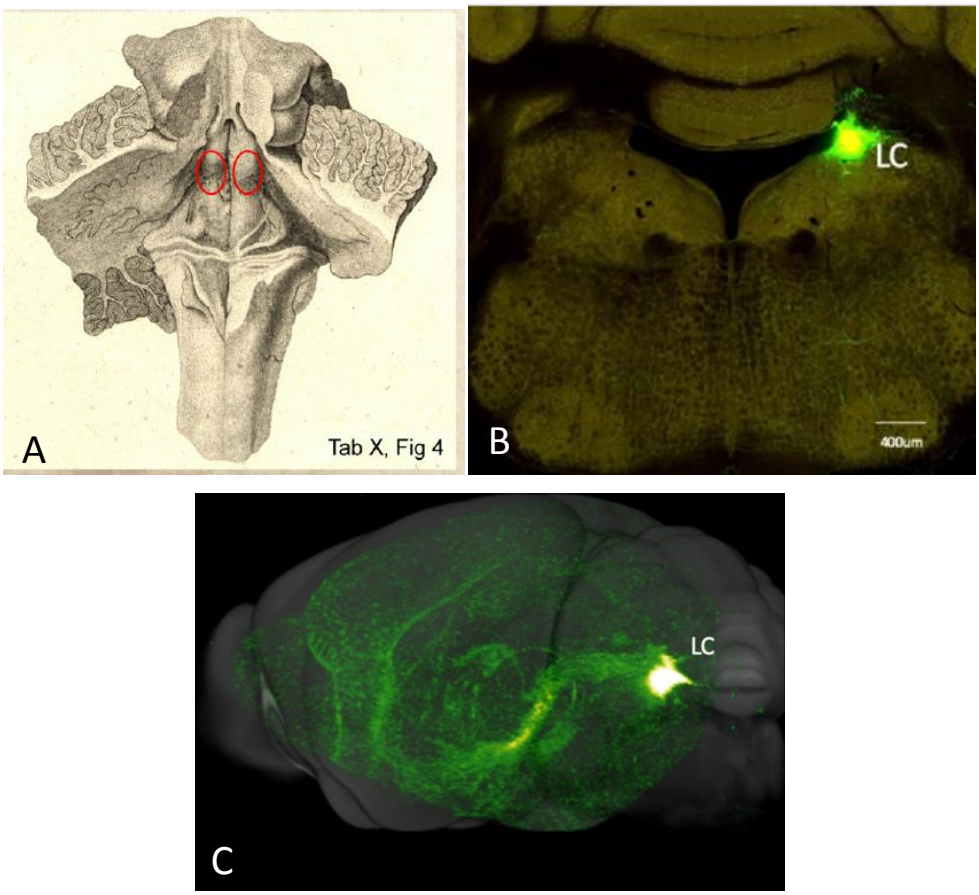

Figure 1. Location of the Locus Coeruleus. *(A) The early drawing of the human pons by Wenzel and Wenzel (1812) reporting a dark bilateral pigmented area on the roof of the pons, just under the 4th ventricle, named Loci caerulei. Red ovals highlight the LC. (B) Expression of the fluorophore in the right LC in a mouse brain. A Cre-dependent recombinant adeno-associated virus (AAV) expressing the fluorophore was injected into the LC of a cre-driver mouse in which Cre recombinase is under the control of Tyrosine Hydroxylase (TH) promoter. (Allen Brain Atlas Connectivity Project Experiment 511971714, TH-Cre_Fl172 mouse). (C) 3D reconstruction of LC widespread axonal projections by two-photon serial tomography. Modified from (Chandler et al., 2019).*

Morphological characteristics

The LC was first described as a dark band of tissue in the human brainstem (Swanson, 1976), located lateral to the wall of the fourth ventricle and medial to the mesencephalic trigeminal nucleus in the pons (Swanson, 1976). The location of the LC is relatively well conserved across mammalian species (Russell, 1955). The borders of the LC nucleus were originally defined by Nissl staining (Russell, 1955), and were later confirmed by catecholamine fluorescence (Fuxe, 1965). The LC-NE neurons can be identified immunohistochemically by antibodies directed against tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu, 1969), or dopamine-β- hydroxylase (DBH), the final enzyme responsible for NE synthesis (Axelrod, 1971) **(Figure 2).** The body of the LC nucleus is subdivided into three main parts: an anterior pole, a compact core that extends in the dorsoventral axis, and a posterior pole (Loughlin et al., 1986).

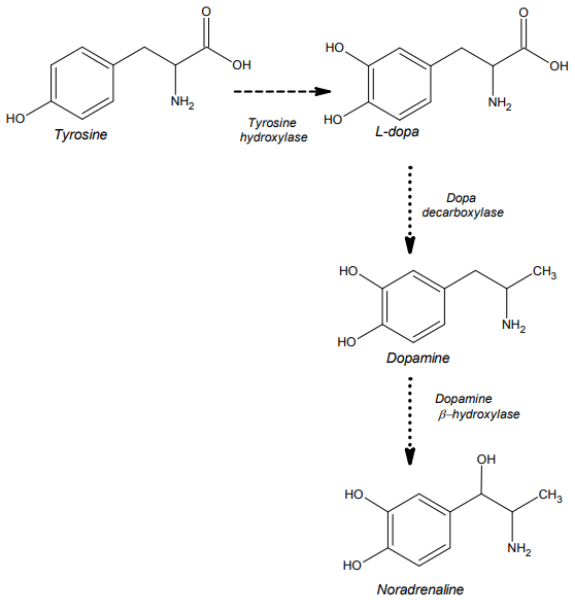

Figure 2. Biosynthesis of catecholamines. *Tyrosine hydroxylase (TH) hydroxylates tyrosine forming L-3,4-dihydroxyphenylalanine (L-DOPA) which in turn is decarboxylated to form dopamine via the catalysis of dopa decarboxylase. Dopamine β-hydroxylase hydroxylates dopamine to produce noradrenaline. Modified from (Costa et al., 2012).*

The diversity of LC cells was first described by early histological examinations (Cintra et al., 1982; Grzanna and Molliver, 1980; Swanson, 1976). Two types of approaches were used to unveil the morphology of LC neurons: Golgi impregnations and immunohistochemistry staining against enzymes for NE synthesis (Grzanna and Molliver, 1980; Swanson, 1976). In the aforementioned studies, it was soon noted that cells in the rat LC are a heterogeneous population (Cintra et al., 1982; Grzanna and Molliver, 1980; Swanson, 1976). Two major LC cell types were identified: 1) multipolar cells (~35 µm), predominantly located in the ventromedial part of the LC, with long dendrites radiating in all

directions and 2) fusiform cells (~20 µm), found in the dorsal division of the LC. The fusiform neurons have long dendrites that extend far beyond the nucleus (Grzanna and Molliver, 1980; Swanson, 1976) (**Figure 3).**

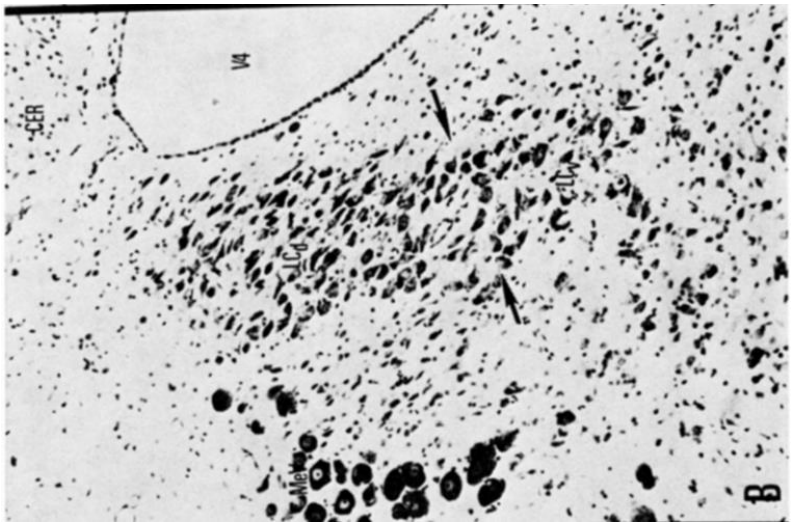

***Figure 3*. Photomicrograph from an early immunocytochemistry study of the rat Locus Coeruleus.** *Two types of LC cells were identified within the dorsal and ventromedial LC. Nissl staining. Modified from (Swanson, 1976).*

The third group of cells - small ovoid cells (~10 µm) - was identified within LC by Golgi staining (Cintra et al., 1982; Swanson, 1976). The small ovoid cells were not DBH^+, suggesting their non-monoaminergic origin. Indeed, later studies had identified the presence of GABAergic interneurons intermingled within and surrounding LC-NE neurons (Breton-Provencher and Sur, 2019a; Jin et al., 2016).

Each morphologically defined subpopulation of LC cells exhibits preferred spatial distribution within the different zones of the nucleus and have distinct efferent projections. However, neither spatial distribution nor morphologic class alone can completely characterize LC-NE efferent projections (Loughlin et al., 1986). (**Figure 4).**

The morphological diversity of LC has been also described in the human brain. As many as four types of LC-NE neurons were observed based on TH^+ and DBH^+ immunostaining, including small and large multipolar, large elliptical bipolar, and small ovoid bipolar neurons (Chan-Palay and Asan, 1989). These cell groups possess topographical organization with large multipolar cells located preferentially in the rostral LC (Chan-Palay and Asan, 1989). Interestingly, in Alzheimer's disease patients, there is a greater loss of LC-NE neurons in the rostral LC compared to other parts of the nucleus (Chan-Palay and Asan, 1989; Marcyniuk et al., 1986).

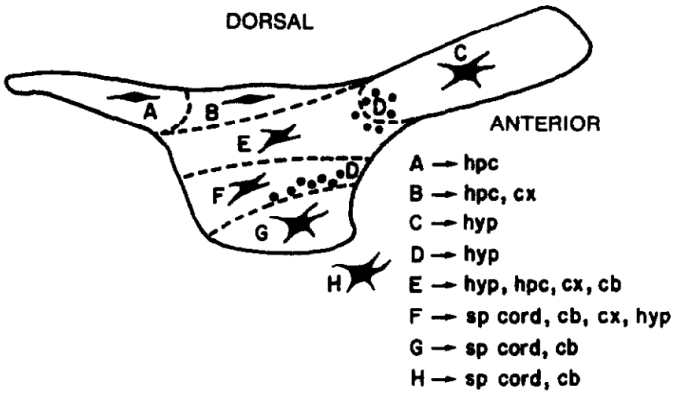

Figure 4. A Sagittal schematic of LC indicating the morphological subpopulations of LC cells with their location and preferred efferents in rodents brain. *Cb Cerebellum; cx, cortex; hpc, hippocampus; hyp hypothalamus; sp spinal cord. Modified from (Loughlin et al., 1986).*

Neurotransmitters of LC neurons

Initially, the LC-NE nucleus was viewed as a one-neurotransmitter system with NE as LC's sole transmitter. Identification of multiple peptides and neurotransmitters co-localized with LC-NE neurons added a new perspective to the "homogeneous" noradrenergic nucleus. In addition to morphological differences, LC cells can be also subdivided into groups according to neuropeptides and neuromodulators that are co-expressed in LC-NE neurons.

Neuropeptide galanin is co-expressed in ~80% of LC neurons, mainly in the dorsal and central part of the nucleus (Holets et al., 1988) **(Figure 5)**. Galanin signaling has been linked to feeding behavior, nociception, active coping behaviors, stress, and anxiety (Karlsson and Holmes, 2006; Lang et al., 2015). To note, both the galaninergic and NE systems are implicated in stress response (Borodovitsyna et al., 2018a; Karlsson and Holmes, 2006). In a recent work by Tillage and colleagues (Tillage et al., 2020), the roles of the LC co-transmitters in stress behavior have been resolved. Authors demonstrated that NE and galanin in the LC nucleus mediate stress-induced anxiety behavior at different timescales - NE is necessary for the expression of acute stress-induced anxiety, whereas galanin regulates more persistent responses following stress termination (Tillage et al., 2020).

The neuropeptide Y (NPY) is the second major neuropeptide co-expressed in 40% LC-NE of neurons, preferentially located at the dorsal part of the nucleus (Xu et al., 1998) (**Figure 5)**. NPY signaling exhibits an anxiolytic effect (Comeras et al., 2019). The co-existence of NPY and galanin within the same LC-NE neurons has been reported but the anatomical distribution or functional significance of these neurons has not been addressed so far (Xu et al., 1998).

Neuropeptides can be released locally within LC and modulate LC neuron's electrophysiological properties. For example, galanin and NPY have been shown to hyperpolarize LC-NE neurons by increasing conductance through potassium channels (Illes and Regenold, 1990; Pieribone and Aston-Jones, 1991). Nonetheless, these small molecules could directly act on LC target brain regions (Domschke et al., 2010; Lang et al., 2015) and/or modulate NE released locally within these regions (Rasmusson et al., 2000; Zini et al., 1993). Smaller subsets of LC-NE neurons co-express other neuropeptides as neurotensin (Jennes et al., 1982; Sutin and Jacobowitz, 1991), somatostatin (Finley et al., 1981), substance P (Léger et al., 1983), vasoactive intestinal peptide (Lorén et al., 1979), vasopressin (Caffé and van Leeuwen, 1983). Besides the vast expression of the neuropeptides, brain-derived neurotrophic factor (Castren et al., 1995; Conner et al., 1997), dopamine (Devoto et al., 2005; Koylu et al., 1999) were also reported to co-express in some LC neurons.

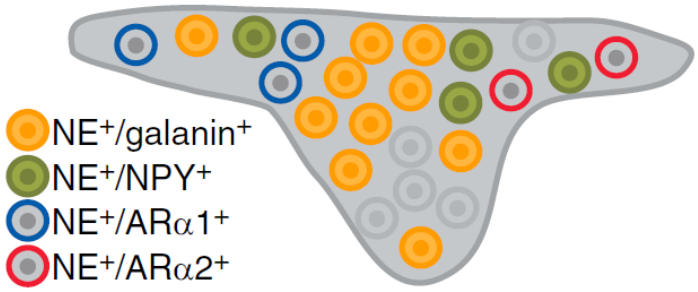

Figure 5. Molecular heterogeneity of LC-NE system. *Although all the LC neurons express NE, spatially defined clusters of LC-NE neurons co-release other peptides (galanin, NPY). Subsets of LC-NE neurons also show higher expression of certain adrenoceptors which may differentially regulate LC activity within anterior and posterior parts of the nucleus. Modified from (Schwarz and Luo, 2015).*

Receptors distribution in the LC

Along with numerous neurotransmitter molecules identified in the LC-NE neurons, the nucleus is also receptive to various neurochemicals as identified by broad receptors profile expressed within the LC (Schwarz and Luo, 2015). The different receptor expression may account for how individual LC neurons respond to homogenous input.

The LC-NE neurons contain several adrenergic receptors (AR) localized on their somatodendritic membranes in a rostrocaudal axis. The G-protein coupled α2-receptor (ARα2) is the most abundantly expressed ARs in the LC, preferentially distributed within the rostrocaudal axis (Chamba et al., 1991; Young and Kuhar, 1980). The ARα2 is coupled to the $G_{i/o}$ signaling cascade, thus the activation of this

receptor inhibits the cAMP production and leads to an autoinhibition of LC neurons. Less abundant AR found in the anterior LC is the α1-receptor (ARα1) (Chamba et al., 1991) **(Figure 5)**.

The two autoreceptors have an opposite effect on LC-NE neurons, where the ARα1 is responsible for LC excitation and ARα2 for inhibition (Nakamura et al., 1988). The activation of the ARα1 by its agonist cirazoline was shown to enhance the release of NE (up to 400% compared to the basal level) (Pudovkina and Westerink, 2005). Accordingly, the activation of ARα2 leads to an inhibition of LC firing and suppression of NE release (Aghajanian et al., 1977). Interestingly, low doses of NE applied iontophoretically to the LC result in excitation of the nucleus in newborn and juvenile rats (P1-34) mediated by ARα1. Meanwhile, high doses of NE, applied to the same age group of rats, resulted in inhibition of LC activity via ARα2. This suggests, that NE has a higher affinity for ARα1 as compared to ARα2 during early development, and later in adulthood ARα2 affinity for NE predominates over that of ARα1 (Nakamura et al., 1988).

Nicotinic cholinergic receptors (nAChRs) are also abundantly expressed within the LC. The application of acetylcholine and nicotine has been shown to depolarize LC-NE neurons and increase their firing rate (Egan and North, 1986). Single-cell reverse transcription-(PCR) experiments revealed two distinct subpopulations of LC-NE neurons according to nAChR subunit expression: small cells expressing high levels of α_3 and β_4 mRNA, and large cells expressing α_6 and β_3 subtypes (Léna et al., 1999). The two subpopulations of LC-NE neurons exhibit different magnitude of the responses upon nicotine application (Léna et al., 1999).

LC-NE neurons also express GABA-, galanin-, opioid-, endocannabinoid-, corticotropin-releasing factor-, orexin-, glutamate -receptors (Dubé and Marshall, 1997; Horvath et al., 1999; Luque et al., 1994; Ma et al., 2001; Mansour et al., 1994; Scavone et al., 2010; Smagin et al., 1996) but their spatial distribution within the LC has not been investigated yet.

As illustrated so far, LC-NE neurons are much more morphologically and molecularly diverse than their primary definition as NE-producing cells. Although many of these molecules were already reported decades ago, still little is known about their roles in the LC.

Heterogeneous LC-NE efferent pathways

Besides the molecular composition of LC neurons, the anatomical organization of the nucleus is crucial to unlocking its function during diverse behaviors. Nearly all brain regions receive LC-NE projections, which makes the LC nucleus a central neuromodulatory hub for the entire brain. Given the long list of brain regions that receive LC projections, describing them all would be out of the scope of this thesis. Therefore in the following chapter, I will focus on the most recent evidence describing heterogeneous LC modules.

Until recently, limited information was available about the organization of heterogeneous efferent projections, or if sub-populations of LC neurons are projecting differentially to specific targets. The use of retrograde tracers had facilitated addressing these questions. The injection of retrograde dye horseradish peroxide in brain regions receiving LC projections revealed the topographical organization of LC neurons according to their output targets (Mason and Fibiger, 1979). The LC neurons projecting to the hippocampus and septum are located in the dorsal LC, whereas neurons projecting to the cerebellum and the spinal cord are preferentially located in the ventral part of the nucleus. Along the anterior-posterior axis, hypothalamic projecting LC neurons are located anteriorly, thalamus-projecting cells posteriorly, and cortical and amygdala-projecting neurons are scattered throughout the nucleus (Loughlin et al., 1986; Mason and Fibiger, 1979). As mentioned in the chapter "Morphological characteristics", some of the output-dependent topography correspond with different LC cell types (Loughlin et al., 1986).

The knowledge of how the topographical LC organization relates to diverse LC functions became accessible along with the advances in viral tract tracing technologies. The catecholamine-selective synthetic promoter, PRS (Hwang et al., 2001), has been particularly helpful for dissecting functionally distinct LC subunits by selective optogenetic or chemogenetic activation of NE cells (Borodovitsyna et al., 2020; Hirschberg et al., 2017; Li et al., 2016). Hirschberg and colleagues (Hirschberg et al., 2017) used PRS-containing canine adenoviral vector (CAV) to dissect LC projections involved in analgesia and aversion-related behaviors. The CAV-PRS virus-containing chemogenetic actuator was selectively expressed in the spinal cord- or PFC-projecting LC neurons. The activation of spinally-projecting LC neurons produced a potent analgesic effect, while activation of PFC-projecting LC cells resulted in aversion and worsening of the pain phenotype (Hirschberg et al., 2017).
A similar strategy was used to dissect LC projections related to anxiety-like behaviors in response to stress (Borodovitsyna et al., 2020). Paired injections of a CAV-PRS virus encoding Cre recombinase at specific LC output sites (PFC or CeA) with Cre-dependent viral vectors containing inhibitory or

excitatory DREADDs into LC, allowed selective activation/inhibition of LC neurons projecting to mPFC of CeA. The CeA-projecting LC neurons promote anxiety-like behavior, whereas those innervating the mPFC promote exploration. The opposing LC efferent roles in mediating anxiety-like behavior are accompanied by changes in projection-specific LC cells excitability after acute stress experience (Borodovitsyna et al., 2020).

Another set of experiments allowed to distinguish the contribution of discrete LC projections to opposing learning states (Uematsu et al., 2017). Optogenetic activation of BLA-projecting ensemble promoted aversive learning by enhancing the association between a conditioned stimulus and unconditioned shock, while mPFC-projecting LC neurons facilitated extinction learning in absence of shock stimulus to enable flexible behavior. Inhibition of LC terminals in mPFC and BLA had the opposite effect: fear learning was reduced, whereas extinction learning got enhanced. Interestingly, the selective inhibition of LC terminals had a stronger effect on fear-conditioning related behaviors compared to the inhibition of the entire LC nucleus, suggesting that the manipulation of the whole LC may activate opposing behaviors and confound the interpretation of selected behaviors (Uematsu et al., 2017).

Because of the critical role NE plays in prefrontal cortical functions, extensive research has been dedicated to understanding NE-dependent modulation in this region. Depending on the behavioral state, the PFC-projecting LC ensembles were shown to mediate aversion to pain (Hirschberg et al., 2017), exploration of a novel environment (Borodovitsyna et al., 2020), extinction learning of a fearful stimulus (Uematsu et al., 2017), disengagement from ineffective behavioral strategy and improved application of new behavioral strategies (Cope et al., 2019), promote stochastic behavioral choices (Tervo et al., 2014). By using retrograde tracers in rats, Chandler and colleagues showed that segregated sub-populations of LC-NE neurons project to the orbitofrontal cortex (OFC), medial prefrontal cortex (mPFC), and anterior cingulate cortex (ACC) (Chandler et al., 2014). These subpopulations of LC-NE neurons also express different mRNAs of glutamate receptors and exhibit diverse electrophysiological properties, with mPFC projecting LC cells being more excitable compared to LC cells projecting to cortical circuits involved in motor control (Chandler et al., 2014). In line with this idea, mPFC projecting LC axon's varicosities were shown to be denser compared to efferents ascending to the motor or somatosensory cortex (Agster et al., 2013). Recently developed method for mapping axon collaterals of genetically defined neuronal population (tracing axon collaterals (trAC)) method allowed to demonstrate that axons of LC-NE efferents have a primary major terminal field which is densely innervated, and widespread minor terminal fields targets with sparser axonal collaterals, than those in the primary region (Plummer et al., 2020). For instance, LC neurons

projecting primarily to the mPFC, are also branching throughout other cortical and subcortical brain regions (Plummer et al., 2020). Whether sparser collaterals found outside of the "primary" zones are coordinated in a functionally significant manner has not been investigated yet (Plummer et al., 2020).

In summary, the current *state-of-the-art* methods facilitated the discovery of clusters among LC neurons that are topographically organized in their axonal projections and regulate specific behaviors.

LC-NE modulation of global brain states

As described so far, there are at least some clusters of LC-NE neurons that project to specific target brain regions to exert a discrete coding. However, the capacity of targeted neuromodulation depends not only on projections specificity but also on how synchronized or desynchronized firing is at the population level (Chandler et al., 2019).

Early correlative studies proposed a global NE release based on LC firing modes, necessary for modulating global brain states as arousal and sleep/wake cycle (Aston-Jones and Bloom, 1981). Neurons of LC display phasic and tonic modes of discharge (Aston-Jones et al., 1980; Florin-Lechner et al., 1996). Phasic bursts of activity are characterized by few action potentials at 10-20 Hz often followed by suppression of spontaneous firing (200-700ms) (Clayton et al., 2004; Devilbiss and Waterhouse, 2011). Phasic discharge is elicited by novel or salient multimodal sensory stimuli or by top-down response-related signals from the prefrontal cortex (Aston-Jones and Bloom, 1981; Berridge and Waterhouse, 2003; Clayton et al., 2004). Tonic activity (0.5 - 10 Hz) (Graham and Aghajanian, 1971) results from the intrinsic peacemaking properties mediated by Na^+ current, Ca^{2+} activated K^+ conductance, and persistent Ca^{2+} current (de Oliveira et al., 2010). Tonic firing mode has been linked to global arousal levels and sleep/wake cycle (Breton-Provencher and Sur, 2019a; Carter et al., 2010; Foote et al., 1980). The tonic LC activity and performance of the tasks that require sustained attention follow an inverted-U relationship, described by the classic Yerkes-Dodson curve (Aston-Jones and Cohen, 2005). At low tonic firing mode, the performance in a given task is poor due to drowsiness and lack of alertness; at moderate tonic firing mode and phasic activation of goal-directed stimuli, the performance is optimal; at high tonic firing level, the performance drops again due to distractibility (Aston-Jones and Cohen, 2005) **(Figure 6)**.

The long-standing consensus that LC neurons fire as a synchronized population and provide global release of NE to alter global brain states (Foote et al., 1980) has been recently challenged by the work of Totah and colleagues (Totah et al., 2018). The authors used single-unit large-scale population

recordings of the LC of anesthetized rats. This technique consists of a high-density electrode recording array that allows isolating a large number of single units (52 single units were isolated in Totah and colleague's (Totah et al., 2018) work). The recording array is a silicone probe and contains 32 recording channels (Totah et al., 2018). The probe was positioned in the coronal plane allowing to isolate single units along the dorsal-ventral LC axis (Totah et al., 2018). The LC population activity was not correlated among single units, on contrary, only limited pairwise synchrony was found. Population spiking was absent even upon nociceptive stimuli, which previously were thought to evoke synchronous discharge of LC nucleus (Aston-Jones and Bloom, 1981). The sharp synchronicity was only found among LC neuron pairs, suggesting that activity correlation in the LC nucleus is limited and spatially confined (Totah et al., 2018). Furthermore, the identified ensemble pairs had similar efferent projections, as was assessed by antidromic activation. The oscillatory synchrony between the pairs occurred over submillisecond, tens of milliseconds, and milliseconds timescales that, authors speculate, may reflect gap junction connectivity, common afferent inputs, mesoscale signals across the brain, respectively (Totah et al., 2018). In summary, while global LC-NE neuromodulation has been proven to be synchronous by nature and provide the control over brain states relevant to arousal and behavior, the evidence of LC ensemble code suggest the existence of more targeted and fine forebrain modulation.

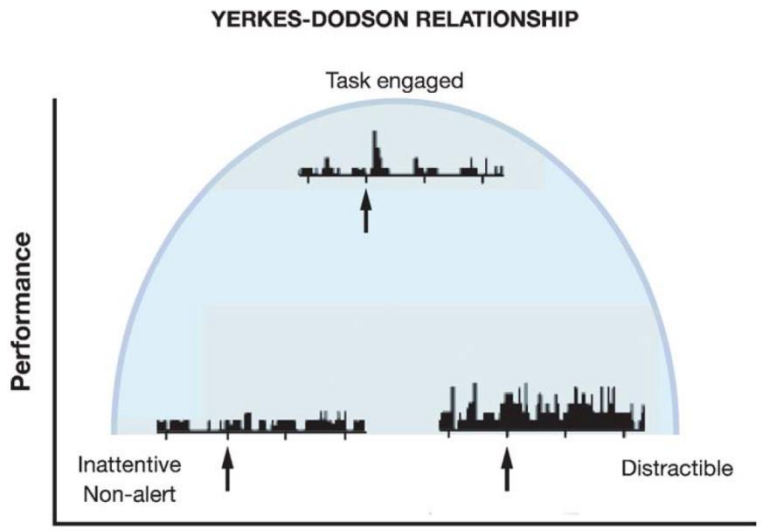

Figure 6. Inverted U-shape relationship between LC activity and performance on a task that requires sustained attention. *Performance is poor at very low levels of LC tonic discharge (indicated by the peri-stimulus histogram in black). Performance is optimal with moderate LC tonic activity and prominent phasic activation when exposed to salient stimuli (black arrow). Adapted from (Aston-Jones and Cohen, 2005).*

Afferent inputs

While extensive work has been dedicated to understanding the modularity of LC efferents, the integration of LC efferent clusters and LC afferent patterning has been largely ignored. To gain a better understanding of how LC functions as a cluster of modules, it is necessary to study how LC afferents integrate within LC microcircuitry and shape the efferent output (Poe et al., 2020).

The LC receives inputs from over 100 brain regions, as assessed in mice (Schwarz et al., 2015) **(Figure 7)**. The development of a viral-genetic tracing strategy called cTRIO, allowed trans-synaptic rabies virus tracing from a subset of neurons chosen by a cell type and their projection pattern (Schwarz et al., 2015). The study showed that the majority of LC neurons receive similar inputs irrespective of their efferent targets (Schwarz et al., 2015). In contrast, recent work by Yackle and colleagues demonstrated that selective LC afferents can induce different LC-mediated behaviors (Yackle et al., 2017). For instance, a sub-set of pre-Botzinger complex neurons (Cdh9/Dbx1) drives the activation of LC neurons under mild arousal conditions of placement in a novel environment (Yackle et al., 2017), providing evidence that different behavioral situation may recruit specific LC afferents.

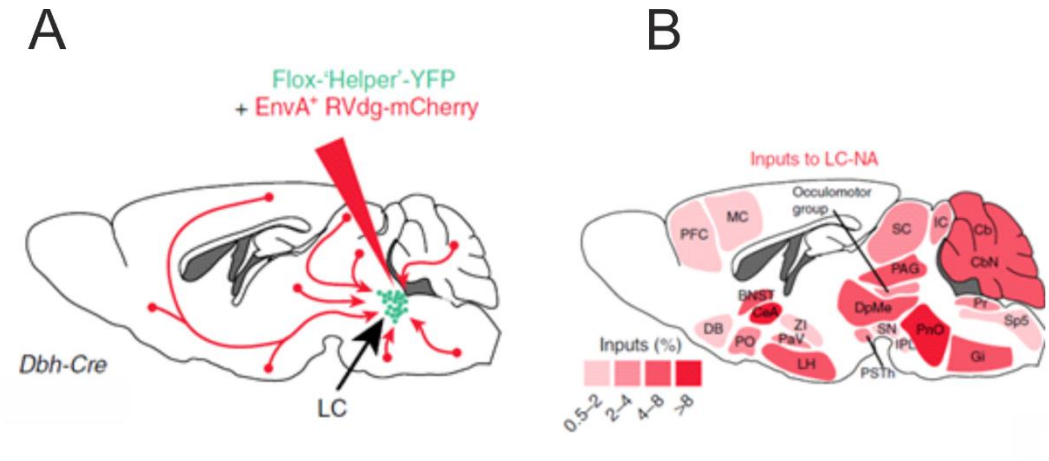

Figure 7. *(A) Schematic for targeting a pseudo-rabies virus to LC-NE neurons in a mouse line expressing Cre recombinase under the Dopamine Beta Hydroxylase promoter (Dbh-Cre). This allows targeting LC neurons as 'starter cells' for retrograde tracing. Only DBH$^+$ starter neurons will be virally infected and allow Rabies virion replication and spreading retrogradely to the mono-synaptically connected pre-synaptic neurons. (B)Transynaptically labeled neurons in various brain regions following the injection of Rabies virus in LC of Dbh-cre mice. LC receives projections from multiple bran regions. BNST, bed nucleus of the stria terminalis; Cb, cerebellum; CbN, cerebellar nuclei; CnF, cuneiform nucleus; DB, diagonal band; DpMe, deep mesencephalic nucleus; Gi, gigantocellular nucleus; IC, inferior colliculus; IPL, interpeduncular nucleus; LH, lateral hypothalamus; LHb, lateral habenular nucleus; MC, motor cortex; PAG, periaqueductal gray; PaV, paraventricular nucleus; PH, posterior hypothalamus; PnO, pontine nucleus; PO, preoptic nucleus; Pr, prepositus nucleus; PSTh, parasubthalamic nucleus; Rt, reticular nucleus; SC, superior colliculus;*

SN, substantia nigra; Sp5, spinal trigeminal tract; SPF, subparafascicular thalamic nucleus; SuM, supramammillary nucleus; ZI, zona incerta. Modified from (Breton-Provencher and Sur, 2019a).

The inputs to LC from diverse brain regions may induce a different effect on LC-NE output depending on where on the nucleus synaptic connections are made. The LC microcircuitry consists of a dense core, enriched by LC-NE cell bodies and their processes, and a pericoeruleus (peri-LC) zone, where LC-NE dendrites extend and ramify (Shipley et al., 1996). A full schematic summarizing the currently known topography and neurochemical identity of LC inputs to the core and peri-LC regions is shown in **Figure 8**. The dendritic contacts in the peri-LC region have been mostly examined from CeA, nucleus tractus solitarius (NTS), and paragigantocellularis nucleus (PGi) inputs (Van Bockstaele et al., 1998, 1996). The CeA terminals were detected in the dorsolateral peri-LC zone where they mostly form symmetrical contacts with TH^+ dendrites. Labeled CeA terminals often made asymmetrical synapses with TH^- dendrites, suggesting that CeA afferents might indirectly modulate the excitatory output of nuclei that project to the LC. This theory was later confirmed by showing that Corticotropin-release factor (CRF), a neuropeptide released in LC by large proportion by the CeA, dose-dependently regulates glutamatergic transmission in LC (Prouty et al., 2017). The NTS and PGi axonal endings were found to innervate LC in the peri-LC zone, but in contrary to CeA inputs, formed symmetrical and asymmetrical synapses with TH immunoreactive dendrites (Van Bockstaele et al., 1998, 1996). As illustrated by the *ex-vivo* electrophysiology experiments in CA1 hippocampal pyramidal neuron slices, the incoming information may be transmitted more effectively if received at the distal part of the dendrites (Spruston, 2000). Accordingly, LC dendrites extending to the peri-LC zone might serve as a site of integration and amplification of various afferent inputs it receives. Along with distant LC inputs, a distinct population of GABA-ergic interneurons resides in the peri-LC zone where they exert local regulation of LC neuron activity (Breton-Provencher and Sur, 2019a).

As depicted in **Figure 8**, the LC nucleus integrates inputs from many brain regions along with various neurotransmitters and neuromodulators released on different parts of the LC dendritic tree. The role of many of these inputs is still unclear. For this thesis, in the following section, I will focus on the brain regions that provide glutamatergic and CRF innervations to the LC.

Glutamatergic synaptic transmission in the LC

The LC receives main excitatory glutamatergic inputs from the PGi (Ennis et al., 1992; Holloway et al., 2013) and the PFC (Arnsten and Goldman-Rakic, 1984; Aston-Jones et al., 1986). Additionally, co-release of glutamate along with neuropeptide orexin and CRF has been described in the literature (Henny et al., 2010; Peyron et al., 1998; Reyes et al., 2005). The PGi nucleus, located in the rostral ventrolateral medulla, is one of the strongest inputs to the LC (Aston-Jones et al., 1986; Schwarz et al., 2015) and activates a large majority (up to 80%) of LC neurons upon a single-pulse electrical stimulation (Ennis et al., 1992). The excitatory action of PGi on LC neurons is mediated via kainate receptors (Aston-Jones et al., 1986). The N-methyl-D-aspartate (NMDA) receptor antagonist, AP7, did not block the PGi stimulation evoked LC response, while the kinate receptor antagonist, kynurenic acid, and γ-D-glutamylglycine, completely abolished the excitatory transmission (Ennis and Aston-Jones, 1986). The PGi inputs to LC are associated with nociceptive sensory response (Chiang and Aston-Jones, 1993), opioid withdrawal-like behaviors (Kaeidi et al., 2015; Liu et al., 1999), autonomic functions as cardiovascular and respiratory control (Van Bockstaele and Aston-Jones, 1995).

The PFC sends the main cortical glutamatergic input to the LC (Breton-Provencher and Sur, 2019a). While LC efferent projections to the PFC have been extensively studied (Chandler et al., 2014; Giustino et al., 2019), relatively little is known about the role of PFC inputs to the LC. The Anterior Cingulate Cortex (part of the PFC) projections to the LC have been implicated in sustained arousal in novel environments (Gompf et al., 2010). Retrograde tracing by injection of the Cholera Toxin subunit B (CTb) into LC showed that ACC sends the most prominent inputs from those of the cortex. The neurons of the Prelimbic and Infralimbic Cortex were also retrogradely labeled, although less abundant (Gompf et al., 2010). The PFC projections target the peri-LC area, where dense LC dendrites reside (Lu et al., 2012; Luppi et al., 1995). Although according to retrograde rabies tracing studies, PFC inputs to the LC account only for up to 2% of total LC inputs (Breton-Provencher and Sur, 2019a; Schwarz et al., 2015), the PFC exerts a potent effect on LC neurons (Jodo et al., 1998; Sara and Hervé-Minvielle, 1995). The PFC excitatory transmission in LC is mediated via NMDA and non-NMDA receptors, since the NMDA receptor antagonist, AP5, and non-NMDA receptor antagonist, 6-cyano-2,3-dihydroxy-7-niroquiinoxaline (CNQX) significantly reduced PFC stimulation-induced response (Jodo et al., 1998). As demonstrated by Breton-Provencher (Breton-Provencher and Sur, 2019a) the PFC can have either a direct excitatory effect on LC neurons or inhibit via local GABA-ergic interneurons.

The nature and directionality of PFC-LC reciprocal connectivity were investigated by Eschenko and Totah (Eschenko et al., 2012; Totah et al., 2020). LC temporal relation to slow (1-2 Hz) PFC

oscillations was studied in non-anesthetized, naturally sleeping rats. Roughly 50% of LC neurons were time-locked to the cortical oscillation cycles, firing preferentially at the rising phase of the slow wave, which represents a transition from Down-to-Up state. Meanwhile, the PFC neurons fired only at the peak of the slow-wave. These results suggest that during slow-wave oscillations LC leads to PFC activity (Eschenko et al., 2012). Reciprocal PFC-LC connectivity should also allow the opposite, top-down control where PFC leads the LC activity. In urethane-anesthetized rats, Totah and colleagues recorded LC multi-unit activity (MUA) along with PFC single unit and local field potential (LFP) activity (Totah et al., 2020). The authors demonstrated cross-regional coupling between the power of PFC LFP oscillation within the high Gamma range (60-200 Hz) and the phase of 5 Hz oscillations in LC population spike rate. The PFC hGamma power increase preceded and was predictive of the 5 Hz LC-MUA oscillations. These results propose that increased PFC population synchrony in the hGamma range may be a sign of top-down control over LC neuron activity. Whether 5 Hz oscillation in the LC is physiological and has a functional significance in behaving animals remains to be established.

Neuropeptide CRF signaling in the LC

The neuropeptide corticotropin-releasing factor (CRF, also named corticotropin-releasing hormone) is one of the key integrators of neuroendocrine, autonomic, and behavioral responses to stress (Inda et al., 2017). LC-NE nucleus, a critical node in the stress response, is innervated by a number CRF containing structures, including CeA (McCall et al., 2015; Van Bockstaele et al., 1998), Paraventricular Nucleus (PVN) (Reyes et al., 2005), the Bed Nucleus of Stria Terminalis (BNST) (Van Bockstaele et al., 1998), the Barrington's Nucleus (Valentino et al., 1996) and the PGi (Valentino et al., 1992) **(Figure 8)**. During stress, CRF acts on LC via G_s-coupled CRF1R (Reyes et al., 2005), which activation stimulates adenylyl cyclase and ultimately leads to LC depolarization via cyclic AMP-dependent decrease in potassium conductance (Jedema and Grace, 2004). Moreover, the acute application of CRF on LC brain slices induces a dose-dependent effect on synaptic transmission. The CRF1 agonist (CRF) at 50μM concentration increases the amplitude and charge transfer of spontaneous excitatory postsynaptic currents (sEPSCs), while at higher concentration (200 μM) CRF significantly decreases excitatory synaptic transmission (Prouty et al., 2017). *In vivo*, CRF facilitates the shift of the phasic LC firing mode to a high tonic state, which blunts sensory stimulus-driven phasic firing (Curtis et al., 2012; Devilbiss et al., 2012) and promotes scanning attention and behavioral flexibility to adapt an optimal behavioral strategy for a given situation (Curtis et al., 2012; Snyder et al., 2012). Long-lasting CRF-mediated LC-NE adaptations after exposure to acute stress were also reported, as increased spontaneous LC-NE discharge rate and strong morphological changes in LC neurons (Borodovitsyna et al., 2018a).

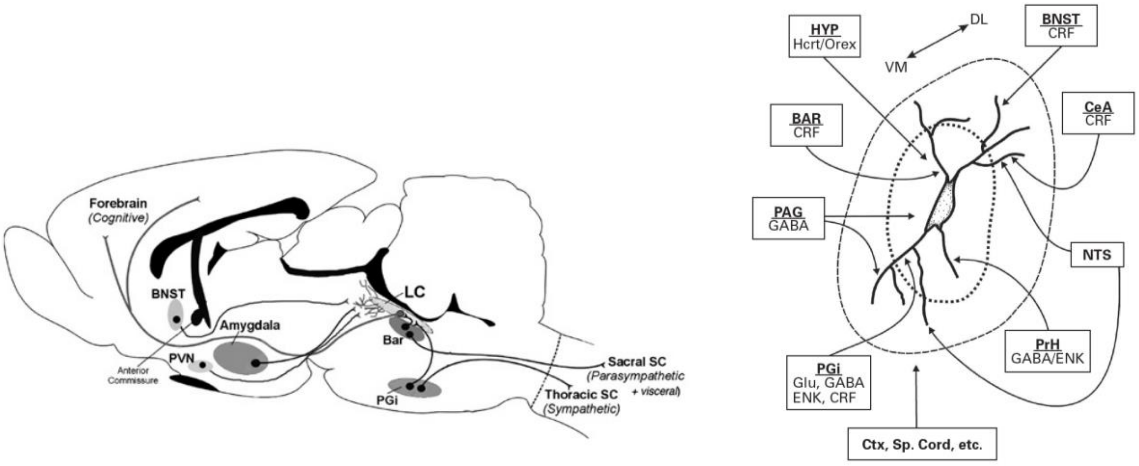

Figure 8*. Schematic depicting the localization of CRF afferents to the LC (left) and sites of termination in the locus coeruleus region (right). CRF neurons from Barrington's nucleus (Bar) and the nucleus paragigantocellularis (PGi) terminate in the nuclear core of the locus coeruleus. These nuclei also project to preganglionic parasympathetic and sympathetic neurons, respectively, and thereby may coordinate autonomic activity with cognitive functions. The central nucleus of the amygdala, bed nucleus of the stria terminalis (BNST), and paraventricular nucleus of the hypothalamus (PVN) terminate outside of the nuclear core in the peri-coerulear region where locus coeruleus dendrites extend (modified from Valentino et al. 2008).*

The terminals from CRF projecting brain structures to the LC are topographically organized. Afferents from PGi and Barrington's nuclei innervate LC core, CeA, and BNST inputs preferentially target rostrolateral peri-LC whereas PVN target both regions (Reyes et al., 2005; Valentino et al., 1996, 1992; Van Bockstaele et al., 2001) **(Figure 8)**. Diverse neurochemical organization of CRF inputs was also demonstrated. Immunohistochemistry studies show that 13% of CRF-containing terminals in the LC are from BNST and 35% from the CeA (Van Bockstaele et al., 2001), while in PGi only 8% of retrogradely labeled neurons were CRF^{+} (Valentino et al., 1992). The output from each CRF-containing nuclei is expected to impact LC activity differentially given the differential representation of CRF and synaptic profiles. Indeed, different CRF brain regions are engaged by distinct stimuli to activate LC. Barrington's nucleus, located adjacent to the LC, activates LC by non-noxious levels of colonic distention thus initiating a coordinated visceral response (Rouzade-Dominguez et al., 2001). The BNST preferentially mediates unconditioned fear responses (Walker and Davis, 1997). PVN is a primary driver of the hypothalamic-pituitary-adrenal (HPA) axis, which activation is pivotal for mediating central stress response (Herman and Tasker, 2016). So, while CRF released from PVN to LC initiates stress response (Valentino and Van Bockstaele, 2008), the role of CRF released in the LC by BNST and PGi has not been closely investigated yet.

CeA is one of the strongest extrahypothalamic CRF sources in the LC (Bouret et al., 2003; Reyes et al., 2008). CeA-CRF inputs to the LC are aversive (at 10 Hz stimulation) and induce anxiety-like behavior (McCall et al., 2015; Paretkar and Dimitrov, 2018), which mediates conditioned fear (Walker and Davis, 1997). This pathway is activated by various stressors including social defeat stress (Reyes et al., 2019) and hypotensive stress (Curtis et al., 2002). McCall and colleagues (McCall et al., 2015) have investigated the effect of CeA-CRF release on LC firing activity *in-vivo*. By using 10 Hz photostimulation of CeA-CRF terminals in the LC, the authors reported a heterogeneous population of LC responses. While a significant proportion of LC cells increased firing, an equal subset of putative LC neurons had shown a decrease in firing rate. Although previous works suggested that CeA to LC inputs are glutamatergic and co-releasing neuropeptides CRF and dynorphin (Reyes et al., 2008), in the more recent study authors did not detect any fast neurotransmission (neither excitatory nor inhibitory) by optogenetic stimulation of CeA-CRF terminals in the LC (McCall et al., 2015).

The understanding of the LC nucleus had undergone a dramatic shift since it was first discovered. The current knowledge of the LC-NE system integrates multiple levels of organization, including molecular/neurochemical variety, global NE release and ensemble activity patterns, and the modular anatomy of LC efferent pathways. Notably, abundant research has been dedicated to elucidating LC-NE efferent connectivity, while still little is known about the LC afferent patterning or their functional significance. For example, although the PFC is one of the main glutamatergic cortical input to the LC, the functional relevance of PFC to LC connection is still sparse. Neither is known how would PFC inputs integrate with other LC afferents or shape LC-NE output on its target brain regions.

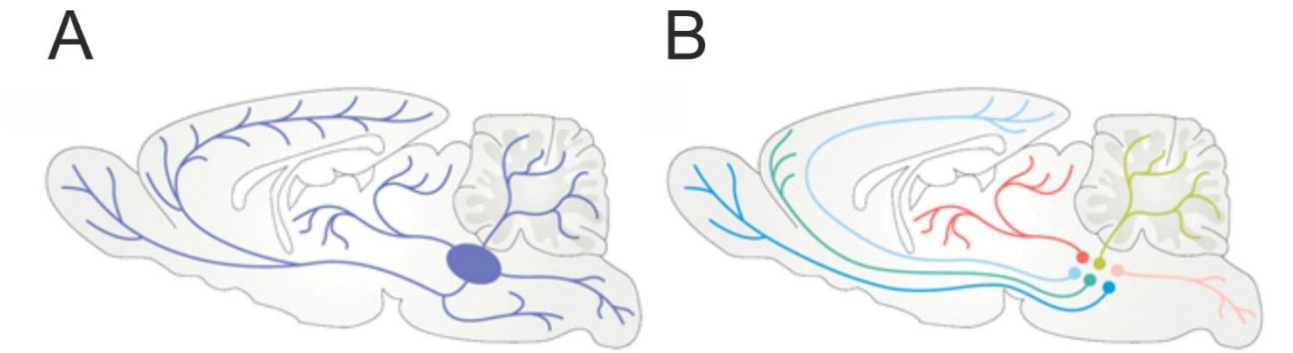

Figure 9. Changing views about the LC synaptic architecture and functional organization. *(A) Historically, the LC was viewed as homogeneous neurons containing structure (depicted here by uniform blue color), whose axons extensively collateralize and indiscriminately innervate functionally diverse brain regions. (B) As recent studies (Chandler et al., 2014; Hirschberg et al., 2017; Kebschull et al., 2016; Uematsu et al., 2017) indicate the axons of LC neurons are less extensive than was previously thought and innervate anatomically and functionally distinct brain target regions. In the schematic, each color represents a population of LC neurons that preferentially project to diverse terminal fields. Modified from (Poe et al., 2020).*

Plasticity of the Locus Coeruleus

Neuronal circuits are not hardwired and can be modified by experience throughout life (Hensch, 2004). The brain's ability to change and adapt to an ever-changing environment is known as synaptic plasticity (Brzosko et al., 2019). LC-NE system gate and modulate synaptic plasticity in several target brain regions, including the hippocampus, visual cortex, olfactory cortex, amygdala (Hansen and Manahan-Vaughan, 2015; Huang et al., 2014; Johansen et al., 2014; Liu et al., 2017; Maity et al., 2016; Sullivan et al., 1989). However, little research has been dedicated to establishing whether and how LC neurons undergo plastic changes.

LC-NE neurons are functional already at birth, yet some of their physiological features undergo maturation process, including changes in receptor coupling (from predominantly gap-junctions to synaptic connections), membrane properties, receptor expression levels, and responsiveness to sensory stimuli (Marshall et al., 1991). In anesthetized newborn rats, LC response is enhanced to innocuous sensory stimuli, whereas in later developmental stages LC neurons appear to be more selective to noxious stimuli (Nakamura et al., 1987). Such enhanced responsiveness in LC of neonatal rats may underlie enhanced infantile learning capabilities (Caldji et al., 1998). Some electrophysiological characteristics in LC neurons change during early development. The conduction velocity and synchronized spiking of LC-NE neurons of neonatal rats are slower compared to adults (Christie, 1997; Marshall et al., 1991; Nakamura et al., 1987). The gap-junctions, that are thought to underlie the high synchrony in LC neurons, disappear at P21 (Christie, 1997; Nakamura et al., 1987). Moreover, there is a transient expression of excitatory synaptic coupling among LC-NE neurons during infancy, which is temporarily mediated by ARα1, in contrast to persistent ARα2 inhibitory coupling (Christie, 1997; Ennis and Aston-Jones, 1986; Williams and Marshall, 1987). Collectively, these studies had demonstrated that LC neurons undergo plastic developmental changes.

Only two documented studies were dedicated so far to assessing synaptic plasticity changes in the LC. First, performed by Martins and Froemke (Martins and Froemke, 2015), investigated whether LC neuronal activity is affected by auditory experiences. The authors made *in-vivo* whole-cell recordings from rat LC while measuring its responsiveness to auditory stimuli. Auditory stimuli alone did not evoke any significant responses in LC neurons. However, the repetitive pairing of the auditory and nociceptive (foot shock) stimuli evoked LC response specifically to the paired tone. Moreover, the substitution of the nociceptive stimulus by LC electrical stimulation was enough to get an LC response to a specific paired auditory stimulus. This form of long-lasting auditory stimulus-driven plasticity was dependent on NMDA receptors, as the application of antagonist APV was enough to block the plastic

changes. The plastic changes of the LC induced further changes in primary auditory cortex (A1) responses and induced long-lasting improvements in auditory perception. This study was the first to demonstrate that LC plasticity can be instrumental for downstream plastic adaptations in LC target regions, like A1. This cascade of plasticity mechanisms between LC-A1 is also instrumental to shape the perception of the external stimuli (Martins and Froemke, 2015).

The second study aimed to test the effect of a single cocaine administration on glutamatergic transmission in the LC (Zhu et al., 2017). The authors showed that a single cocaine administration increases the AMPA/NMDA ratio in LC excitatory synapses. AMPA/NMDA ratio is a measure of postsynaptic changes in synaptic strength and it can be used to infer whether long-term potentiation (LTP) or long-term depression (LTD) had occurred at postsynaptic glutamatergic synapses (Counotte et al., 2014; Kauer and Malenka, 2007). Cocaine administration leads to the insertion of new Ca^{2+} permeable AMPA receptors, as measured by decreased amplitude of EPSC recorded at -70mV (represents AMPAR component) upon application of a selective Ca2+ permeable AMPA receptor blocker. GluR2-lacking AMPA-receptors are Ca2+ permeable and display inward rectification (i.e. they show a reduced outward current at depolarizing membrane potentials). Inward rectification can be inspected by the rectification index (RI; AMPA current -70mV to that at +40mV), which is a measure of the AMPA receptor subunit composition, and it is used to indicate the presence of GluR2-lacking AMPARs (Bellone and Lüscher, 2006). Accordingly, the AMPA currents measured in cocaine-treated rats had a stronger inward rectification index compared to vehicle-treated animals, further supporting the cocaine-induced insertion of GluA2 lacking Ca2+ permeable AMPA receptors on the post-synaptic membrane. Finally, the authors demonstrated that antagonism of α1-adrenergic receptors before cocaine administration was sufficient to block the changes in AMPA/NMDA ratio and inward rectification. This suggests that α1-adrenergic receptors mediate potentiation of synaptic transmission upon a single cocaine administration (Zhu et al., 2017). Overall, this study provides evidence of the plastic properties of LC glutamatergic synapses.

In summary, the two studies described above confirmed that LC can undergo diverse forms of synaptic plasticity. The changes in LC plastic properties are closely related to life experiences. The LC-NE system is involved in myriad brain functions but still little is known about the importance of LC synaptic plasticity in modulating them. Neuroplastic changes of the LC might affect the NE release on its target brain regions and in turn influence plasticity mechanisms in multiple LC-NE efferent regions.

Adolescence: a critical period for neural system remodeling

Adolescence is a critical developmental period of physical, behavioral, and cognitive transition from childhood to adulthood (Larsen and Luna, 2018). During this period, adolescent individuals experience profound changes in brain architecture, sexual maturation, social reorientation from parents to peers, and novelty-seeking behaviors. The physical and behavioral manifestation of the adolescence period is conserved across mammalian species, facilitating the study of this developmental period in depth while using rodent models (Schneider, 2013; Spear, 2000). This developmental period is of particular interest because it is a time when major psychopathologies start to manifest (Larsen and Luna, 2018). Additionally, adolescence in both humans, and rodents is marked by heightened stress, anxiety-like behavior, and enhanced sensitivity of the hypothalamic-pituitary-adrenal axis (HPA axis) in response to stress (Spear, 2009).

During adolescence, neuronal circuitry and synapses are sculpted to scale up the developing brain to adult-like cognition from basic skills to more complex tasks involving executive functions (Eiland and Romeo, 2013). The core of neurobiological mechanisms that underlie developmental brain changes during adolescence are synaptic pruning and stabilization of synapses (Drzewiecki et al., 2016), increased inhibitory neurotransmission (Piekarski et al., 2017), myelination (Paus, 2010), increase in perineuronal nets (Gogolla et al., 2009), and establishment of local and long-range connections (Arruda-Carvalho et al., 2017). Together these neurobiological factors support reliable and stable higher-order function development during adolescence.

Developmental plasticity: Unbalanced development between PFC and Amygdala

The dynamic period of adolescence is marked by the final maturation phase of the PFC in humans and rodents (Gogtay et al., 2004; Laube et al., 2020; Monk et al., 2003; Van Eden and Uylings, 1985). The loss of gray matter density occurs in anterior-to-posterior trajectory, therefore maturation first takes place in sensorimotor areas and lastly in higher-order regions as the PFC (Gogtay et al., 2004) **(Figure 10)**. The PFC is one of the most functionally advanced areas of the associative cortex and mediates several higher-order cognitive functions, including decision-making, working memory, planning, reasoning, impulse control, organization, and execution of goal-directed tasks (Bossong and Niesink, 2010; Fuster, 2015). The acquisition of these higher brain functions depends on the development and maturation of the PFC, which involves diverse biological processes, including local and long-range network formation, integration of the neuromodulatory networks, myelination, pruning, increase in perineuronal nets, and activity-dependent synaptic plasticity (Dow-Edwards and Silva, 2017;

Koppensteiner et al., 2019; McDougall et al., 2018; Ueda et al., 2015). The proteomics analysis of adolescent mouse PFC indicates developmentally linked alterations in protein networks that regulate neuronal signaling, synaptic plasticity, and cellular organization/structure (Agoglia et al., 2017). Importantly, many of the proteins identified in Agoglia and colleagues study (Agoglia et al., 2017) were previously observed in the postmortem human adolescent cortex (Harris et al., 2009) highlighting the translational value of the mouse model.

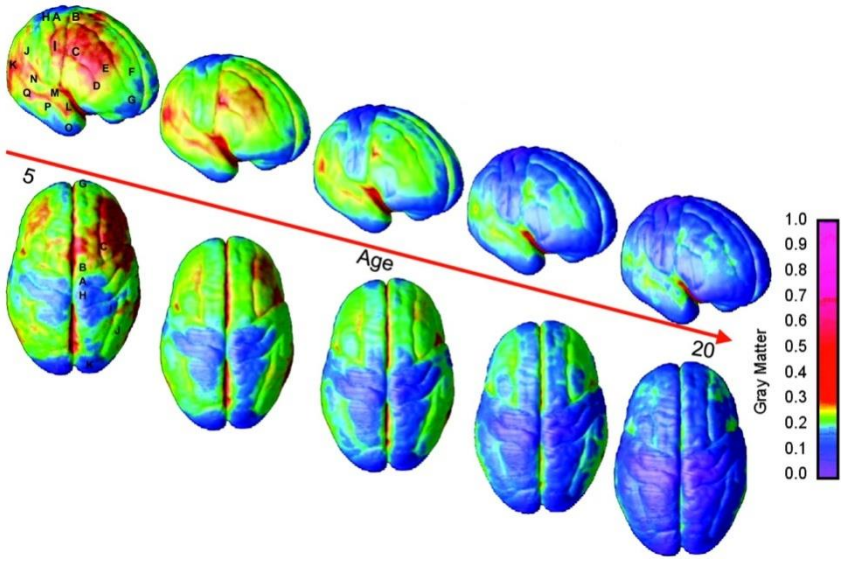

Figure 10*. The sequence of gray matter maturation over the cortical surface of the human brain (lateral and top views). The sidebar represents units of gray matter volume. Gray matter loss is associated with maturation of brain regions. Modified from (Gogtay et al., 2004).*

Amygdala, compared to the PFC, shows early structural and functional development (Gee et al., 2013; Giedd et al., 1996; Guyer et al., 2008; Hare et al., 2008; Payne et al., 2010; Ulfig et al., 2003). Amygdala is associated with emotional significance assignment to stimuli, it mediates affective responses, and emotional learning (Brenhouse et al., 2008). Amygdala-PFC projections arise before PFC-to-amygdala projections (Bouwmeester et al., 2002) and it continues developing throughout adolescence (Cressman et al., 2010; Kim and Richardson, 2009). In the context of emotion regulation, emotions can be generated from the 'bottom-up' (in response to inherently emotional perceptual properties of the stimulus) or 'top-down' (in response to cognitive processes.) (McRae et al., 2012). Accordingly, the ‘bottom–up’ and ‘top–down’ components of emotional evaluation may be mediated by different neural systems. Studies of emotional evaluation using fMRI have associated ‘bottom–up’ processing with the amygdala and ‘top–down’ processing with the orbitofrontal and ventromedial prefrontal cortices (Wright et al., 2008).

The developmental imbalance between the PFC and Amygdala results in less 'top-down' control over Amygdala by the PFC, resulting in greater impulsiveness, risk-taking, and other emotionally prompted behaviors in adolescence (Ernst et al., 2006).

Experience-dependent plasticity in adolescence

External stimuli play an essential role in brain maturation and shape the brain in a unique way for every individual. During adolescence, experience-induced patterns of neural activity promote a cascade of events that refine the initially coarse connectivity into refined circuits (Bossong and Niesink, 2010). Experience plays an important role in the strengthening and pruning of the synapses, dendritic, and axonal arbors (Tau and Peterson, 2010). This continuous process of neuronal network sculpting in response to experience is referred to as experience-dependent plasticity (Fandakova and Hartley, 2020).

The developmental plasticity of each brain region will be optimally refined if exposed to functionally relevant experiences for that brain area (e.g. visual input for visual cortex, auditory input for auditory cortex) (de Villers-Sidani et al., 2008; Prusky and Douglas, 2003). The PFC maturation is at its peak during adolescence, hence higher-order cognitive processes are the optimal prerequisites to drive the development of this brain region. Indeed, during adolescence, there is a shift to behavioral patterns and experiences that primarily engage the PFC and other associative cortexes (Larsen and Luna, 2018). Increased autonomous exploration, novelty-seeking, exploration of mating behavior, and heightened seek for reward are typical behaviors emerging during adolescence in both humans and rodents (Logue et al., 2014; Spear, 2000; Stansfield and Kirstein, 2006). These behaviors allow the accumulation of experiences that are distinct from previous developmental periods as they are more psychologically, socially, and cognitively demanding. As the complexity of these autonomous behaviors increase, the brain regions involved, as the PFC, become more engaged and able to meet the environmental demands (Larsen and Luna, 2018). Altogether, increasingly complex adolescent behavior drives the experience-dependent plasticity of the maturing brain.

Experience and neurobiology are interconnected; adolescents' experiences shape their developing brain, but also the neurobiological mechanisms dominating in this particular period can strongly influence the perception and behavior during new experiences (Hensch, 2005). An ever-changing environment constantly challenges individuals to attend to novel stimuli. As mentioned above, among all mammalian species, the adolescence period has been highlighted by novelty-seeking behavior (Spear, 2013, 2000; Stansfield and Kirstein, 2006). A novel environment or experience represents ambiguous valence since it has never been encountered before. Hedonic valence is the basic building block of emotions and refers to the degree to which something is pleasurable or aversive (Tye, 2018).

The concept of valence emerged from psychology a century ago and has more recently been applied in neuroscience to study specific neuronal substrates of the corticolimbic system (Beyeler et al., 2018; Tye, 2018). Valence can refer to a positive internal state (promoting approach behavior) or negative (supporting avoidance behavior) (Lustberg et al., 2020b; Russell, 1980; Tye, 2018). Does still developing adolescent brain process novelty differently compared to an already mature adult brain? Are there any brain regions that preferentially dominate this process during adolescence? These fundamental questions have been a major drive in the research activity I have performed.

Novelty processing in adolescence versus adulthood: focus on PFC to LC connection

Novelty processing is based on two main components: the motivational component which defines the engagement with a stimulus (whether it is salient/arousing) and the cognitive component which processes the information about the identity of a new stimulus (Petrulis et al., 2005).

The LC-NE system plays an important role in vigilance, arousal, and salience processing behaviors (Aston-Jones et al., 1994; Aston-Jones and Cohen, 2005; Breton-Provencher and Sur, 2019a; Carter et al., 2010; Vazey et al., 2018; Ventura et al., 2008). The LC is sensitive to contextual novelty as its firing rate increases in novel environments (Aston-Jones and Bloom, 1981; Vankov et al., 1995). Recently, Lustberg and colleagues (Lustberg et al., 2020b) demonstrated a critical role for LC-NE transmission in the expression of avoidance and approach behaviors in a novel environment. Interestingly, LC activation in response to novelty may not depend on NE signaling as DBH knockout and heterozygous mice showed similar levels of *c-fos* activation upon exposure to novelty (Lustberg et al., 2020a). In turn, lesions in the ACC (part of the PFC) reduced *c-fos* expression in the LC during exploration of a novel environment, suggesting that PFC inputs to LC might be involved (Gompf et al., 2010). Indeed, the PFC is associated with novelty detection (Berns et al., 1997) and processing (Løvstad et al., 2011), in both humans and rodents (Berns et al., 1997; Daffner et al., 2000; Dias and Honey, 2002; Løvstad et al., 2011). The PFC is one of the cortical circuits involved in valence assignment in a novel environment through the "top-down" mechanism of cognitive appraisal (Ochsner et al., 2002). Cognitive appraisal is defined as a perception or/and evaluation of a stimulus and assessment of the degree to which a stimulus is potentially stressful or threatening (Alhurani et al., 2018). Appraisal processes may be dominant in unfamiliar or ambiguous situations (Lazarus, 1999), where the valence of the stimulus is not clear. In rodents, the appraisal index relies on motor and associated physiological responses, like arousal, avoidance/approach, and defense behaviors (Britton et al., 2011).

During adolescence, the cognitive appraisal center PFC is still developing and emotional responses are mostly driven by the Amygdala without the supervision of cortical regions (Hare et al., 2008; Monk et al., 2003; Stephanou et al., 2016). Limited PFC regulation over Amygdala during adolescence has been documented by ex-vivo electrophysiology in rats (Selleck et al., 2018) and fMRI recordings in humans (Silvers et al., 2017, 2017). This negative connectivity is associated with greater sensitivity to emotional cues (Guyer et al., 2008), poor cognitive appraisal and emotion regulation (Gee et al., 2013; Guyer et al., 2008; Hare et al., 2008; Monk et al., 2003; Vink et al., 2014). In turn, the development of 'top-down' regulatory efficiency occurs linearly with PFC maturation throughout the adolescence period (Stephanou et al., 2016). The developmental shift in the functional connectivity of PFC-Amygdala was associated with the decline of amygdala reactivity. This may explain the age-related improvement in cognitive task performance and decline in anxiety (Gee et al., 2013).

Together, the immaturity of emotional regulation in adolescence may result from a combination of ‘bottom-up’ processing, which highlights the dominance of subcortical brain regions, such as the amygdala and immaturity of the PFC system, responsible for ‘top-down’ regulatory control (Somerville et al., 2010; Steinberg, 2008; Stephanou et al., 2016). Thus, novelty processing might be different between adult and adolescent individuals due to the unsynchronized maturation of the PFC and Amygdala. PFC inputs to the LC may facilitate the cognitive appraisal upon conscious re-evaluation of a novel situation in adults but not in adolescents.

Endocannabinoid neuromodulation of synaptic plasticity

Introduction

Behavior arises from the current activity state of brain networks. Neuronal networks underlying any higher-order behavior or cognition integrates animal internal states, such as motivation or attention, that shapes animal interaction with its surrounding environment. For example, in a classroom full of students, one can easily observe different behaviors shaped by various internal states: a motivated student will stay attentive and will carefully follow a lecture, meanwhile a non-motivated student may become drowsy and disengaged from what is happening in a classroom. Different internal states will have an impact on adaptation to environmental demands, memory, and learning processes. Increasing experimental evidence indicates that synaptic plasticity and neuromodulation are two brain mechanisms that together allow the dynamic brain integration of external cues and internal states.

Synaptic plasticity is the brain ability to make experience-dependent long-lasting changes in the strength of neuronal connections, whereas neuromodulation refers to changes in the functional properties of synapses, induced by the momentary release of long-range (NE, serotonin, acetylcholine, dopamine) or local signaling molecules (endocannabinoids, nitric oxide), named neuromodulators (Brzosko et al., 2019). Neuromodulators set the conditions for the induction of synaptic plasticity and shape its outcome (Blackwell et al., 2019; Brzosko et al., 2019; Foncelle et al., 2018; Gerstner et al., 2018; Melis et al., 2014).

At the cellular level, neuromodulators typically act via G-protein-coupled signaling pathways to alter neuronal excitability and spiking dynamics, modify ion channel activity and activate processes shaping synaptic strength (Choi et al., 2005; Melis et al., 2014; Scheiderer et al., 2004; Seol et al., 2007). Since neuromodulators receptors are coupled to intracellular signaling cascades, they can also influence nuclear transcription mechanisms involved in synaptic plasticity (Brzosko et al., 2019). Through the described mechanisms, neuromodulators can shape short-term and long-term synaptic adaptations.

For this thesis, I will focus on the lipid molecules endocannabinoids (eCBs), which play an essential role in the neural control of learning and memory by modulating the plasticity of long-range connected neuronal circuits.

Endocannabinoid system

The eCB system is a neuromodulatory lipid system, which consists of cannabinoid receptors CB1 and CB2, two major endogenous ligands, N-arachinolyl ethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), and a set of eCBs synthesizing and degrading enzymes (Freund et al., 2003; Marsicano and Lutz, 2006). Unlike canonical neurotransmitters, AEA and 2-AG are synthesized postsynaptically "on-demand", then in a retrograde fashion they travel to bind the presynaptically located CB1-receptors (CB1R) (Wilson and Nicoll, 2002). The lifespan of AEA and 2-AG in the synapse is regulated by uptake processes and intracellular hydrolysis by fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG-L), respectively (Hillard, 2000). CB1Rs are mainly expressed in neurons and represent the most abundant G-protein-coupled receptors in the CNS (Herkenham et al., 1990). CB2-receptors (CB2R) are mainly located in peripheral and immune cells (Munro et al., 1993; Pertwee, 2005), although there is evidence about CB2R presence in neurons of the brainstem (Atwood et al., 2012). CBRs are generally coupled to $G_{i/o}$ proteins, which activation leads to an inhibition of adenylyl cyclase, activation of potassium channels, and inhibition of voltage-gated calcium channels (Howlett et al., 2002). Since CB1R is primarily localized presynaptically to axon terminals, the activation of CB1R leads to suppression of neurotransmitter release (Kano et al., 2009) **(Figure 11)**. This unique feature of eCBs to act as a retrograde messenger represent the fundamental means for a postsynaptic neuron to fine-tune the synaptic gain from its afferents, thus shaping the transmission of behaviorally relevant information (Freund et al., 2003; Melis et al., 2014). However, there is evidence suggesting a non-retrograde eCBs signaling, that regulates the neuronal function and synaptic transmission by targeting transient receptor potential vanilloid receptor type 1 (TRPV1) and CB1Rs located at the postsynaptic site to provide an autocrine signal (Bacci et al., 2004; Grueter et al., 2010; Marinelli et al., 2008; Musella et al., 2014; Puente et al., 2011).

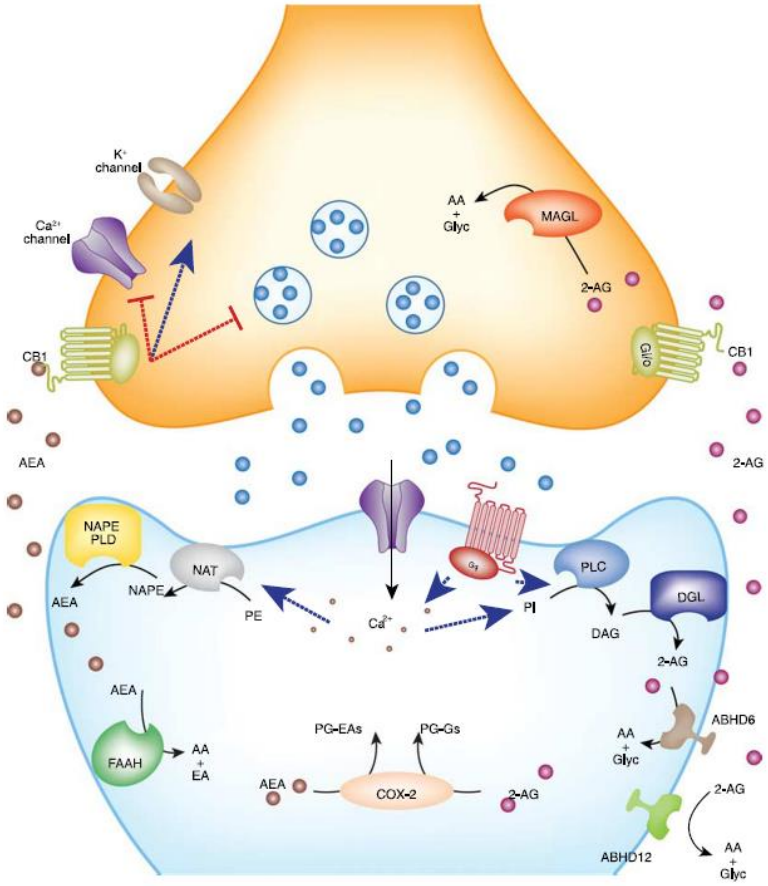

Figure 11. *Schematic illustrating the retrograde endocannabinoid signaling. Upon release of neurotransmitter (for example, glutamate), postsynaptic depolarization causes increased intracellular Ca^{2+} levels through activation of AMPA, NMDA receptors, and/or G_q-coupled receptors (eg, mGluR1/5) and voltage-gated Ca^{2+} channels. Intracellular Ca^{2+} elevation increases endocannabinoid biosynthesis. This model illustrates the two primary biosynthetic pathways for anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), respectively. AEA is synthesized from phospholipid precursors (as phosphatidylethanolamine, PE) by a Ca^{2+}-dependent transacylase, N-acyltransferase (NAT), yielding N-arachidonoyl PE (NAPE). NAPE is then hydrolyzed by a phospholipase D (NAPE-PLD) to yield AEA. Ca^{2+} influx and/or the activation of G_q-coupled receptors stimulate phospholipase C (PLC), which hydrolyses phosphatidylinositol (PI) into diacylglycerol (DAG). DAG is converted to 2-AG by diacylglycerol lipase (DGL). AEA and 2-AG then migrate from postsynaptic neurons to bind presynaptic-located cannabinoid type 1 (CB1) receptors. Once activated, CB1 receptors couple through $G_{i/o}$ proteins to inhibit adenylyl cyclase and regulate ion channels, and ultimately suppress neurotransmitter release. Endocannabinoid signaling is then terminated by degrading enzymes. AEA is mainly hydrolyzed to arachidonic acid (AA) and ethanolamine (EA) by fatty acid amide hydrolase (FAAH), located postsynaptically. 2-AG is hydrolyzed presynaptically to AA and glycerol (Glyc) by monoacylglycerol lipase (MAGL), which accounts for ~ 85% of 2-AG hydrolysis, and postsynaptically by alpha-beta-hydrolase 6/12 (ABHD6/12), which accounts for the remainder of 2-AG hydrolysis. AEA and 2-AG are also oxygenated by cyclo-oxygenase 2 (COX-2) to form prostaglandin-ethanolamides (PG-EAs) and prostaglandin-glycerols (PG-Gs). Modified from (Morena et al., 2016).*

eCBs signaling in synaptic plasticity

Through the discrete synaptic signaling mechanisms, eCBs signaling contributes to many forms of short-term (i.e. short-term depression, depolarization-induced suppression of inhibition/excitation) and long-term plasticity (mainly long-term depression (LTD) but also long-term potentiation (LTP) at both excitatory and inhibitory synapses (Castillo et al., 2012; Gerdeman and Lovinger, 2003; Heifets and Castillo, 2009). LTP and LTD are considered as long-lasting brain adaptations underlying behavioral changes, whereas short-term plasticity is a form of rapid, activity-dependent modulation of synaptic strength (Abbott et al., 1997; Hennig, 2013; Melis et al., 2014).

Short-term plasticity

Short-term plasticity is thought to underlie information processing and it lasts from tens of milliseconds to several minutes (Fioravante and Regehr, 2011). The most-well studied form of eCBs-mediated short-term plasticity is depolarization-induced suppression of excitation (DSE) and inhibition (DSI), depending on whether the silenced input is glutamatergic or GABA-ergic (Diana and Marty, 2004). DS-I/-E lasts for tens of seconds and its induction requires depolarization of postsynaptic cell that subsequently activates voltage-gated Ca^{2+} (VGCC) channels and a rise in intracellular Ca^{2+} levels, eventually leading to eCBs mobilization (Diana and Marty, 2004; Kano et al., 2009). eCBs mobilization can be also triggered by activation of G_q-coupled GPCRs (i.e. group 1 metabotropic glutamate receptors) to induce short-term depression (STD) (Kano et al., 2009; Uchigashima et al., 2007; Varma et al., 2001). The transient suppression of synaptic transmission by STD and DSE/DSI is an important mechanism to modulate neuronal activity by altering neuronal excitability of neuronal responsiveness to incoming stimuli (Augustin and Lovinger, 2018). These forms of eCBs-mediated short-term plasticity have been observed in many brain regions, including the striatum (Narushima et al., 2006), hippocampus (Hu et al., 2011), cerebellum (Safo et al., 2006), and amygdala (Zhu and Lovinger, 2005).

Long-term depression

eCBs can induce long-lasting changes in synaptic strength, lasting from hours to weeks that are referred to as LTP or LTD. The eCBs-mediated induction of long-lasting changes in synaptic connection strength relies on a pattern of afferent stimulation (Hebbian high-, moderate-, low-frequency stimulation) or timing of neuronal output and input spikes (Spike-Timing-Dependent Plasticity (STDP)). The most-well characterized form of eCB-LTD is triggered by patterns of afferent

stimulation in the range of 10-100 Hz. This type of stimulation typically recruits glutamatergic inputs which in turn act on postsynaptic group 1 metabotropic glutamate receptors (mGluR) and increase intracellular calcium concentration. eCB-LTD triggered by pattern stimulation has been reported in many brain regions, including the dorsal striatum, amygdala, hippocampus, PFC, ventral tegmental area, and nucleus accumbens (Heifets and Castillo, 2009; Melis et al., 2014). The STDP is a synaptic learning rule in which the synaptic weight is adjusted based on the relative timing of presynaptic neuron output and postsynaptic neuron spikes (Brzosko et al., 2019; Markram et al., 2012). eCBs are also involved in the regulation of spike-timing-dependent LTD (t-LTD), in which postsynaptic back-propagating action potentials precede the presynaptic stimulation within a critical time window (Shen et al., 2008). The t-LTD has been best described at cortico-cortical and corticostriatal synapses (Melis et al., 2014). Following either way of eCB-LTD induction (frequency-dependent or timing-dependent), eCB signaling leading to the LTD is induced by similar mechanisms: a transient increase in neuronal activity leading to calcium increases at the postsynaptic neuron resulting in the release of eCBs to bind the presynaptic CB1Rs (Calabresi et al., 1994; Choi and Lovinger, 1997; Gerdeman et al., 2002). eCB-LTD can also occur in response to activation of metabotropic receptors (i.e. glutamate type 5 receptors) that stimulate diacylglycerol formation initiating 2-AG production and augments the release of Ca^{2+} from intracellular stores to further increase eCBs levels (Augustin and Lovinger, 2018). LTD mediated by eCBs persists long after CB1R activation has ended. It is still unclear how CB1R activation can differentially mediate short-term and long-term plasticities. Differential involvement of intracellular signaling mechanisms, such as cAMP/PKA and Rim1α signaling, have been proposed to account for long-lasting plasticity changes in contrast to the short plasticity (Chevaleyre et al., 2007).

Long-term potentiation

Besides depressing synaptic transmission, eCBs are also able to increase neurotransmitter release at numerous synapses in the brain (Piette et al., 2020). Using the STDP induction protocol, few presynaptic and postsynaptic pairings induced eCBs-dependent LTP at corticostriatal synapses, which was mediated by CB1R and TRPV1 (Cui et al., 2018, 2016, 2015). 2-AG levels and subsequent CB1R activation can have a bidirectional effect on eCB-plasticity: CB1R activation by approx., 10-15 post-pre pairings together with high levels of eCBs synthesis can induce eCB-LTP, whereas low levels of eCBs produced by pre-post pairings result in eCB-LTD (Cui et al., 2018, 2016, 2015). Hence, few post-pre pairings promote an eCB synthesis for maximal CB1R activation, combined with minimal CB1R desensitization (Cui et al., 2016). The expression of eCB-LTP or eCB-LTD at the corticostriatal synapses was determined by the balance of presynaptic PKA/calcineurin, such that eCB-LTP relied on active PKA, while eCB-LTD required calcineurin activation (Cui et al., 2016). Similar bidirectional

eCB-mediated plasticity was reported also at neocortical pyramidal neurons (Cui et al., 2018). A CB1R-dependent eCB-LTP was induced by high-frequency stimulation in hippocampal granular cells of the dentate gyrus. This long-term potentiation was dependent on CB1R activation of presynaptic focal adhesion molecules (FAK)/ Rho-associated kinase (ROCK) signaling pathways favoring glutamate release (Wang et al., 2018). In detail, FAK is a non-receptor tyrosine kinase that mediates integrin effects on the actin cytoskeleton and plays a prominent role in synaptic plasticity (DeMali et al., 2003; Fabry et al., 2011). Activated integrin-associated FAK acts on its downstream effector ROCK, which leads to presynaptic cytoskeletal changes required for a long-lasting increase in transmitter release (Wang et al., 2018). This results in enhanced glutamate release probability by improved vesicle docking (Wang et al., 2018). eCB-mediated LTP can also occur from activities of distinct synapses/pathways, known as heterosynaptic plasticity (Piette et al., 2020). Heterosynaptic eCB-LTP can be mediated by depression of inhibitory transmission, astrocyte, and dopamine signaling (Cachope et al., 2007; Chevaleyre and Castillo, 2003; Navarrete and Araque, 2010, 2008; Piette et al., 2020; Zhu and Lovinger, 2007). Moreover, the eCB-LTP expression is also regulated by other neuromodulators, which act on eCB synthesis, release, or downstream signaling of CB1Rs (Piette et al., 2020). For example, at corticostriatal synapses, the competition for $G_{i/o}$ protein availability between CB1R and Dopamine type 2 receptor (D2R) can restrict of $G_{i/o}$ protein availability, promoting CB1R coupling to G_s protein signaling and activation of PKA pathway, thus favoring eCB-LTP (Gonzalez et al., 2009; Piette et al., 2020). Together, eCB system can act as bidirectional regulators of synaptic transmission and plasticity mechanisms.

Metaplasticity of eCBs signaling

As described so far, the eCB system regulates LTP and LTD expression by directly contributing to the cellular mechanisms required for the induction of plasticity. Previous synaptic activity and neuromodulatory events at one point in time can influence the subsequent induction of synaptic plasticity. This is known as 'metaplasticity' or priming of synaptic plasticity (Abraham, 2008; Abraham and Bear, 1996; Seol et al., 2007). The 'meta' part of the term refers to the higher-order of plasticity - which is, the plasticity of the synaptic plasticity (Abraham, 2008). Metaplasticity entails an alteration in the physiological and/or biochemical state of synapses that affects the subsequent ability to generate synaptic plasticity. An essential feature of metaplasticity is that the change of neuronal function outlasts the priming stimulation and persists until the subsequent activity induces synaptic plasticity (Abraham, 2008; Melis et al., 2014). Metaplasticity is regulated by a myriad of mechanisms, some of which include modification of postsynaptic membrane potential, altered calcium dynamics, altered states of kinases or phosphatases function, gene expression, and priming of protein synthesis

machinery (Abraham and Tate, 1997). Essentially, metaplasticity serves as a way for synapses to integrate responses that occur in a temporally spaced manner and prevent synapses from entering the states of saturated LTP or LTD (Abraham and Tate, 1997).

Environmental stimuli, such as salience or stress, can affect neuronal plasticity, thus could be considered a form of metaplasticity. However, it may be not easy to distinguish if plasticity was affected by neuromodulators and hormones, from metaplasticity phenomena. One widely accepted approach is to use *ex-vivo* brain preparation after removing tissue from animals that were exposed to environmental stimuli *in-vivo* (Abraham, 2008). This approach has revealed that eCB-mediated plasticity may also vary depending on neuronal activation history. Network activity induced by *in-vivo* experience can cause homeostatic adaptations of eCB signaling, which are reflected by changes in eCBs production, degradation, CB1R expression, and function. For instance, acute stress enhances glutamate release in a reciprocal BLA-PFC circuitry by impairing 2-AG signaling (Marcus et al., 2020). On the other hand, chronic stress cause glucocorticoid elevation which enhances eCBs breakdown at inhibitory synapses of the BLA (Sumislawski et al., 2011).

In summary, eCB system is a key regulator of synaptic function and contributes to the adaptive neuronal responses via diverse signaling cascades at both sides of the synaptic compartment. The eCB-mediated LTD can exert inhibitory control over neuronal circuits in macro and micro levels to suppress unnecessary neural activity, while the eCB-LTP may strengthen the synapses to promote or maintain certain behaviors. On top of that, the eCB system is highly sensitive to the ongoing neuronal activity related to behavior and cognitive functions, and can accordingly shape the subsequent synaptic plasticity rules.

Endocannabinoid role in regulating LC physiology

The eCBs are known to regulate the activity of LC neurons (Gobbi et al., 2005; Muntoni et al., 2006). CB1R mRNA and proteins were localized within the LC (Herkenham et al., 1991; Matsuda et al., 1993). Further studies showed that CB1R is localized at the presynaptic axon terminal of glutamatergic and GABA-ergic neurons, which form synapses with LC cells. Moreover, CB1R was also localized postsynaptically in the somatodendritic compartment of LC neurons (Scavone et al., 2010). In other brain regions, activation of postsynaptically located CB1Rs induces auto-inhibition in so-called non-retrograde eCB signaling (Bacci et al., 2004; Castillo et al., 2012). The same auto-inhibition seems plausible in the LC neurons. Postsynaptic CB1Rs are positioned in an optimal place to fine-tune glutamatergic transmission without altering presynaptic release probability, although the role of postsynaptic CB1R in the LC as in other brain regions has not been fully elucidated yet.

Electrophysiological studies have demonstrated that CB1R can influence LC activity (Wyrofsky et al., 2019). CB1R agonists (WIN 55212-2 or CP 55940), administered systemically or centrally, dose-dependently increased LC tonic firing rate in anesthetized rats. The effect was blocked by the selective CB1R antagonist SR 141716A further supporting the link between CB1R and regulation of tonic firing rate in LC neurons (Mendiguren and Pineda, 2006; Muntoni et al., 2006). Inhibition of the AEA hydrolysis enzyme, FAAH, with the selective blocker URB597, increases the spontaneous activity of LC-NE neurons (Gobbi et al., 2005; Muntoni et al., 2006). Moreover, a single application of CB1R antagonist can decrease LC-NE activity (Carvalho and Van Bockstaele, 2012; Muntoni et al., 2006) indicating a tonic-regulation of LC neurons by eCBs. Interestingly, various doses of CB1R agonists and antagonists yield opposing effects on LC activity, hence highlighting the biphasic regulation of the eCB system in the LC nucleus (Carvalho et al., 2010; Wyrofsky et al., 2019). It has been shown that tonic eCBs activity is affected differently depending on where CB1R is expressed: basal tonic CB1R activation has a stronger effect on GABA-ergic terminals, while glutamatergic CB1Rs are preferentially activated by phasic eCB release (Katona and Freund, 2008; Marsicano et al., 2003). Since CB1Rs in the LC have been identified on both glutamatergic and GABA-ergic presynaptic neurons, the biphasic nature of CB1R activation may be explained by tonic eCB release primarily acting on GABA-ergic neurons, while phasic eCBs release may further gate glutamatergic excitation of LC neurons. Hence, the net effect of CB1R activation on LC activity may depend on the levels of CB1R agonist (Wyrofsky et al., 2019).

As discussed in previous chapters, intracerebroventricular CRF administration increases the excitability of LC-NE neurons (Curtis et al., 1997). An immunofluorescent study by Wyrofsky and colleagues (R. Wyrofsky et al., 2017) demonstrated that CB1R is co-localized with CRF-containing axon terminals in the LC (R. Wyrofsky et al., 2017), suggesting that CB1Rs are positioned to directly regulate CRF release (R. Wyrofsky et al., 2017). These findings support eCB involvement in the modulation of CRF signaling in the LC, which is associated with anxiety-like and stress behavior (McCall et al., 2015).

In summary, eCB signaling is an important modulator of LC physiology with a potential role in shaping and modulating glutamatergic and CRF inputs. Whether these inputs can undergo plastic changes and what would be the role of eCB system, remains largely unexplored.

eCBs guide developmental PFC trajectories

As discussed in the previous chapter, brain development continues during adolescence, particularly in the prefrontal brain areas. Along with dramatic changes in gross brain morphology (loss of gray matter and increase of white matter (Giedd and Rapoport, 2010; Tau and Peterson, 2010)), the maturation process occurs also in different neurotransmitter systems, including the eCB system. Functionally active eCBs and their receptors emerge in early development (Fernández-Ruiz et al., 2004), although the expression levels undergo specific patterns of development during adolescence (Dow-Edwards and Silva, 2017). In the male rat brain, AEA levels progressively increase in the PFC to adult levels, meanwhile, 2-AG is already at the maximal level during early adolescence. 2-AG levels decrease in mid-adolescence but rise back again in late adolescence (Ellgren et al., 2008). The PFC CB1R levels are at a maximum during early adolescence and gradually decrease towards adulthood (Ellgren et al., 2008) **(Figure 12)**. Along these lines, developmental increase in AEA levels has been associated with changes in the direction of synaptic plasticity in the striatum (Ade and Lovinger, 2007). In response to a high-frequency stimulation (HFS) protocol, a developmental shift from eCB-LTP in P12-14 rats to eCB-LTD in P16-34 rats was reported. The application of synthetic AEA during the HFS on brain slices from P12-14 rats allowed for LTD induction at striatal glutamatergic synapses, while blocking CB1R in P16-34 rats during the HFS resulted in LTP. Blocking 2-AG synthesis with DAGL inhibitor did not alter the plasticity outcome in either developmental stage. Additionally, synaptic depression produced by a synthetic CB1R agonist WIN 55,212 was similar in P12-14 and P16-34 rats, excluding the possibility of differential CB1R sensitivity during this developmental window (Ade and Lovinger, 2007).

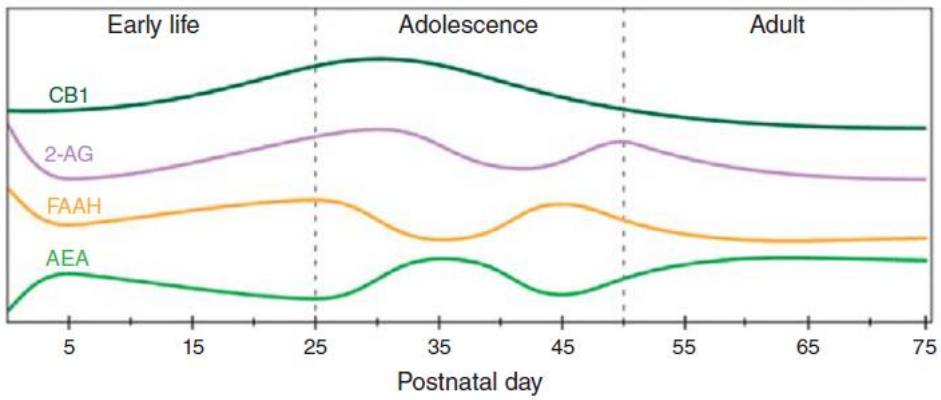

***Figure 12**. Relative changes in the eCB system during postnatal development of a rodent. Modified from (Meyer et al., 2018).*

The maturation of cortical neurocircuitries during adolescence is associated with the thinning of the gray matter (Gogtay et al., 2004). It is suggested, that the changes in the gray matter volume are a consequence of synaptic pruning (Giedd et al., 1999; Gogtay et al., 2004). Pruning preferentially occurs at asymmetric junctions, on dendritic spines (Brenhouse and Andersen, 2011), that are primarily excitatory. Thus, pruning strongly impacts glutamatergic neurotransmission (Bourgeois and Rakic, 1993), particularly in the maturing PFC. The eCB system is involved in the homeostatic regulation of glutamatergic and GABA-ergic terminals, thus during adolescence eCB system contributes to the maintenance of excitatory/inhibitory balance and pruning of cortical excitatory synaptic connections (Bossong and Niesink, 2010; Katona and Freund, 2008; Marsicano et al., 2003). Together, these eCB signaling-mediated mechanisms contribute to the functionally relevant synchronization of developing neural circuits and refinement of information processing in the maturing PFC (Freund et al., 2003; Gerdeman and Lovinger, 2003; Meyer et al., 2018).

Similarly, perturbations of the eCB system during adolescence can impede the structural and functional maturation of the PFC (Renard et al., 2016). The CB1R exogenous activation during adolescence has been shown to alter the dendritic arborization of pyramidal neurons in the medial PFC (Renard et al., 2016). Moreover, blocking eCB activity by CB1R antagonist AM251 in this period prevents normal decrease of post-synaptic density- 95, GluA2, and GluN2A (subunits of AMPA and NMDA receptors, respectively) (Rubino et al., 2015). Given the GluA2 and GluN2A subunit involvement in the stabilization of excitatory synapses, the eCB tone could play a role in the pruning of glutamatergic synapses during adolescence (Rubino et al., 2015).

Aims and relevance

The LC is an anatomically and functionally heterogeneous nucleus, integrating over a hundred monosynaptic inputs from all over the brain (Breton-Provencher and Sur, 2019a; Schwarz et al., 2015). In contrast to LC efferents, the role of the impinging inputs to the LC remains largely unexplored. Investigating the synaptic physiology of LC-NE neurons by focusing on how LC afferents shape LC neuronal activity is instrumental for understanding LC's diverse functions. Moreover, it remains to be established how local and long-range neuromodulators shape the activity of LC synapses, and thereby affecting LC-NE output.

One of the main input to the LC is represented by cortical projections originating from the PFC. (Jodo et al., 1998; Sara and Hervé-Minvielle, 1995). Anatomical studies provide evidence for monosynaptic projections from the PFC to the LC (PFC→LC) (Breton-Provencher and Sur, 2019a; Schwarz et al., 2015), but the functional significance of this connection is unclear. The PFC is involved in valence assignment in a novel environment through the 'top-down' mechanism of cognitive appraisal (Ochsner et al., 2002), which is dominant in unfamiliar or ambiguous situations (Lazarus, 1999). Thus, PFC→LC input may exert ‘top-down‘ executive control over LC activity and guide behavior in unfamiliar and ambiguous circumstances. As the LC nucleus is swiftly recruited by the salient and arousing conditions (Aston-Jones et al., 1994; Breton-Provencher and Sur, 2019a; Vazey et al., 2018; Ventura et al., 2008), PFC input to the LC may grant the cognitive appraisal of a novel environment, thus shaping behavioral response (Ochsner and Gross, 2005). To fully elucidate the functional role of the PFC→LC inputs, it is important to study not only the features of the hardwired connections between the two brain regions but also synaptic plasticity mechanisms, which can shape synaptic efficacy. Notably, the PFC is one of the last brain regions to reach maturation across multiple species (Gogtay et al., 2004; Laube et al., 2020; Monk et al., 2003). Plastic changes at PFC→LC synapses may occur upon exposure to salient or arousing experience, or during postnatal development, like adolescence. During adolescence, the cognitive appraisal center - PFC - is still developing and emotional responses are mostly driven by the Amygdala without the supervision of cortical regions (Hare et al., 2008; Monk et al., 2003; Stephanou et al., 2016). Thus, novelty processing might differ between adult and adolescent individuals due to unsynchronized maturation times of the PFC and the Amygdala. PFC inputs to the LC may facilitate the cognitive appraisal upon conscious re-evaluation of a novel situation in adults but not in adolescents.

Notably, the developmental maturation processes occur also in other neuromodulatory systems (Meyer et al., 2018). Hence, the plasticity mechanisms occurring at PFC→LC inputs during adolescence to adulthood transition may be differentially shaped by local neuromodulators such as endocannabinoids and long-range impinging inputs, like CRF.

In my Ph.D. project, I have been testing the ***hypothesis*** that experience-dependent plasticity at PFC→LC inputs differentially modulates behavioral response to novelty in adult and adolescent mice, and this modulation is shaped by the activity of local LC neuromodulators as well as via long-range neuromodulatory afferents.

To test this hypothesis, I have been developing the following aims:

1. Assess developmental changes in synaptic strength at PFC→LC inputs.

2. Investigate synaptic plasticity and its molecular determinants at PFC→LC inputs at the two developmental stages.

3. Address how exposure to a novel environment results in different synaptic adaptations at PFC→LC inputs in adolescent mice compared to adults.

To develop the project, I combined electrophysiological, pharmacological, and optogenetic approaches with behavioral paradigms.

Results

Young and adult mice behavior in a novel environment

We first assessed the behavior of adult (P60-75) and young (used interchangeably with adolescent in this dissertation) (P23-28) mice upon exposure to a novel environment. To this aim, we exposed mice to a previously unexplored open field (OF) which is a salient stimulus known to trigger phasic LC activity (Moorman and Aston-Jones, 2014) and it represents a situation of ambiguous valence where rodents face an internal conflict between danger avoidance and novel environment exploration (Crawley, 2008; La-Vu et al., 2020; Post et al., 2011). We found that young mice show more negative valence-associated behaviors, like avoidance, compared to adults when exposed to a novel environment (La-Vu et al., 2020; Powell et al., 2004). Young mice spent significantly more time in the corners (young = 347.1 s ± 33.61 s, n=15 versus adult = 232.7 s ± 17.15 s, n=18; p=0.003) and entered the center zone less times than adult mice (young = 37.4 ± 3.73, n=15 versus adult = 52.4 ± 5.2, n=18, p=0.03). Thigmotaxis index (young = 68.2 ± 2.1, n=15 versus adult = 52 ± 3.4, n=18, p=0.0006) and thigmotaxis time (young = 75.2 ± 2.1, n=15 versus adult = 58.8 ± 3.8, n=18, p=0.001) was significantly higher in young compared to adult mice **(Figure 13)**.

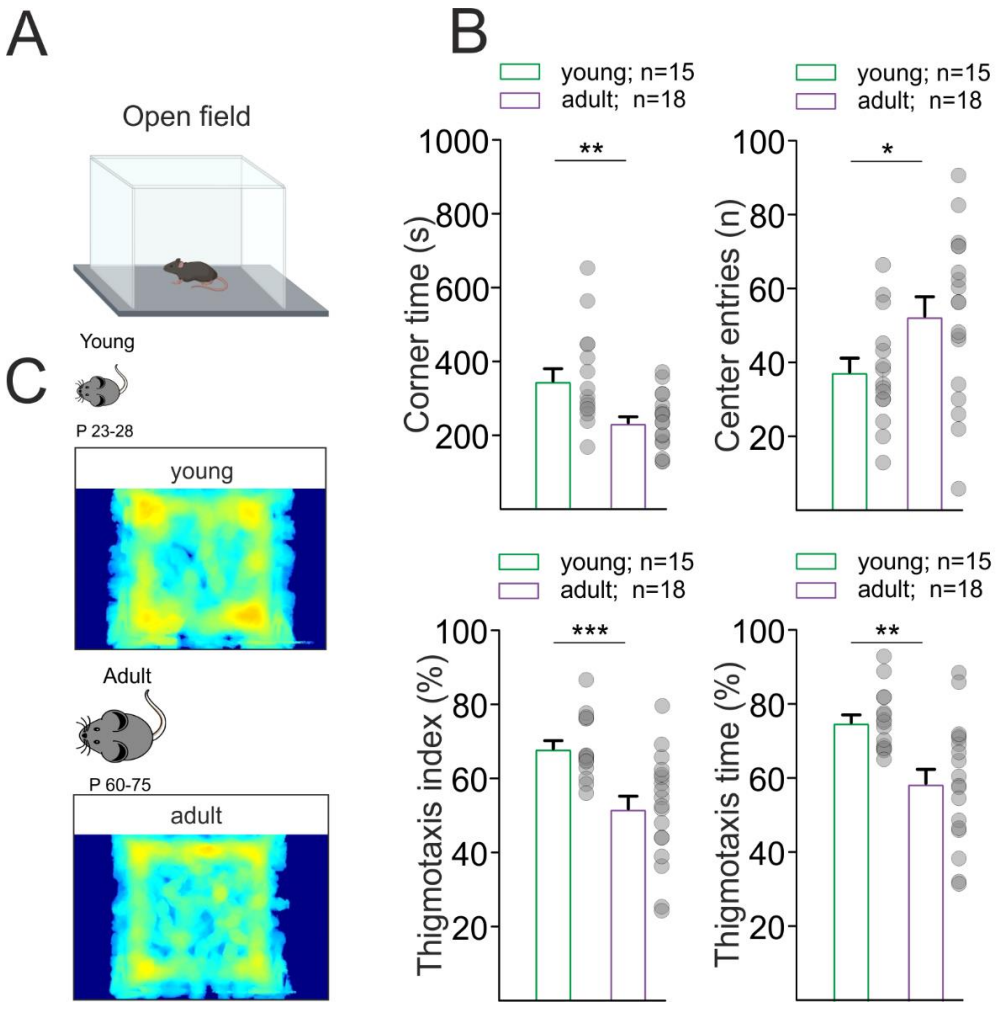

Figure 13. *(A) Schematic representing a novel open field test. (B, top, left) Scoring of the corner time (young, n=15 versus adult, n=18; p=0.003; 2sided unpaired t-test).(B, top, right) Scoring of center entries (young, n=15 versus adult, n=18; p=0.03; 2sided unpaired t-test). (B, bottom, left) Scoring of the thigmotaxis index (young, n=15 versus adult, n=18; p=0.0006; 2sided unpaired t-test). (B, bottom, right) Scoring of the thigmotaxis time (young, n=15 versus adult, n=18; p=0.001; 2sided unpaired t-test). (C) Representative heat maps of motor behavior in the open field of young (top) and adult (bottom) mice.*

To verify that the exposure to a novel OF activates LC neurons, we measured the immediate early gene (*c-fos*) expression as a readout for neuronal activity (Bullitt, 1990; Perrin-Terrin et al., 2016). We found that in the adult group, mice that underwent exposure to the novel OF had a significantly higher percentage of *c-fos* $^{+}$/TH^{+} cells compared to the naive control group (adult naive = 2.6% ± 1 %, n=5 versus adult OF = 22.1% ± 3.7, n=6; p=0.001) **(Figure 14 A)**. This data confirms that exposure to a novel OF activates LC neurons in adults.

Because of the behavioral differences observed in young and adult mice upon exposure to the OF, we compared the levels of *c-fos* activation in the two age groups. We found that young mice had less *c-fos* positive LC neurons, compared to adults (young OF = 8.5% ± 1.6%, n=7 versus adult OF = 22.1% ± 3.7, n=6; p=0.005) **(Figure 14 B)**. We still have to evaluate the baseline level of *c-fos* activation in the LC of young mice that were not exposed to a novel environment, as we demonstrated in adult mice **(Figure 14 A)**. Our data suggest that novelty exposure more strongly activate LC neurons in adult compared to young mice.

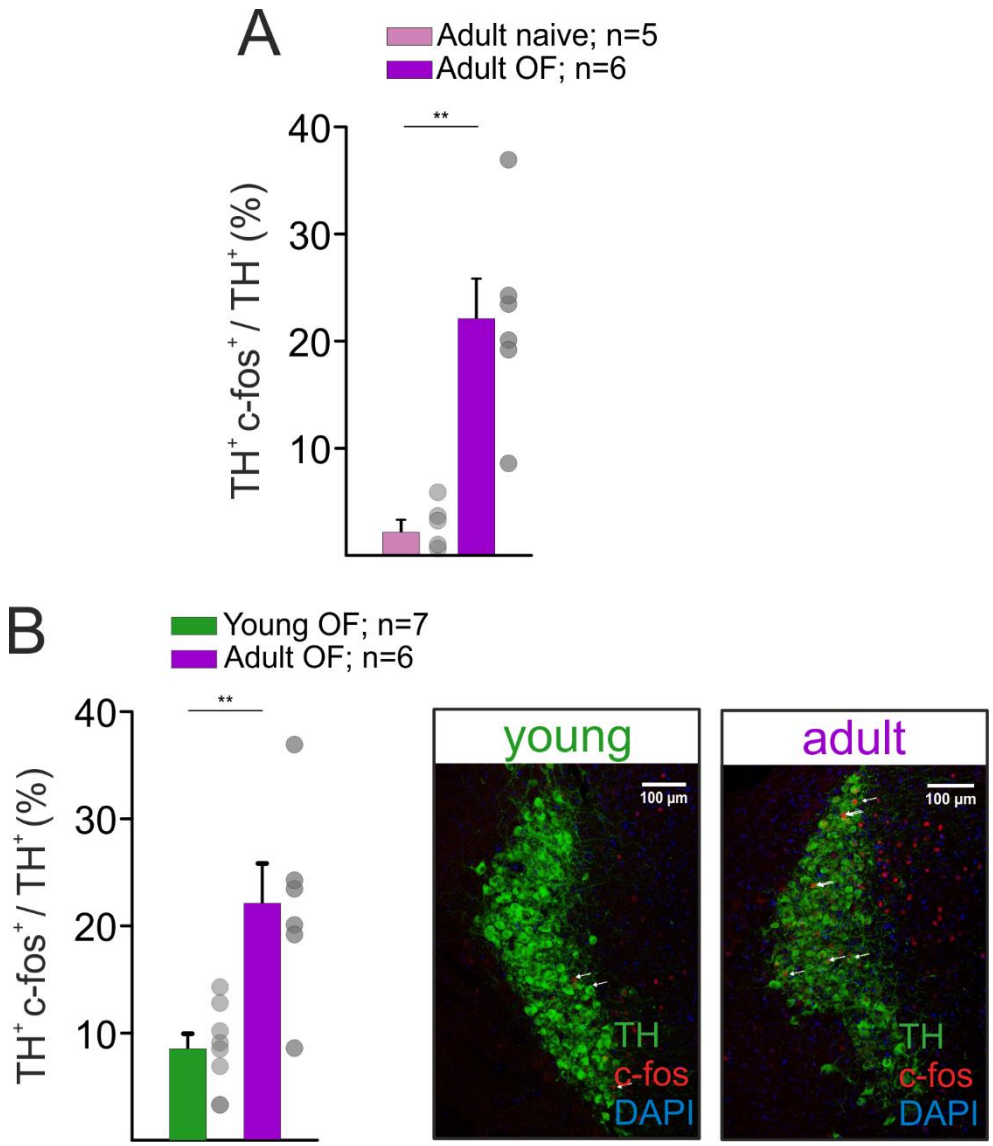

Figure 14. *(A) Percentage of TH⁺- c-fos ⁺ neurons in adult naive and adult OF group (adult naive, n=5 versus adult OF, n=6; p=0.001; 2sided unpaired t-test). (B, left) Percentage of TH⁺- c-fos ⁺ neurons in young and adult groups after exposure to a novel OF (young OF, n=7 versus adult OF, n=6; p=0.005; 2sided unpaired t-test). (B, right) Representative immunofluorescent c-fos staining of LC obtained from animals that were exposed to a novel OF. TH (green), c-fos (red), DAPI (blue), the arrows indicate TH⁺- c-fos ⁺ neurons.*

PFC→LC input dissection at two developmental stages: adolescence and adulthood

Synaptic strength at PFC→LC synapses changes during adolescence to adulthood transition

In previous studies, lesion of the rat Anterior Cingulate Cortex (a part of the PFC) has been associated with lower LC *c-fos* levels after exposure to novelty, compared to rats with an intact ACC (Gompf et al., 2010). Thus, we reasoned that lower LC activation in young mice may reflect still maturing PFC→LC inputs. Therefore, we aimed to investigate developmental changes in synaptic strength at PFC→LC synapses in young and adult mice *ex-vivo*. To study the PFC→LC synapses we probed LC-NE circuitry with optogenetics. We bilaterally injected an adeno-associated virus (AAV) vector encoding ChR2-eYFP under the control of the CaMKII promoter [AA9-CaMKIIa-ChR2(H134R)-eYFP] into the PFC of naive adult (P30) and a newborn (P0) mice (C57BL/6J). 5-7 weeks after injection for adults and 3 weeks for young mice, we obtained strong expression of the reporter fluorophore eYFP in the injection site as well as in the PFC terminals in the LC and peri-LC region **(Figure 16)**.

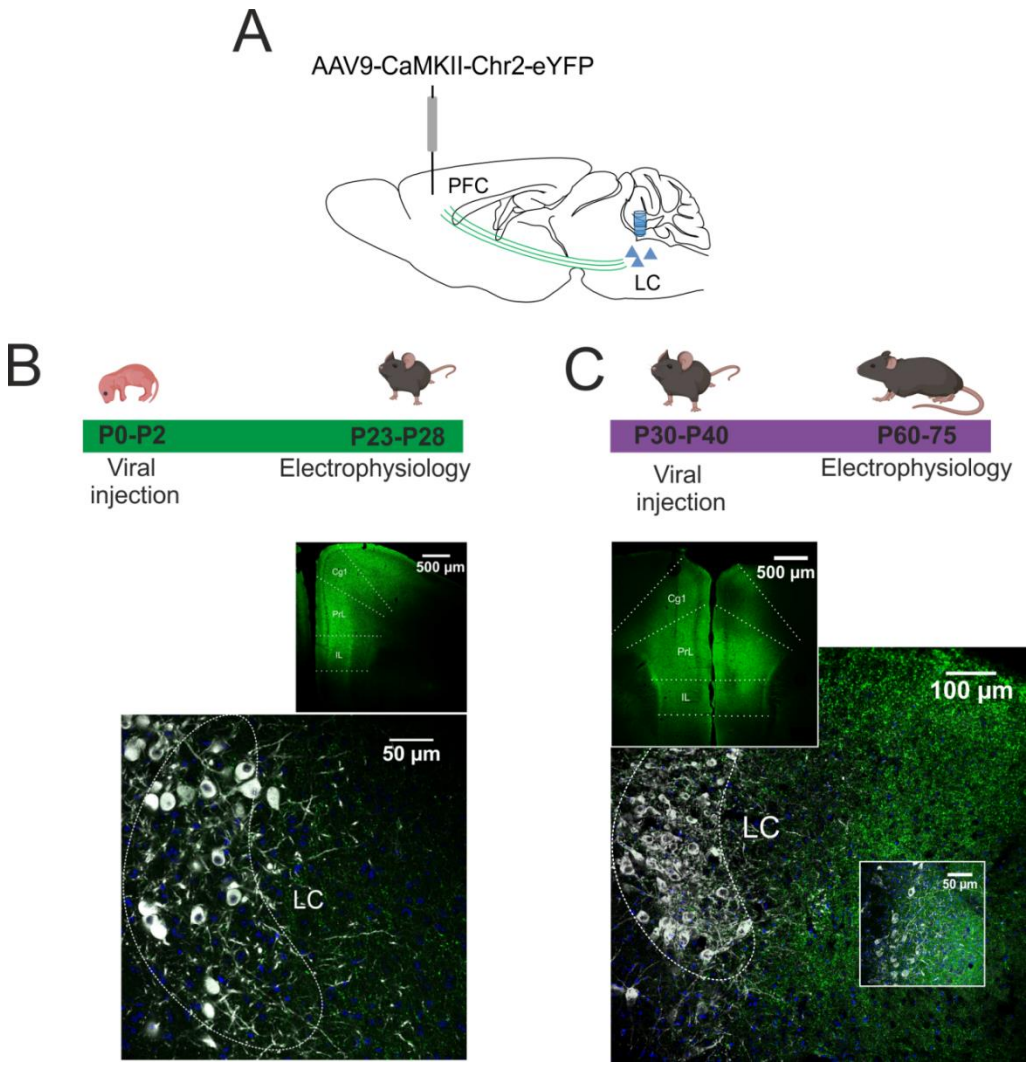

Figure 16. *(A) Schematic of the virus injection site and optogenetic stimulation strategy of PFC→LC afferents. (B, top) Injection timeline of young mice and (B, bottom) AAV9-CamKIIa-ChR2(H134R)-eYFP expression targeting the PFC (inset). PFC terminals are visible in the LC and peri-LC. eYFP (green), TH (silver). (C, top) Injection timeline of adult mice and (C, bottom) AAV9-CamKIIa-ChR2(H134R)-eYFP expression targeting the PFC (inset). PFC terminals are visible in the LC and the peri-LC. eYFP (green), TH (silver). LC (Locus Coeruleus).*

LC-NE cells were identified by the location (near the IV ventricle), soma morphology and size (20-30 µm), and their tonic firing rate (Williams and Marshall, 1987). After recordings, LC cell identity was confirmed by immunoreactivity for the marker tyrosine hydroxylase (TH), the enzyme for the biosynthesis of catecholamines (Axelrod, 1971) **(Figure 17)**.

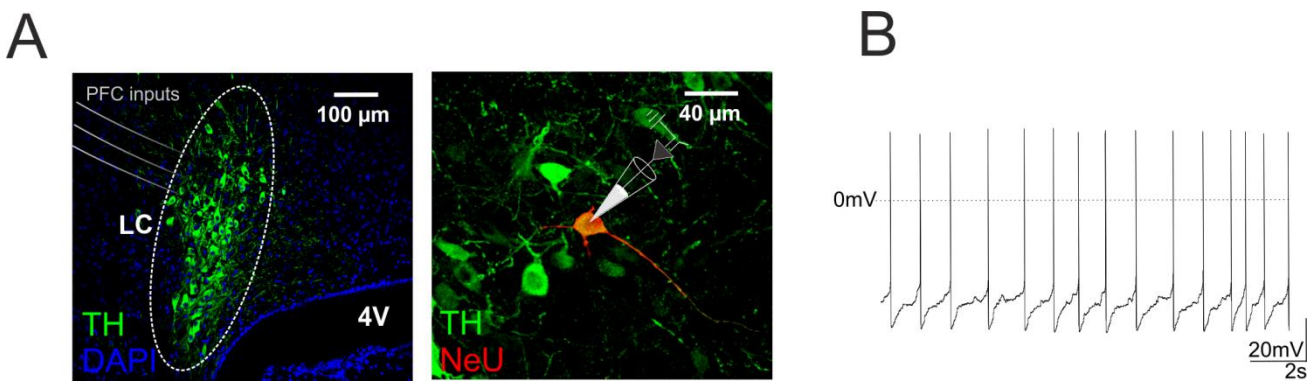

Figure 17. *(A) Representative immunofluorescent staining of LC containing slice, PFC→LC inputs are depicted in grey. (TH) in green and DAPI in blue. (A, right) TH staining in green and patched cell filled with Neurobiotin in red. A patch pipette is illustrated in white. Locus Coeruleus (LC). Fourth ventricle (4V). (B) Representative trace of LC neuron tonic firing.*

First, in the acute brain slices, we assessed the synaptic strength at PFC→LC synapses in adult (P60-75) and young (P23-28) mice by recording AMPA/NMDA ratio at PFC→LC synapses. On *ex-vivo* LC brain slices, a short blue light pulse through the microscope objective (1ms at 470 nm) evoked excitatory postsynaptic currents (EPSCs-Opto$_{PFC}$) in LC neurons. We found that AMPA/NMDA ratio was higher in adult compared to adolescent mice (young = 1.9 ± 0.3, n=7 versus adult = 3.4 ± 0.4, n=16, p=0.04) **(Figure 18)** suggesting higher synaptic strength in the adults. These results are in line with the hypothesis that the adolescent brain undergoes functional remodeling of PFC→LC synapses during the adolescent to adulthood transition.

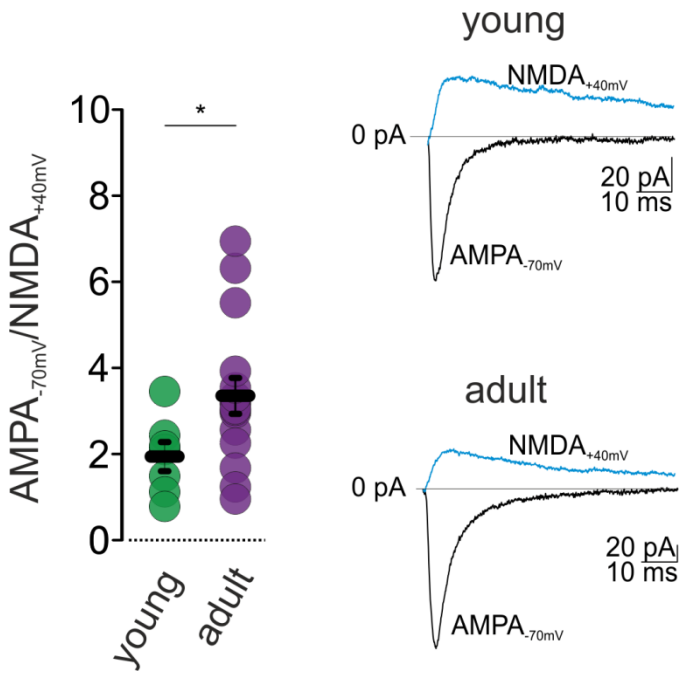

Figure 18. *(left) AMPA/NMDA ratio at PFC→LC synapses of young and adult mice (young, n=7 versus adult, n=16, p=0.04; 2sided unpaired t-test). (right) representative current traces.*

eCBs regulate synaptic plasticity at PFC→LC synapses

So far, we described a different propensity for novel exploration in the two age groups we analyzed. This predisposition is associated with a higher proportion of *c-fos*-positive LC cells and AMPA/NMDA ratio in adults compared to young mice. As previously described, the eCB system, by modulating synaptic plasticity, is crucial in regulating the balance between novelty seeking and safety assessment and in defining the developmental trajectories of different brain regions (Alpár et al., 2014; Cusulin et al., 2014; Lafenêtre et al., 2009; Lee and Gorzalka, 2012; Meyer et al., 2018). Therefore, we focused on the potential role of the eCB system in modulating LC synaptic plasticity in the two age groups. To probe the eCB system we used the negative Spike-Timing dependent plasticity paradigm, as in many brain regions spike-timing-dependent Long-Term Depression (t-LTD) relies on eCB signaling (Heifets and Castillo, 2009). This type of plasticity can be studied in *ex-vivo* acute brain slice preparations by a post-pre STDP paradigm, in which postsynaptic spikes precede the presynaptic stimulation by a critical time window (Brzosko et al., 2019; Markram et al., 2012) **(Figure 19)**. Modulation of this form of plasticity can fine-tune the synaptic gain of PFC→LC input, thereby affecting the release of NE in target brain regions, and ultimately shaping behavioral reactivity to relevant salient stimuli.

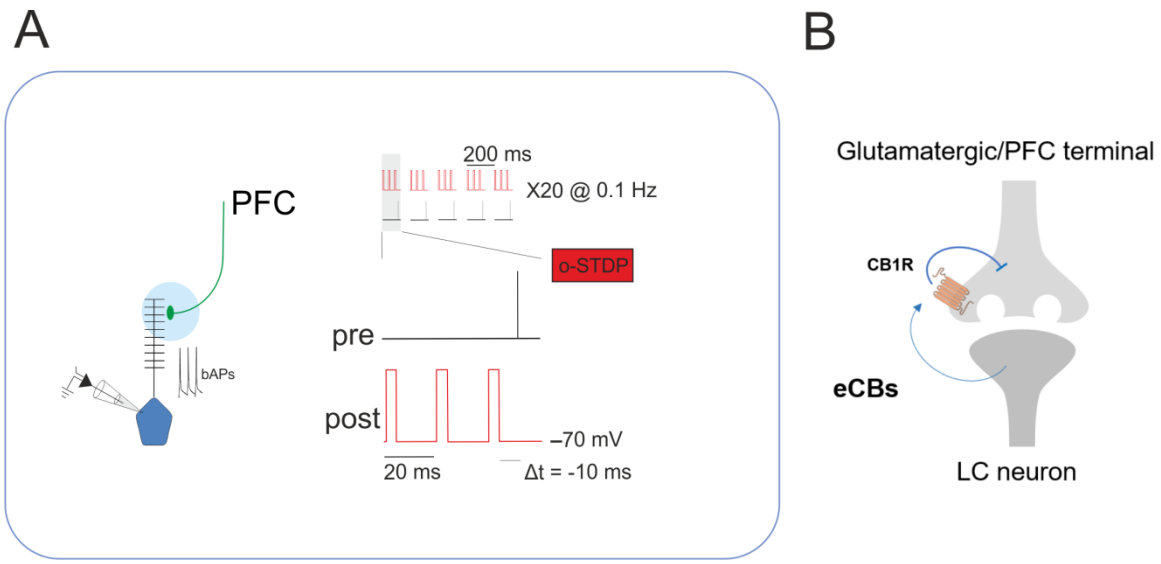

Figure 19. *(A) Burst pairing protocol for the induction of negative-STDP. The protocol consists of 20 bouts, delivered 10 s apart. Each bout consists of five bursts (200ms apart) each composed of three*

action potentials at 50 Hz followed by one EPSP (negative timing). (B) Schematic representation of the eCB signaling. eCBs are produced postsynaptically, then they travel retrogradely to decrease the presynaptic release of neurotransmitters acting on CB1R.

We monitor EPSCs-Opto$_{PFC}$ before and after the induction of the negative STDP. During the post-pre pairing paradigm, presynaptic EPSP was evoked by optogenetic stimulation of PFC terminals **(Figure 20 B)**. In this experimental setting, the Opto$_{PFC}$-STDP induced t-LTD at PFC→LC synapses of young mice (61% ± 7% of baseline; n=8; $p<0.05$ Tukey) **(Figure 20 C)**. To test whether this t-LTD is mediated by eCB signaling, we bath applied the selective CB1R antagonist AM251. On average, AM251 did not prevent PFC-LTD (82% ± 9% of baseline; n=11; $p<0.05$; Tukey; data not shown). However, we observed a non-normal distribution of the plasticity ratio values ($EPSC_a/EPSC_b$) ($p=0.03$, Shapiro-Wilk) upon AM251 application. This suggests that inhibition of CB1R does not univocally affect the plasticity outcome in LC neurons. Therefore, we segregated data into two subgroups by using K-means cluster analysis **(Figure 20 A)** (LC Opto$_{PFC}$+AM251 cluster 1, cluster center 51.9, average distance from cluster center 6.6; n=5; LC Opto$_{PFC}$+AM251 cluster 2, cluster center 107.2; average distance from cluster center 6.07; n=6;). In one subgroup of LC neurons t-LTD was completely prevented by CB1R antagonist AM251 (Opto$_{PFC}$-AM251 cluster 2, 107% ± 3% of baseline, n=6; $p>0.05$ Tukey). In a second subgroup, t-LTD was not affected by AM251 application (Opto$_{PFC}$-AM251 cluster 1, 52% ± 3 of baseline, n=5; $p<0.05$ Tukey) **(Figure 20 C)**. These results suggest that t-LTD at PFC→LC synapses might rely on different expression mechanisms that segregate in two LC neuronal subgroups. These subgroups could result from different molecular charateristics of LC cells or different synaptic states.

CB1R-dependent and non-dependent t-LTD may be indicative of subgroups of neurons in a different internal neuromodulatory state.

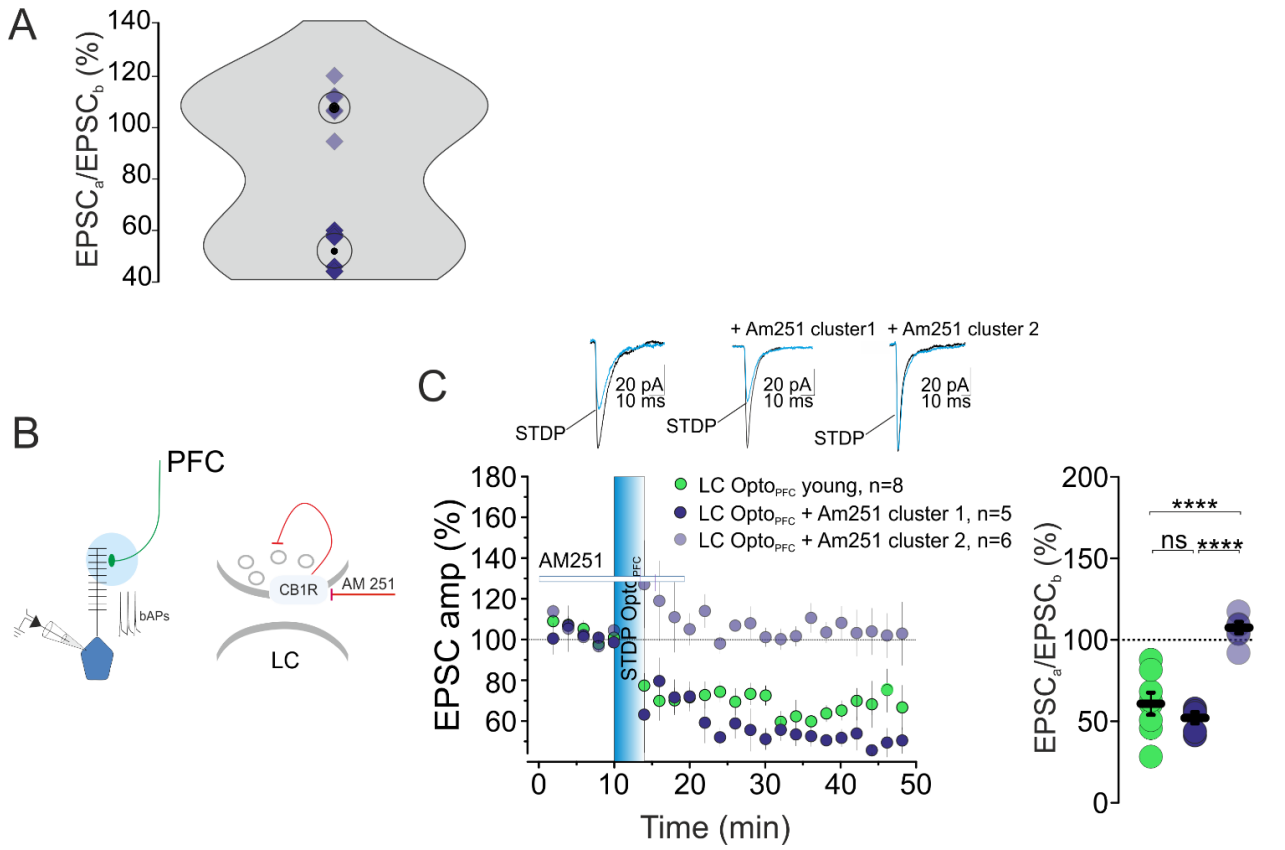

Figure 20. *(A) Violin plot representing a bimodal distribution of LC Opto$_{PFC}$+AM251 group EPSC$_a$/EPSC$_b$ values. Different color coding represents the two groups divided by K-means analysis. Cluster centers (black dots) and average distance from the cluster center (equivalent to the radius of the hallowed circle). (B, left) Schematic of the recording configuration in LC-NE neurons. (B,right) Representation of the AM251-mediated CB1R antagonism. (C, left) Time course and (C, right) scatter plot showing the LTD induced by post-pre pairing (solid green circles), and (C, top) representative current traces. CB1R antagonism by AM251 (4µM; empty horizontal bar) blocked the t-LTD evoked by the Opto$_{PFC}$-STDP in a subgroup of LC neurons (transparent dark blue circles), while Opto$_{PFC}$-STDP was not affected by AM251 in a second group of neurons (solid blue circles) (1WA; $F_{2,16}$=26.24; p<0.0001; LC Opto$_{PFC}$ young versus LC Opto$_{PFC}$ +AM251 cluster 1, p=0.5; Opto$_{PFC}$ young versus LC Opto$_{PFC}$ +AM25 cluster 2, p<0.0001; LC Opto$_{PFC}$+AM251 cluster 1 versus LC Opto$_{PFC}$ +AM251 cluster 2, p<0.0001; Tukey). Insets represent superimposed averaged recordings (30 traces) before (black) and after (blue) the delivery of the STDP protocol (vertical bar).*

(C) In these figures, and in analogous plots following throughout the manuscript, data are presented as a time course (mean ± SEM) of normalized EPSC amplitudes. The EPSC time courses were obtained in presence of 10 µM gabazine, and in whole-cell configuration. Scatter plots summarize the ratios of synaptic responses 20-25 min after (a) and 5-10 min before (b) the STDP. Insets represent superimposed averaged recordings (30 traces) before (black) and after (blue) the delivery of the STDP pairing protocol (blue vertical bar).

Since AM251 did not prevent depression at PFC→LC synapses in all the recorded LC neurons, we asked whether different plasticity mechanisms mediate the expression of t-LTD in distinct LC

subpopulations. Different signaling pathways (NMDA, mGluR1/5, etc) converge on AMPA receptor endocytosis (Beattie et al., 2000; Grueter et al., 2010; Henley and Wilkinson, 2013; Waung et al., 2008), thus, we investigated the possibility that t-LTD in young mice is expressed postsynaptically in the subpopulation of neurons insensitive to AM251. Notably, intracellular dialysis of the peptide $GluA2_{3Y}$ (100µg/ml), which blocks regulated AMPA endocytosis (Brebner et al., 2005; Collingridge et al., 2010), prevented t-LTD ($Opto_{PFC}$-$GluA2_{3Y}$, 86% ± 5% of baseline, n=7, p>0.05 Tukey) **(Figure 21)**. Confirming $GluA2_{3Y}$ specific action, the control peptide $GluA2_{3A}$ (100 µg/ml) resulted in the t-LTD ($Opto_{PFC}$-$GluA2_{3Y}$, 50% ± 7% of baseline, n=7, p<0.05 Tukey) **(Figure 21)** similar to naive young mice **(Figure 21)**. $GluA2_{3A}$ is a control containing peptide (3A) in which the tyrosine residues were replaced by alanines (Rao-Ruiz et al., 2011). The control peptide shows that the results acquired with the $GluA_{3Y}$ peptide are not due to DMSO in the intracellular solution but rather due to the action of the $GluA_{3Y}$ peptide. Knowing that CB1R at PFC→LC synapse mediates t-LTD in a subpopulation of LC cells, we expected only a fraction of LC neurons to be sensitive to $GluA2_{3Y}$. However, blocking AMPA receptor internalization prevented t-LTD without resulting in a bimodal distribution indicating that, at PFC→LC synapse, AMPA endocytosis accounts for t-LTD in the whole LC population regardless of AM251 sensitivity. This can suggest that eCB mediated t-LTD at PFC→LC synapse is expressed through AMPA receptor internalization. Although other eCB receptors, such as TRPV1, can mediate AMPA endocytosis (Castillo et al., 2012; Chávez et al., 2010; Grueter et al., 2010; Puente et al., 2011), this mechanism has never been described for CB1R. Nevertheless, combined activation of CB1R and TRPV1R could be involved, as it was previously shown that the co-activation of these two eCBs receptors is needed for high-frequency stimulation-induced LTD in the striatum (Nazzaro et al., 2012). Further experiments using TRPV1 antagonists will help to elucidate this hypothesis.

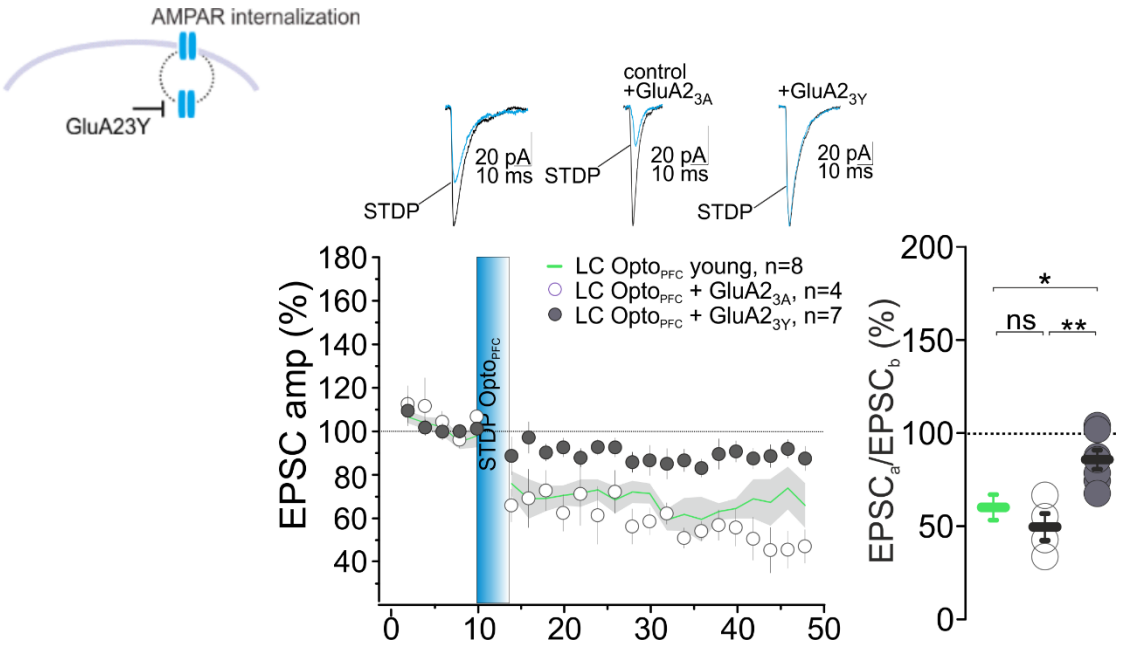

Figure 21. *(top, left) Representation of the GluA2$_{3Y}$ -mediated blocking of AMPAR internalization. (bottom, left) Time course and (bottom, right) scatter plot showing that intracellular application of the endocytosis-disrupting GluA2$_{3Y}$, blocked the t-LTD (100 µg/ml; grey circles), while the control peptide GluA2$_{3A}$ did not affect t-LTD (100 µg/ml; empty circles). The green line shows the time course of the plasticity from the Opto$_{PFC}$ young group (Figure 20 C) (1WA; $F_{2, 16}$=7.6; p=0.004; Opto$_{PFC}$ young versus Opto$_{PFC}$+GluA2$_{3A}$, p=0.5; Opto$_{PFC}$+GluA2$_{3A}$, versus Opto$_{PFC}$-+GluA2$_{3Y}$, p=0.007; Opto$_{PFC}$ young versus Opto$_{PFC}$-+GluA2$_{3Y}$, p=0.02; Tukey). Insets represent superimposed averaged recordings (30 traces) before (black) and after (blue) the delivery of the STDP protocol (vertical bar).*

Similar to what we obtained in young mice, in adult mice, the Opto$_{PFC}$-STDP induced t-LTD at PFC→LC synapses (71% ± 4% of baseline, n=8; p<0.05 Tukey). In this age group, t-LTD was dependent on eCB signaling since it was prevented by CB1R antagonist AM251 (90% ± 5% of baseline, n=11; p>0.05 Tukey) **(Figure 22)**.

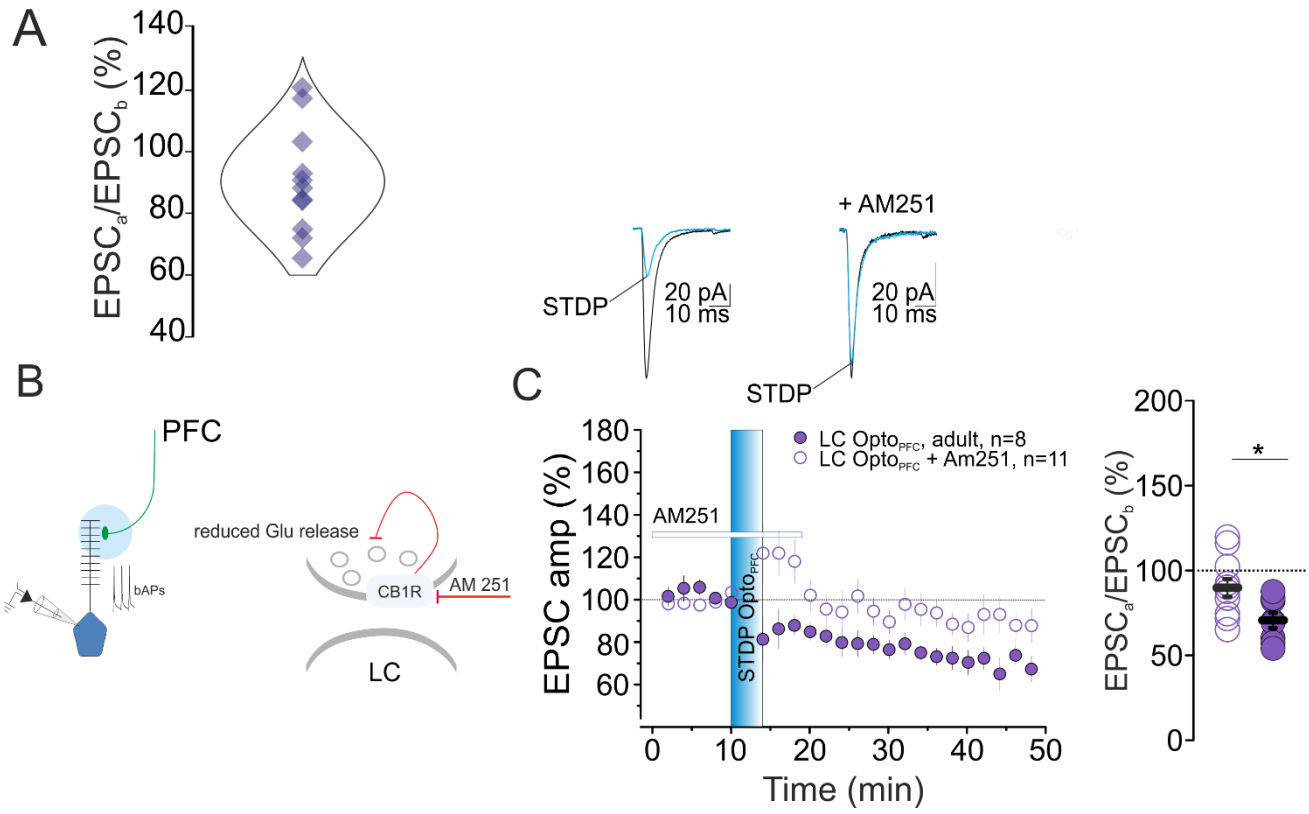

Figure 22. *(A) Violin plot representing a distribution of LC Opto$_{PFC}$+AM251 group EPSC$_a$/EPSC$_b$ values. (B, left) Schematic of the recording configuration in LC-NE neurons. (B, right) Representation of the AM251-mediated CB1R antagonism. (C, left) Time course and (C, right) scatter plot showing the LTD induced by post-pre pairing (solid purple circles), and (C, top, left) representative current traces. CB1R antagonism by AM251 (4µM; empty horizontal bar) blocked the t-LTD evoked by the Opto$_{PFC}$-STDP (empty purple circles)(Opto$_{PFC}$ adult; n=8 versus Opto$_{PFC}$ +AM251; n=11, p=0.01; 2sided unpaired t-test). Insets represent superimposed averaged recordings (30 traces) before (black) and after (blue) the delivery of the STDP pairing protocol (blue vertical bar).*

Exposure to novelty modulates synaptic plasticity in adult, but not young mice PFC→LC synapses

So far, we have demonstrated that eCB signaling is involved in the induction of PFC→LC t-LTD in a subpopulation of LC NE-neurons in young mice, and it is required for the induction of PFC→LC t-LTD in adult mice. We next sought to assess whether this plasticity mechanism is modulated by *in-vivo* experience. For this purpose, we investigated how t-LTD is modified in response to novel environment exposure. Our behavioral data **(Figure 13)** demonstrated that young and adult mice show diverse coping strategies when exposed to a novel open field. Young mice are more avoidant while adults show more approaching behavior. In other brain regions, the eCB system is engaged during exposure to novel environments (Lafenêtre et al., 2009) and CB1R signaling correlates with the salience of an experience (Cusulin et al., 2014). Hence, we aimed to assess how t-LTD at young and adult mice PFC→LC synapses is modulated by exposure to novelty. After assessing young and adult mice behavior in a novel OF, a subset of mice from each cohort were sacrificed for *ex-vivo* electrophysiology 24h after the OF test **(Figure 23 A)**. We found that $Opto_{PFC}$-STDP induced t-LTD was significantly impaired by exposure to a novel open field in adults (90% ± 6% of baseline, n=16; $p>0.05$ Tukey) but not in young mice (71% ± 8% of baseline, n=9; $p<0.05$ Tukey) **(Figure 23 B, C)**.

Together these results suggest that, in the LC, changes in presynaptic eCB-mediated signaling capacity reflect experience- and developmental stage-dependent modulation of PFC→LC synapses. Nevertheless, in young mice, presynaptic CB1R signaling is involved in the expression of PFC→LC t-LTD only in a subgroup of LC NE-neurons **(Figure 20)**. Therefore, the t-LTD we observe in young mice after the exposure to a novel OF could be induced by postsynaptic AMPA endocytosis-mediated mechanisms, while the presynaptic CB1R-mediated t-LTD might be blocked, as in adult mice. We will test this by using CB1R antagonist and AMPA-endocytosis blocking intracellular peptide in the young mice LC recordings after the exposure to a novel OF.

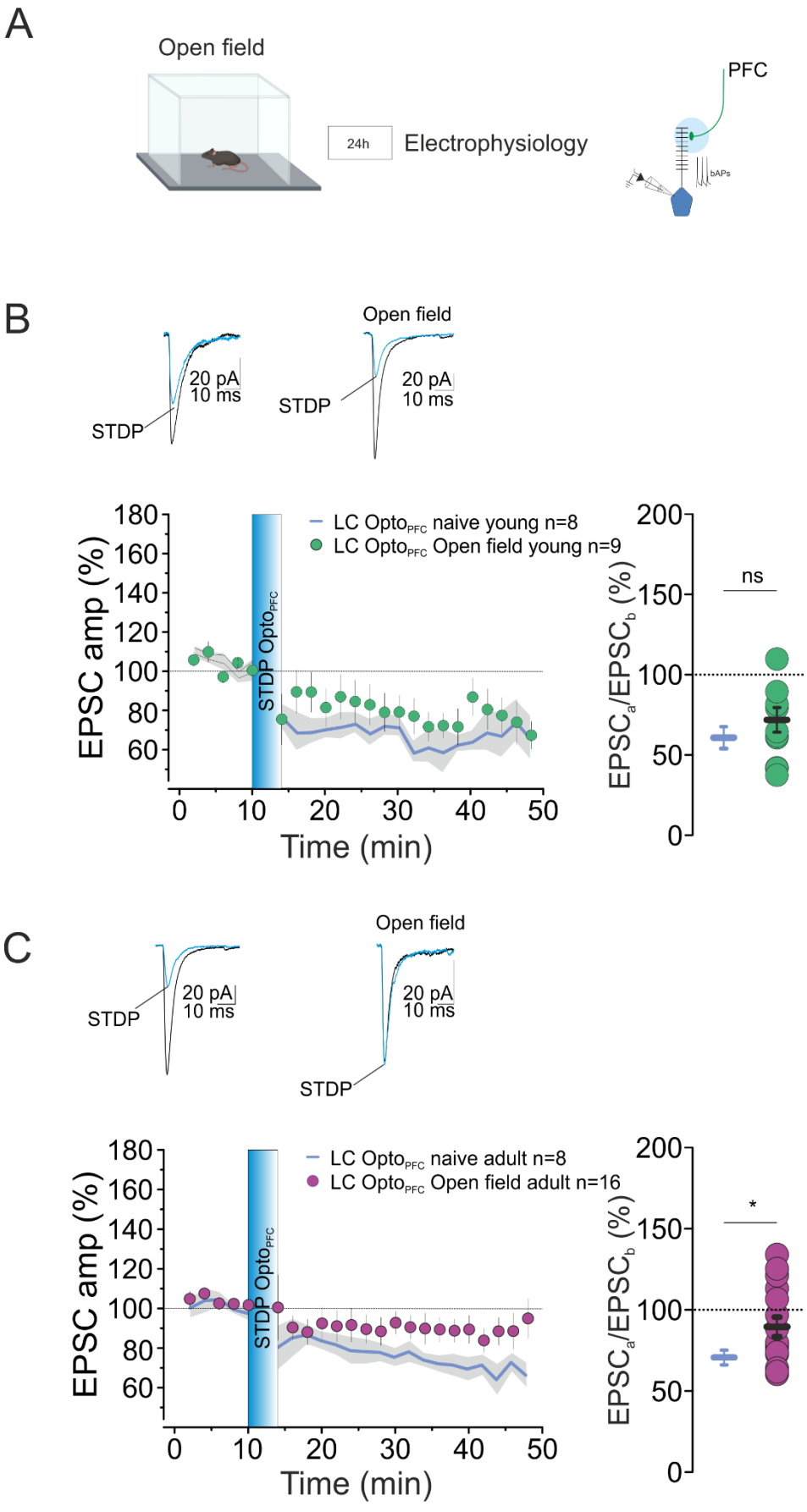
A
Open field
24h
Electrophysiology
PFC
bAPs
B
Open field
20 pA
10 ms
STDP
20 pA
10 ms
STDP
LC Opto$_{PFC}$ naive young n=8
LC Opto$_{PFC}$ Open field young n=9
STDP Opto$_{PFC}$
EPSC amp (%)
Time (min)
EPSC$_a$/EPSC$_b$ (%)
ns
C
Open field
20 pA
10 ms
STDP
20 pA
10 ms
STDP
LC Opto$_{PFC}$ naive adult n=8
LC Opto$_{PFC}$ Open field adult n=16
STDP Opto$_{PFC}$
EPSC amp (%)
Time (min)
EPSC$_a$/EPSC$_b$ (%)
*

Figure 23. *(A, left) Schematics of the experimental pipeline. (A, right) Schematics of the experimental settings used for EPSCs-Opto$_{PFC}$ recordings in LC-NE neurons. (B) Time course and scatter plot demonstrating that the novel OF does not modify the t-LTD at PFC→LC synapses of young mice. The shadowed line shows the time course of plasticity from the LC Opto$_{PFC}$ group (Figure 20 C) (LC Opto$_{PFC}$ Open field young; n=8 versus LC Opto$_{PFC}$ naive young; n=8, p=0.3; 2sided unpaired t-test). (C) Time course and scatter plot showing that the novel OF impairs t-LTD at PFC→LC synapses of adult mice. The shadowed line shows the time course of plasticity from the LC Opto$_{PFC}$ group (Figure 22 B) (LC Opto$_{PFC}$ Open field adult; n=16 versus LC Opto$_{PFC}$ naive young; n=8, p=0.04; 2sided unpaired t-test). Insets represent superimposed averaged recording (30 traces) before and after (blue) the delivery of the STDP protocol (indicated by the blue vertical bar).*

In summary, our data show that exposure to a novel environment activates LC neurons to a higher extent in adult mice compared to young animals, and this is accompanied by differences in valence assignment to novel stimuli. We show that differences in novelty processing occurs in parallel to dynamic modulation of the endocannabinoid signaling only in adult mice PFC→LC synapses. Moreover, our data show that PFC→LC inputs undergo developmental changes in synaptic strength and, at least in a subpopulation of cells, in the mechanisms regulating t-LTD.

Synaptic mechanisms of plasticity at non-dissected glutamatergic LC synapses: adolescence and adulthood

We have demonstrated that PFC→LC synapses undergo developmental changes in synaptic plasticity mechanisms and in the ability to recruit eCB-mediated signaling during salient behavioral experiences. Along with the eCB system, other long-range neuromodulators, such as CRF, undergo developmental transitions during the adolescence period (Korosi and Baram, 2008). We, therefore, aimed to further characterize synaptic plasticity mechanisms at LC synapses by assessing the impact of simultaneous activation of glutamatergic and neuromodulatory inputs impinging on this noradrenergic nucleus. For this purpose, the previously used optogenetic stimulation of PFC inputs was replaced by conventional electrical stimulation in order to recruit glutamatergic and neuromodulatory fibers **(Figure 24)**. We recorded EPSCs in LC neurons upon delivery of twin electrical stimuli, in presence of the $GABA_A$ receptor antagonist gabazine (10µM).

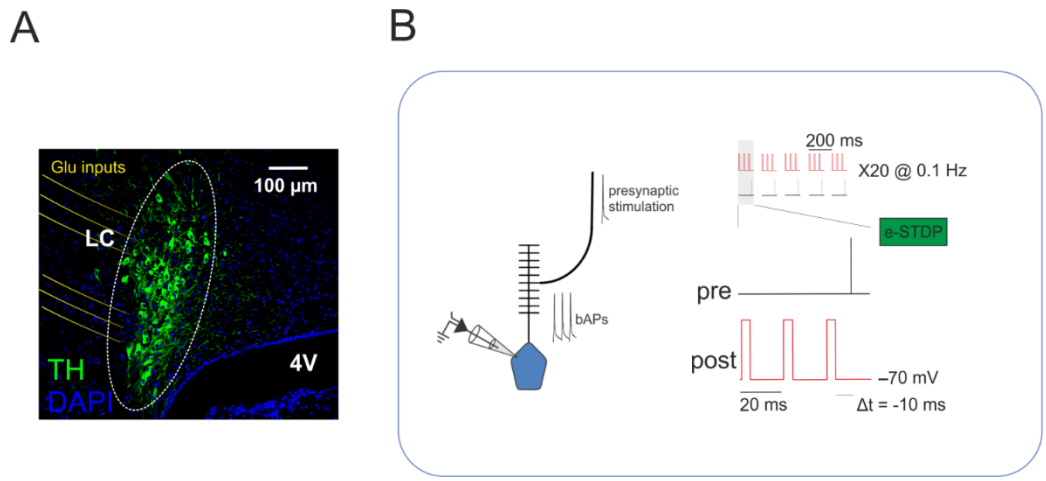

***Figure 24**. (A) Representative immunofluorescent staining of LC containing slice, glutamatergic inputs to LC are depicted in yellow. TH in green and DAPI in blue. (B) Burst pairing protocol for the induction of the electric negative-STDP. The protocol consists of 20 bouts delivered 10 s apart. Each bout consists of five bursts (200ms apart) each composed of three action potentials at 50 Hz followed by one EPSP (negative timing).*

eCB-mediated plasticity at LC glutamatergic synapses undergoes developmental regulation during adolescent to adulthood transition

Upon delivery of post-pre pairing of STDP protocol (e-STDP), we observed t-LTD in adult mice (78% ± 6% of baseline, n=7; $p<0.05$; Tukey) **(Figure 25 B)**, which, consistently with our previous results obtained at PFC-LC dissected inputs **(Figure 22)**) was blocked by the CB1R antagonist AM251 (4µM) (119% ± 8% of baseline n=9; $p>0.05$; Tukey) **(Figure 25 B)**. We next investigated the locus of

plasticity expression by measuring the paired-pulse ratio (PPR), a hallmark of presynaptic plasticity. Adult t-LTD was accompanied by an increase in PPR, ($^{5\text{-}10min}$Pre-STDP = 1.37 ± 0.16 versus $^{20\text{-}25}$Post-STDP = 1.47 ± 0.17; $p<0.05$). No changes in PPR were found in the presence of AM251 ($^{5\text{-}10min}$Pre-STDP = 1.57 ± 0.16 versus $^{20\text{-}25}$Post-STDP = 1.41 ± 0.10; $p<0.05$) **(Figure 25 C D)**. Together, these results are consistent with a canonical form eCB-LTD previously described in other synapses (Chevaleyre et al., 2007; Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Lafourcade et al., 2007).

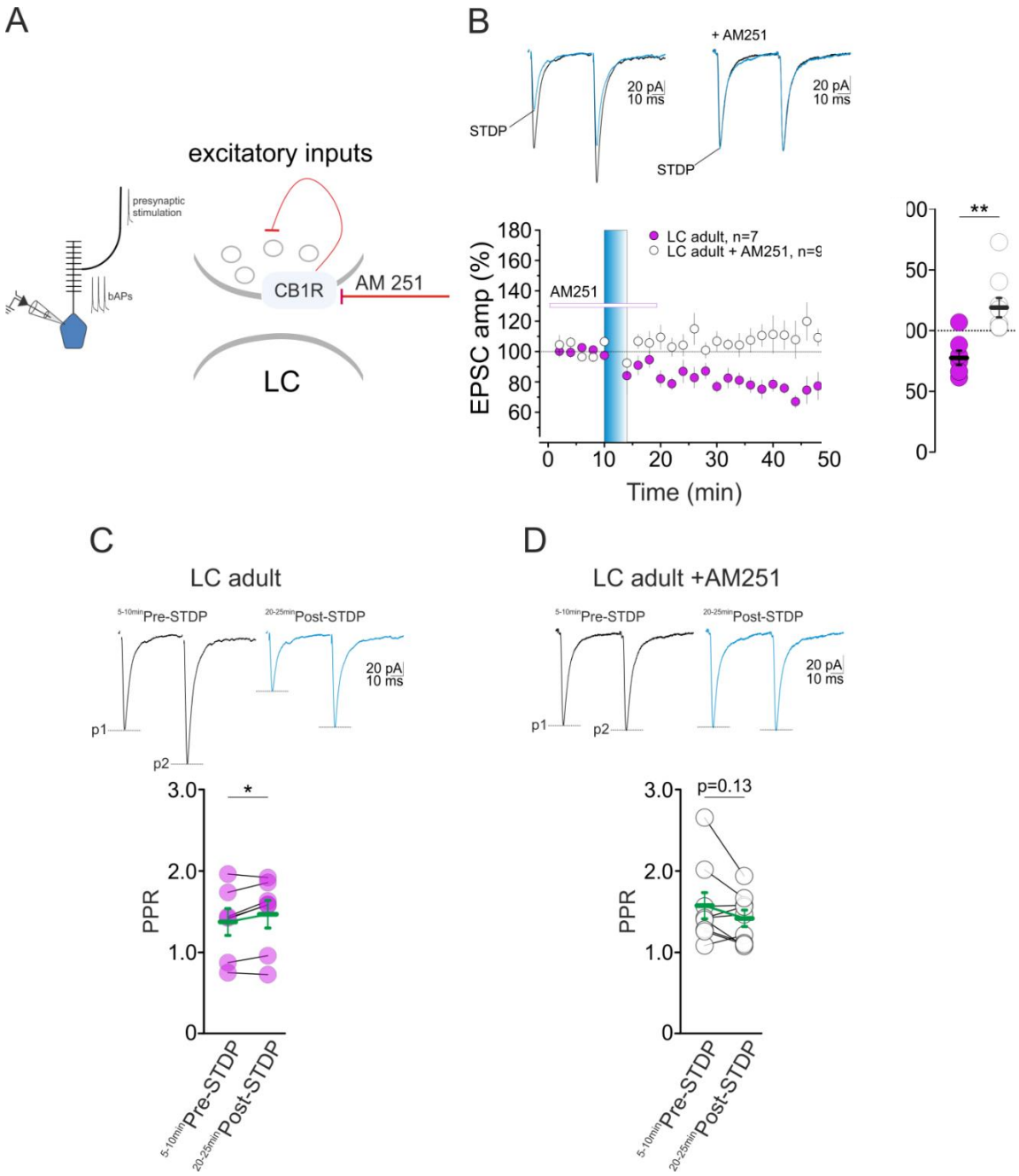

Figure 25. *(A, left) Schematic of the experimental setting used to record el.EPSCs in LC neurons. (A, right) Representation of AM251-mediated CB1R antagonism. (B) Post-pre pairing in adult LC neurons induced t-LTD (solid purple circles). Bath perfusion of AM251 (4µM; empty horizontal bar) prevented*

t-LTD (empty circles) (LC adult, n=7 versus LC adult+AM21, n=9, p=0.003; Mann-Whitney test). (C) Scatter plot indicating the paired-pulse ratios of the last 5 min of baseline and 20-25 min after STDP. PPR is calculated as the ratio between the amplitude of the second (p2) and the first averaged EPSCs (p1) ($^{5\text{-}10min}$Pre-STDP versus $^{20\text{-}25\ min}$ Post-STDP, p=0.04; 2tailed paired t-test). (D) AM251 scatter plot indicating the paired-pulse ratios of the last 5 min of baseline and 20-25 min after STDP ($^{5\text{-}10min}$Pre-STDP versus $^{20\text{-}25\ min}$ Post-STDP, p=0.13; 2tailed paired t-test). (B-D) Insets represent averaged recordings (30 traces) before and after (blue) the delivery of the STDP protocol (blue vertical bar). Dots represent single paired values.

In contrast to adult mice, the post-pre STDP protocol resulted in LTP of postsynaptic currents in adolescents (138% ± 13% of baseline; n=11; $p<0.05$ Tukey) **(Figure 26 B)**. The LTP was still dependent on eCB signaling, as it was blocked by CB1R antagonist AM251 (4µM) (98% ± 10% of baseline n=8; $p>0.05$; Tukey) **(Figure 26 B)**. No significant changes in PPR were associated with this form of plasticity ($^{5\text{-}10min}$Pre-STDP = 1.86 ± 0.18 versus $^{20\text{-}25}$Post-STDP = 1.8 ± 0.2; p=0.6) **(Figure 26 C)**.

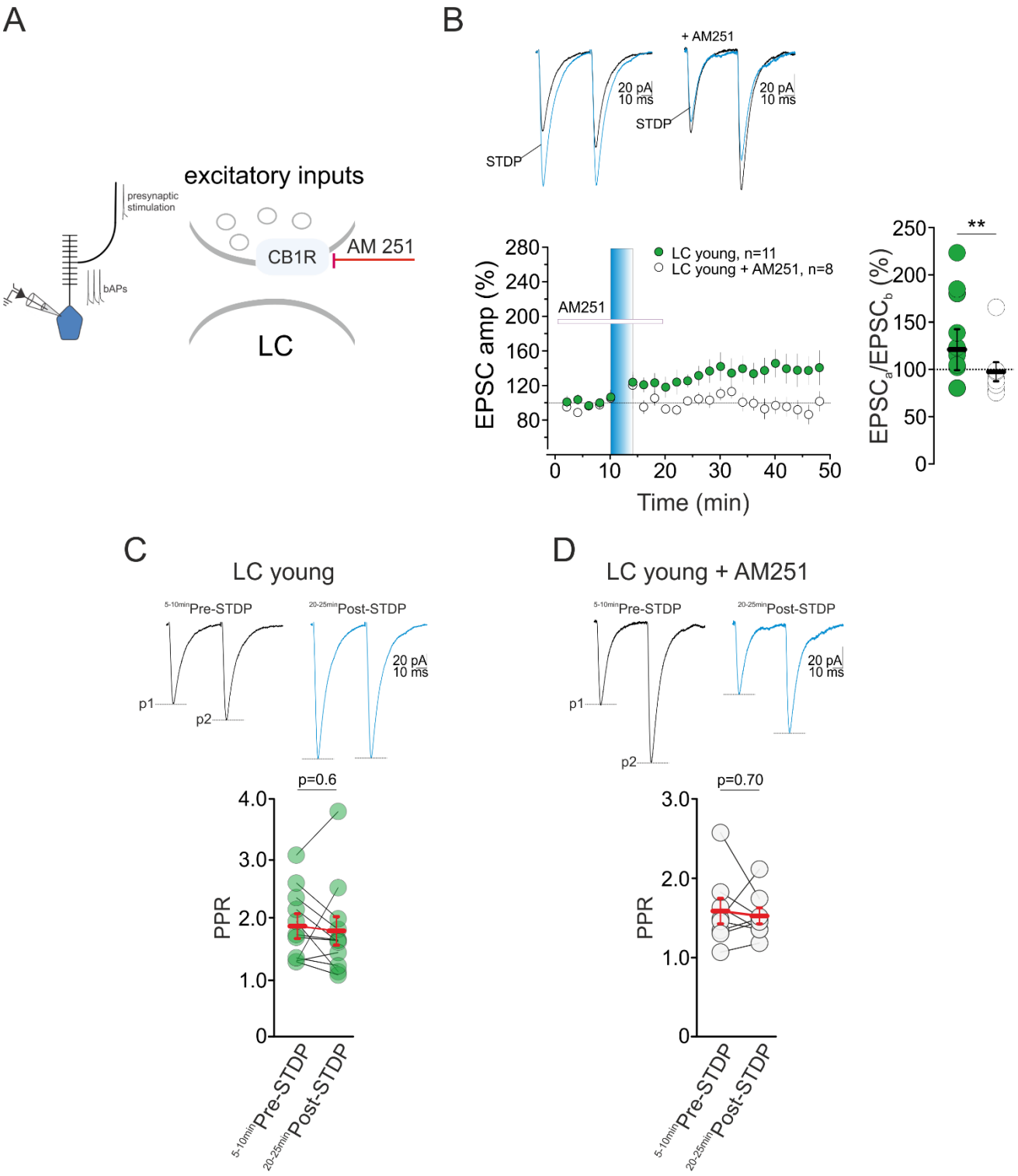

Figure 26. *(A, left) Schematic of the experimental setting used to record el.EPSCs in LC neurons. (A, right) Representation of AM251-mediated CB1R antagonism. (B) Post-pre pairing in young LC neurons induced t-LTP (solid green circles). Bath perfusion of AM251 (4µM; empty horizontal bar) prevented the t-LTP (empty circles) (LC young, n=11 versus LC young+AM21, n=8, p=0.009; Mann-Whitney test). (C) Scatter plot indicating the paired-pulse ratios of the last 5 min of baseline and 20-25 min after STDP. PPR is calculated as the ratio between the amplitude of the second (p2) ad the first averaged EPSCs (p1) ($^{5\text{-}10min}$Pre-STDP versus $^{20\text{-}25\ min}$ Post-STDP, p=0.6; 2tailed paired t-test). (D) AM251 scatter plot indicating the paired-pulse ratios of the last 5 min of baseline and 20-25 min after STDP ($^{5\text{-}10min}$Pre-STDP versus $^{20\text{-}25\ min}$ Post-STDP, p=0.7; 2tailed paired t-test). (B-D) Insets represent*

averaged recordings (30 traces) before and after (blue) the delivery of the STDP protocol (blue vertical bar). Dots represent single paired values.

Previously reported uncanonical forms of eCB-mediated LTP (Cui et al., 2018, 2016; Piette et al., 2020) mostly rely on GABA-ergic disinhibition, which facilitates LTP (Chevaleyre and Castillo, 2003; Lin et al., 2011; Pan et al., 2011; Zhu and Lovinger, 2007). This mechanism cannot explain the LTP we observed because our recordings were performed in presence of $GABA_A$ antagonist gabazine (10µM). The differences in synaptic plasticity outcomes upon the same stimulating protocol may be explained by differential involvement of long-range neuromodulator release in the two age groups (Brzosko et al., 2019, 2017; Cachope et al., 2007). In our experimental configuration, intra-LC electric stimulation is likely engaging projections from the CeA (McCall et al., 2015), which is one of the strongest extrahypothalamic CRF sources for the LC (Bouret et al., 2003; Reyes et al., 2008). In the LC, CRF activates CRF1R (Jedema and Grace, 2004; Reyes et al., 2006), which is coupled to G_s signaling pathway. Activation of CRF1R has been demonstrated to trigger LTP in other brain regions (Blank et al., 2002; Krishnan et al., 2010; Pollandt et al., 2006). We thus reasoned that there might be a developmentally regulated interplay between CRF and eCBs mobilization in response to presynaptic activity. To test this, we bath applied the selective CRF1R antagonist NBI 35965 during the induction of post-pre STDP protocol. CRF1R antagonism blocked t-LTP induction (NBI 35965, 1µM; 89% ± 6% of baseline; n=6; $p>0.05$ Tukey) in young mice **(Figure 26 B)**. In adult mice, NBI 35965 application did not affect t-LTD (71% ± 9% of baseline; n=7; $p<0.05$; Tukey) **(Figure 26 C)**.

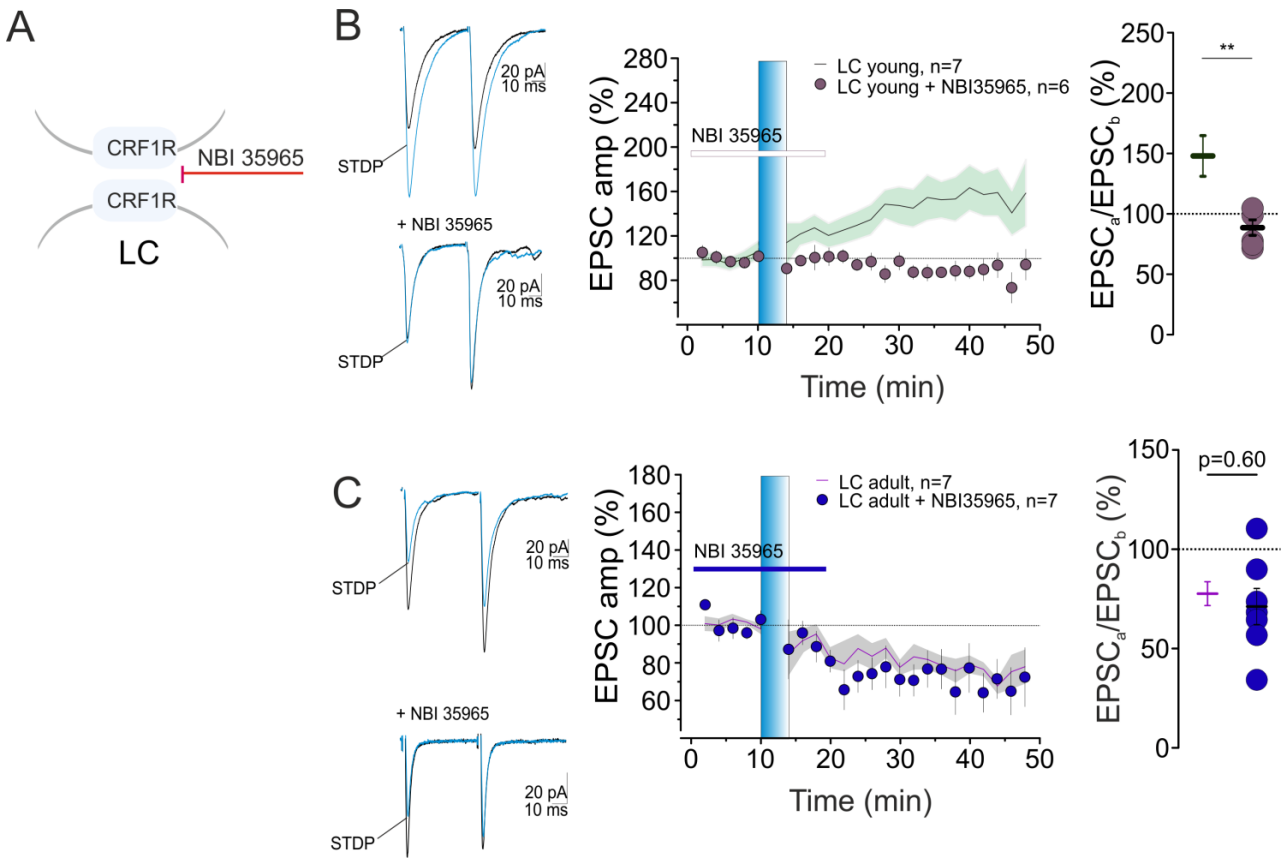

Figure 27. *(A, left) Representation of NBI 35965-mediated action on CRF1R. (B) Perfusion of the CRF1R selective antagonist NBI 35965 (1µM; empty horizontal bar) prevented t-LTP in young mice (LC young, n=7 versus LC young+NBI 35965, n=6; p=0.005; Mann-Whitney test). (C) NBI 35965 (1µM; empty horizontal bar) did not affect t-LTD in adult mice (LC adult, n=7 versus LC adult+ NBI 35956, n=7; p=0.6; Mann-Whitney test). (B-C) the shadow line shows the time course of plasticity from young* ***(Figure 26 B)*** *and adult mice* ***(Figure 25 B)****. Insets represent averaged recordings (30 traces) before and after (blue) the delivery of the STDP protocol (blue vertical bar).*

LC neurons in young mice show heterogeneous response to CB1R activation

So far our results show that, in LC neurons, CB1R activation is involved in opposite forms of plasticity in adolescent versus adult mice. Next, we asked whether it is sufficient to activate CB1R signaling to induce these two opposite forms of plasticity. To test this, we examined the effect of the CB1R agonist CP 55,940 on the EPSCs magnitude evoked by twin electrical stimuli.

Bath-applied CP 55,940 (1 µM) induced a significant depression of the glutamatergic responses in adult mice (adult CP 55,940; 43% ± 7 % of inhibition, n=9; p<0.05; Tukey) **(Figure 28 C)** but not in young subjects (data not shown, -34% ± 21 % of inhibition, n=17; p>0.05; Tukey). Since we observed a non-normal distribution of our data collected from young subjects (p=0.01, Shapiro-Wilk), we used a K-means cluster algorithm to segregate our data in two subgroups (young CP 55,940 cluster 1; cluster center 23.8; average distance from cluster center 20.2; n=11; young CP 55, 940 cluster 2, cluster center

-142, average distance from cluster center 24.9; n=6). We found that CP 55,940 (1µM) induced either potentiation (young CP 55, 940 cluster 2; -142% ± 12% of inhibition, n=6; p<0.05, Tukey) or depression (young CP 55,940 cluster 1; 23.8% ± 9% of inhibition, n=11; p<0.05; Tukey) of el.EPSCs in two different subgroups of young mice LC neurons. Overall, our results show that it is sufficient to activate CB1R to get a depression in adult mice LC neurons and in a subset of young animal NE-cells and to obtain a CB1R-dependent non-canonical potentiation in a subgroup of young mice LC neurons. Hence, CB1R is an important regulator of LC synaptic strength in both age groups.

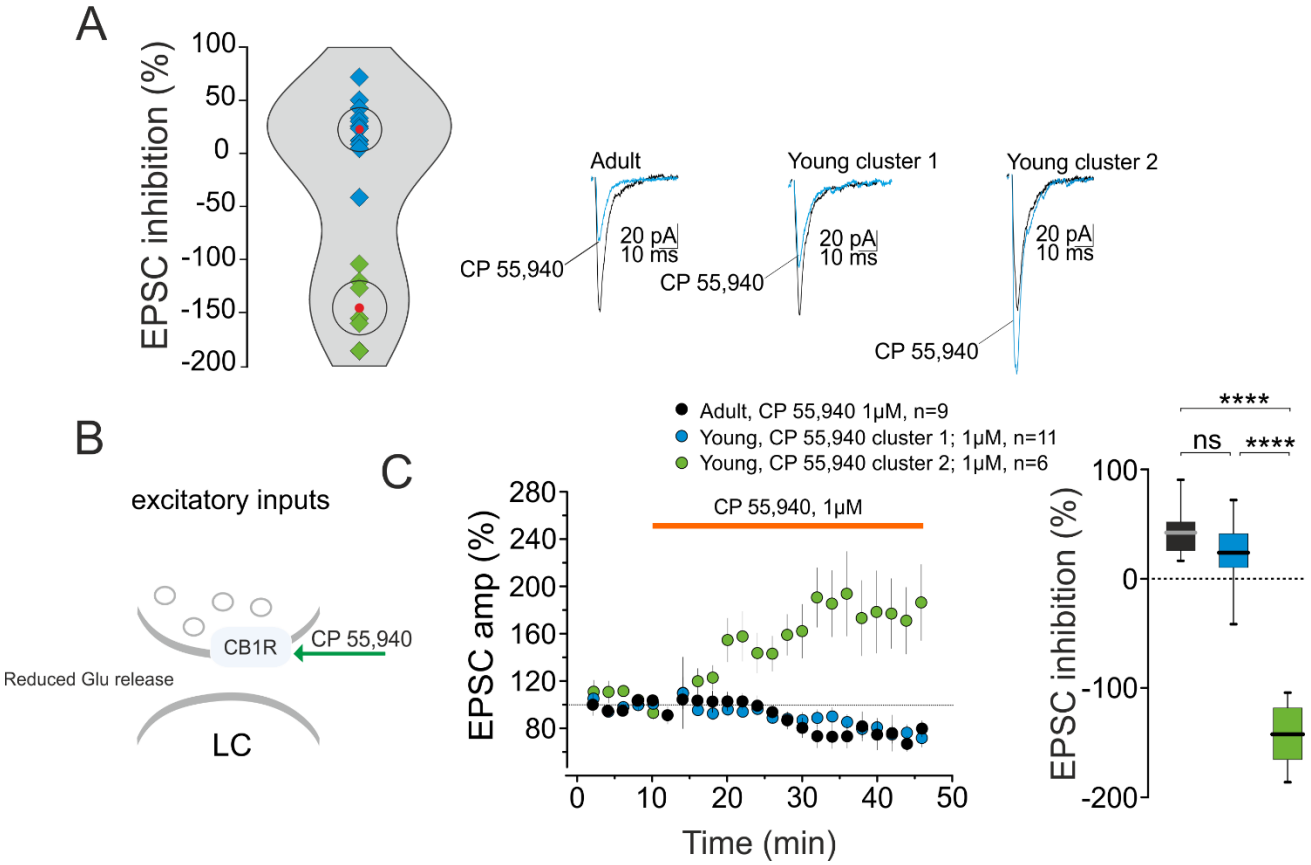

Figure 28. *(A) Violin plot representing a bimodal distribution of Young CP 55,940 1 µM group EPSC inhibition values. Different color- coding represents the two groups divided by K-means analysis. Cluster centers (red dots) and average distance from the cluster center (equivalent to the radius of the hollowed circle). (B, left) Representation of CP 55,940-mediated CB1R activation. (C) Time course showing the ESCP amplitude and scatter plot showing EPSC inhibition in response to CP 55, 940. The application of CP 55, 940 (1µM; orange horizontal bar) reduced EPSC amplitude in adult mice (black circles). In young mice, application of CP 55, 940 (1µM) resulted in a decrease of EPSC amplitude (blue circles) in cluster 1, while in young mice cluster 2 CP 55,940 (1µM) significantly increased EPSC amplitude (green circles).(1WA; $F_{2,23}$=97.47; p<0.0001; young CP 55,940 cluster 2 versus young CP 55,940 cluster 1, p<0.0001; young CP 55,940 cluster 2 versus adults CP 55,940, p<0.0001; young CP 55,940 cluster 1 versus adults p=0.2; Tukey). Whisker box plot extends from the 25th to 75th percentiles. Values are the minimum, mean (bar inside the box), and the maximum.*

CRF1R activation is necessary for CB1R-mediated potentiation of glutamate release

Our data indicate that t-LTP in LC neurons of young mice is mediated by both, CB1R and CRF1R. While it is established that CRF and eCB systems independently regulate the activity of LC neurons (Herkenham et al., 1990; Page et al., 2007; Reyes et al., 2012; Valentino et al., 1992), Wyrofsky and colleagues (R. Wyrofsky et al., 2017) provided ultrastructural evidence for CB1R co-localization with CRF-containing axon terminals in the LC. This anatomical evidence supports our previous finding regarding the involvement of CRF1R in the eCB mediated young animal's LTP. Therefore we next aimed to test whether CRF1R signaling is involved in CP 55,940-mediated potentiation in young mice LC neurons. Bath co-application of CP55,940 and CRF1R antagonist NBI 35965 resulted in a depression of glutamatergic response (19% ± 6% of inhibition; n=16; p<0.05; Tukey) **(Figure 29)**, the potentiation found in a subpopulation of LC cells upon CP 55,940 (1µM) application alone was no longer observed. These results suggest that the potentiation of el.EPSCs mediated by CB1R activation require the activation of CRF1R signaling.

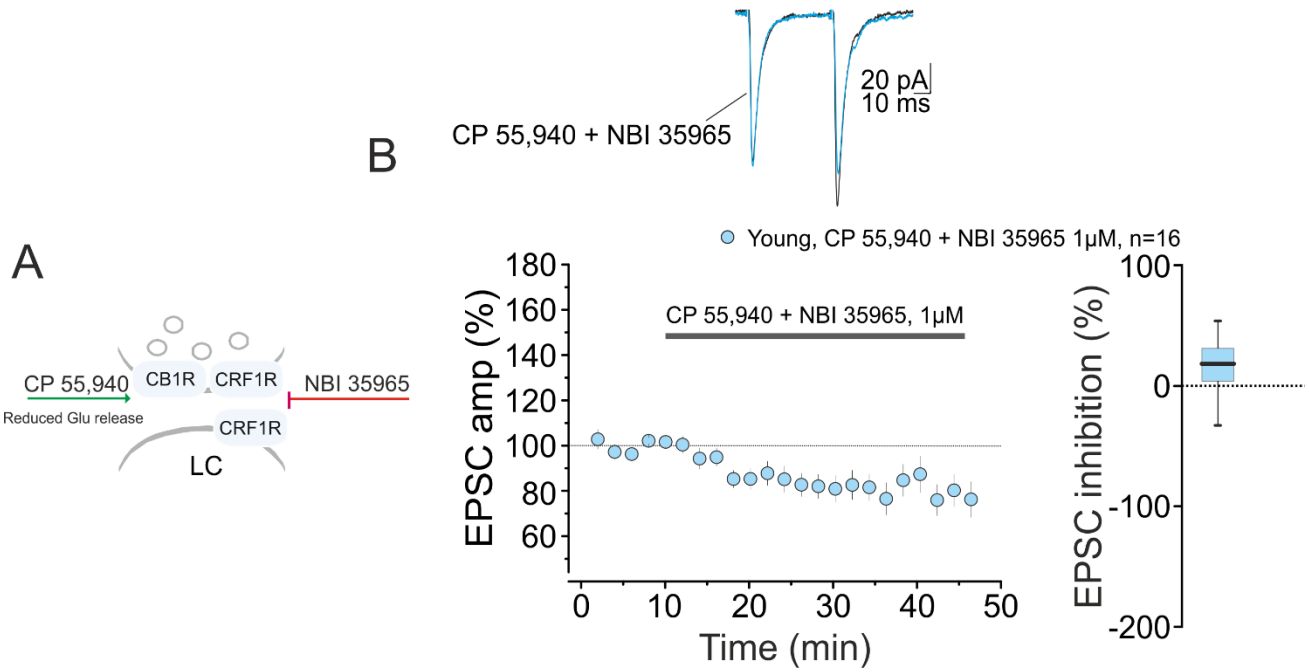

Figure 29. *(A) Representation of CP55,940-mediated CB1R activation and NBI 35965-mediated action on CRF1R. (B) Time course of EPSC amplitude in response to bath application of CP 55,940+NBI 35965 (1 µM; grey horizontal bar) and scatter plot of EPSC inhibition. Insets represent superimposed averaged recording (30 traces) before and after (blue) the application of CP 55,940+NBI 35965. Whisker box plot extends from the 25th to 75th percentiles. Values are the minimum, mean (bar inside the box), and the maximum.*

Previous studies had identified tonic and constitutive activation of CRF1Rs in other brain regions (Liu et al., 2004). We thus investigated whether CRF1R in the LC synapses is tonically active. We bath

applied CRF1R antagonist NBI 35965 and monitored EPSCs (el. EPSC) in the LC neurons. We did not observe changes in el.EPSCs magnitude (-6% ± 10% of inhibition; n=8; p>0.05; Tukey), after the application of NBI 35965 showing that NBI alone does not significantly affect EPSC magnitude in LC cells **(Figure 30)**.

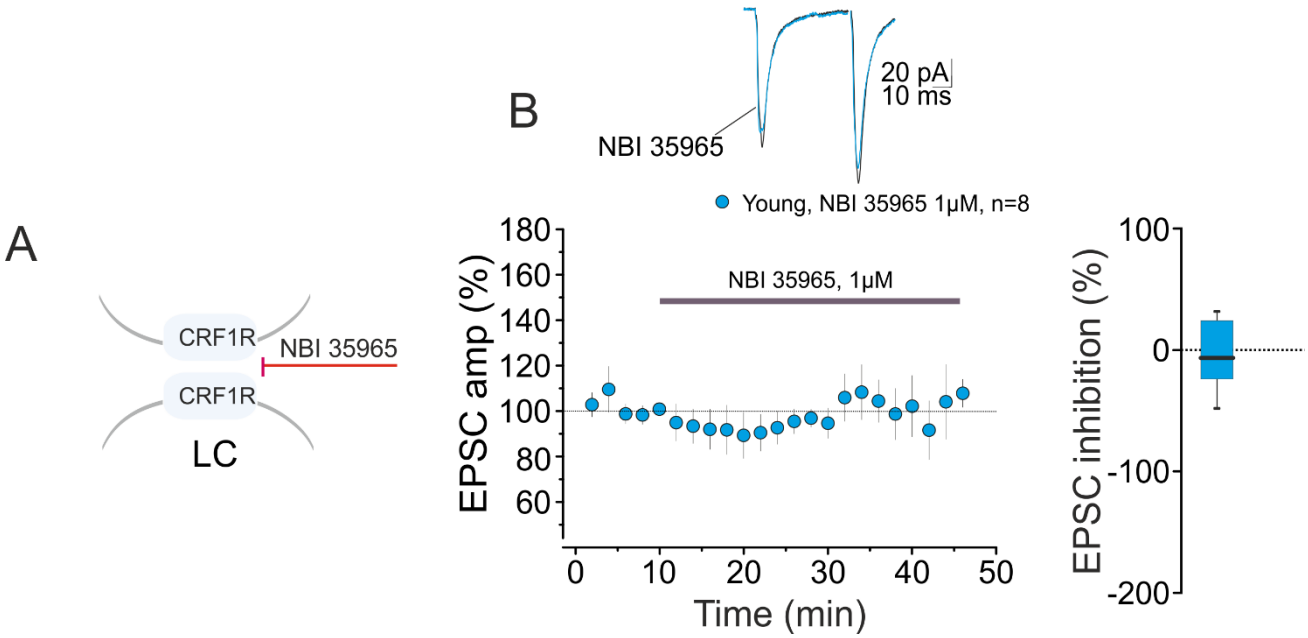

Figure 30. *(A) Representation of NBI 35965-mediated action on CRF1R. (B) Time course of el.EPSC amplitude in response to bath application of NBI 35965 (1 µM; grey horizontal bar) and scatter plot of EPSC inhibition. Insets represent averaged recordings (30 traces) before and after (blue) application of NBI 35965. Whisker box plot extends from the 25th to 75th percentiles. Values are the minimum, mean (bar inside the box), and the maximum.*

In conclusion, we show that the negative STDP paradigm induces t-LTD in adult mice, and surprisingly, t-LTP in young mice at non-dissected glutamatergic LC inputs. While in adult mice t-LTD relies on CB1R signaling, young mice‘s t-LTP is mediated by both, CB1R and CRF1R signaling. Interestingly, the activation of CB1R alone is enough to potentiate a subgroups of LC neurons, and this potentiation requires CRF1R activation.

Project implementation

The main findings of this study can be summarized as follow:

1) LC neurons are more strongly entrained in adult mice compared to young ones upon novelty exposure. This is associated with different exploratory behavior in the former age group **(Figure 13; Figure 14)**.

2) PFC→LC inputs undergo changes in synaptic strength and, at least in a subpopulation of cells, in the mechanisms regulating t-LTD **(Figure 18; Figure 20; Figure 21; Figure 22)**.

3) In adult but not young subjects, eCB signaling at PFC→LC is modulated by novel experiences **(Figure 23)**.

4) At glutamatergic LC inputs, postnatal development induces changes in the synaptic mechanisms regulating plasticity **(Figure 25; Figure 26; Figure 27).**

Together, the first three points suggest a link between the maturation of PFC→LC connection and the expression of different patterns of exploratory behavior in adults versus adolescents. Thus, project implementation requires establishing a causal relationship between PFC→LC input activation and exploratory behavior at the two developmental periods. Secondly, to further clarify the role of the eCB system in the expression of exploratory behavior, we will monitor the eCB dynamics during PFC→LC dependent behaviors. Lastly, our findings suggest an uncanonical form of eCB-mediated long-term potentiation at young mice LC glutamatergic synapses that need to be further characterized.

Establishing the causal link between PFC→LC input activation and exploratory behavior

Our results demonstrate that upon exposure to a novel OF, young and adult mice exert different coping strategies: young mice are more avoidant, while adult mice show more approaching behavior **(Figure 13)**. To test whether PFC→LC inputs are causally involved in these different exploratory strategies we will use optogenetic inhibition of PFC→LC inputs during exposure to novelty. For this, we will use ArchT, a light-driven outward-proton pump, which enables inhibition of neurons in response to pulses of light (Han et al., 2011). We will inject ArchT expressing virus [AAV9-CamKIIa-ArchT-eYFP] into the PFC of adult mice and implant optical fibers in the LC. We will optogenetically inhibit PFC→LC synapse during exposure to a novel OF. Preliminary data from our laboratory suggest that PFC→LC input may be anxiolytic in adult mice, as the inhibition of PFC→LC input just before the exposure to a novel OF was related to the expression of an avoidant behavior during OF exploration (data not shown). Moreover, young mice showed reduced activation of LC cells upon novelty compared to

adults **(Figure 14)**. Therefore, if our hypothesis is correct, adult mice should display more avoidant behavior in a novel OF during PFC→LC inputs inhibition.

Young mice exposed to a novel OF field show more avoidant behavior compared to adults. Our AMPA/NMDA ratio results suggest functional remodeling of PFC→LC inputs during adolescence to adulthood transition **(Figure 18)**. We hypothesize the weaker synaptic strength at the PFC→LC connection observed in adolescence might contribute to more avoidant behavior during novelty experience. To test this, we will activate PFC→LC inputs during exposure to novelty. Specifically, we will inject ChR2 expressing virus [AA9-CaMKIIa-ChR2(H134R)-eYFP] in the PFC of newborn mice (P0) and at P18 implant optic fibers in the LC. We will optogenetically activate PFC→LC synapse during exposure to a novel OF, expecting to overcome the weaker PFC→LC synaptic strength of young mice. This, in turn, should lead to a reduction in the avoidant behavior during novelty exposure, similar to naive adult mice.

Establishing a direct involvement of the eCB system and exploring its dynamics at PFC→LC synapses during exposure to a novel environment

Our *ex-vivo* electrophysiology data indicate that the eCB system modulates PFC→LC synapses. Moreover, the eCB signaling capacity reflects experience-dependent modulation at PFC→LC synapses of adult mice, but not young ones **(Figure 23)**. However, we still need to validate that t-LTD in young mice upon novel OF exposure is, similar to naive animals, mediated by eCBs in a subpopulation of LC neurons **(Figure 20)**. To test this, upon OF exposure, we will use the selective CB1R antagonist AM251 on *ex-vivo* brain slices to prevent LTD. If novelty exposure does not affect PFC→LC synapse in young subjects we expect to see an effect in a discrete LC subpopulation.

To causally test whether CB1R expressed at PFC→LC synapse is necessary for the expression of different coping behaviors upon exposure to a novel environment, we will selectively delete this receptor at PFC→LC input and monitor the explorative behavior in a novel environment in young and adult mice. To achieve a specific CB1R deletion at PFC→LC synapse we will employ an intersectional viral approach (Kakava-Georgiadou et al., 2019), in which CB1R deletion is dependent on a retrograde CRE-expressing FLP-dependent virus injected in the LC [retroAAV-EF1a-FRT-CRE] and FLP recombinase expressing virus injected in the PFC of CB1 Flox/Flox mice. CB1R lacking mice specifically at PFC→LC synapses will be subjected to a novel OF test. We expect to see an impaired exploratory pattern at least in adult animals since novel OF exposure completely prevented eCB dependent t-LTD at PFC→LC synapses, possibly indicating involvement of CB1R signaling during exposure to novelty.

Next, to confirm that eCBs are released during novel environment exploration, we will assess the *in-vivo* eCB dynamics of LC neurons in this task. We will image the signal dynamics of a genetically encoded eCB sensor $GRAB_{eCB}$ (Dong et al., 2020) by using fiber photometry in freely-moving dopamine β-hydroxylase Cre driver mice. Given that our plasticity results point at an occluded LTD upon novel OF exposure in adult mice only **(Figure 23)**, we expect to see higher eCBs released at the LC level in adult mice compared to young ones.

Investigating the mechanism of CRF1R mediated t-LTP in young mice

Our opto-STDP protocol induced t-LTD in PFC→LC synapses of young and adult mice **(Figure 20, 22)**, however when we engaged multiple glutamatergic and neuromodulatory afferents by electrical stimulation, el.STDP protocol resulted in t-LTP only in young mice LC neurons **(Figure 26)**. We hypothesized that, by recruiting neuromodulatory afferents, electrical stimulation induces the release of neuromodulatory molecules pushing towards synaptic potentiation. Our data indicates CRF, through CRF1R signaling, as a possible candidate able to promote LTP. Yet the remaining question is how CRF is more readily recruited in young compared to adult mice?

CeA is one of the strongest extrahypothalamic CRF sources in the LC (Bouret et al., 2003; Reyes et al., 2008), and compared to the PFC, CeA shows early structural and functional development (Gee et al., 2013; Giedd et al., 1996; Guyer et al., 2008; Hare et al., 2008; Payne et al., 2010; Ulfig et al., 2003). Thus, we speculated a stronger connection between CeA-CRF^+ neurons and LC in young compared to adult subjects. To test this, in collaboration with M. Pasqualetti we started an anatomical tracing study by using retrograde rabies virus (for strategy explanation see: **Figure 7**). As a proxy of input strength, we will count the number of CeA-CRF^+ neurons projecting to the LC in the two age groups. We expect a higher number of CeA-CRF^+ neurons retrogradely labeled reflecting a stronger CeA-CRF^+→LC connection in young compared to adult mice.

To verify that CRF contributing to the t-LTP in young mice is released by the CeA, we will chemogenetically activate CeA inputs to the LC during the opto-STDP paradigm. In detail, we will inject AAV-hSyn-DIO-hM3D(G_q)-mCherry virus in the CeA and AA9-CaMKIIa-ChR2(H134R)-eYFP in the PFC of the CRF-cre transgenic mouse at P0. During *ex-vivo* electrophysiological experiments, we will engage hM3D(G_q) expressing CeA-CRF inputs to the LC by bath applying the hM3D(G_q) cognate ligand clozapine-N-oxide (CNO) during opto.STDP paradigm. We expect to recapitulate the t-LTP at PFC→LC inputs mediated by CRF release from the CeA.

Discussion

While extensive research focused on unveiling Locus Coeruleus (LC) efferent pathways and the role of Norepinephrine (NE) in target brain regions, the contribution of LC afferents in modulating LC activity remains unknown. The LC is a highly integrative neuromodulatory nucleus (Breton-Provencher and Sur, 2019a; Schwarz et al., 2015; Szabadi, 2013), thus studying synaptic physiology of LC-NE neurons could advance the understanding of how changes in LC-NE signaling affect LC-dependent behaviors in health, and disease. The cognitive and emotion control center PFC sends glutamatergic projections to the LC (Jodo et al., 1998; Sara and Hervé-Minvielle, 1995) through which the PFC may exert top-down control over sub-cortical LC during salient novel experiences (Arain et al., 2013; Selleck et al., 2018; Silvers et al., 2017). However, how this connection is regulated through postnatal development and its behavioral role is still unclear.

Behavioral role of PFC→LC inputs

Our results show that LC neurons are recruited upon novelty exposure. These observations are in line with previous findings showing LC-NE neuron activation in exposure to novel objects, enriched novel environments, and during neophilic, and neophobic behaviors (Gompf et al., 2010; Lustberg et al., 2020b; Vankov et al., 1995). Following exposure to a novel environment, we found that LC neurons are recruited to a higher extent in adult mice compared to adolescents. This difference in LC neurons activation occurred in parallel to markedly different exploratory behaviors in a novel open field (OF). Young animals demonstrated negative valence-related behavior, as they were avoidant to explore the novel environment, and spent more time in the periphery of the OF. In contrast, adult mice seemed to assign more positive valence when exposed to a novel environment, as they approached the center of the OF arena more often (Powell et al., 2004; Tye, 2018). Higher negative emotional reactivity and emotional cues-driven behavior are commonly reported in human and rodent adolescents (Casey et al., 2019; Cunningham et al., 2002; Gee et al., 2013; Guyer et al., 2008; Hare et al., 2008; Monk et al., 2003; Vink et al., 2014). This behavioral propensity is associated with protracted PFC and accelerated Amygdala maturation (Hare et al., 2008; Kiss et al., 2007; Monk et al., 2003; Stephanou et al., 2016). The PFC is involved in valence assignment to a novel environment through a top-down mechanism known as a cognitive appraisal (Britton et al., 2011; Ochsner et al., 2002). We found lower synaptic strength at PFC→LC synapses of young mice compared to adults, pointing to an ongoing functional maturation of PFC→LC synapses during postnatal development. Weaker PFC→LC inputs in adolescence may also result in a lower number of LC cells entrained by exposure to a novel environment. Accordingly, we found a lower number of *c-fos* positive LC neurons after novel OF in

adolescents compared to adults. Thus, lack of top-down cognitive control from the PFC during adolescence may explain more negative valence-associated behavior, compared to adult mice, when exposed to novelty.

In the peri-LC region, we observed some *c-fos*$^{+}$/TH^{-} neurons. The peri-LC region closely approximates the Barrington's (Bar) nucleus (Verstegen et al., 2017), which neurons show immunoreactivity for Glut2 antibody, and roughly half of Bar cells express corticotropin-releasing factor (CRF) (Verstegen et al., 2017). Besides Bar nucleus, TH^{-} GABA-ergic interneurons (LC-GABA) are intermingled with and surround TH^{+} LC neurons (Breton-Provencher and Sur, 2019b). LC-GABA neurons are mainly located in the anterior and medial parts of the LC. The *c-fos*$^{+}$/TH^{-} neurons in the peri-LC zone could be either Bar or LC-GABA neurons, but further immunohistological examination is needed to confirm this hypothesis. In addition to staining against *c-fos* and TH, we could immunolabel for Vglut2, Vgat, CRH in the peri-LC zone to elucidate the identity of *c-fos*$^{+}$/TH^{-} neurons.

In our study, stronger LC *c-fos* activation was related to more positive valence-related behavior (adult mice), while weaker LC activation was associated with more avoidant behavior (young mice). These findings are at odds with previous studies demonstrating that chemogenetic/optogenetic activation of the LC-NE nucleus (McCall et al., 2015; Zerbi et al., 2019) or of CeA-CRF^{+}→LC projections promote anxiety-like behavior and acute aversion (McCall et al., 2015). As discussed in the first chapter of this thesis (see: 'Heterogeneous LC-NE efferent pathways'), LC is a highly heterogeneous nucleus, and NE release from distinct subpopulations of LC neurons can play functionally diverse or even opposite roles on behavior (Borodovitsyna et al., 2020; Hirschberg et al., 2017; Li et al., 2016). LC cells receiving inputs from CeA could display a different output pattern compared to the ones receiving from the PFC potentially explaining these opposite behavioral outcomes. Although PFC activation has a potent effect on LC neurons (Jodo et al., 1998; Sara and Hervé-Minvielle, 1995), it accounts only for 2% of total LC inputs. In contrast, CeA represents one of the strongest inputs to the LC (McCall et al., 2015). Thus, activation of the entire LC nucleus may recapitulate the role of stronger LC afferents while occluding the effect of weaker LC inputs. Additional studies are needed to assess the PFC→LC→output pathways to elucidate the net circuit effect of LC activation by selective inputs.

High arousal and negative valence are commonly regarded as anxiety (Russell, 1980). In the OF test, the rodents' tendency to avoid the OF center zone can be interpreted as anxiety-related, under the assumption that the center of the arena is more threatening in respect to the periphery (Lipkind et al., 2004; Sestakova et al., 2013). Thus, in our study, the behavioral differences observed in young and adult mice upon exposure to previously unexplored OF can also be regarded as different levels of anxiety (Simon et al., 1994). The anxiety induced by forced exposure to a novel environment (i.e. OF test) reflects a transient emotional state, rather than pathological trait anxiety (Rodgers, 1997). Classical anxiety tests, as elevated plus maze or light-dark box could be performed to confirm our

results about higher state anxiety in young mice compared to adults but these tests would not rule out whether young mice exhibit trait anxiety (as both tests represent inescapable novelty condition). Trait anxiety can be tested by a Novelty Induced Place Preference test (Griebel et al., 1993) in which a mouse is confronted with a familiar and a novel compartment during free exploration of an experimental arena (Belzung and Griebel, 2001). This free-exploration-based test is considered to be devoid of anxiogenic stimuli. To distinguish between state and trait anxiety, we will subject mice to the Novelty Induced Place Preference test and measure their voluntary exploration of the novel compartment. The adolescence period is characterized by impulsive behavior and excessive risk-taking in rodents (Laviola et al., 2003; Macrì et al., 2002). These studies are in contrast with our results showing the reduced exploration of the central area of the OF in young mice, suggestive of reduced risk-taking. This discrepancy can be explained by the different adolescence period in which mice were tested in previous studies, compared to ours. We perform experiments on young adolescents at P23-28, while risk-taking behavior is reported only in older adolescents (P31-60) (Laviola et al., 2003). In line with our study, young adolescent mice (P35) show higher anxiety-like behavior assessed in the elevated plus-maze, compared to older adolescents (P48) and adult (P61) mice (Laviola et al., 2003).

Developmentally-regulated transition in long-term synaptic plasticity mechanisms at LC synapses

In this study, we investigated the long-term synaptic plasticity of LC neurons *ex-vivo* by applying a post-pre STDP paradigm. At PFC→LC synapses in young and adult mice, the STDP pairing resulted in t-LTD. In adult mice, the t-LTD was mediated by eCB signaling, as for other brain regions (Bosch-Bouju et al., 2016; Cavaccini et al., 2018; Chevaleyre and Castillo, 2003; Crozier et al., 2007; Cui et al., 2018, 2015; Foncelle et al., 2018; Heifets and Castillo, 2009; Sjöström et al., 2004). Interestingly, in young mice, we found that t-LTD at PFC→LC synapses is mediated by CB1R signaling and postsynaptic AMPAR endocytosis-mediated mechanism. This suggests a developmentally-regulated transition of synaptic plasticity mechanisms from AMPAR endocytosis-to CB1R-dependent depression expressed at PFC→LC synapses respectively from early adolescence (P23-28) to adulthood (P60-75). In our experiments, we record LC neurons from young mice between P23 to P28. We did not find a correlation between the recording day and the plasticity mechanism preferentially regulating the t-LTD in young mice. Recordings at different time windows are necessary to assess when this an age-dependent transition from AMPAR internalization- to solely CB1R-mediated t-LTD takes place.
We also found that eCB-mediated signaling capacity reflects experience-dependent plasticity at PFC→LC synapses. Exposure to a novel environment impaired t-LTD in the LC of adult mice. These results are in line with previous findings demonstrating recruitment of the eCB signaling upon salient

events (Cusulin et al., 2014). In contrast to adult mice, eCB-dependent t-LTD was not significantly affected in the LC of young mice. Since, in young subjects, t-LTD at PFC→LC synapses relies on CB1R only in a fraction of PFC-receiving LC cells, it is tempting to speculate that presynaptic eCB-LTD at this synapse is needed in the whole LC population to cope optimally with a novel environment and reduce avoidant behavior. Nonetheless, further experiments are needed to causally prove this point. As we further explored the plasticity mechanisms at LC synapses by engaging multiple excitatory and neuromodulatory inputs, we found that postnatal development has a significant impact on the post-pre STDP outcome. The same electrical STDP protocol induced t-LTD in adult mice and t-LTP in young animals. The t-LTP we observed is dependent on CB1R- and CRF1R-mediated signaling. Moreover, in young mice, it was enough to activate CB1R signaling in a subpopulation of LC neurons to have a strong potentiation of glutamatergic signaling. The downstream signaling of CB1R is crucial for the direction of synaptic plasticity expression (Piette et al., 2020). The view of an exclusive coupling of CB1Rs with $G_{i/o}$ proteins was challenged when a functional interplay between CB1R and dopamine D2 receptors was identified, showing that restricting $G_{i/o}$ protein availability (e.g. in presence of active D2 receptor) in presynaptic terminals favors CB1R switching from $G_{i/o}$ to G_s (Busquets-Garcia et al., 2018; Glass and Felder, 1997; Kearn et al., 2005). Such context-dependent eCB signaling pathway may also be influenced by the process of brain maturation. eCB system undergoes developmental maturation during the adolescence period (Meyer et al., 2018). Supporting this, the maturing eCB system has been shown to play a role in the switch of plasticity rules in other brain regions such as the striatum (Ade and Lovinger, 2007). Limited $G_{i/o}$ protein availability in presynaptic terminals can facilitate the switch of CB1Rs to G_s protein coupling and stimulate pkA pathway, thus leading to an LTP (Glass and Felder, 1997; Gonzalez et al., 2009). Hypothetically, during adolescence there may be a different ratio of available $G_{i/o}$ proteins for CB1Rs in PFC terminal impinging on the LC, compared to adulthood, thus pushing CB1R coupling to G_s proteins.

We found that CB1R activation-induced potentiation of synaptic responses by the agonist CP 55,940 requires CRF1R activation, as bath co-application of CP 55,940 and CRF1R antagonist NBI 35965 resulted in depression of synaptic responses, and the potentiation of synaptic responses induced by application of CP 55,940 alone was no longer observed. CRF1R activation does not occur through tonic or constitutive CRF signaling, since we showed that CRF1R antagonist NBI 35965 does not affect the magnitude of synaptic responses. These results point at additional mechanisms that may activate CRF1R during the CB1R-induced potentiation. Immunofluorescence studies report co-localization of CB1R with CRF-containing axon terminals profiles in the LC (R. Wyrofsky et al., 2017) which raises the possibility for coordinated receptor signaling. This could occur through functional heterodimerization of the two receptors or signaling through a shared pool of secondary messengers (Haack and McCarty, 2011; Terrillon and Bouvier, 2004). Alternatively, CP 55,940 may also have a

CB1R independent action on synaptic glutamate release, as demonstrated for other synthetic cannabinoids that can affect evoked EPSCs in absence of CB1R (Hájos et al., 2001). To examine if CP 55,940 effect is mediated solely through activation of CB1R, we will have to test whether the effect of the cannabinoid is prevented by CB1R antagonism.

Compared to increasing data showing molecular, neurochemical, and anatomical heterogeneity of LC neurons (Chandler et al., 2014; Schwarz and Luo, 2015; Uematsu et al., 2017, 2015), modules of synaptic plasticity mechanisms in the LC were not reported so far. At young mice PFC→LC synapses, we found that different mechanisms might mediate the expression of t-LTD in distinct LC subgroups. Moreover, at non-dissected glutamatergic LC synapses, we found heterogeneous responses to CB1R agonist (potentiation or depression of synaptic responses). Additional molecular, morphological or electrophysiological evidence is necessary to elucidate whether these are truly distinct populations of LC neurons. Differences in synaptic properties, including neuromodulatory state and previous synaptic activity could also account for the heterogeneous response to CB1R agonist and antagonist on LC slices of young mice. These results may add to the list of LC physiological features that undergo postnatal developmental alterations, along with previously reported changes in adrenergic receptor expression, cellular coupling, membrane properties, and responsiveness to sensory stimuli (Marshall et al., 1991; Nakamura et al., 1987).

In summary, this study suggests that the maturation of PFC→LC connection is linked to the expression of different exploratory behavior in a novel environment in adult and adolescent mice. We found that eCB signaling regulates PFC→LC synapses during adulthood and only in a cluster of neurons, during adolescence. Consistently, eCB-mediated signaling capacity engagement by a novel salient environment is developmentally regulated. The LC-NE neurons undergo several major physiological alterations during the adolescence developmental period, including changes in synaptic strength and shift in plasticity rules where CRF and eCB systems act in synchrony to regulate the synaptic plasticity at LC glutamatergic synapses.

Our results not only reveal a new mechanism of experience-dependent neuromodulated plasticity at PFC-LC synapses but also uncover diverse molecular players involved in shaping this synapse during adolescence to adulthood transition. Moreover, these results contribute to our understanding of how LC activity is regulated by cortical and subcortical inputs during different neurodevelopmental stages.

Materials and methods

Animals

Male C57BL/6J mice (postnatal day 23-28 (young/adolescent) and postnatal day 60-75 (adult)) were used in this study. Mice were kept in standard cages with food and water ad libitum at 22 ± 1°C under artificial 12/12-h light (7-19) / dark cycle.

Ex-vivo electrophysiology

Slice preparation

Mice were anesthetized under isoflurane, intracardially perfused with a dissecting artificial cerebrospinal fluid (aCSF) solution containing: 87mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4, 7mM $MgCl_2$, 75mM sucrose, 25mM $NaHCO_3$, 25mM D-glucose, 0.5mM $CaCl_2$, saturated with a mix of 95%O_2 5%CO_2. Mice were subsequently decapitated; their brains were kept in the same ice-cold dissecting aCSF used during the successive vibratome slicing procedure. Horizontal slices containing pons sections (200 µm thickness) were obtained using a Vibratome 1000S (Leica) then transferred to aCSF containing: 115mM NaCl, 3.5mM KCl, 1.2mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2mM $CaCl_2$, 25mM $NaHCO_3$ and 25 mM D-glucose, aerated with 95% O_2 and 5% CO_2. Slices were incubated for 20 min at 32°C, then kept at 22-24 °C. During electrophysiological experiments, slices were continuously superfused with aCSF at a rate of 2mL/min at 28-30 °C.

LC recordings

Whole-cell patch-clamp recordings were performed on LC-NE neurons in horizontal brain slices. Neurons were visualized under IR-DIC (infrared differential interference contrast) and selected by their anatomical location, their morphology, by their electrophysiological properties (Zhang et al., 2010). The intracellular solution contained: 130 mM KMeSO4, 5mM KCl, 5mM NaCl, 10mM HEPES, 0.1 mMEGTA, 2mM $MgCl_2$, 0.05 mM $CaCl_2$, 2mM Na2-ATP and 0.4 mM Na3-GTP (pH 7.2-7.3, 280-290 mOsm/kg). The excitatory postsynaptic currents (EPSCs) were evoked at 0.1 Hz in the presence of the GABA-A receptor antagonist gabazine (10 µM). by local electrical stimulation using a theta electrode connected to a constant current insulator unit (Digimeter Ltd) or by optogenetic

stimulation of prefrontal cortex afferents using short blue light pulses (1ms at 470 nm) of LED light delivered through the microscope objective (CoolLED, pE-100, Andover, Hampshire, United Kingdom). To induce plasticity a negative STDP paradigm was used: 20 EPSPs bouts paired with bAPs, delivered 10 s apart. Each bout consisted of five bursts (200 ms apart) each composed of three bAPs at 50 Hz followed by one EPSP (negative timing). The onset of the EPSPs followed the last postsynaptic stimulation by 10 ms (Δ t= -10 ms). During plasticity induction, the postsynaptic neuron was held at -70mV in between the bAPs. AMPA and NMDA receptor-mediated EPSC neurons were recorded at -70 mV and +40 mV, respectively. NMDA currents were recorded in presence of the AMPA receptors-antagonist NBQX disodium salt (20 µM). Data were excluded when the access resistance (Ra) changed >20%. Data are reported without corrections for liquid junction potentials Data were acquired using a Multiclamp 700B amplifier controlled by pClamp 10 software (Molecular Device), filtered at 2.4 kHz and sampled at 10 kHz (voltage clamp), or filtered at 10 kHz and sampled at 20 kHz (current clamp) with (Digidata 1322, Molecular Device).

Data analysis

The occurrence and magnitude of synaptic plasticity were evaluated by comparing EPSC normalized amplitudes from the last 5 min of baseline recordings with the values between 20-25 min after conditioning. The plasticity loci (pre- vs post-synaptic) were deducted from the change in the paired-pulse ratio (PPR) after the delivery of the stimulation protocol in the same time periods. AMPA and NMDA ratio for each neuron was calculated as the ratio between AMPA EPSC peak and the NMDA EPSC peak amplitude (pA) of the subtracted current upon NBQX application.

Substances

Triton-X, NaCl, KCl, NaH2PO4, MgCl2, CaCl2, NaHCO3, D-glucose, Sucrose, KMeSO4, HEPES, Na2-ATP, Na3-GTP, Paraformaldehyde (PFA), were purchased from Sigma Aldrich. Gabazine (SR 95531 hydrobromide), AM251 were purchased from HelloBio. NBI 35965 was purchased from Abcam. C-Fos antibody was purchased from Cell Signaling Technology, Inc. Neurobiotin was purchased from (DBA, Italy). DAPI and Steptavidin conjugated with Alexa 568 (Thermofisher Scientific). CP 55, 940 was purchased HelloBio.

Animal surgeries

Adult mice viral injections

C57BL/6J (P30-45) were anesthetized with isoflurane (4-5% for induction, 1-2% for maintenance) and O_2 mix. Subsequently, mice were mounted on a stereotaxic frame (Stoelting). To target the PFC 0.5 µL of AAV9.CaMKIIa.hChR2.eYFP (AddGene) was delivered at the following stereotaxic coordinates from bregma: AP+1.94; ML ± 0.4; DV -2.4., the virus volume was delivered at a 0.1 µL/min rate using a syringe (WPI, Nanofil 10 µL) connected to a Micro Pump (WPI, UMP3 UltraMicroPump). Electrophysiological recordings were performed at least 5 weeks after surgery.

P0-P3 viral injections

C57BL/6J (P0-3) were anesthetized through hypothermia and placed on a stereotaxic apparatus (Stoelting). To target the PFC 0.15 µL of AAV9.CaMKIIa.hChR2.eYFP (AddGene) was delivered at the following stereotaxic coordinates from bregma: AP+0.5; ML ± 0.1; DV -0.9., the virus volume was delivered at a 0.1 µL/min rate using a syringe (WPI, Nanofil 10 µL) connected to a Micro Pump (WPI, UMP3 UltraMicroPump). Electrophysiological recordings were performed between P23-28.

Behavior

Open field test

Mice were habituated to the experimental room and handling for 5 consecutive days. The control mice used for *c-fos* staining underwent the same habituation routine as the experimental group, except for the exposure to the novel open field. The OF apparatus consisted of a 30cmx30cmx40cm dark plastic box. Mice were allowed to freely explore the apparatus for 10 min, during which their activity was filmed with an infrared camera situated above the OF. 90 min., after the test, a subset of mice were sacrificed for c-Fos staining. Alternatively, mice used for electrophysiological recordings were sacrificed 24h after the OF test. Behavior was scored using AnyMaze behavioral tracking software (Stoelting). Thigmotaxis index was calculated as a ratio of distance traveled in the periphery and total distance traveled, expressed as a percentage. Thigmotaxis time was calculated as a ratio of total time spent in the periphery and total time in the apparatus expressed as a percentage.

Immunofluorescence

Tyrosine Hydroxylase (TH) immunostaining

To confirm the LC-NE identity of the recorded neurons, the patched cells were filled with Neurobiotin (0.3mg/mL, DBA Italia) and processed post-hoc for their immunoreactivity for the Tyrosine Hydroxylase marker. Following the electrophysiological recordings, brain slices were kept for 24-48h in 4% PFA in 0.1M Phosphate buffer 7.4pH (PB). Brain slices were then rinsed 3 times in PB saline (PBS) for 15 min and then permeabilized and blocked (PBS containing 3% Bovine Serum albumin (BSA) [w/v]; 0.3 Triton-X [v/v]). Slices were then incubated at 4°C overnight in the same permeabilization and blocking medium containing the anti-TH rabbit polyclonal antibody (1:500, Merk-Millipore). Brain sections were then rinsed 3x15min in PBS and incubated 4h RT in the permeabilization and blocking buffer containing Alexa568-streptavidin (1:1000, Thermofisher) to label the Neurobiotin filled patched cells. Subsequently, slices were rinsed 3x15min in PBS and incubated 2h RT in the permeabilization and blocking medium containing Alexa488 conjugated secondary antibody (1:500, Themofisher). After rinsing the slices 3x15min, the nuclei were stained with DAPI solution (1:500 in PBS, Sigma-Aldrich), rinsed again, mounted on glass slides, and coverslipped with ProLong Gold antifade reagent (Invitrogen).

c-fos immunostaining

Briefly, mice were transcardially perfused with 25mL PBS and 25 ml 4% PFA in 0.1M PB. Brains were extracted, post-fixed 24h in PFA 4%, cryoprotected (sucrose 20%), and then sliced in the coronal plane to obtain brain sections containing LC.

Free-floating sections were rinsed in 0.1 PBS 3 times, incubated in a solution containing 10% BSA and 0.2% Triton in 0.1M PBS for 1h at RT. Subsequently, sections were incubated in 3% BSA with anti-TH chicken polyclonal antibody (1:500, Merk-Millipore) and rabbit anti-c-Fos (1:500, Cell signaling Technology) at 4°C overnight, rinsed again 3 times in 0.2% Triton in 0.1M PBS, incubated in a solution of 3% BSA and 0.2% Triton in 0.1M PBS with AlexaFluor 568 goat anti-rabbit (1:500, Invitrogen) and AlexaFluor 488 goat anti-chicken (1:500, Invitrogen) secondary antibodies. Washed 3x in 0.2% Triton in 0.1 PBS. After rinsing the slices 3x15min, the nuclei were stained with DAPI solution (1:500 in PBS, Sigma-Aldrich), rinsed again, mounted on glass slides, and coverslipped with ProLongGold antifade reagent (Invitrogen). From each brain, 5 images of LC-containing slices were acquired with an inverted Leica TCS SP5 confocal microscope. TH^+ cells and $TH^+C\text{-}FOS^+$ were counted using ImageJ software.

Statistics

Appropriate parametric statistics were used to test our hypothesis unless the data did not meet the assumptions of the intended parametric test (normality test). In that case, appropriate nonparametric tests were used. Power analysis assumptions (the probability that the test correctly rejects the null hypothesis) was as follows: 0.9 alpha, 0.05; two-tailed and expected difference 50% greater than the observed s.d. Data were analyzed by one-way repeated measures ANOVA for comparison within a group, by one-way ANOVA for between-group comparisons. Two-way was used in the case compared respectively two independent factors (GraphPad Prism 7 software). Post-hoc analysis (Tukey or Dunnett, as indicated) was performed only when ANOVA yielded a significant main effect. Two-group comparisons were performed t-test, for normally distributed data, or Mann-Whitney U nonparametric test (GraphPad Prism 7 software). Statistical details of experiments are shown in the results, figures, and figure legends. Data are reported as mean ± s.e.m. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

The violin plot outlines illustrate Kernel probability density (i.e. the width of the area represents th proportion of the data located there). The Bandwith has been selected using Scott's rule (SCOTT, 1979).

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Publications

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