AGE DEPENDENT DIFFERENTIAL EFFECT OF NOREPINEPHRINE ON THE PYRAMIDAL NEURONS OF THE ANTERIOR PIRIFORM CORTEX IN MOUSE MODEL OF EARLY ODOR PREFERENCE LEARNING

By

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ABSTRACT:

Rodent pups show preference to an odor when it is paired with a tactile stimulation- stroking the back with a paintbrush- only within a critical period of postnatal (P) 10-12 days of age. Norepinephrine (NE) released from the locus coeruleus during stroking plays a crucial role in this learning. Here we established the learning model in mouse pups and showed that it was β-adrenoceptor dependent. Next we investigated the developmental changes in pyramidal cell excitability and NE responsiveness in the anterior piriform cortex layer II neurons. Two concentrations (0.1 and 10 μM) of norepinephrine did not alter intrinsic properties in either group, although there was an age-related difference in those properties. In P8–11 pups, norepinephrine at 0.1 μM presynaptically decreased miniature inhibitory postsynaptic current (IPSC) and increased miniature excitatory postsynaptic current (EPSC) frequencies, opposite to the effect of norepinephrine at 10 μM. This suggested involvement of different receptors with different concentrations. In P14 and older pups both concentrations promoted inhibition.

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For Titli

We did it together…

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-Abhinaba Ghosh

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LIST OF ABBREVIATIONS:

2-DG	2-deoxyglucose
aCSF	artificial Cerebro Spinal Fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Action Potential
aPC	anterior Piriform Cortex
APV	(2R)-amino-5-phosphonovaleric acid
cAMP	cyclic Adenosine Monophosphate
CREB	cAMP Response Element-Binding Protein
CS	Conditioned Stimulus
EPL	External Plexiform Layer
EPSC	Excitatory Post Synaptic Current
GC	Granule Cell
GCL	Granule Cell Layer
GL	Glomerular Layer
IPL	Internal Plexiform Layer
IPSC	Inhibitory Post Synaptic Current
JG	Juxtaglomerular Cells
LC	Locus Coeruleus
LC-NE	LC-noradrenergic

LOT	Lateral Olfactory Tract
LTP	Long Term Potentiation
MC	Mitral Cell
MCL	Mitral Cell Layer
mEPSC	miniature Excitatory Post Synaptic Current
mGluR	metabotropic Glutamate Receptor
mIPSC	miniature Inhibitory Post Synaptic Current
M/T	Mitral/Tufted
mV	millivolt
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NE	Norepinephrine
OB	Olfactory Bulb
ONL	Olfactory Nerve Layer
ORN	Olfactory Receptor Neurons
O/S^+	Odor with Stroking
O/S^-	Odor without Stroking
OT	Olfactory Tubercle
P8	Postnatal day 8
pA	picoampere
PBS	Phosphate Saline Buffer
PC	Piriform Cortex

pCREB	Phosphorylated CREB
PG	Periglomerular
PKA	Protein Kinase A
PNDs	Postnatal Days
PP	Peppermint
RMP	Resting Membrane Potential
s.c.	Subcutaneous
SA	Short Axon
sAHP	slow After Hyper Polarisation
SEM	Standard Error of Mean
SEL	Subependymal layer
TBS	Theta Burst Stimulation
UCS	Unconditioned Stimuli

CO-AUTHORSHIP STATEMENT:

A version of the research presented in this thesis has been published-

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I, Abhinaba Ghosh, hold a first author status for the manuscript. It is co-authored by my supervisors and colleague. Qi Yuan and Nicole C. Purchase performed experiments of figure 6. Rest of the experiments were performed and analysed by me and Qi Yuan. Research question and experimental design was originally developed by Xihua Chen and Qi Yuan. Subsequent adjustment was done by me with proper guidance from Xihua Chen and Qi Yuan. Manuscript was written by me, Nicole C. Purchase and Qi Yuan.

Introduction

Procuring food, finding shelter and running away from danger - these traits are common to all animals. Our system is hard-wired to follow these instincts even in the early days of life. However, procuring food and shelter in adult life is very different from that in early life, when the babies are dependent on their mothers. Whether babies learn to associate their mothers with source of food and shelter or they are born with this "system-setting" is arguable; but this maternal dependence fades away when they grow up.

It is surprising to see how the whole system works towards obtaining maternal care in the early postnatal life. Newborns have only a few senses to rely on at this stage. Rodent pups are devoid of auditory or visual clues and they only depend on the olfactory and somatosensory systems to survive. Pups learn to associate olfactory and somatosensory cues to reach their mother. Certain odors that are associated with their mother are better remembered by them. This is called early odor preference learning.

Interestingly, this learning does not take place when the pups are in their second week of postnatal life. Although they continue to learn olfactory cues and use them for survival and procuring food throughout their life, this particular maternal-care-dependent-olfactory-learning ceases to occur after a certain time period – this is what we call "critical/sensitive period" of this learning.

Why does it happen? To understand this marvel of nature, first we need to take a look at the anatomy of the olfactory system.

1. Anatomy

1.1 Brief anatomy of the olfactory bulb

Odor molecules bind at the nasal epithelium and then the information travels down the olfactory nerve to reach the olfactory bulb- the first station of odor information processing. A significant portion of information processing happens here and different neurons with different network-organisations take part in that.

The olfactory bulb (OB) is one of the primitive brain structures evolutionarily conserved and even can be seen in lower animals. It is considered a part of the primitive cortex called allocortex. The OB is a small structure having a volume of ~7.53 cubic millimetre in adult mouse (Parrish-Aungst et al., 2007). It has a laminar organisation and the layers are listed here from superficial to deep (Price and Powell, 1970a; Price and Powell, 1970b; Pinching and Powell, 1971a, b, c)- Olfactory nerve layer (ONL), Glomerular layer (GL), External plexiform layer (EPL), Mitral cell layer (MCL), Internal plexiform layer (IPL), Granule cell layer (GCL) and Subependymal layer (SEL). These layers are not isolated, rather different types of the member neurons connect to each other by means of synaptic connections. For example, each glomerulus is a hub of extensive glutametargic synaptic connections between the axons of several (to the scale of thousands) olfactory receptor neurons' (ORN) and the dendrites of 10-70 mitral/tufted (M/T) cells (Mori et al., 2006; Sosulski et al., 2011; Ke et al., 2013). These synapses are under modulation of juxtaglomerular (JG) cells, which includes periglomerular cells, short axon cells, and external tufted cells (Pinching and Powell, 1971a, b, c). Granule cells are roughly 30 times more numerous than M/T cells and they shape the activity of these output neurons by dendrodendritic GABAergic modulation. Odor information coming from the odorant molecules

via ORN reaches glomerular circuitry and finally activates a set of M/T cells. After further processing by the GCL, M/T cells send off the information downstream. The long axons of these neurons form the lateral olfactory tract, a part of which reaches the piriform cortex (Figure 1).

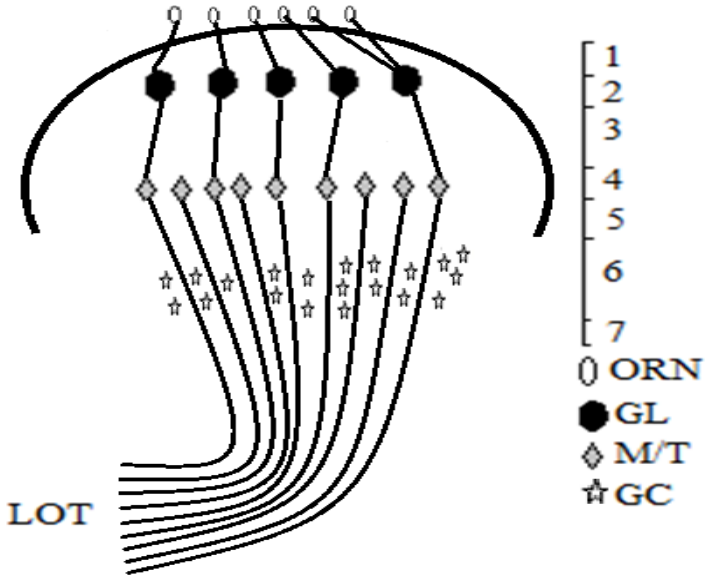

Figure 1. Cytoarchitecture of the olfactory bulb

Olfactory bulb has seven layers from superficial to deep -

1.Olfactory nerve layer (ONL); 2.Glomerular layer (GL); 3.External plexiform layer (EPL); 4.Mitral cell layer (MCL); 5.Internal plexiform layer (IPL); 6.Granule cell layer (GCL) and 7.Subependymal layer (SEL). Olfactory receptor neurons (ORN) sends their axons to the glomerulous (GL) where the excitatory synapse is formed with dendrites of the output neurons of the olfactory bulb- known as- mitral/tufted cells (M/T). The output from these neurons gets modified by granule cells (GC) and reaches piriform cortex and other areas by lateral olfactory tract (LOT).

1.2 Brief anatomy of the piriform cortex

Partially processed information travels down the olfactory tract from the olfactory bulb to reach the olfactory cortex or piriform cortex (and also to some other structures).Major processing of the incoming information takes place in the piriform cortex and then it is sent off to several other cortical and subcortical regions.

Similar to the OB, the piriform cortex is also a part of a phylogenetically older cortex called paleocortex. It lies downstream to the olfactory bulb in olfactory information processing. Being an older cortex, it has a relatively simple three layered, cytoarchitecture which remains a fascinatingly complex processing system researchers have been trying to dissect out for decades.

The piriform cortex consists of layers I, II and III; layer I is further subdivided in Ia and Ib. Layer II consists of different pyramidal neurons, namely semilunar and superficial pyramidal neurons with the latter being present in deeper segments. Layer III has deep pyramidal neurons. These neurons send long apical dendrites to receive LOT inputs at layer Ia. At layer IIb, they receive cortical association inputs (Figure 2). Semilunar neurons have multiple long dendrites reaching the LOT and thereby rendering the typical “fork” shape. They lack the presence of basal dendrites compared to the pyramidal neurons. A small population of multipolar neurons with spiny dendrites are also observed in deeper segment of layer III. All the neurons described so far are excitatory in nature (Haberly and Price, 1978 a,b; Tseng and Haberly, 1989).

Inhibitory interneurons are abundant in the piriform cortex- horizontal cells in layer I, bipolar neurons in superficial layer II; small and large multipolar neurons present in layers II and III,

respectively. These GABAergic neurons take part in feed-forward and feedback inhibition mechanisms to modulate odor information processing. Deep to layer III, there is endopiriform nucleus consisting of excitatory multipolar neurons with spiny dendrites (Hoffman and Haberly, 1993).

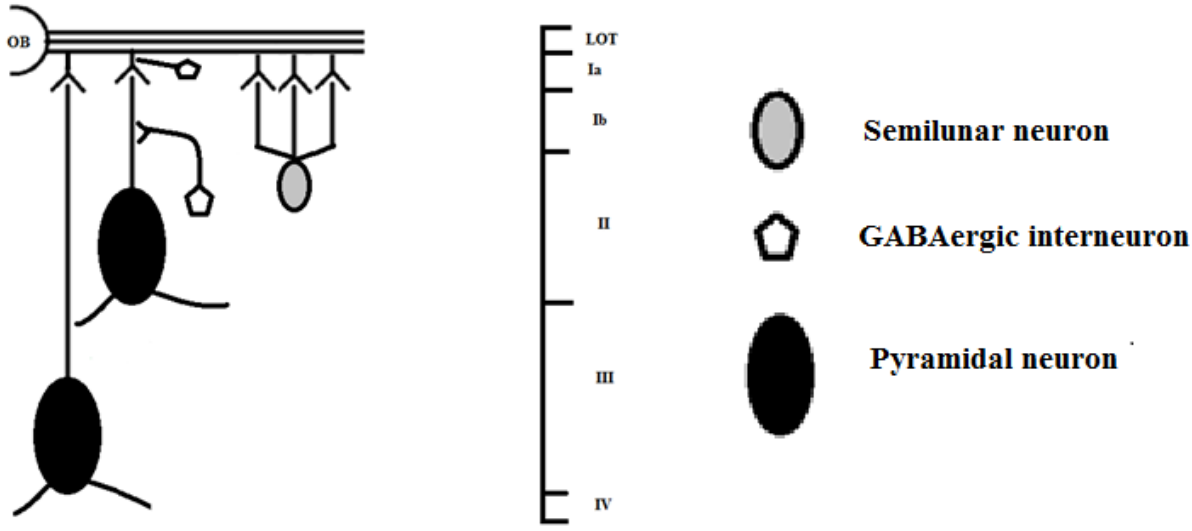

Figure 2. Simplified cytoarchitecture of piriform cortex

Output neurons from the olfactory bulb (OB) send their axons as lateral olfactory tract (LOT) to the piriform cortex and other brain areas. The afferents from LOT make excitatory synapses onto the dendrites of semilunar neurons and pyramidal neurons at layer Ia. Associational fibres make synaptic contacts at layer Ib. Semilunar cells are differentiated from pyramidal cells by their relatively superficial positioning at layer II and lack of the basal dendrites.

The piriform cortex is broadly divided into the anterior and posterior piriform cortex with respect

to the LOT (Shepherd, 2003). The anterior piriform cortex is known to have more afferent inputs from the LOT whereas the posterior piriform cortex has more associational inputs (Hagiwara et al., 2012). The internal circuitry of the piriform cortex is extensive and roughly ten times heavier than the afferent connections. This is evident by the fact that each pyramidal neuron receives ~200 afferent inputs compared to ~2000 associative inputs from other pyramidal neurons. This provides a strong computational ability which is necessary for the piriform cortex to perform complex processing of odor information (Davison and Ehlers, 2011; Hagiwara et al., 2012).

Output from the piriform cortex goes to several areas in the brain including the anterior olfactory nucleus, prefrontal cortex, entorhinal cortex, amygdala etc. and a heavy back projection to the olfactory bulb as well (de Olmos et al., 1978; Haberly and Price, 1978b, a; Luskin and Price, 1983a; Luskin and Price, 1983b; Carmichael et al., 1994; Haberly, 1998; Boyd et al., 2015). Neurons from deeper layers like IIb or III have their axons back projecting on GL and GCL in OB (Nicoll, 1971; Matsutani, 2010). This exhaustive circuitry renders a dynamic control of the incoming information and is useful for understanding the sensory stimuli.

Different neuromodulators (dopamine, serotonin, acetylcholine and noradrenaline/ norepinephrine) modify information processing in the brain. They reach different areas of the brain through the long axons of the corresponding neurons that produce them. These long axons are popularly termed as centrifugal fibres as they travel from centre to the periphery of the brain. One such neuromodulator is norepinephrine (NE). Noradrenergic centrifugal fibres innervate different parts of the brain including the piriform cortex and participate in olfactory information processing. This will be discussed in the following section.

1.3 Brief anatomy of the noradrenergic innervation of the olfactory system

Noradrenergic innervation of the olfactory system has been dissected out quite well. The OB receives heaviest noradrenergic innervation from a deep brain structure containing noradrenergic neurons called locus coeruleus (LC) (Smythies, 2005). The Piriform cortex is also known to receive a bulk of this centrifugal NE innervation (Figure 3). The LC is the major source of NE in the brain although the lateral tegmental field also provides some of it. Noradrenergic LC neurons are situated at the floor of fourth ventricle within the dorsal wall of the rostral pons (Smythies, 2005). Dopamine β- hydroxylase acts on dopamine to produce norepinephrine which gets released as a neuromodulator in different parts of the brain. Rat LC has ~1500 noradrenergic neurons (Sara, 2009). NE was one of the earliest discoveries in neuromodulatory systems in the brain and hence has received a lot of attention over the years in deciphering its functional role and impact on behavior. NE is known to regulate attention, arousal, emotional state, learning and memory etc. by modulating the functions of different parts of the brain (Harley, 1987). Regarding olfaction, NE is known to modulate different types of olfactory learning, such as early odor preference learning (Yuan and Harley, 2014; Yuan et al., 2014), adult odor discrimination learning (Doucette et al., 2007), habituation (Guérin et al., 2008), non-associative learning (Mandairon et al., 2008; Escanilla et al., 2010) etc.

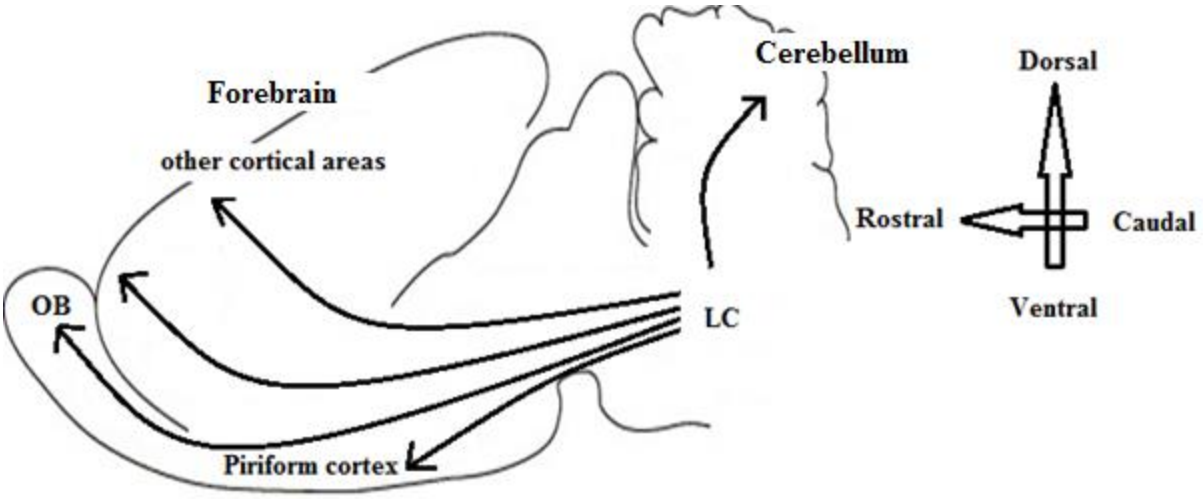

Figure 3. Locus Coeruleus projection to different brain areas

Cartoon shows Locus Coeruleus (LC) and its noradrenergic projections to many brain areas including olfactory bulb (OB), piriform cortex, cerebellum, hippocampus and other cortical and subcortical areas in a sagittal view of hemisected mouse brain.

2. Early Odor Preference Learning

Now that we understand the basic pathways of olfactory information processing (and one important neuromodulator for that processing), let's have a look at the principles and different varieties of odor preference learning, especially early odor preference learning.

Different behavioral paradigms attempt to test the involvement of different brain regions in various aspects of learning and memory. The salience of the incoming stimulus can be either positive or negative -giving rise to preference or aversion learning. Spatial, visual or auditory memories are the common ones to be tested apart from olfactory memory. All of these approaches ask a bigger

question- how specific information is learned and retained in the corresponding brain area and how it is retrieved when required.

Odor preference learning is an integral part of a rodent's life and it is particularly crucial for survival in early life as it helps young pups to find their mother for food and shelter. Later in life, it becomes important for acquisition of food, finding mating partner etc. Researchers have been trying to understand the principles of learning, new memory acquisition and retrieval using this as a model system.

Like many other odor preference learning paradigms, early odor preference learning is viewed as classical conditioning where a conditioned stimulus (CS), say an odor, can be associated with an unconditioned stimulus (UCS). Owing to repeated pairing of the CS with UCS during training phase, the subject "learns" that CS is important and readily identifies and shows preference to it during a testing session later.

This model was first described by Leon and colleagues (Leon et al., 1977; Alberts and May, 1984; Coopersmith and Leon, 1984). Neonatal rat pups exposed to peppermint odor 3-4 hrs daily till P19 (postnatal day 19) show robust preference to the odor at P20. Another group demonstrated that daily 3 min exposure is effective enough to induce preference (Caza and Spear, 1984). A similar type of odor preference learning was found in human neonates as well (Balogh and Porter, 1986; Sullivan et al., 1991). However, no CS and UCS association was required for these learning paradigms. An associative learning paradigm showed that a single 10 min pairing of odor and stroking in rat pups can induce preference memory lasting a day (Sullivan and Leon, 1986).

A wide range of UCS have been used to demonstrate early odor preference learning in rat pups including stroking and tactile stimulation (Pedersen et al., 1982; Sullivan and Leon, 1986; Sullivan and Hall, 1988; Moore and Power, 1992; McLean et al., 1993), tail pinch (Sullivan and Leon, 1986), mild foot shock (Camp and Rudy, 1988; Roth and Sullivan, 2001, 2003; Sullivan, 2003; Moriceau et al., 2006), odor of maternal saliva (Sullivan and Leon, 1986), milk (Johanson and Hall, 1979; Johanson and Teicher, 1980; Johanson and Hall, 1982) etc.

It has been proven that this learning is purely associative in nature. Pups do not show any preference to the odor if we present only CS, only UCS, random CS-UCS pairing/ backward UCS-CS presentation (Sullivan et al., 1989a, b). Scientists have used different training paradigms for early odor preference learning. Ten minutes of odor+stroke pairing has been used most extensively. However extended periods of training have showed longer trace of memory, even in some cases extending up to the adulthood to influence mating behaviors (Fillion and Blass, 1986).

To simplify, when an odor is presented, rat pups will remember it depending on the associated reward. Odor preference learning occurs throughout the life, but, early life odor preference learning, based on UCS related to maternal presence or care (for example, stroking), has some unique feature- a “Critical Period“ of learning.

3. Critical period

Learning window for this early odor preference learning is relatively short and specific. It varies across the models and paradigms; but the variation is only marginal and thus it is a hallmark of this learning paradigm.

The concept of critical period is common in several behavioral paradigms. Generally it is viewed as a phase of heightened plasticity of the network that renders higher learning abilities in younger age. Functional competition between inputs and subsequent electrical activity leads to consolidation of selected parts of the neuronal network. This type of experience-driven reorganisation of the circuitry is common across multiple systems but usually varies greatly in duration, time course and underlying molecular mechanism. Existence of critical periods has been demonstrated in different sensory modalities- somatosensory, auditory, visual etc, as well as in olfaction (Hensch, 2004).

In early odor preference learning, interestingly, only young pups up to 10-12 postnatal days of age, are able to learn this paradigm. Several studies have been aimed at determining the critical period (or sensitive period) for early odor preference learning. Woo and Leon (1987) have shown that stroking does not work as UCS after the first postnatal week (~10 days). Other researchers have shown that mild foot shock provokes aversive response to the presented odor beyond the critical period (Camp and Rudy, 1988; Sullivan et al., 2000a; Moriceau et al., 2006). This feature makes this learning paradigm unique, and scientists have been trying for decades to solve the mystery of this critical period.

4. Role of norepinephrine

Norepinephrine is known to be associated with olfactory information processing. Researchers have found that it is strongly related to early odor preference learning as well. Here we will learn more about the involvement of this neuromodulator in olfactory behavior and in electrical activities of neurons.

Norepinephrine acts via different receptors namely α and β adrenoceptors. There are different subtypes of them and they can perform many downstream functions ranging from exciting neurons to inhibiting them. This can potentially alter the electrical activity of the neurons and thus shaping the information processing in the neuronal network. Thus engagement of the adrenoceptors by norepinephrine has the potential to alter animal behaviours as evident by the extensive research work listed below.

4.1 Role of norepinephrine in behavior

Norepinephrine has different roles in peripheral systems (autonomic nervous system and endocrinal system) and in brain. Coincidental surge in maternal and neonatal NE levels during parturition (Sperling et al., 1984) was an early clue to the importance of NE in early life. This catecholamine surge has been considered a potential facilitator of early odor preference learning (Sulyok, 1988). This has been demonstrated in animals as well as humans. Increased umbilical cord blood NE level is known to be associated with better learning for head-turning towards trained odor in human infants (Varendi et al., 2002). Early odor preference learning in rodents is also greatly modulated by LC-driven release of NE.

We see various effects of NE in brain as well. Different tactile stimuli (stroking, air puff, mild tail pinch) showed responsive electrical activity at the LC even in early neonatal life (Nakamura et al.,

1987). Researchers have shown that this release of NE is crucial for conditioned odor preference (Nakamura et al., 1987; Rangel and Leon, 1995). Direct LC stimulation paired with odor presentation created preference in rat model while blocking β-adrenoceptors in the OB prevented odor preference learning (Sullivan et al., 2000b). This created a model where LC-dependent NE release was rendered necessary and sufficient for early olfactory learning. Other researchers had already explained the decrease in NE release from the LC following the sensitive period is because of the development of α2 autoreceptor mediated inhibition in the LC (Kimura and Nakamura, 1987b; Nakamura et al., 1987; Nakamura and Sakaguchi, 1990). This convinced the scientists that NE has a strong association with the critical period. But how exactly does it exert its effects locally? The LC has noradrenergic projections to cortices and also to the deeper brain structures. An α2 receptor mediated autoinhibition of NE release from the LC beyond the critical period would lead to a global decrease of NE, not just specific to the olfactory system. What unique function does NE serve to the olfactory system that undergoes a radical change around the critical period? The question remains open, so is the investigation!

4.2 Role of norepinephrine in modulating electrical activity of neurons

NE is known to have a major influence in shaping electrical activity of neurons throughout the brain. A change in electrical activity would alter the processing of the incoming information in the circuitry. Although the exact pattern of this change to promote learning is unknown, it is generally believed that an increase in input-specific excitation, or a decrease in background spontaneous activity and thereby increasing the signal-to-noise ratio. NE is known to contribute to a better signal-to-noise ratio as well as to convert subthreshold stimuli to suprathreshold spiking

(Waterhouse et al., 1990; Mouradian et al., 1991; McLean and Waterhouse, 1994; Devilbiss and Waterhouse, 2000; Hirata et al., 2006). NE performs similar functions in the olfactory system including the OB and PC (Jiang et al., 1996; Ciombor et al., 1999; Hayar et al., 2001a; Bouret and Sara, 2002) as it does in other sensory modalities (Linster et al., 2011). This gives us a good reason to look at the OB and PC and learn about role of NE in modulating their function in the backdrop of early odor preference learning.

5. The enlightened bulb

Being the first station of olfactory information processing and a recipient of major noradrenergic projections from the LC, the OB has received much attention as a crucial site for early odor preference learning to happen. There is a large body of evidence available; major milestones will be discussed in the following section.

5.1 Norepinephrine receptor distribution in the bulb

Adrenoceptors transduce NE's cellular effects. α1, α2, β1 and β2 adrenoreceptors are present in the OB. They play a significant role in adrenoceptor mediated plasticity in the bulb. Different adrenoceptors mediate a range of cellular effects in different types of neurons giving rise to complex information processing. α1 and α2 adrenoreceptors are mostly concentrated on the MCs and GCs (McCune et al., 1993; Pieribone et al., 1994; Hayar et al., 2001a; Nai et al., 2010) whereas radiographic techniques have demonstrated the presence of β1-adrenoreceptors in GCL, IPL and GL and β2-adrenoreceptors in the EPL (Woo and Leon, 1995). A more recent study described β1-adrenoceptor distribution in MCs, PGs, and GCs as well (Yuan et al., 2003b).

5.2 Norepinephrine modulating behavior in the bulb

Because of the varied receptor distribution in the bulb and complex connectivity, it is difficult to determine how exactly NE performs its crucial role in learning. To seek the answer, researchers resorted to several pharmacological interventions combined with behavioural studies.
Investigations reveal that NE is crucial for odor preference learning at the level of the OB. Alpha adrenoceptors have received less attention; though evidence suggests that systemic α1 agonist injection or bulbar infusion of α2 adrenoceptor agonist may reliably serve as UCS for odor preference learning (Harley et al., 2006; Shakhawat et al., 2012a).
Beta adrenoceptor blockade in the OB leads to the inability to learn odor preference (Sullivan et al., 1991; Sullivan et al., 2000b). Also β adrenoceptor agonists can serve as UCS and renders odor preference learning when paired with odor presentation (CS) (Sullivan et al., 1989b; Langdon et al., 1997; Sullivan et al., 2000a; Yuan et al., 2003a; Harley et al., 2006; Lethbridge et al., 2012). Crucial findings regarding β adrenoceptors role in the OB lead to a hypothesis that NE at the OB is necessary and sufficient for odor preference learning (Sullivan et al., 2000b).

5.3 Norepinephrine modulating electrical properties of the neurons at olfactory bulb

NE is known to modulate excitation-inhibition balance in the OB. GABAergic inhibition of MC is regulated by NE in a concentration-dependent fashion involving different receptor subtypes (Nai et al., 2010). Also an age-dependent alteration in α2 adrenoceptor-mediated disinhibition of the MC cells- thus switching MC output around critical period- was found in a study by Pandipati and

Schoppa (2012). Increase in MC spiking activity and decrease in PG cell EPSP onto MC by β agonist suggested stronger evidence for NE providing more efficient ORN-MC transmission (Yuan, 2009a; Lethbridge et al., 2012).

6. Rise of the piriform cortex

6.1 Why look at the piriform cortex?

Indirect evidence that the piriform cortex is involved in odor preference learning comes from a study by Hall and colleagues. They had shown that lateralization of odor preference memory is possible in PND 6 pups, before the anterior commissure connects the two hemispheres. After training PND6 or younger pups with one naris blocked in odor + milk paradigm, they show odor preference only for the open naris, not for the blocked one. However at PND 12 blocked and open naris behaves in similar way suggesting that anterior commissure connections have been established. Severing the anterior commissure restores lateralization of odor preference memory, strongly suggesting a cortical trace of memory formation (Kucharski et al., 1986; Kucharski and Hall, 1987). In line with their work, Sullivan and colleagues showed that c-fos an immediate early gene, gets activated in both the OB and aPC, after odor preference learning in rat pups (Roth et al., 2006). All these findings are indicative of a bigger role of the aPC in early odor preference learning. More direct manipulations of cortical memory have been done recently. Morrisson et al. (2013) showed that odor preference memory cannot be acquired if the aPC is non-functional following local infusion of lidocaine or muscimol. As OB was intact in this experiment, it clearly suggested that aPC is necessary for odor preference learning as is OB. This study had another

interesting finding. They have shown that blocking NMDAR activity or β adrenoceptor activity locally at aPC prevents early odor preference learning although the OB was intact. Following the previous logic, it suggests that the aPC holds an important role in this learning irrespective of the OB. It is possible that an NMDAR antagonist could have changed the odor perception altogether. But in another experiment DAPV, NMDAR antagonist, was infused in the aPC prior to testing but not during / before training. As odor preference learning was intact, it could be inferred that NMDAR did not alter odor perception, hence aPC indeed holds an important role in early odor preference learning irrespective of the OB (Morrison et al., 2013).

Calcium imaging study of pyramidal neurons in rodent pups' aPC shows a reduction in firing threshold of the pyramidal neurons within the memory window, thereby making these cells more responsive to LOT input (Fontaine et al., 2013). This gives us a good rationale to look at the aPC and learn how NE modulates behaviour or the electrical activity in there, just as we learned in the OB.

6.2 Norepinephrine modulating behavior in the anterior piriform cortex

As we have already discussed, abolishing β adrenoceptor effect by antagonist infusion at the aPC prevents odor preference memory. Also, β adrenoceptor agonists serves as UCS (instead of stroking) during the training and rendered odor preference memory concentration-dependent. As Sullivan and colleagues found NE in the OB to be necessary and sufficient for odor preference learning- borrowing the same rationale- NE (β adrenoceptor) in the aPC now could be tagged as necessary and sufficient for odor preference learning. It is probably safe to state that both the OB and the aPC are crucial for this learning and NE plays an important role at both sites.

6.3 Norepinephrine modulating electrical properties of the neurons at the anterior piriform cortex

6.3.1 A sketch of electrical activity and network function at the anterior piriform cortex

Pyramidal neurons receive incoming odor information through the LOT. This signal is subjected to a rigorous processing by the complex neuronal network comprised of both inhibitory and excitatory neurons. That processed signal then travels far in other cortical and subcortical areas. Processing at the PC is crucial to modulate the behavioral outcome and NE plays an important role in that. It is understandable that increased signal-to-noise ratio or converting a subthreshold stimulus to a suprathreshold one (these are important functions of NE, as we discussed previously) would have an impact in modulating the behavioral outcome.

6.3.2 Electrical activity and physiology

A typical neuron makes thousands of synaptic connections with other neurons. All these inputs are integrated for the neuron's response in the form of action potential firing. As a result, alterations in electrical property of the pyramidal neurons are known to be associated with physiological changes and behavioral outcomes. The OB feeds olfactory information directly to the aPC; this connection is excitatory. Enhanced synaptic transmission between principal neurons of OB and PC drives up the activity of aPC and is therefore conducive to olfactory learning (Roman et al., 1987; Litaudon et al., 1997; Saar et al., 2002; Cohen et al., 2008). Similarly, changes that alter the way a pyramidal

neuron fires a train of spikes also lead to functional consequences. For example, the ability to fire repetitively has been recorded following rule learning (Knafo et al., 2001; Saar et al., 2002), suggesting that learning is a result of increased firing of pyramidal neurons in the aPC. After conditioning there is a higher activity of PC neurons as evident from multi-site recording using voltage-sensitive dye (Litaudon et al., 1997). However this may not always be the case since contradictory results showed inhibition of PC pyramidal neurons after learning (Brosh and Barkai, 2009). Although it is not clear how olfactory information is coded in the temporal pattern of action potentials, it is widely accepted that all changes to a connected circuitry will eventually be reflected by the electrical activities of the member neurons.

6.3.3 Short term plasticity

Neuronal networks can change the strength of synapses depending on the need and activity. This ability to change is called plasticity, which is an absolute requirement for learning-related changes to occur in the circuitry and to store the memory. The plasticity can be short-term or long-term depending on the nature of the incoming input and the further downstream molecular cascades taking place in the neuron. NE has been known to modulate short term plasticity which is a reflection of the alteration of synaptic efficacy owing to the past presynaptic activity. β adrenoceptor activation rescues spike frequency accommodation (a sign of short term plasticity) of odor evoked response in aPC. LOT-evoked EPSPs decreases with continued odor stimulation because of a decrease in presynaptic glutamate release. β adrenoceptor activation is known to prevent this (Best and Wilson, 2004).

6.3.4 Long term plasticity

Besides short term plasticity, NE is known to be involved in long term plasticity as well. Long term plasticity implies heightened synaptic strength of the selected synapses for longer time periods. This process is generally thought as a correlate of long term memory. Evoked field EPSP recording in aPC slices show that β adrenoceptor agonist isoprotenerol increases the amplitude of LOT LTP during theta burst owing to enhanced presynaptic glutamate release as evident by reduced paired pulse ratio (Morrison et al., 2013). Isoprotenerol induces PKA mediated phosphorylation by activating cAMP. mGluR activation by theta burst is reversed by this PKA mediated phosphorylation of the receptor (Cai et al., 2001). This mechanism may explain the role of NE in NMDAR dependent associative learning happening at the olfactory cortex.

7. The gap in plot

Neurons are connected to each other by synapses. Information reaches a neuron through multiple synapses and then gets conveyed to the next neuron by an action potential. Electrophysiological properties of a neuron are thus divided in two sections – intrinsic and synaptic properties. Intrinsic property depends on the physical properties of the membrane itself, including passive membrane properties (cable parameters such as resistance and capacitance) and active conductions (due to ion channels in the membrane). These physical properties determine the threshold, amplitude, width of action potential, input resistance (this is a measure of how

excitable the neuron is), resting membrane potential etc. Synaptic property deals with the "communication" system between the presynaptic and postsynaptic neuron and generally the read-out is amplitude and frequency of the postsynaptic current. For example, a strengthened synapse would have different synaptic properties compared to a regular one. It gives us a good understanding whether presynaptic and/or postsynaptic changes have occurred in a synapse in response to conditioning. It applies to both excitatory and inhibitory synapses. Like any neuronal circuitry, aPC pyramidal neurons have these properties. It is understandable that if early odor preference learning is happening in the aPC, there should be a change in the output of these neurons. Also, if NE is crucial in making this learning happen in younger animals but not in older animals, we should see a change in these electrophysiological properties of these neurons.

In spite of thorough research over the past four decades on this behavioral paradigm, little is known how NE acts at the single cell level and alters these intrinsic and synaptic properties in the aPC to make "NE in the aPC necessary and sufficient" for early odor preference learning. My research in this thesis is aimed at answering this question.

8. The Question(s)

If the aPC is an independent site of memory formation and NE at the aPC is indispensable for learning, NE must have some effect in shaping the circuitry function at the aPC. It is possible that intrinsic and synaptic properties in neurons and/or their modulation by NE is different in two age groups (P8-11 and P14+)- thus providing the basis of loss of ability to learn early odor preference beyond a critical period. Also it is possible for the neurons to undergo a

developmental change in their electrophysiological properties which may be the underlying cause of their inability to learn in this particular paradigm beyond the critical period. We thus sought answers to the following questions step by step after establishing the behavioral model in mouse-pups along with β adrenoceptor antagonist mediated prevention of learning:

- Is there a difference between the electrophysiological properties of the two age groups?
- Does NE differentially alter these properties in the two age groups?
- If there is a difference in NE modulation, which receptor(s) plays a major role?

9. Overview of the conducted experiments

In this work, we first replicated the critical period odor learning model in mice (Roth et al., 2013) and establish the causal role of β-adrenoceptors in learning. We then characterized the concentration-dependent effects of NE on the intrinsic and synaptic properties of aPC pyramidal cells and the potential roles of β-adrenoceptors by whole cell electrophysiological recording of aPC layer II pyramidal neurons in acute slices.

Materials and Methods

1. Experimental subjects and ethics statement

C57B1/6J mouse pups (Charles River, Canada) of either sex were used in this study. All animals were bred in Memorial University of Newfoundland's Animal Care Facility. Dams were provided with *ad libitum* access to food and water. Day of parturition was considered 0 days of age; i.e. P0. Subjects aged P8-11 or P14-21 (P14+) were used for experimentation. Pups were arbitrarily selected and assigned to training conditions from each litter. Conditioning and testing were performed in a temperature-controlled room (~28°C). All experimental procedures were approved by the Institutional Animal Care Committee at the Memorial University of Newfoundland in accordance with the Canadian Council on Animal Care guidelines.

2. Odor preference training and testing

Each pup was placed individually for behavioral training and testing. Following a 10 min habituation in a training box with unscented corn-cob bedding, pups were placed in a training box with peppermint-scented corn cob bedding (0.3 mL of odorant extract in 500 mL of bedding). For odor preference training group (O/S^+), each subject was stroked on the back with a paintbrush for 30 s, followed by a 30 s interval, repeating for a total of 10 minutes. Control group (odor only, O/S^-) was placed on the peppermint-scented corn cob bedding (without stroking) for

10 minutes. Pups were returned to home cage until testing 24 hours later.

Twenty-four hours after training, pups were individually tested for odor preference. The order of pups tested with respect to their experimental condition was random. The testing apparatus was a stainless steel box (30 cm x 20 cm x 18 cm) placed over two training boxes with a mesh floor. One box contained peppermint-scented corn cob bedding and the other contained unscented corn cob bedding. There was a 2 cm neutral zone in the apparatus separating two boxes. Each test involved placing a pup in the neutral zone, and recording the time spent over each box (peppermint vs. unscented). This was recorded for five trials of 60 s each, with a 60 s interval in between trials. Starting orientation of the pup was counterbalanced over the trials. Strips of floor mesh, that pups came in contact with, were cleaned with 70% ethanol after each trial. The percentage of time spent over the peppermint bedding with respect to the unscented bedding was calculated (Figure 4).

Propranolol Injection

Mouse pups (P8) were randomly assigned to experimental groups and weighed. Pups were injected subcutaneous (s.c.) with 25 µl of either saline or propranolol (20 mg/kg; Sigma-Aldrich). Pups were returned to the home cage for 30 min before habituation and training (Table 1).

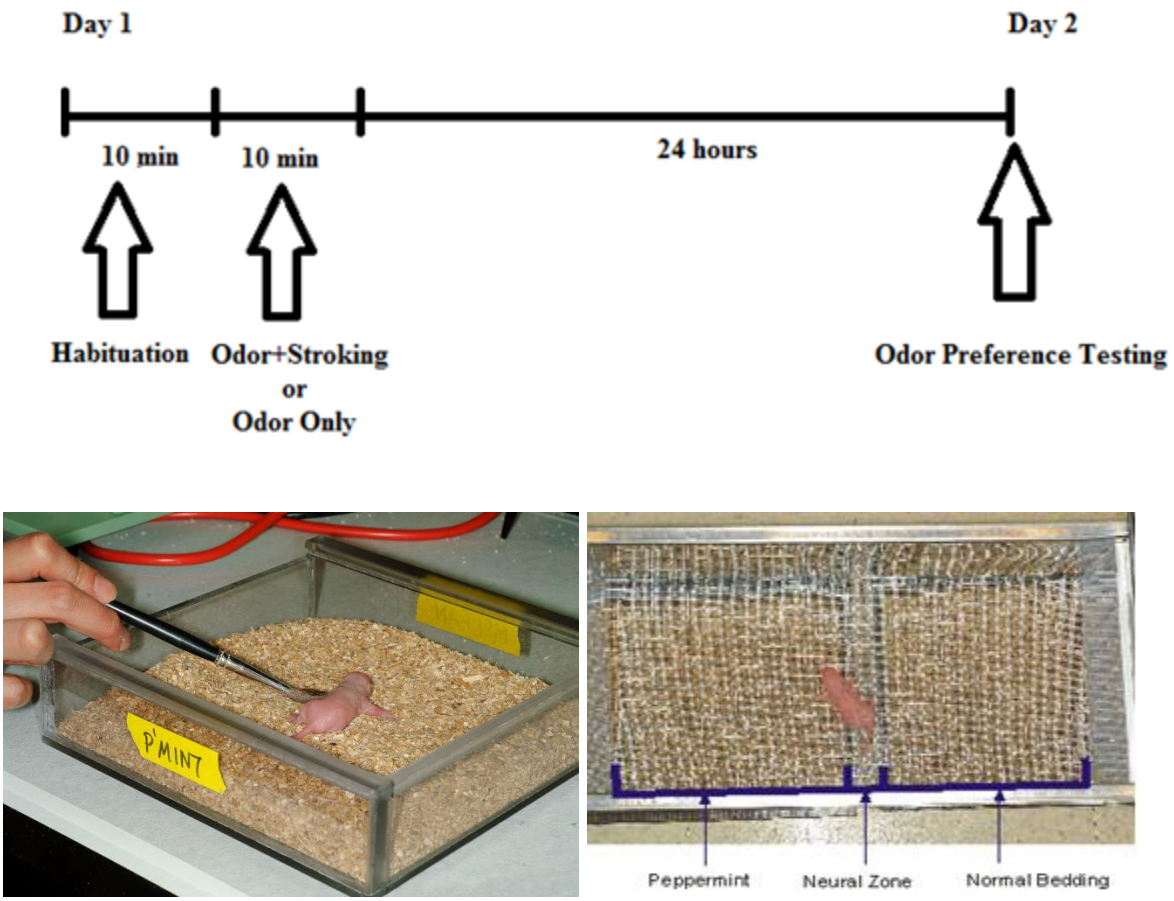

Figure 4. Odor Preference Training and Testing Apparatus

Top: Following 10 min habituation, each subject (O/S^+) was stroked on the back for 30 s, followed by a 30 s interval, repeating for a total of 10 minutes and returned to the cage until testing 24 hours later. Bottom Left: A mouse pup is placed on peppermint scented bedding in the training box and its back is stroked with a paint brush (O/S^+) for 30s following a 30s interval, repeating over a total period of 10 min. Bottom Right: 24 hours later the pup is placed at the neutral zone on the testing apparatus where one side is has peppermint scented bedding and the other side has normal bedding. Pup shows preference towards the peppermint side.

Table 1. Number of animals used in different groups for behavioral training and testing

Groups	Number of pups used in P8 age group	Number of pups used in P14 age group
O/S^-	10	8
O/S^+	10	6
O/S^- with saline s.c. injection	9	X
O/S^+ with saline s.c. injection	18	X
O/S^+ with propranolol s.c. injection	15	X

3. Ex vivo electrophysiology

Naive pups from both age groups were anaesthetized with isofluorane, followed by decapitation. Brain was removed quickly and placed in cold (2-4˚C) sucrose based solution containing: (in mM) 83 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 72 sucrose, 0.5 $CaCl_2$, bubbled with 95% O_2 & 5% CO_2 (Morrison et al., 2013). Para-sagittal slices (300 µm) were cut in a vibrtatome (Leica) and incubated in the same sucrose based solution for 30 min at 35˚C before experiment.

An open bath recording chamber was continuously perfused with warm (30-32˚C) artificial CSF

(aCSF) containing (in mM): 110 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 2.5 $CaCl_2$, at the rate of 2-3 mL/min. For miniature EPSC recording, aCSF containing (in mM) 119 NaCl, 5 KCl, 4 $CaCl_2$, 4 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose were used in the presence of the $GABA_A$ receptor antagonist picrotoxin (Poo and Isaacson, 2007). Olympus BX51WI upright microscope was used for viewing the slices in differential interference contrast with 40X magnification.

Recordings of layer II pyramidal cells in the anterior piriform cortex were performed with glass micropipette (resistance 3-6 MΩ) pulled on a Flaming/Brown micropipette puller (P-97, Stutter Instrument Co., USA) and filled with intrapipette solution containing (in mM): 123 K-gluconate; 2 $MgCl_2$,$6H_2O$; 8 KCl; 0.2 EGTA; 10 HEPES; 4 Na_2-ATP; 0.3 Na-GTP for recording intrinsic properties and miniature EPSC. For IPSC experiments, micropipettes were filled with intrapipette solution containing (in mM): 123 KCl; 2 $MgCl_2$,$6H_2O$; 8 KCl; 0.2 EGTA; 10 HEPES; 4 Na_2-ATP; 0.3 Na-GTP; thus chloride reversal potential being 0 mV. Data were included only from healthy cells which was defined as cells having a resting membrane potential (RMP) negative than -50 mV and good patch quality (access resistance <25 MΩ) throughout the experiment. Cells which had an access resistance change of more than 30% were excluded from analysis (Figure 5).

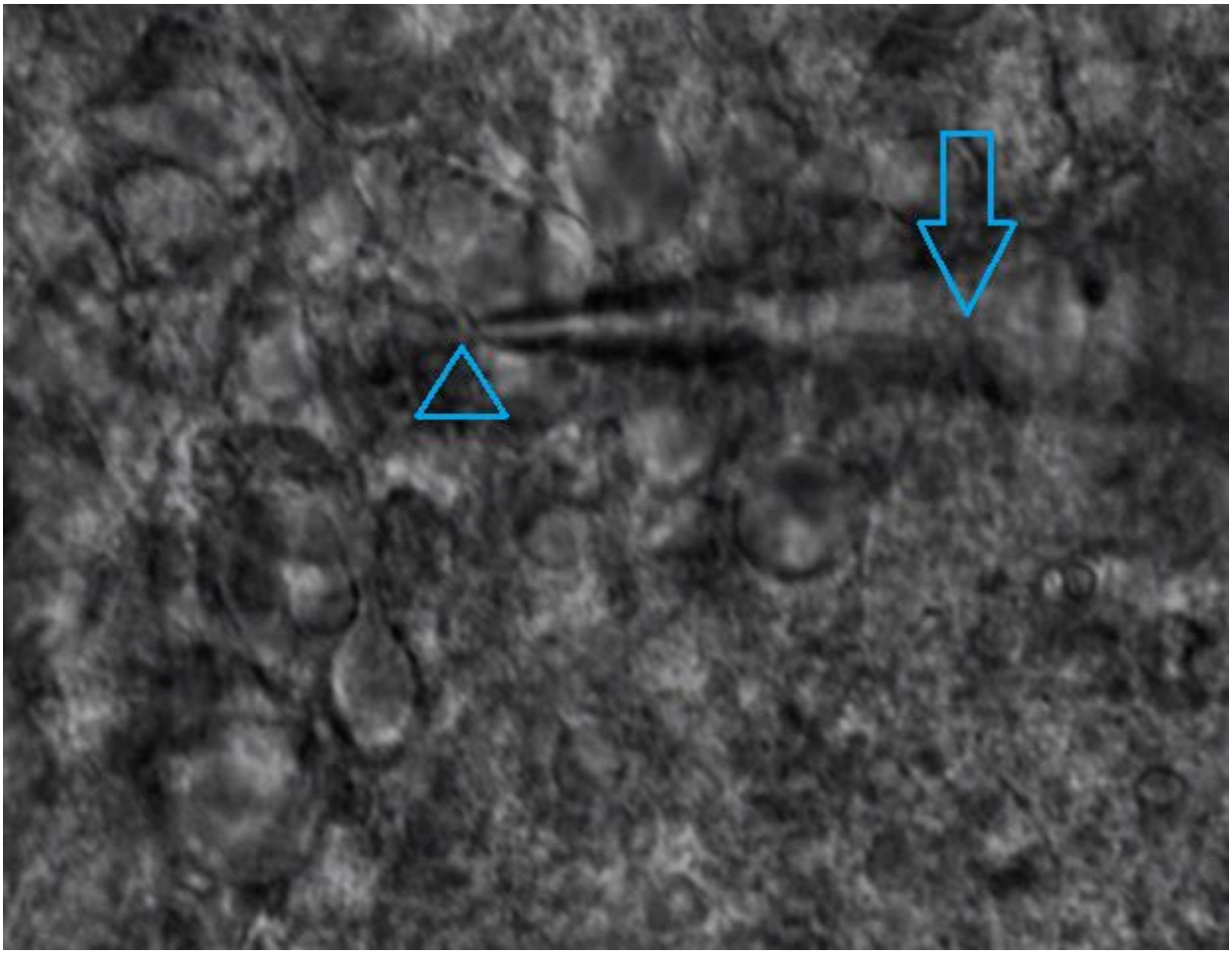

Figure 5. *Ex vivo* electrophysiology

In a parasagittal slice of anterior piriform cortex, a layer II superficial pyramidal neuron's (arrowhead) electrophysiological property is being recorded in "whole-cell" configuration through the glass electrode (arrow).

For measuring intrinsic properties, depolarization currents of increasing amplitude (10 pA steps) were injected into the cell through the patch-pipette in current clamp mode. The action potential (AP) evoked by the smallest current injection was used for measuring threshold, amplitude, half width, rise time and decay time. Input resistance was measured by taking the average of multiple (5 or more) traces following 10 pA hyperpolarizing current injection.

For synaptic properties, cells were held at -70 mV in voltage-clamp mode. Synaptic properties can be measured in three different ways-

1. Evoked – Here the synaptic activity is induced by stimulating the afferent fibres or presynaptic neuron and the response is measured from the post synaptic neuron.
2. Spontaneous- Here no stimulation is induced presynaptically. The spontaneous synaptic activity is recorded postsynaptically.
3. Miniature- In spontaneous synaptic events, we cannot exclude the network effect. For example, if there is a spontaneously spiking cell directly/indirectly connected to our cell of interest, it will influence the spontaneous synaptic transmission. This problem is addressed in miniature synaptic recording, where the network is silenced using the voltage sensitive Na+ channel blocker tetrodotoxin. Thus miniature synaptic events reflect spontaneous activity of synapses (either excitatory or inhibitory) excluding the network effect. This was apt for our purpose.

Miniature IPSC (mIPSC) was measured in the presence of tetrodotoxin (0.5 µM; Tocris), D-APV (50 µM; Tocris) and NBQX (40 µM; Tocris) in aCSF. Miniature EPSC (mEPSC) was measured in the presence of tetrodotoxin (0.5 µM) and picrotoxin (100 µM; Tocris) in the high

divalent aCSF. Cells were recorded for 5 min in each NE concentration (0.1, 1 and 10 μM; norepinephrine bitartrate salt monohydrate, Sigma-Aldrich) in an accumulative manner. β-adrenoceptor antagonist propranolol hydrochloride (20 μM: Sigma-Aldrich) and α-adrenoceptor antagonist phetolamine hydrochloride (50 μM; Sigma-Aldrich) were added to the aCSF to test the specificity of NE at various concentrations on adrenoceptors. Antagonists were washed in for >10 min before adding NE. The specific effects of β-adrenoceptors were tested with propranolol in the presence of NE.

Multiclamp 700B amplifier and pClamp10 software (Molecular Devices) were used for data acquisition (filtered with 2 kHz low pass filter) and digitization (10 kHz sampling frequency). Electrophysiological data were analysed by Clampfit (Molecular Devices) and Igor Pro (Wave Metrics) software. For miniature IPSC and EPSC, Mini Analysis Program (Synaptosoft Inc.) was used to analyze the frequency and amplitude of the events.

4. Post hoc histology

In a subset of recorded cells, biocytin (0.1%) was added to the intrapipette solution and the recorded cell was filled up during the experiment. Slices were transferred to 4% Paraformaldehyde solution, left at 4˚C until further processing. Slices were washed with phosphate buffer saline (PBS) afterwards. Then the slices were incubated with CY3-conjugated streptavidin (1:1000 in PBS; Sigma Aldrich) at room temperature for 2 hours, washed with PBS, mounted onto slides, and imaged at 40X magnification with an Olympus Fluoview FV1000 confocal microscope (Olympus Lifesciences).

5. Statistics

OriginPro 9.0 software (OriginLab Corp.) was used to analyze all datasets. Data were reported as mean ± SEM (Standard Error of Mean). One way Analysis of Variance (ANOVA) and Student's t-test (two-sample, two-tailed) were used for behavioral studies and comparisons of intrinsic electrophysiological properties. Paired two-sample, two-tailed t-test was used for all experiments characterizing concentration-dependent NE synaptic effects. Significance level was set at $p<0.05$.

Results

1. Critical period early odor preference learning in mice

We first replicated critical period odor learning in mice, which was well established in rat pups (Wilson and Sullivan, 1994; Yuan et al., 2014) and recently in mice (Roth et al., 2013). Establishing this model in mice pups is important as we can take advantages of transgenic mice lines for future experiments. P8 mouse pups in the odor + stroking (O/S^+) group spent significantly more time in the peppermint bedding (57.25 ± 3.92%, n = 10) than the odor only (O/S^-) group (32.56 ± 5.14%, n = 10, t = 3.82, p = 0.001; Figure 6A). However, P14 pups with O/S^+ training (52.86 ± 7.95%, n = 6) did not show significant difference from those in the O/S^- group (61 ± 4.55%, n = 8, t = 0.94, p = 0.363; Figure 6B). Interestingly, mouse pups at P14 lost mild aversion to peppermint which was evident in P8 pups.

When the β-adrenoceptor blocker propranolol was injected intraperitoneally before training, mouse pups failed to form odor preference compared to saline O/S^+ controls. A one-way ANOVA revealed a significant difference among groups ($F_{2,39} = 5.73$, p = 0.006; Figure 6C). *Post hoc* Fisher test demonstrated significant differences between the propranolol injected O/S^+ group (28.13 ± 5.18%, n = 15) and the saline O/S^+ group (50.71 ± 4.97%, n = 18, t = 3.18, p = 0.002), while the propranolol group was not different from the saline O/S^- group (31.64 ± 6.32%, n = 9, t = 0.40, p = 0.684). These results suggest early odor preference learning in mouse pups is dependent on NE release acting *via* β-adrenoceptors.

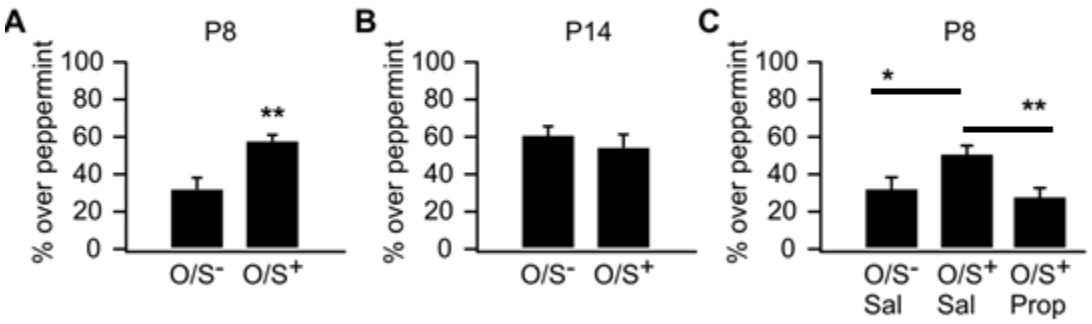

Figure 6. Odor preference learning in mice. (A) Percentage of time spent over peppermint-scented bedding in P8 pups. **(B)** Percentage of time spent over peppermint-scented bedding in P14 pups. **(C)** Percentage of time spent over peppermint-scented bedding in P8 animals with either propranolol or saline intraperitoneal injections. O/S^+: odor paired with stroking. O/S^-: odor only without stroking. $^*p < 0.05$; $^{**}p < 0.01$.

2. Effects of norepinephrine on pyramidal cells in the piriform cortex

To understand the role of NE in the piriform cortex that may underlie early odor preference learning, we compared the effects of NE on both intrinsic and synaptic properties of pyramidal cells in the anterior piriform cortex. We selectively recorded from layer II pyramidal cells which receive excitatory afferent inputs from the OB and associative cortical inputs, as well as local inhibitory inputs. To distinguish from semilunar cells, pyramidal cells were selected by somatic

morphology under differential interference contrast microscope (oval shaped *vs.* semilunar shaped), depth in the layer II (deeper *vs.* superficial), and in some cases, paired pulse ratios of evoked EPSCs were measured (facilitation *vs.* depression) (Suzuki and Bekkers, 2011). A subset of cells with biocytin staining (n = 23) were assessed by *post hoc* histology to be pyramidal cells because of the presence of both apical and basal dendrites (Suzuki and Bekkers, 2011).

2.1 Basic neuronal properties of pyramidal cells in two age groups

First we asked whether there is any difference in the two age groups with respect to the intrinsic neuronal properties. We restricted our experiments to maximum two cells/animal. Compared to the P14+ group, the P8-11 group showed different intrinsic properties (Table 2); such as- higher AP threshold (-31.30 ± 1.7 mV, n =16, *vs.* -37.72 ± 1.80 mV, n = 15, t = 2.63, p = 0.014), smaller AP amplitude (65.55 ± 2.24 mV, n =16, *vs.* 77.07 ± 2.11 mV, n = 15, t = 3.73, p = $8.38E^{-4}$), wider AP half-width (1.51 ± 0.06 ms, n =16, *vs.* 0.99 ± 0.04 ms, n = 15, t = 6.70, p = $2.39E^{-7}$). Also the rising time was slower (0.40 ± 0.02 ms, n =16, *vs.* 0.29 ± 0.02 ms, n = 15, t = 4.19, p = $2.41E^{-4}$) as well as decay time (1.25 ± 0.08 ms, n =16, *vs.* 0.73 ± 0.03 ms, n = 15, t = 5.91, p = $2.07E^{-6}$). Additionally, P8-11 animals exhibited a more depolarized RMP than the P14+ group (-59.53 ± 1.09 mV, n =22, *vs.* -64.30 ± 1.83 mV, n = 18, t = 2.33, p = 0.025). The membrane resistances were not different between the two age groups (P8-11: 211.44 ± 18.79 MΩ, n =23, *vs.* P14+: 213.57 ± 21.12 MΩ, n = 17, t = 0.075, p = 0.941). Thus our result suggests clear differences in the intrinsic neuronal properties before and after the critical period.

Table 2: Pyramidal cell intrinsic properties in P8–11 and P14+ mice.

	P8-11	P14+	t	P
AP Thresold (mV)	-31.3 ± 1.7	-37.7 ± 1.8	2.63	0.01 *
AP Amplitude (mV)	65.6 ± 2.2	77.1 ± 2.1	3.72	<0.01 **
AP Half Width (ms)	1.51 ± 0.07	0.99 ± 0.04	6.7	<0.01 **
AP rising time (ms)	0.40 ± 0.02	0.29 ± 0.02	4.19	<0.01 **
AP decay time (ms)	1.25 ± 0.08	0.73 ± 0.03	5.91	<0.01 **
V resting (mV)	-59.5 ± 1.1	-64.3 ± 1.8	2.33	0.03 *
Rm (MΩ)	211.4 ± 18.8	213.6 ± 21.1	0.08	0.94

Despite differences in the intrinsic properties between the two age groups, NE at two concentrations (0.1 and 10 μM) had no effect on the parameters measured in either age group (Table 3 and 4), except that the AP decay time showed significantly different group effects in the P14+ group with 10 μM NE. NE significantly reduced AP decay time (0.70 ± 0.02 ms in NE *vs.* 0.76 ± 0.03 ms in control, n = 9, t = 3.05, p = 0.016, paired t-test). However, this effect was not reversed after 10 min NE washout (0.63 ± 0.11, compared to control, t = 3.11, p = 0.014).

There were no significant differences in the synaptic properties between the two age groups. We

analysed mEPSC amplitude (16.4±0.49 pA in P 8-11 v/s 15.43±0.58 pA in P14+; p>0.05), mEPSC frequency (1.46±0.18 Hz in P 8-11 v/s 1.88±0.3 Hz in P14+; p>0.05), mIPSC amplitude (54.4±2.8 pA in P 8-11 v/s 56.9±4.3 pA in P14+; p>0.05) and mIPSC frequency (1.31±0.1 Hz in P 8-11 v/s 1.44±0.11 Hz in P14+; p>0.05). As we did not find any major differential effect of NE on intrinsic neuronal properties between two age groups that can possibly explain occurrence of the critical period in this model, we shifted our focus to the NE-dependent changes in the synaptic properties.

Table 3 : The effects of 10 µM NE on pyramidal cell intrinsic properties

	P8-11 (n = 8)					P14+ (n = 9)				
	Control	NE 10 µM	NE Wash	$F_{(2.21)}$	P	Control	NE 10 µM	NE Wash	$F_{(2.24)}$	P
AP thresold (mV)	-36.1 ± 1.5	-35.4 ± 1.9	-36.3 ± 2.1	0.07	0.93	-38.2 ± 2.4	-38.4 ± 2.4	-40.8 ± 2.1	0.37	0.69
AP amplitude (mV)	68.1 ± 2.9	66.8 ± 2.3	62.9 ± 2.5	1.11	0.35	75.7 ± 2.8	72.9 ± 3.3	70.5 ± 3.7	0.63	0.54
AP half Width (ms)	1.40 ± 0.09	1.35 ± 0.06	1.37 ± 0.05	0.12	0.89	1.02 ± 0.04	1.00 ± 0.03	0.94 ± 0.04	1.25	0.3
AP rising time (ms)	0.37 ± 0.03	0.36 ± 0.02	0.40 ± 0.02	0.73	0.49	0.30 ± 0.02	0.30 ± 0.01	0.31 ± 0.01	0	1
AP decay time (ms)	1.18 ± 0.10	1.13 ± 0.09	1.05 ± 0.05	0.66	0.53	0.76 ± 0.03	0.70 ± 0.02	0.63 ± 0.04	4.69	0.02 *
RMP (mV)	-63.4 ± 3.1	-63.2 ± 2.8	-65.2 ± 3.7	0.12	0.89	-64.7 ± 3.8	-63.9 ± 4.0	-65.1 ± 4.2	0.02	0.98
Rm (MΩ)	305.3 ± 23.4	338.1 ± 26.7	299.6 ± 27.4	0.64	0.54	243.7 ± 28.4	245.1 ± 32.7	241.2 ± 29.8	0	1

Table 4: The effects of 0.1 µM NE on pyramidal cell intrinsic properties

	P8-11 (n = 10)					P14+ (n = 8)				
	Control	NE 0.1 µM	NE Wash	$F_{(2.27)}$	P	Control	NE 0.1 µM	NE Wash	$F_{(2.21)}$	P
AP thresold (mV)	-34.9 ± 1.0	-35.4 ± 0.8	-34.8 ± 1.5	0.08	0.92	-36.7 ± 1.1	-36.4 ± 1.2	-36.4 ± 1.5	0.02	0.98
AP amplitude (mV)	66.6 ± 2.7	63.6 ± 3.6	61.1 ± 4.5	0.55	0.58	72.9 ± 2.4	69.6 ± 3.6	64.7 ± 4.5	1.28	0.30
AP half Width (ms)	1.66 ± 0.08	1.66 ± 0.09	1.52 ± 0.16	0.43	0.66	1.27 ± 0.11	1.31 ± 0.11	1.27 ± 0.13	0.04	0.96
AP rising time (ms)	0.51 ± 0.02	0.52 ± 0.02	0.50 ± 0.02	0.16	0.85	0.39 ± 0.02	0.41 ± 0.03	0.42 ± 0.04	0.33	0.72
AP decay time (ms)	1.27 ± 0.08	1.23 ± 0.11	1.13 ± 0.11	0.48	0.62	0.95 ± 0.11	0.95 ± 0.11	0.87 ± 0.11	0.21	0.81
RMP (mV)	-60.8 ± 1.28	-61.1 ± 2.1	-63.9 ± 2.16	0.79	0.46	-63.1 ± 2.3	-63.6 ± 2.0	-61.7 ± 2.5	0.19	0.82
Rm (MΩ)	251.1 ± 14.3	258.1 ± 19.9	274.8 ± 34.3	0.25	0.78	246.4 ± 17.0	246.5 ± 19.0	262.3 ± 26.2	0.19	0.83

2.2 Bi-directional norepinephrine modulations of mEPSCs in P8-11 and the effect of β-adrenoceptor blockade

We asked whether NE can change mEPSC properties in younger pups, and, if it can, how significant is the contribution of the β-adrenoceptor to that. Figure 7A shows example traces of mEPSCs recorded in one cell throughout various concentrations of NE. NE had no effect on mEPSC amplitudes (Figure 7B1 and 7B2), however, mEPSC frequency was bi-directionally modulated (Figure 7C1 and 7C2). Low concentration NE (0.1 µM) increased mEPSC frequency (1.84 ± 0.28 Hz *vs.* 1.51 ± 0.29 Hz in control, n = 7, t = 2.56, p = 0.043), whereas a high concentration (10 µM) reduced mEPSC frequency (1.01 ± 0.23 Hz) compared to control (t = 3.48, p = 0.01; Figure 7C2). A moderate concentration of NE (1 µM) had no effect on mEPSC

frequency (1.38 ± 0.32 Hz, t = 1.09, p = 0.32; Figure 7C2). The NE effects at both low and high concentrations were blocked by a mixture of α- and β-adrenoceptor blockers (phentolamine 50 μM and propranolol 20 μM; Figure 7D and 7E). Thus we found that there is indeed NE dependent change in mEPSC properties in P8-11 pups.

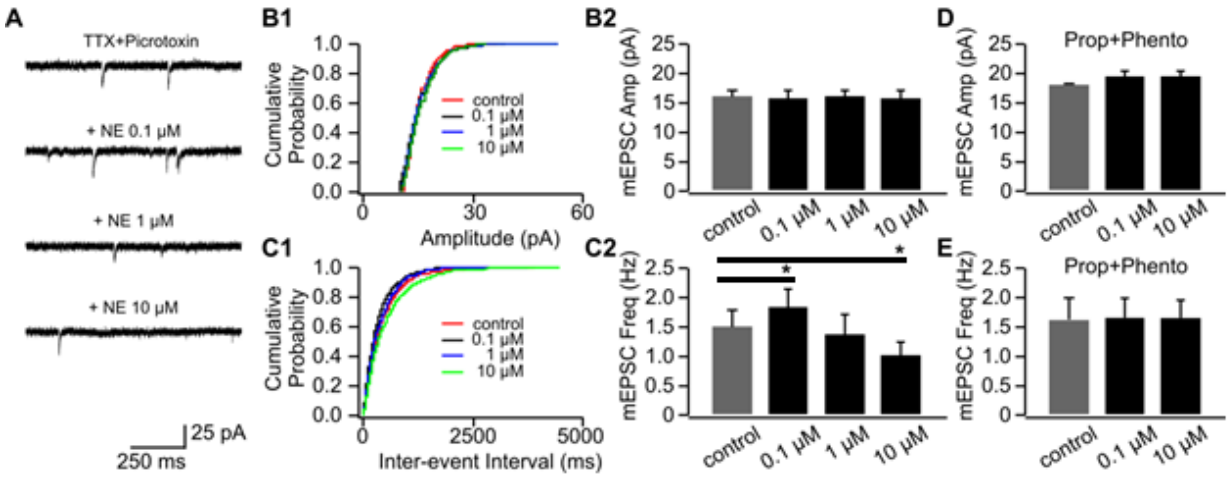

Figure 7. The effects of NE on mEPSCs in P8–11. (A) Example mEPSC traces of a cell with various concentrations of NE. **(B1)** Cumulative probability of mEPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mEPSCs at various NE concentrations. **(C1)** Cumulative probability of mEPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mEPSCs at various NE concentrations. **(D)** Amplitudes of mEPSC at various NE concentrations in the presence of 20 μM propranolol (Prop) and 50 μM phentolamine (Phento). **(E)** Frequencies of mEPSCs at various NE concentrations in the presence of propranolol and phentolamine. $^{*}p < 0.05$.

Next we asked to what extent β-adrenoceptor contributed to NE-dependent modulation of mEPSC. Application of β-adrenoceptor antagonist propranolol had no effect on mEPSC amplitude (Figure 8A, 8B1 and 8B2), however, it abolished the effect of 0.1 μM NE on mEPSC frequency (1.07 ± 0.16 Hz *vs.* 1.34 ± 0.32 Hz in control, n = 8, t = 1.43, p = 0.20, Figure 8C1 and 8C2). Propranolol did not affect the suppressive effect of 10 μM NE on mEPSC frequency (0.67 ± 0.12) compared to control (t = 2.55, p = 0.038; Figure 8C2). Additionally, β-adrenoceptor blockade uncovered the inhibitory effect of NE on mEPSC frequency, since the moderate concentration of NE (1 μM) reduced mEPSC frequency in the presence of propranolol (0.89 ± 0.15) compared to control; although statistical test did not reveal any significant difference (t = 2.28, p = 0.055; Figure 8C2). Thus our result suggests that β-adrenoceptor activation promotes mEPSC frequency in younger pups.

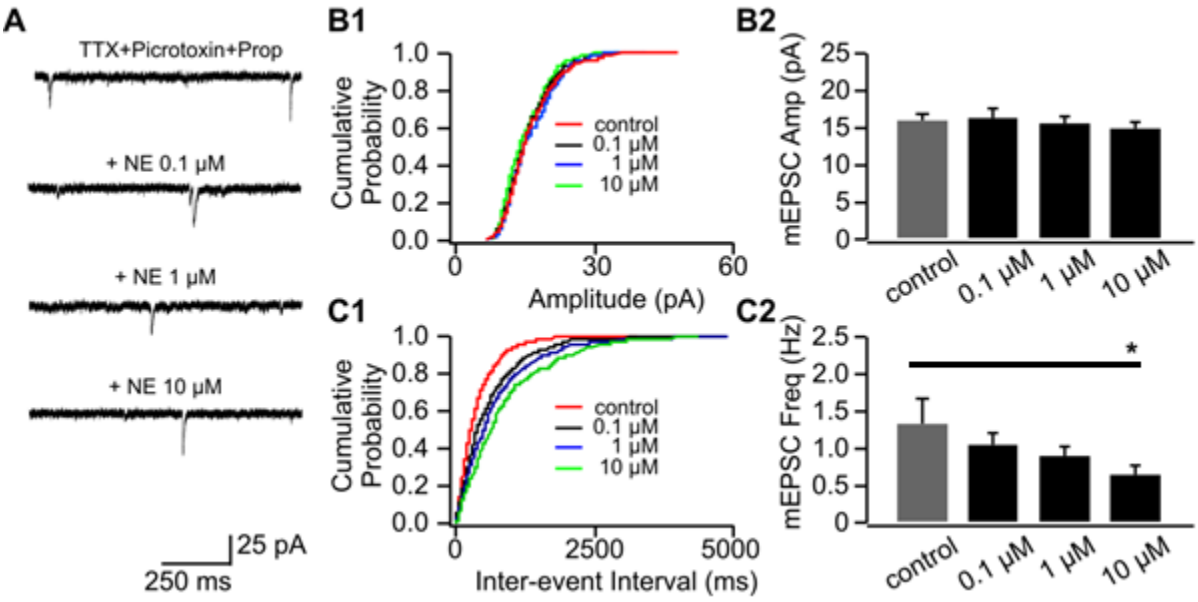

Figure 8. The effects of NE in the presence of propranolol on mEPSCs in P8-11.

(A) Example mEPSC traces of a cell with various concentrations of NE in the presence of propranolol. **(B1)** Cumulative probability of mEPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mEPSCs at various NE concentrations. **(C1)** Cumulative probability of mEPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mEPSCs at various NE concentrations. $^{*}p < 0.05$.

2.3 Suppression of mEPSCs in P14+ by norepinephrine

After testing NE-effect on mEPSC in younger pups, we asked whether NE has similar effects on mEPSC in older pups beyond critical period. We found that NE had no effect on mEPSC amplitudes in P14+ animals (Figure 9A, 9B1 and 9B2). However, in contrast to cells in the P8-11 group, NE at the low concentration (0.1 μM) did not increase mEPSC frequency (1.5 ± 0.40 Hz *vs.* 1.83 ± 0.50 Hz in control, n = 9, t = 1.95, p = 0.087; Figure 9C1 and 9C2). Furthermore, both the moderate (1 μM; 1.37 ± 0.38 Hz, n = 9, t = 3.07, p = 0.015) and the high concentration NE (10 μM; 1.18 ± 0.26 Hz, n = 9, t = 2.54, p = 0.035) suppressed mEPSC frequency compared to the control (Figure 9C2). These suppressive effects by NE were again, blocked when both α- and β-blockers were added to the aCSF before NE application (Figure 9D and 9E).

Together, these results suggest α- and β-adrenoceptors have differential effects on the pyramidal cell mEPSCs in the piriform cortex. The effect of β-adrenoceptor activation is more dominant at the low concentration of NE and increases presynaptic release, while α-adrenoceptor mediates

inhibitory effect on presynaptic release and is predominate when NE concentration is high. The lack of facilitatory effect of NE on mEPSC frequency in older pups implies that there may be developmental changes in adrenoceptor subtype expressions which alter the balance of α- and β-adrenoceptor mediated effects.

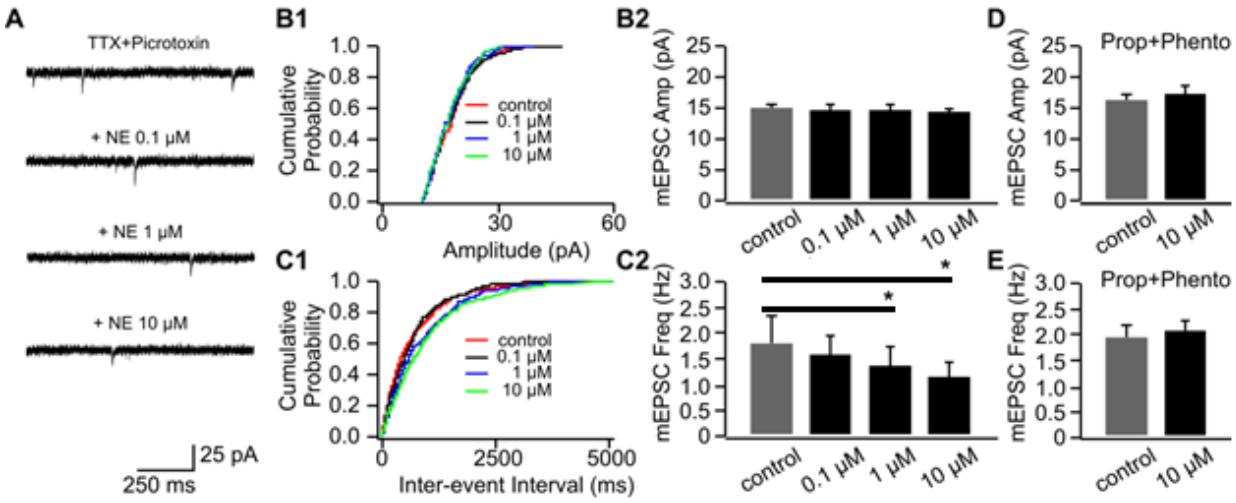

Figure 9. The effects of NE on mEPSCs in P14+. (A) Example mEPSC traces of a cell with various concentrations of NE. **(B1)** Cumulative probability of mEPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mEPSCs at various NE concentrations. **(C1)** Cumulative probability of mEPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mEPSCs at various NE concentrations. **(D)** Amplitudes of mEPSC at various NE concentrations in the presence of propranolol (Prop) and phentolamine (Phento). **(E)** Frequencies of mEPSCs at various NE concentrations in the presence of propranolol and phentolamine. $^{*}p < 0.05$

2.4 Bi-directional norepinephrine modulations of mIPSCs in P8-11 and the effect of β-adrenoceptor blockade

After checking mEPSC, we asked whether NE can change mIPSC properties in younger pups, and, if it can, how significant is the contribution of the β-adrenoceptor to that. Example traces of mIPSCs with various concentrations of NE were shown in Figure 10A. NE had no effect on mIPSC amplitudes (Figure 10B1 and 10B2), however, similar to its effect on mEPSCs, NE bi-directionally modulated mIPSC frequencies (Figure 10C1 and 10C2). NE at the low concentration (0.1 μM) significantly decreased mIPSC frequency (0.96 ± 0.09 Hz *vs.* 1.17 ± 0.09 Hz in control, n = 9, t = 3.87, p = 0.005), whereas a high concentration (10 μM) increased mIPSC frequency (1.44 ± 0.15 Hz) compared to control (t = 2.66, p = 0.03; Figure 10C2). A moderate concentration of NE (1 μM) had no effect on mIPSC frequency (1.17 ± 0.12 Hz, t = 0.06, p = 0.95; Figure 10C2). The NE effects at both low and high concentrations were blocked by a mixture of 20 μM propranolol (Prop) and 50 μM phentolamine (Phento). (Figure 10D and 10E). Thus we found that there is indeed NE dependent change in mIPSC properties in P8-11 pups.

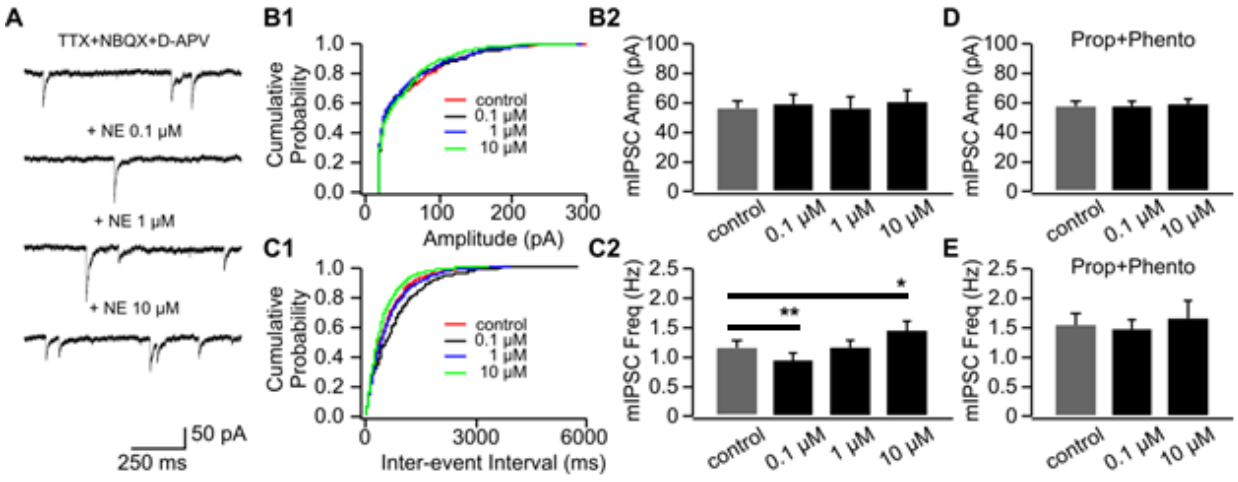

Figure 10. The effects of NE on mIPSCs in P8–11. (A) Example mIPSC traces of a cell with various concentrations of NE. **(B1)** Cumulative probability of mIPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mIPSCs at various NE concentrations. **(C1)** Cumulative probability of mIPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mIPSCs at various NE concentrations. **(D)** Amplitudes of mIPSC at various NE concentrations in the presence of 20 μM propranolol (Prop) and 50 μM phentolamine (Phento). **(E)** Frequencies of mIPSCs at various NE concentrations in the presence of propranolol and phentolamine. $^{*}p <$ 0.05; $^{**}p < 0$.

01.

Next we asked to what extent β-adrenoceptor contributed to NE-dependent modulation of mIPSC in younger animals. Application of propranolol itself had no effect on mIPSC amplitude (Figure 11A, 11B1 and 11B2), however, the effect of 0.1 μM NE on mIPSC frequency was abolished in the presence of propranolol (1.26 ± 0.24 Hz *vs.* 1.28 ± 0.22 Hz in control, n = 8, t = 0.21, p =

0.84, Figure 11C1 and 11C2). Propranolol did not alter the effect of 10 μM NE on mIPSC frequency (1.62 ± 0.26Hz) compared to control (t = 3.28, p = 0.014; Figure 11C2). Moderate concentration of NE (1 μM) did not affect mIPSC frequency significantly in the presence of propranolol (1.38 ± 0.24Hz) compared to control (t = 1.60, p = 0.153; Figure 11C2). Thus our result suggests that β-adrenoceptor activation can inhibit mIPSC frequency in younger pups.

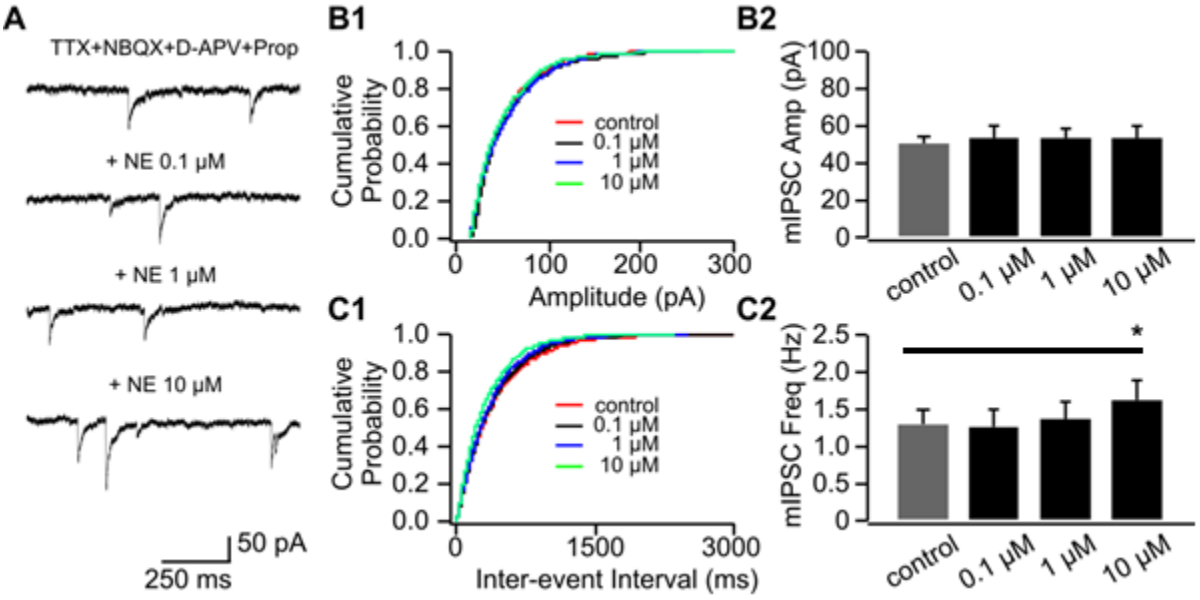

Figure 11. The effects of NE in the presence of propranolol on mIPSCs in P8–11. (A) Example mIPSC traces of a cell with various concentrations of NE in the presence of propranolol. **(B1)** Cumulative probability of mIPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mIPSCs at various NE concentrations. **(C1)** Cumulative probability of mIPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mIPSCs at various NE concentrations. $^{*}p < 0.05$

2.5 Enhancement of mIPSCs in P14+ by norepinephrine

After testing NE-effect on mIPSC in younger pups, we asked whether NE has similar effects on mIPSC in older pups beyond critical period. Interestingly, NE consistently increased mIPSC amplitudes in P14+ animals with the higher concentrations (1 and 10 μM; Figure 12A, 12B1 and 12B2). The mIPSC amplitude increased to 53.98 ± 10.08 pA with 1 μM NE from 50.59 ± 9.53 pA in control condition ($n = 7$, $t = 4.18$, $p = 0.006$) and further increased to 55.55 ± 10.24 pA with 10 μM NE ($t = 5.24$, $p = 0.002$; Figure 12A, 12B1 and 12B2). However, in contrast to cells in the P8-11 group, NE at the low concentration (0.1 μM) did not decrease mIPSC frequency (1.45 ± 0.13 Hz *vs.* 1.48 ± 0.15 Hz in control, $n = 7$, $t = 0.26$, $p = 0.802$; Figure 12C1 and 12C2). The high concentration of NE (10 μM; 1.89 ± 0.13 Hz) increased mIPSC frequency compared to the control ($t = 2.76$, $p = 0.033$; Figure 12C2). The effects of NE on both mIPSC amplitude and frequency were blocked when both α- and β-adrenoceptor blockers were blocked in the presence of both alpha and beta adrenoceptor blockers. (Figure 12D and 12E).

Consistent with mEPSC recording, β-adrenoceptors were activated by lower concentrations of NE compared to α-adrenoceptors at inhibitory synapses and the β-adrenoceptor effect (lowering mIPSC frequency) was more dominant in younger mouse pups.

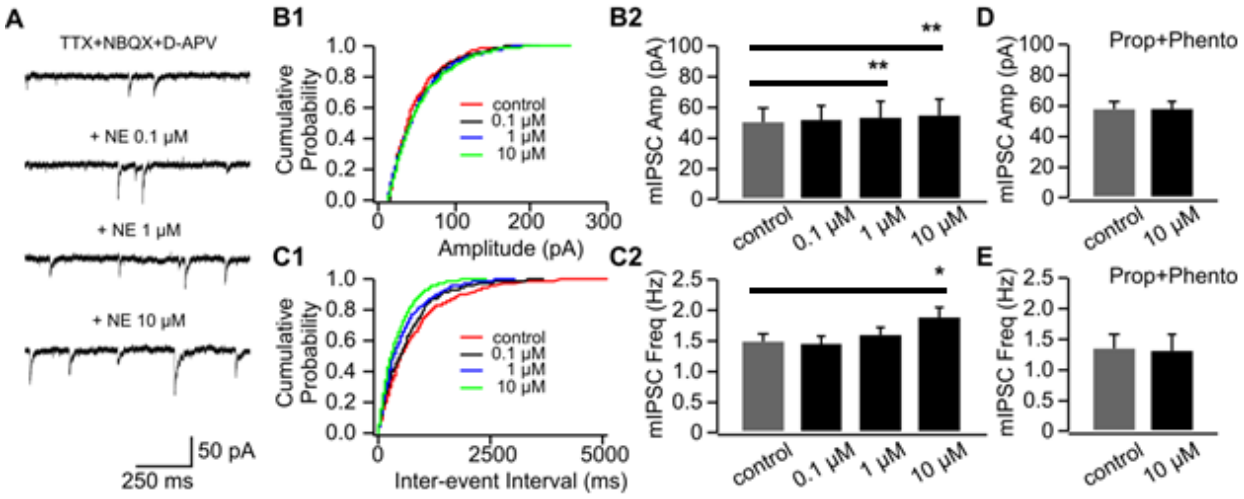

Figure 12. The effects of NE on mIPSCs in P14+. (A) Example mIPSC traces of a cell with various concentrations of NE. **(B1)** Cumulative probability of mIPSC amplitudes in one cell. **(B2)** Amplitudes (Amp) of mIPSCs at various NE concentrations. **(C1)** Cumulative probability of mIPSC inter-event intervals in one cell. **(C2)** Frequencies (Freq) of mIPSCs at various NE concentrations. **(D)** Amplitudes of mIPSC at various NE concentrations in the presence of propranolol (Prop) and phentolamine (Phento). **(E)** Frequencies of mIPSCs at various NE concentrations in the presence of propranolol and phentolamine. $^{*}p < 0.05$; $^{**}p < 0.01$. paired t-test.

Discussion

In this work we established β-adrenoceptor mediated early odor preference learning in mice. Odor preference learning was seen at P8, but not P14. We characterized the effects of NE in aPC layer II pyramidal cells in two age groups (P8-11 and P14+). Pyramidal cells in the aPC undergo developmental changes in their intrinsic electrophysiological properties (RMP and APs) from P8 to weaning age (P21). Two different concentrations of NE did not have clear effects on intrinsic properties in either age group. However, NE differentially modulates synaptic properties in two age groups in a concentration dependent manner. Low concentration (0.1 μM) of NE promoted excitation of the pyramidal cells only in P8-11 pups by increasing mEPSC frequency and by decreasing mIPSC frequency *via* β-adrenoceptor. However, in a higher concentration (10 uM), NE facilitated inhibition by decreasing mEPSCs and increasing mIPSCs in both age groups. Individual aspects of these results and their significance in the context of the existing literature will be discussed below.

1. Developmental changes and their significance

1.1 Intrinsic properties

Other cortical areas, such as somatosensory and visual cortex, are known to undergo developmental changes in the intrinsic properties of pyramidal neurons. For example, layer 5 pyramidal neurons in the rat visual cortex keep maturing until ~4 weeks of age. During this time RMP hyperpolarizes, input resistance decreases and AP profile (amplitude, waveform, threshold etc.) changes as well (Kasper et al., 1994). In the barrel cortex, the change in spike adaptation

(owing to sAHP changes) are directly related to termination of the critical period of sensory map formation (Maravall et al., 2004).

Here, for the first time, we showed the mouse olfactory cortex undergoes similar changes in postnatal development of pyramidal neurons. We found that beyond the critical period neurons were more hyperpolarized and had more negative action potential threshold. Consequently, neurons from the older age group ended up with larger amplitude action potentials. The shape of the action potential changed as well. In P14+ group, action potentials were shorter in width. This was reflected in both AP rise time and decay time. But the input resistance of the neurons remained unaltered across the critical period. Normally a more hyperpolarized RMP is expected to be accompanied with lower excitability. However it is possible that a more negative action potential threshold compensates for that in the older age group. Thus we end up having neurons with similar excitability before and after the critical period. Unaltered intrinsic excitability of pyramidal neurons shifts the focus to the synaptic property related changes.

1.2 Synaptic properties

Earlier research shows a decrease in interneuron number but an increase in the number of inhibitory synapses with development (P0-P60) in aPC which overlaps the critical period of early odor preference learning as well (Sarma et al., 2011). They counted Glutamic acid decarboxylase-green fluorescence protein positive cells for counting the numbers of cells. For measuring inhibitory synapses they counted gephyrin positive puncta. However we did not find a difference in either frequency or amplitude of IPSC between the two age groups. It is possible

that this alteration in the inhibitory synapses is restricted to the semilunar neurons only and as we recorded from the pyramidal neurons it was not reflected in our results. Future experiments can shine more light on cell-type specific differences in IPSC properties.

Learning-related changes in the amplitude of EPSC has been reported in pyramidal neurons in the PC (Ghosh et al., 2016). In this connection we wanted to see if there is any basal level difference in the EPSC properties in two age groups. We did not find any difference between the two age groups in terms of frequency or amplitude of the EPSC. Thus it can be safely concluded that there is no age-related differences in the electrophysiological properties of the pyramidal neurons that can attribute to any general excitability of the neuronal network in younger animals.

2. Noradrenergic modulation of electrical properties and its significance

2.1 Noradrenergic modulation of intrinsic properties

As we have already learned, NE is known to be crucial to early odor preference learning and it has the ability to alter the electrical activity of the neurons to corroborate physiological changes. Hence after discovering the difference in intrinsic properties of neurons between the two age groups, we wanted to look at NE's differential modulation of the same.

Both higher and lower concentrations of NE were mostly ineffective in changing the intrinsic properties in both age groups. But, interestingly, 10 uM NE decreased decay time of AP in P14+ group significantly, but not in younger animals. Possible involvement of potassium or calcium

conductance (Dunlap and Fischbach, 1978; Slack, 1986) has been suggested earlier for similar changes in AP feature. However, the exact mechanism and significance of shortening of AP width following NE application in the older age group is currently unknown. It is another possibility that the decrease in AP decay time is independent of NE as it continued to decrease in wash. However, it is possible that the widened AP will lead to a higher calcium influx and thereby initiate different molecular cascades in the neuron to promote learning. However that does not explain why this mechanism would apply to early odor preference learning but not to other types of odor learnings.

Also NE's overall ineffectiveness in modulating the intrinsic properties may result from either the inability of NE to modulate voltage-gated Na+ channels or K+ channels or from the opposing action of different receptors of NE engaged at these concentrations. Future investigations in this aspect would be helpful in answering these questions. This leaves us with synaptic properties and NE's modulation as the crucial factors to be investigated next.

2.2 Noradrenergic modulation of synaptic properties

After ruling out NE modulation on the intrinsic properties in this model, we looked at how NE can modulate excitatory and inhibitory synapses of the layer II pyramidal neurons. Input specific-changes in a network are better correlated with synaptic properties than intrinsic properties. From the past few decades of research we know different effects of NE on the PC network. One of the earliest work on NE modulation of synaptic transmission was from Collins et al 1984 showing that lower concentrations (0.1–5 μM) of NE promotes transmission from

LOT to pyramidal neurons whereas higher concentration (20–250 μM) prevents this. In a similar line of work, Hasselmo et al (1997) has demonstrated that 10uM or higher concentration of NE will result in a greater decrease in synaptic transmission in layer Ib (associational input) than layer Ia (afferent input). This raises the possibility of improving the signal-to-noise ratio by NE and thereby promoting more efficient olfactory information processing and possibly olfactory learning too (Linster and Hasselmo, 2001). Even in *in vivo* experiments NE release from LC has proven to alter the firing pattern of piriform cortex neurons following odor exposure (Bouret and Sara, 2002).

In spite of the decades-long focus on NE modulation, how exactly NE exerts it effect on the neurons at the single cell level, is unclear. In line with previous findings, we found that at low concentration NE promotes excitation in P8-11 mouse pups. At 0.1 μM concentration NE enhanced frequency of mEPSC and decreased the same for mIPSC, suggesting that both effects are presynaptic in nature. This goes hand-in-hand with the view that the increase in input-specific excitation will lead to learning. Thus NE dependent enhancement of mEPSC and decrease in mIPSC frequency is suggestive of an increase in the signal and thereby an increase in signal-to-noise ratio which is thought to be useful for learning (Linster et al., 2011).

If NE-dependent signal augmentation is important for early odor preference learning, we would expect that this phenomenon should disappear or at least decrease beyond the critical period as older pups are unable to learn the paradigm. Indeed in older pups (P14+), NE was ineffective at low concentration on either mEPSC or mIPSC. This addressed one of our original questions and provided support for the age-dependent change in adrenergic modulation being critical for aPC network activity. Also, it suggested NE modulation of synaptic properties are critical for this

learning, which is consistent with the network properties of learning.

In contrast with the lower concentration, the higher concentration of NE was found to be inhibitory in both age groups. NE at 10 μM suppressed mEPSC and enhanced mIPSC. While presynaptic actions were responsible for NE effects in P8-11mice, modulation of both pre-synaptic and post-synaptic sites were found in inhibitory synapses in P14+ mice. Previous work has shown NE-dependent increase in inhibitory transmission onto the pyramidal neurons in PC (Marek and Aghajanian, 1996). Thus, a major adrenergic modulation of the post synaptic site in older animals would be quite understandable as we found in our experiments. Next, we addressed the last question, which adrenergic receptor subtype is involved in the described response.

3. Concentration dependence and α vs. β adrenoceptor

Our results suggested a clear concentration-dependent change in NE's effect on pyramidal neurons. At different concentrations NE is known to involve different receptors preferentially and thereby exert very different effects on the circuitry. For example, at low concentration (0.1-5 μM) NE is known to enhance synaptic transmission from the LOT to pyramidal neurons; whereas, at higher concentrations (20-250 μM) it reduces transmission (Collins et al., 1984). More recent research shows NE at concentrations of greater than 10 μM causes more reduction in synaptic transmission at the associational inputs (layer Ib) compared to afferent inputs (layer Ia) (Hasselmo et al., 1997). This finding indicates that there is a NE-dependent enhanced signal-to-noise ratio that would promote learning (Linster and Hasselmo, 2001). Also it is known that

alpha adrenoceptor has lower affinity to NE than beta adrenoceptor. For example, in the OB, α_1-adrenoceptor is activated by higher concentration of NE compared to α_2- or β-adrenoceptors (Nai et al., 2009).

We found that at low concentration NE promoted excitation in P8-11 mouse pups. At 0.1 μM concentration NE enhanced frequency of mEPSC and decreased the same for mIPSC, suggesting that both effects are presynaptic in nature. Blockade of β-adrenoceptor abolished both NE effects on mEPSC and mIPSC, suggesting that at this low concentration NE preferably engaged β adrenoceptors and promotes excitation of the pyramidal neurons.

The higher concentration of NE, in younger mice (P8-11), did not involve β adrenoceptor preferentially and hence was not affected by β-adrenoceptor blockade. It implies that higher concentration of NE preferentially engaged α-adrenoceptors. Together, these results suggested that α- and β-adrenoceptors mediate the inhibitory and the excitatory effects of NE respectively, consistent with the opposing actions of these receptor subtypes reported in other brain areas (Kobayashi, 2007a; Kobayashi, 2007b; Salgado et al., 2012b; Salgado et al., 2012a).

We know that α_1-adrenoceptor-mediated activation of GABAergic interneurons exerts inhibition onto the pyramidal neurons in the PC (Marek and Aghajanian, 1996). It is possible that the age-dependent change in NE's action is a result of increased α- and/or decreased β-adrenoceptor expression or function. Future experiments with subtype specific adrenergic agonists will be crucial to test this view.

Different subtypes of β adrenoceptors may exert different actions. Opposing effects of β_1- and

β_2-adrenoceptors on synaptic transmission have been observed in layer V/VI pyramidal cells of the rat prefrontal cortex (Ji et al., 2008; Zhou et al., 2013; Luo et al., 2014b). Presynaptically acting PKA-dependent pathway is known to be involved in β_1-adrenoceptor-induced suppression of glutamate release in the prefrontal cortex (Luo et al., 2014a). Hence it will be important to dissect out the roles of β_1- and β_2-adrenoceptors in promoting excitation by low concentration NE in younger animals.

In older mice, α-adrenoceptor-mediated inhibitory effects appear to dominate. Increased inhibition of pyramidal cells by NE coincides with reduced plasticity and termination of the critical period learning. In older animals, tactile stimulation ceases to promote LC NE release (Kimura and Nakamura, 1987a) because of an increase in α2 adrenoceptor mediated inhibition at the LC level. In the OB a different picture develops. With increased age there is a loss of function of α2-mediated inhibition of GC leading to greater inhibition of the MC (Pandipati et al., 2010). Thus, it is possible that α adrenoceptor-mediated inhibition seems to potentiate with increasing age in different locations throughout the olfactory processing system. It would be interesting to see the contribution of the individual adrenoceptor subtypes in modulating early odor preference learning.

4. The bulb v/s the anterior piriform cortex: where do we stand?

NE-dependent MC excitation and gamma oscillation creates a stronger and more synchronized output to the piriform cortex (Hayar et al., 2001b; Yuan, 2009b; Shakhawat et al., 2012b). It is understandable that NE would be crucial in promoting olfactory learning in the OB. NE at the

level of the OB has been shown to be necessary and sufficient for early odor preference learning (Sullivan et al., 2000b).

In a similar line of argument, Morrisson et al (2013) have shown that applying β adrenoceptor antagonist at the aPC blocks the early odor preference learning whereas β adrenoceptor agonist serves as an UCS for it. This makes NE at the aPC necessary and sufficient for early odor preference learning. This study also shows that transient silencing of the aPC makes the animal unable to learn, even though the OB is intact. Early odor preference learning is also known to increase c-fos and Arc expression in the aPC following exposure to the conditioning odor (Roth and Sullivan, 2005).

The current project emphasizes the crucial role of NE in modulating synaptic properties at the aPC. We discovered that β adrenoceptors are important for promoting excitation of the pyramidal neurons as well as learning in younger animals. And this function is either lost or over-compensated with the opposing action of other adrenoceptors beyond the critical period. In accordance with previous research, this current study explains how the aPC can be a crucial site for retaining odor preference memory.

5. Other behavioural phenomenon and possible involvement of norepinephrine

Researchers have shown that NE is crucially involved in the specificity of odor habituation memory (Mandairon et al., 2008). It is also known to be involved in spontaneous odor discrimination and detection (Doucette et al., 2007; Escanilla et al., 2010). More recent work has

shown that adrenergic modulation at the level of the aPC is crucial for highly similar odor discrimination learning and pattern separation ability is compromised following adrenergic blockade (Shakhawat et al., 2015).

In light of this research, our current study explores the mechanistic part of NE's action in the aPC pyramidal neurons. Knowing that NE-dependent inhibition increases with age, it would be interesting to dissect out and compare the roles of interneurons and pyramidal neurons in the PC and study NE's effect on them individually and with respect to specific receptor subtypes, after the subjects undergo different behavioral training regimes.

6. Glutamate receptors, β-adrenoceptor and learning

The β adrenoceptor agonist, isoprotenerol, is known to have actions on presynaptic glutamate release. It increases the amplitude of LOT LTP when applied to aPC slices during theta burst stimulation (Morrison et al., 2013). This increase is attributable to a cAMP-dependent reversal of the depression of presynaptic release in response to TBS, which otherwise decreases the release by activation of mGluR2/3 (Cai et al., 2001; Best and Wilson, 2004; Morrison et al., 2013). Thus a role of β adrenoceptor in promoting LTP has been established before.

It has been shown that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated response increases at 24 hrs following one trial learning(Morrison et al., 2013). Synaptic strengthening and thereby resultant presynaptic release enhancement was seen only in the early hours after conditioning; but not at 24 hours. However, in more extensive training paradigms, the AMPAR-dependent response is shown to increase even at 48 hours (Fontaine et al., 2013). It is possible that more extensive training involves more noradrenergic

activity. It will be interesting to investigate if higher levels of NE alters AMPAR-dependent changes.

Our current work provides some insight into the modulation of glutamate activity by β adrenoceptors. An increase in the frequency of mEPSC by β-adrenoceptor mediated action is highly suggestive of a mechanism facilitating release of glutamate, not one negatively regulated by the action of mGluR2/3. Although we did not find any β adrenoceptor dependent post synaptic changes in either age group, it is possible that learning induced changes could render a different picture regarding the effects of β adrenoceptors on glutamate receptors. Reasonable extension of our current work would be to assess the effect of Isoprenaline on EPSC frequency and amplitude and on NMDAR/AMPAR following learning.

7. Other monoamines and norepinephrine : difference and similarities in functional roles

In general all the monoamine-neuromodulators (norepinephrine, dopamine, serotonin and acetylcholine) are considered to have a significant effect on the GABAergic interneurons in the piriform cortex. Most of them have the ability to exert inhibitory effects on the pyramidal neurons by enhancing IPSC. However, the magnitude of the effect is different for different neuromodulators. Serotonin has the highest pro-inhibitory effect on the piriform cortex followed by NE and dopamine (Gellman and Aghajanian, 1993). Acetylcholine has a more complex function. It suppresses both excitatory and inhibitory neurotransmission (Collins et al., 1984; McIntyre and Wong, 1986; Williams and Constanti, 1988; Hasselmo and Bower, 1992;

Hasselmo et al., 1997; Patil and Hasselmo, 1999). Acetylcholine also causes depolarization of pyramidal neurons (Tseng and Haberly, 1989; Barkai and Hasselmo, 1994). In our results, we did not find a depolarization by NE in any of the cells. But we saw enhancement of excitatory potentials and suppression of inhibitory potentials with low concentrations of NE, preferably engaging β adrenoceptors. However, consistent with other research works showing NE (α1B adrenoceptor) dependent enhancement of GABAergic interneuron activity, we observed increased inhibitory synaptic transmission with high concentrations of NE, which do not engage β adrenoceptors preferentially (Marek and Aghajanian, 1996). Network simulation studies propose that NE enhances the response to an afferent input by maximizing signal-to-noise ratio (Linster and Hasselmo, 2001). Our current study provides a mechanistic basis of the same via β-adrenoceptor.

8. Conclusion

In this study, we have investigated the role of norepinephrine in differentially modulating electrophysiological properties of anterior piriform cortex pyramidal neurons during and after the critical period for early odor preference learning. Although we have discovered several differences in electrophysiological properties between the two age groups, we have not seen any obvious difference in cellular excitability developing across the critical period. In line with other research works, we have found a crucial role for norepinephrine in this context. Our result suggests that β-adrenoceptor mediated increase in excitation and decrease in inhibition is in effect in younger pups. This effect ceases to exist beyond the critical period. Thus we provide a possible mechanism how β-adrenoceptor may crucially modulate the time-sensitive early odor

preference learning in mouse pups.

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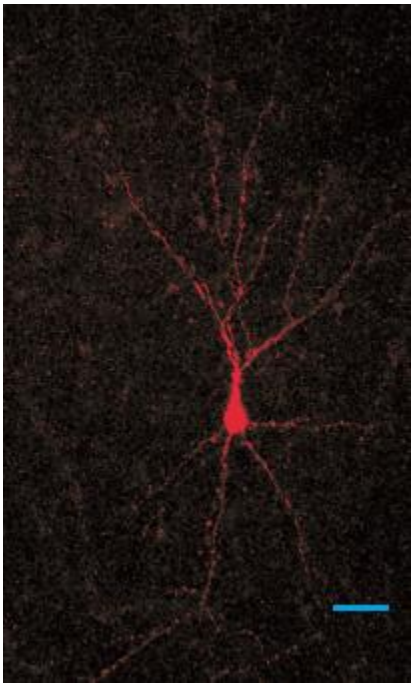
Appendices

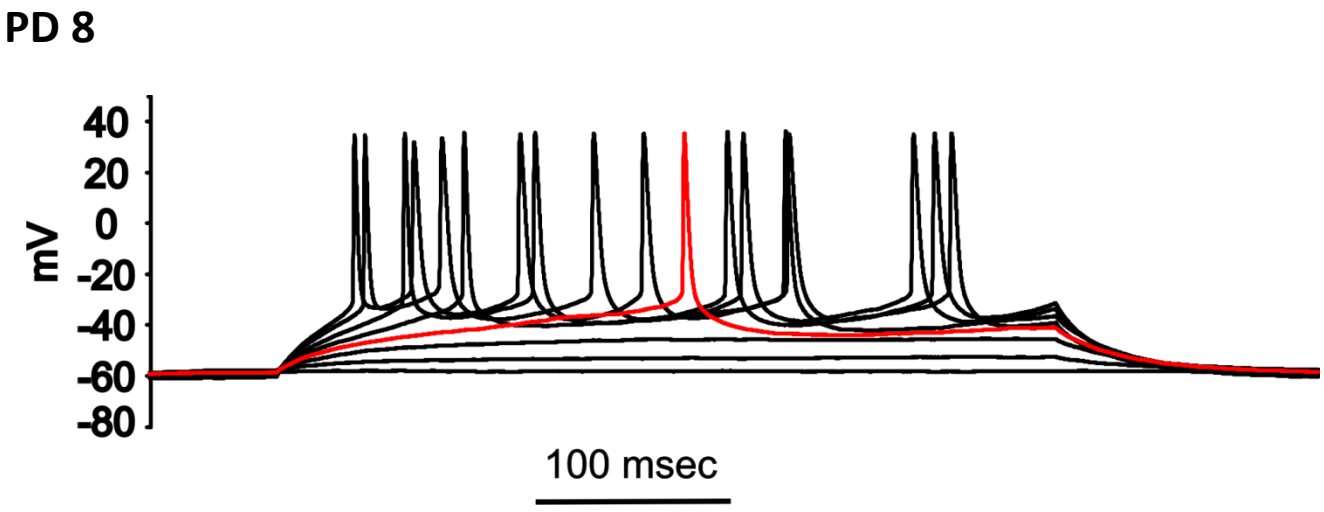
A. Superficial pyramidal neuron

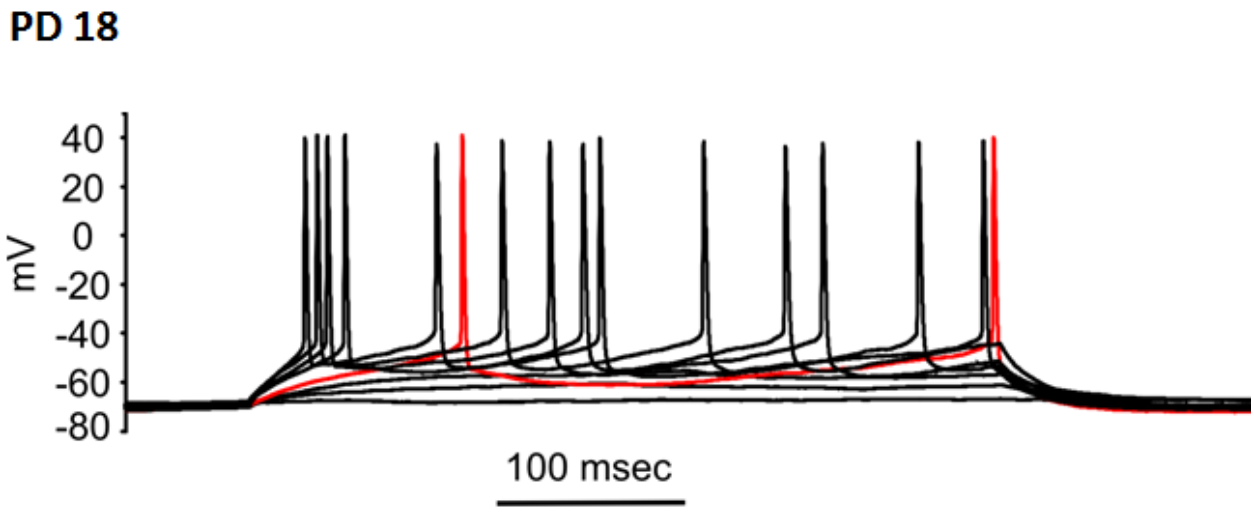


A superficial pyramidal neuron is shown here following electrophysiological recording and biocytin staining. Scale bar: 50µm

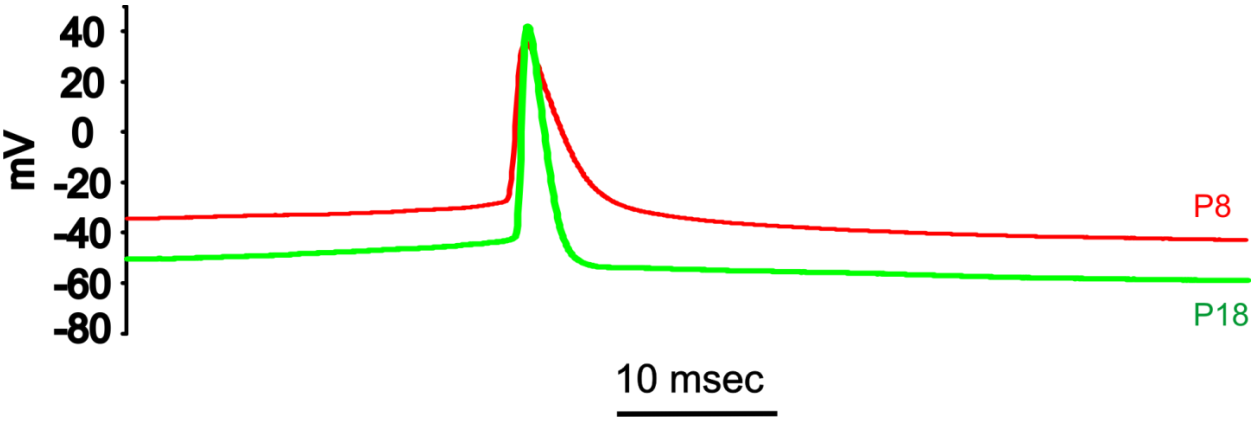
B. Current clamp recordings in two age groups

B1. Recording traces following multiple steps of current injection





B2. Action potential resulting from smallest current step



B1. Depolarization currents of increasing amplitude (10 pA steps) were injected into the cell through the patch-pipette in current clamp mode. First action potential evoked by the

smallest current injection step (highlighted in red) was used for analysis. PD: age in postnatal days.

B2. First action potential evoked by the smallest current injection step is magnified to show the relative difference in their shapes in two age groups. Red indicates younger age group- postnatal day 8 (P8) and green indicates older age group- postnatal day 18 (P18).