Noradrenergic Modulations of Odor Learning and Odor Representation in the Rat

By

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Abstract

How experience alters neuronal ensemble dynamics and how locus coeruleus-mediated norepinephrine release facilitates memory formation in the brain are the topics of this thesis. Here we employed a visualization technique, cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH), to assess activation patterns of neuronal ensembles in the olfactory bulb (OB) and anterior piriform cortex (aPC) to repeated odor inputs. Two associative learning models were used, early odor preference learning in rat pups and adult rat go-no-go odor discrimination learning.

With catFISH of an immediate early gene, *Arc*, we showed that odor representation in the OB and aPC was sparse (~5-10%) and widely distributed. Odor associative learning enhanced the stability of the rewarded odor representation in the OB and aPC. The stable component, indexed by the overlap between the two ensembles activated by the rewarded odor at two time points, increased from ~25% to ~50% (p = 0.004-1.43E^{-}4; Chapter 3 and 4).

Adult odor discrimination learning promoted pattern separation between rewarded and unrewarded odor representations in the aPC. The overlap between rewarded and unrewarded odor representations reduced from ~25% to ~14% (p = 2.28E^{-5}). However, learning an odor mixture as a rewarded odor increased the overlap of the component odor representations in the aPC from ~23% to ~44% (p = 0.010; Chapter 4).

Blocking both α- and β-adrenoreceptors in the aPC prevented highly similar odor discrimination learning in adult rats, and reduced OB mitral and granule ensemble stability to the rewarded odor. Similar treatment in the OB only slowed odor discrimination learning. However, OB adrenoceptor blockade disrupted pattern separation and ensemble stability in the aPC when the rats demonstrated deficiency in discrimination (Chapter 5).

In another project, the role of α_2-adrenoreceptors in the OB during early odor preference learning was studied. OB α_2-adrenoceptor activation was necessary for odor learning in rat pups. α_2-adrenoceptor activation was additive with β-adrenoceptor mediated signalling to promote learning (Chapter 2).

Together, these experiments suggest that odor representations are highly adaptive at the early stages of odor processing. The OB and aPC work in concert to support odor learning and top-down adrenergic input exerts a powerful modulation on both learning and odor representation.

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Abbreviations

2-DG	2-deoxyglucose
ACh	Acetylcholine
AD	Alzheimer disease
AMPARs	AMPA receptors
AON	Anterior olfactory nucleus
aPC	Anterior piriform cortex
Arc	Activity-**r**egulated **c**ytoskeleton-associated protein
catFISH	Cellular compartment analysis of temporal activity by fluorescence in situ hybridization
CR	Conditioned response
CS	Conditioned stimulus
DA	Dopamine
DAPI	4′-6-diamidino-2-phenylindole
dSA	Deep SA cells
DEPC	Diethylpyrocarbonate
EEG	Electroencephalography
EPL	External plexiform layer
ET	External tufted
FRET	Fluorescence resonance energy transfer
GCaMPs	Genetically-encoded calcium reporters
GCL	Granule cell layer
GL	Glomerular layer
GPCR	G-protein coupled receptors
IHC	Immunohistochemistry
HDB	Horizontal diagonal band of Broca (HDB)
HSV-CREB	Herpes simplex virus expressing either CREB
HSV-mCREB	Dominant-negative mutant CREB (HSV-mCREB)
HZs	Horizontal interneurons
ICC	Immunocytochemical staining

IEG	Immediate early genes
IPL	Internal plexiform layer
ISH	*In situ* hybridization
JG	Juxtaglomerular cells
LC	Locus coeruleus
LC-NE	LC-noradrenergic
LOT	Lateral olfactory tract
LTD	Long term depression
LTP	Long term potentiation
MC	Mitral cell
MCL	Mitral cell layer
MEG	Magnetoencephalography
M/T	Mitral/Tufted
NG	Neurogliaform
NE	Norepinephrine
NIR	Near-infrared
NMDARs	NMDA receptors
OB	Olfactory bulb
OD	Optical density
OLR	Overlap ratio
ONL	Olfactory nerve layer
ORs	Odorant receptors
OSNs	Olfactory sensory neurons
O/S^+	Odor paired with stroking
O/S^-	Odor only
OT	Olfactory tubercle
PC	Piriform cortex
pCREB	Phosphorylated CREB
PG	Periglomerular
PND	Postnatal day
pPC	Posterior piriform cortex

PP	Peppermint
RODs	Relative optical densities
ROIs	Regions of interest
SA	Short axon
sSA	Superficial SA
SEL	Subependymal layer
SNc	Substantia nigra pars compacta
SSC	Saline-Sodium Citrate
TSA	Tyramide signal amplification
UCS	Unconditioned stimuli
VA	Vanillin

Co-authorship Statement

I, Amin Md. Shakhawat, hold a first author status for all the manuscripts used in this thesis as chapters (Chapter 2-5). However, each manuscript is co-authored by my supervisors and colleagues, whose mentorship greatly influences the generation of my hypotheses stated in the manuscripts, the conduction of corresponding experiments and finally the writing of the manuscripts.

Contribution from peers in each manuscript is elaborated below.

Manuscript titled “Olfactory bulb α_2-adrenoceptor activation promotes rat pup odor-preference learning via a cAMP-independent mechanism"(chapter 02) in this thesis is co-authored by Carolyn W. Harley and Qi Yuan. As a principle author, I was partly involved in experimental design, conducted all experiments and wrote the first draft of the paper. Qi Yuan and QinLong Hou performed part of figure 1B. Research question and experimental design was originally developed by Qi Yuan. Subsequent adjustment was done by me with proper guidance from Qi Yuan. Whole manuscript was edited and polished by Qi Yuan and Carolyn W. Harley.

My second paper titled "Visualizing the Engram: Learning Stabilizes Odor Representations in the Olfactory Network" (chapter 03) in this dissertation is co-authored by Ali Gheidi, Qinlong Hou, Sandeep K. Dhillon, Diano F. Marrone, Carolyn W. Harley and Qi Yuan. Hypothesis and research involved in this manuscript was developed by Diano F. Marrone, Carolyn W. Harley and Qi Yuan. As the first author of this manuscript I conducted the majority of the experiments in the final figures, data analysis and method writing. The behavioral experiments were conducted by Qi Yuan. Ali Gheidi conducted control experiments. Qinlong Hou, Ali Gheidi and I established the *Arc* visualization technique. Qinlong Hou, Ali Gheidi, Sandeep K. Dhillon and Diano F. Marrone

conducted pilot experiments. Ali Gheidi and Qi Yuan also contributed to data analysis. The manuscript was mostly written by Carolyn Harley and Qi Yuan with partial input from all other co-authors of this manuscript.

My 3rd paper titled “Arc Visualization of Odor Objects Reveals Experience-dependent Ensemble Sharpening, Separation, and Merging in Anterior Piriform Cortex in Adult Rat"(chapter 04) is co-authored by Carolyn W. Harley and Qi Yuan. Hypothesis and research design was developed by Qi Yuan. As the first author of this manuscript I performed all experiments and wrote part of the first draft. Full manuscript was written by Qi Yuan and Carolyn W. Harley.

My 4thpaper titled "*Arc*-expressing neuronal ensembles supporting pattern separation require adrenergic activity in anterior piriform cortex: an exploration of neural constraints on learning" (chapter 05) is co-authored by Ali Gheidi, Iain TK. MacIntyre, Melissa L. Walsh, Carolyn W. Harley, and Qi Yuan. As the first author of this article I conducted most of the experiments, analyzed data and wrote the method section. Ali Gheidi performed part of the experiments and Iain TK. MacIntyre, Melissa L. Walsh helped to conduct pilot experiments at the beginning of the project. Qi Yuan designed the research project and wrote the manuscript together with Carolyn W. Harley.

Chapter-01: Introduction

1.1 Overview

Memories connect the past with the present and influence our decisions about the future – both consciously and unconsciously. In doing this, memories allow for the uninterrupted continuation of life. Without memories, an individual's existence can be jeopardized. For example, a dementia patient who forgets to turn off a stove can place him and his family in a life-or-death situation. In the last 50 years, we have witnessed an unprecedented "explosion" in memory research. With a simple flash of light, we can now recapitulate a fear memory in rodents with more precision (Liu et al., 2012) than a science fiction writer would have dared to dream of a century ago. We are also now able to implant artificial memories **(**de Lavilléon et al., 2015; Ramirez et al., 2013), enhance existing memories, and add new information during sleep (Arzi et al., 2012; Ngo et al., 2013; Oudiette and Paller, 2013; Barnes and Wilson, 2014). To conceptualize this last point, imagine the PhD student who wakes up one morning with all the memories which can be utilize to write comprehensive exam that same day. The in-depth understanding of memory at the cellular, molecular, and circuit level is not only necessary to understand how a PhD student's dreams will be realized, but it is also necessary to delineate the neurobiology of disease conditions.

Studying memory presents a daunting task. Being continuously bombarded with information, our brains have the capacity to store many different memories throughout our lives; yet, perhaps even more remarkable is that they have the ability to recall these same memories decades after they were originally formed. It takes a fraction of a second for a coffee connoisseur to tell the difference between a Tim Horton's and a Starbuck's coffee. A proud parent of twin babies is able to detect the subtle differences between the twins which might otherwise place a stranger in an embarrassing situation. Despite the fact that we have had much success in inducing,

manipulating, implanting, and retrieving a specific memory event in the rodent, the following mechanisms have remained elusive: (1) how the brain encodes and stores different events and (2) how the circuit dynamics of multiple memory engrams within the brain are modified and interleaved following learning. Moreover, how the brain distinguishes very similar objects from each other, how it recalls memory from degraded input, and how different brain regions complement each other during encoding and the modifying of sensory representations following learning requires further investigation.

Memory researchers have been using different sensory modalities to investigate the neurobiological underpinnings of learning and memory. To investigate complex, but interesting questions like those mentioned above, an experimentally tractable sensory model is necessary. The olfactory system offers a unique sensory platform for studying the neurobiology of learning and memory (Davis, 2004). Unlike other sensory modalities, the design and function of the olfactory system is preserved between species (Brennan and Keverne, 1997; Hildebrand and Shepherd, 1997; Laberge and Hara, 2001; Laurent et al., 2001; Mombaerts, 2001; Eisthen, 2002). Moreover, the circuitry involved in processing odor information is well established, which is particularly important for researchers who wish to document the corresponding changes at each level of computation that occurs following learning (i.e., from the periphery to the cortex). Furthermore, odors are believed to be a powerful cue for autobiographical experiences (Chu and Downes, 2000, 2002).

Evolutionarily speaking, the sense of smell has been imperative to mammalian survival, including that of humans. Although we human beings do not rely on our sense of smell as much as other mammals, we can certainly all share poet Diane Ackerman's sentiment that "nothing is more memorable than smell." This becomes especially apparent when we visit our parents and the

smell of mother's cooking elicits vivid memories of childhood. Even science supports Ms. Ackerman's statement as it has been shown that memories evoked by odor cues are more vivid than those triggered by corresponding words (Chu and Downes, 2002). But, for the human, odor memories serve more functions than simply creating sentimental value. Memories of the smell of smoke alert us to the presence of fire before we see a flame, which induces a fight-or-flight response. Memories of the smells of food allow us to distinguish safe food from that which is spoiled. For the rodent, olfaction is particularly vital for many reasons. For example, in the rodent, olfaction plays key roles in reproductive function (Brennan and Keverne, 1997), mother-infant interaction (Kendrick et al., 1992; Leon, 1992; Wilson and Sullivan, 1994; Fleming et al., 1999; Sullivan et al., 2000a), physiological regulation (Leon and Moltz, 1971; Pager, 1974; Leon et al., 1977; Alberts, 1978; Galef and Kaner, 1980; Alberts and May, 1984; Coopersmith and Leon, 1986; Fillion and Blass, 1986; Moore et al., 1996b; Shah et al., 2002; Lledo et al., 2005; Galef, 2013); finding food (Doty, 1986; Leon, 1992; Sullivan, 2003), locating mom for shelter (Doty, 1986; Leon, 1992; Sullivan, 2003) and avoiding predators (Doty, 1986; Leon, 1992; Sullivan, 2003). Thus, studying olfaction is not only necessary for exploring the basic science of sensory processing, but it is also important for other biological reasons.

One of the most important features of the olfactory system is that it is enriched with centrifugal inputs from multiple classical neuromodulatory centres such as cholinergic and GABA-ergic inputs from the basal forebrain (Ichikawa and Hirata, 1986; Ojima et al., 1988; Nunez-Parra et al., 2013; Rothermel and Wachowiak, 2014), serotonergic inputs from the raphe (McLean and Shipley, 1987c; Petzold et al., 2009), and noradrenergic inputs from the locus coeruleus (LC;(Halasz et al., 1977; Shipley, 1985; McLean et al., 1989; Shea et al., 2008)). These neuromodulators, by virtue of their widespread efferent projections, influence information

processing throughout the central nervous system. These centrifugal inputs have been proposed to be involved in decision making, motivation, general arousal, vigilance, prediction errors or unexpected uncertainty, attention, and learning and memory (Robbins, 1997; Schultz et al., 1997; Saper, 2000; Doya, 2002; Bouret and Sara, 2005; Hasselmo, 2006; Doya, 2008; Bethus et al., 2010; Tully and Bolshakov, 2010; Noudoost and Moore, 2011). Mechanistically, they alter functional cortical networks by manipulating synaptic efficacy (i.e., excitatory and inhibitory synaptic transmission), intrinsic properties of neurons, adaptability of cortical pyramidal cells, membrane potential of neurons, rates of synaptic modification, and many other cortical parameters (Frey et al., 1990; Hasselmo and Barkai, 1995; Berridge and Waterhouse, 2003; Tully and Bolshakov, 2010). As in other sensory modalities, these neuromodulators play a major role in odor information processing itself in addition to olfactory learning and memory (Matsutani and Yamamoto, 2008; Shea et al., 2008; Petzold et al., 2009; Fletcher and Chen, 2010; Kato et al., 2012; Nunez-Parra et al., 2013; Wachowiak et al., 2013; Nunez-Parra et al., 2014; Rothermel and Wachowiak, 2014). Among all of these neuromodulators, however, the role of the LC-noradrenergic (LC-NE) system has been the most intensely studied in all sensory modalities, including the olfactory system. It was the first neuromodulator to be characterised both anatomically and neurochemically (Reil, 1809; Maeda, 2000; Sara, 2009). Consequently, it is not surprising that the LC-NE system is also the most well-defined neuromodulatory system in olfaction. Interestingly, Shipley *et al* (1985) has shown that the olfactory bulb (OB) receives the densest projections from LC (40% of LC neurons (Shipley et al., 1985)), suggesting a prominent role for this neuromodulator in olfactory-mediated tasks. LC-NE fibers also innervate other olfactory structures such as the piriform cortex (PC), anterior olfactory nucleus, and olfactory

tubercle (Sara, 2009). Thus, one might suspect a concerted influence of the LC- NE system on all olfactory structures to facilitate olfactory learning and perception.

The LC nucleus is situated deep in the pons and is comprised of 1,500 neurons in the rat, several thousands in the monkey, and 10,000-15,000 in the human (Berridge and Waterhouse, 2003). Almost half a century ago, *Kety* proposed that emotional arousal activates the LC resulting in the diffuse release of NE to different brain regions where it exerts its actions via β-adrenoreceptors (*kety*, 1970). Since then, the pharmacological activation and blockade of different adrenoreceptor sub-types has been a useful tool in delineating the role of the LC-NE system in different sensory modalities.

Odor learning alters odor representations in the OB and PC, the two most studied regions in the olfactory system (Yuan and Harley, 2014; Yuan et al., 2014). The results of numerous experiments have shown unique cellular and behavioral functions of the LC-NE system for each receptor sub-type in neonate and adult rat odor learning (Fletcher and Chen, 2010; Yuan et al., 2014). One learning model that is responsible for many contributions in the field is early odor preference learning. This model takes advantage of the altricial rat's limited sensory and motor functions and its nearly-exclusive reliance on olfaction during post-natal day (PND) ages 1-9. With this model, researchers have been able to trace the plastic changes at the physiological, cellular, and molecular level in the OB and PC (Yuan et al., 2014). Interestingly, the heightened sensitivity of the LC-NE system in neonates makes this model particularly useful in understanding how NE manipulates olfactory processing in both OB and anterior piriform cortex (aPC) in the developing brain. In adult rodents, it has been shown that adrenoreceptors play a major role in odor habituation (Guerin et al., 2008; Mandairon et al., 2008b; Escanilla et al., 2010), spontaneous odor discrimination (Escanilla et al., 2010) and forced choice odor discrimination learning (Doucette et

al., 2007; Mandairon et al., 2008b). Currently, there is a pressing demand for understanding how multiple adrenoreceptors act in concert to influence adaptive behavior. Moreover, much of the previous research was focused on studying the physiological changes at the cellular level following odor-guided behavior, but how noradrenergic modulation influences odor representation at the systems' level to support adaptive behavior has not yet been addressed. Before we decipher how noradrenergic modulation affects networks, it is important to understand how the olfactory circuit represents odors and how odor representation is altered to support behavior. Thus, this thesis aims to clarify the following phenomena in the neonate and adult rat: (1) how multiple adrenoreceptors act concomitantly in odor learning; (2) how odor information is processed via activity of neuronal ensembles in the OB and PC; (3) how odor associative learning modifies ensemble activities in both neonate and adult rats; (4) how noradrenergic modulation influences odor discrimination learning and shapes odor representations in both the OB and PC. Elucidating these mechanisms will provide fundamental insights into how the brain represents sensory information and forms memories.

According to Crick "In biology, if seeking to understand function, it is usually a good idea to study structure"(Crick and Koch, 2005). In the following sections of the introduction, I will first discuss the neuroanatomy of the olfactory system particularly focusing on the OB and PC.

1.2 Olfactory System focusing on the OB and PC

Historically, the journey to anatomically trace the olfactory circuitry began more than a century ago. Camilo Golgi and Cajal were the first investigators to visualize the olfactory circuitry in various species using the Golgi staining method (Ramón y Cajal, 1890; Shepherd et al., 2011; Figueres-Onate et al., 2014; Imai, 2014). A century later, the discovery of the genes that encode olfactory receptor proteins by Buck and Axel (1991) paved a way for pursuing the organization of the olfactory pathway in the brain (Buck and Axel, 1991). Invisible odor becomes a meaningful substance when its odorant molecules first come in contact with odorant receptors that reside in the nasal epithelium. Volatile odorant molecules first dissolve in the nasal mucosa and then initiate odor information processing by binding to one of the thousand different odorant receptors (ORs) (Buck and Axel, 1991; Reed, 1992; Ressler et al., 1993; Krautwurst et al., 1998). These ORs give rise to a multidimensional odor map in the brain (Amoore, 1970, 1971; Buck and Axel, 1991; Reed, 1992; Ressler et al., 1993; Krautwurst et al., 1998). This is made possible by the fact that one odor can activate multiple ORs and one OR can interact with multiple odors, giving the olfactory system the capacity to detect and discriminate the thousands of different odors that exist in nature (Ressler et al., 1994; Malnic et al., 1999; Shepherd, 2004; Hallem and Carlson, 2006).

Although other sensory systems maintain a spatially segregated input, the OR coding patterns of odorant molecules are not topographically segregated in each of the four broad circumscribed zones of the olfactory epithelium (Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994). Once the molecular features of an odor are encoded by ORs, unique olfactory-specific bipolar cells called olfactory sensory neurons (OSNs) transmit this information centrally (Fig-1.1) (Pinching and Powell, 1971a; Morrison and Costanzo, 1990).

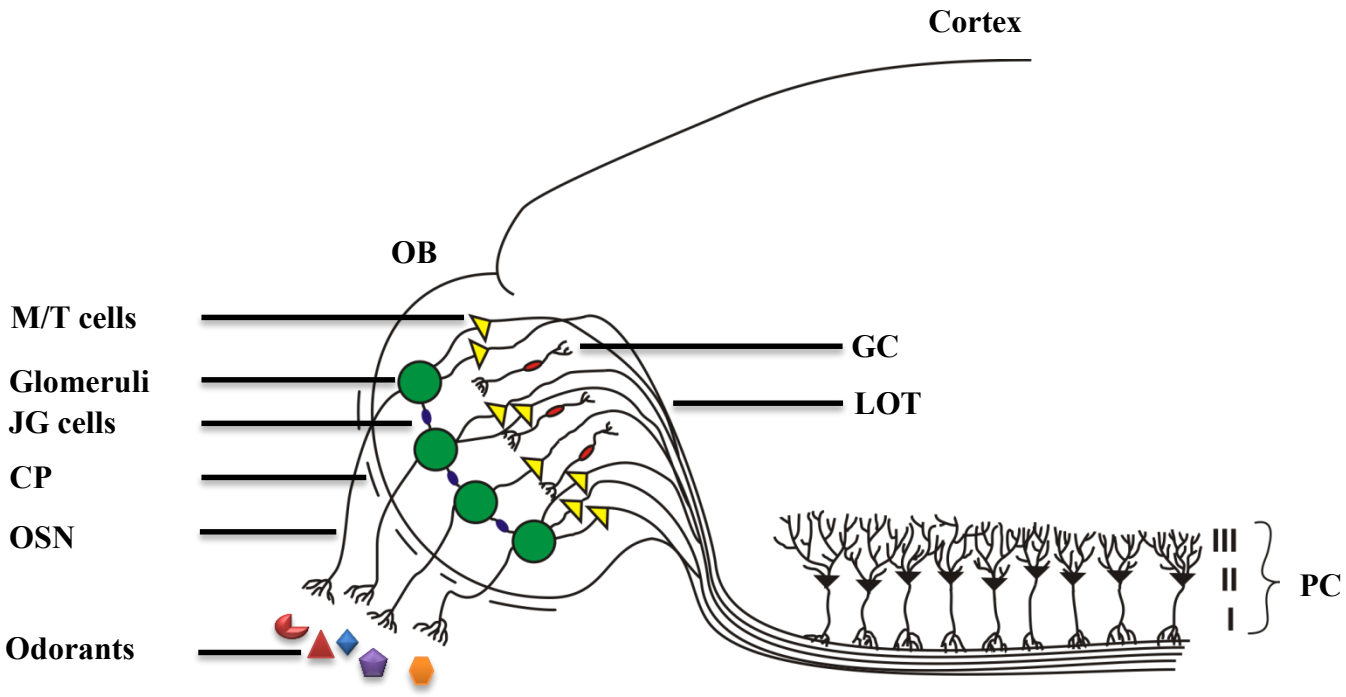

Figure 1.1 Simple schematic of olfactory circuitry involving the olfactory bulb and piriform cortex
Olfactory sensory neurons (OSN) first received odor information from the external world by interacting with odorant molecules in the air. Then the OSN send that informatin to glomeruli where the principle neurons of the olfactory bulb (OB) receive the information and relay it to the piriform cortex (PC) via the lateral olfactory tract (LOT). Odor information is subject to modulation by two types of interneurons in the OB which include granule cells (GC) and juxtaglomerular (JG) cells. Drawing courtesy of Christine Fontaine and usage permitted by her.

This transduction process occurs by conformational changes of ORs, a family of G-protein-coupled receptors (GPCR), which then initiates a cascade of intracellular molecular events to generate an action potential in the OSN (Jones and Reed, 1989; Bruch and Teeter, 1990). This action potential then propagates via the unmyelinated axon of the OSN to the OB – the first relay station of the central olfactory system (Fig-1.1) (Cajal, 1911b; Pinching and Powell, 1971c; Mori et al., 1999; Shepherd et al., 2004). OSNs synapse with the principle neurons of the OB in a spherical structure called a glomerulus, which is encompassed by glial sheets (Pinching and Powell, 1971a; Bailey et al., 1999; Kasowski et al., 1999). Glomeruli are hubs for the first synapses to occur between OSNs and the principle neurons of the OB, namely Mitral/Tufted (M/T) cells (Fig 1.1 & 1.2). Each OSN expresses only one type of OR and projects to a few topographically-fixed glomeruli (Fig-1.1& 1.2; Vassar et al., 1994; Mori and Yoshihara, 1995; Buck, 1996; Mombaerts et al., 1996). Thus, a "one glomerulus—one receptor rule" is used by the OB to detect molecular features of odorants (Mori et al., 1992; Chess et al., 1994; Mori et al., 1999). Such precise axonal projections of OSNs to glomeruli form spatial OR maps in the glomerular layer (GL) of the OB (Mori et al., 2006; Imai et al., 2010; Mori and Sakano, 2011). Initially Laurent (1997) demonstrated an apparent spatial organization of glomerular odor maps in the OB, which later has been widely accepted (Rubin and Katz, 1999; Xu et al., 2000; Wachowiak and Cohen, 2001; Leon and Johnson, 2003; Soucy et al., 2009). However, recent precise imaging techniques with single glomerular resolution (Ma et al., 2012) together with theoretical analyses (Cleland, 2010) challenge the idea of chemotopic mapping existing in the glomerular layer.

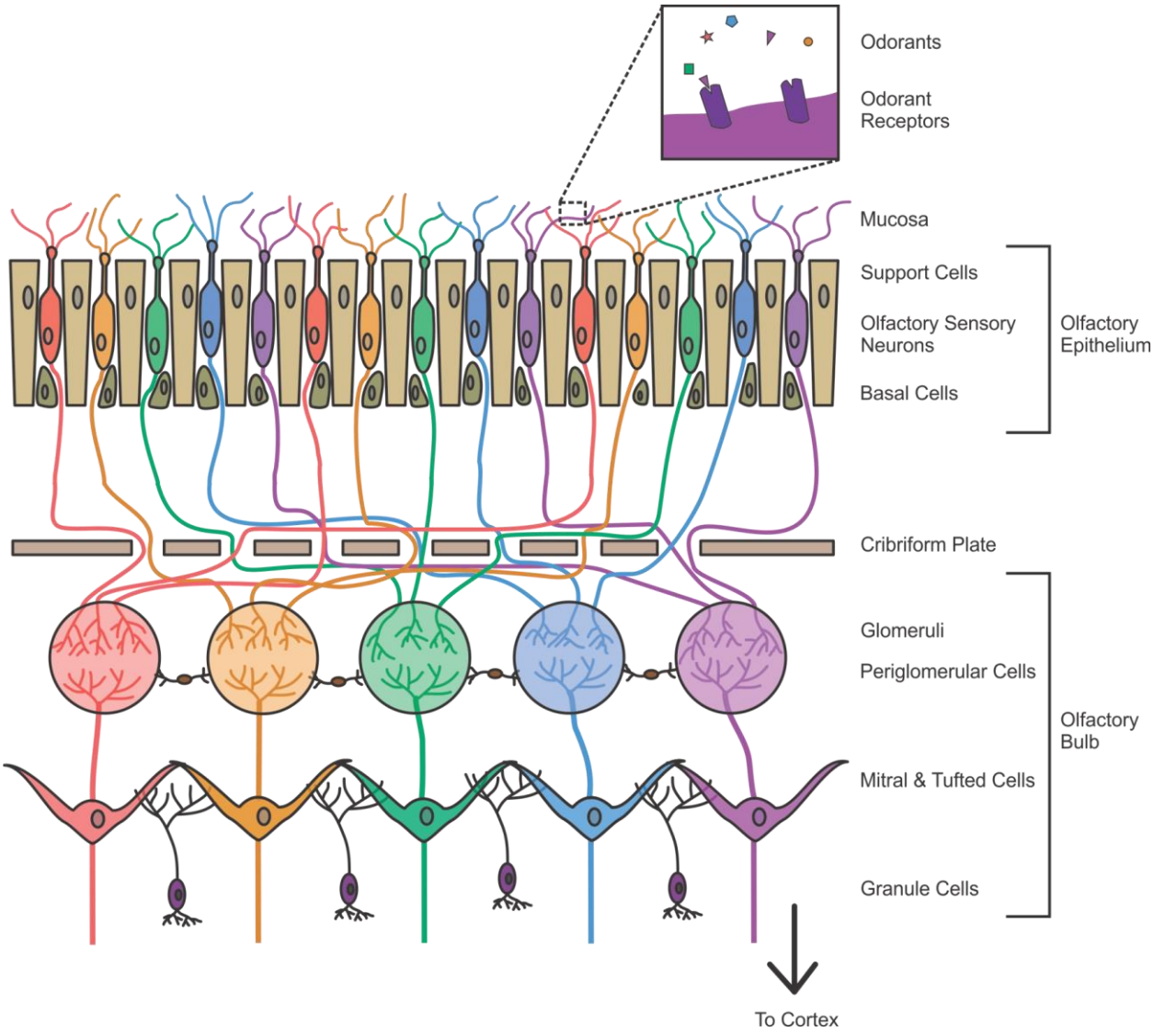

Figure 1.2 Organization of neuronal circuitry in the olfactory bulb

1.2.1 The olfactory bulb

The OB is an allocortex that comprises the most rostral part of the brain. In humans, it lies on the ventral aspects of the frontal lobes. The cribriform plate of the ethmoid holds the two bulbs inside the skull (Fig 1.1 &1.2). The typical volume of an eight-week-old mouse OB has been measured to be 7.53 mm^3 (Parrish-Aungst et al., 2007). The volume of the rat OB has been measured to be approximately 3 times that of the mouse (Frazier and Brunjes, 1988). Using the nuclear dye Sytox Green, Parrish-Aungst and colleagues (2007) histologically estimated the number of cells present in the mouse OB: an eight-week-old mouse main OB was found to contain approximately 3.22 X 10^6 cells.

Similar to other cortical structures, the OB has a characteristic laminar organization (Fig 1.3). Although Golgi (1875) originally considered the OB to be a three layered structure, Cajal and colleagues eventually showed that the bulb consists of seven layers using histological methods (Schwalbe, 1881; Ramón y Cajal, 1890; Blanes, 1898; Shipley and Ennis, 1996; Figueres-Onate et al., 2014; Nagayama et al., 2014). These seven layers, organized superficial to deep, are: the olfactory nerve layer (ONL), GL, the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL), and the granule cell layer (GCL) (Price and Powell, 1970b, a; Pinching and Powell, 1971a, c, b). The deepest layer of the bulb is referred to as the subependymal layer (SEL).

Each OB contains several thousands of glomeruli and each glomerulus allows synaptic communication to take place between thousands of OSN axons and the dendritic branches of approximately 10-70 M/T cells (Mori et al., 2006; Sosulski et al., 2011; Ke et al., 2013). Glutamatergic synapses between OSNs and M/T cells are subject to modulation by three types of neurons present in the GL: periglomerular cells (PG), short axon cells (SA), and external tufted

cells (ET) (Pinching and Powell, 1971a,b,c). These neurons are collectively known as juxtaglomerular cells (JG; Fig-1). Another cell type called the granule cell, which outnumbers the excitatory M/T cells by roughly 30:1, also greatly shapes odor representation in the OB via two-way dendrodendritic GABAergic modulation (Allison, 1953; Shepherd, 1972; Woolf et al., 1991). All of the aforementioned local bulbar circuitry dynamically tune olfactory information and convert it into a spatiotemporal neural code. M/T cells then directly or indirectly relay that information for higher-order information processing, culminating in odor object perception (Price and Sprich, 1975; Miyamichi et al., 2011; Sosulski et al., 2011; Igarashi et al., 2012).

The GL and GCL account for the highest percentage volume of the bulb; in fact, approximately 50% of the bulb is composed of GL and GCL (GL: ~26%; GCL: ~29%). SEL accounts for the lowest percentage volume of the bulb (~1%); EPL and ONL account for ~19% and ~16%, respectively; and MCL and IPL account for ~10% (MCL: ~6%; IPL: ~ 4%). The number of cells in each layer also varies according to the size (percent volume of the bulb) of each layer. As such, the GL and GCL contain the highest number of cells – ~1.23 x $10^6$and 0.87 x 10^6, respectively – while the remaining cell layers contain cell numbers ranging from 0.05 x 10^6 – 0.66 x 10^6 (Parrish-Aungst et al., 2007).

Each bulb consists of heterogeneous populations of cell types that include principle neurons (M/T cells), interneurons (PG, SA, ET, Granule cells, Van Gehuchten cells, and Blanes cells), and glial cells (astrocytes, oligodendrocytes, olfactory ensheathing cells, NG2, and microglia). An extensive review of the diverse cell populations of the OB is provided in a recent paper by Nagayama and colleagues (2014).

1.2.1.1 The olfactory nerve layer

The ONL is the most superficial layer of the OB (Fig 1.3). It consists of axons from the OSN and glial cells. One interesting cell type that is also found in this layer is the olfactory ensheathing cell (Doucette, 1989, 1990; Valverde and Lopez-Mascaraque, 1991). Additionally, the presence of astrocytes in the ONL has also been confirmed from several studies (Doucette, 1990; Bailey and Shipley, 1993; De Carlos et al., 1996; Blanchart et al., 2011). It is important to note that olfactory ensheathing cells possess progenitor characteristics that allow for the continuous turnover of these cell types (90 day half-life) throughout a rodent's lifespan and that, despite this regeneration, OSNs have the ability to precisely reconnect with their target glomeruli to maintain olfactory topographic maps (Gogos et al., 2000; Schwarting et al., 2007).

1.2.1.2 The glomerular layer

The immediate deep layer to the ONL is the GL (Fig 1.3). This layer contains the most diverse cell population within the OB. In rodents, it is composed of approximately 2000-6300 spherical-to-ovoid glomeruli per bulb (Shipley and Ennis, 1996; Mori et al., 2006). Each mouse bulb contains approximately 1800 glomeruli (Allison, 1953; White, 1972; Brunjes, 1983; Royet et al., 1988), whereas numbers in the rat and rabbit have been estimated to be ~2,400-4,200 (Allison and Warwick, 1949; Meisami and Safari, 1981; Meisami et al., 1990; Royet et al., 1998). The process of glomerulus formation involves heterogeneous cell types such as radial glia, astrocytes, OSNs, JG cells, M/T cells, and olfactory Schwann cells throughout the embryonic and early postnatal development stages (Bailey et al., 1999). Structurally, the spheroid-shaped glomeruli are surrounded by a shell of small neurons and astrocyte cell bodies. Their centres are enriched with neuropil and the thick processes of wedge-shaped astrocytes, one of the principle astrocyte

subtypes in the GL (Bailey and Shipley, 1993; Shipley and Ennis, 1996). Neuropil accommodates synapses among the axons of OSNs, the apical dendrites of M/T cells, and the dendrites of JG. The size of an individual glomerulus may vary from 40-190 μm (Royet et al., 1988) and there are, on average, 680 cells per glomerulus (Parrish-Aungst et al., 2007). Interestingly, the number of OSN axonal arbors that penetrate each glomerulus outnumbers that of any other cell type that exists within glomeruli by 1-2 orders of magnitude (Schoenfeld and Knott, 2004).

Glomeruli are anatomically and functionally unique network units in the bulb that are proposed to be very similar to "barrels" and "columns" present in the cerebral cortex (Shepherd et al., 2004). Using early Golgi and electron microscopy techniques, classical neuroanatomists postulated the presence of three morphologically distinct interneurons – PG, SA, and ET – within each glomerulus (Golgi, 1875; Blanes, 1898; Cajal, 1911b; Pinching, 1970; Price and Powell, 1970a; Pinching and Powell, 1971a, c, b, 1972b, a). Modern techniques such as chemoanatomical methods, *in vitro* slice preparation, and whole-cell recording have revealed that these cells can also be equally distinguishable in terms of their physiological properties such as receptor expression pattern, types of neurotransmitter they release, calcium binding proteins, and synaptic characteristics (Kosaka et al., 1995; Nickell et al., 1996; Kosaka et al., 1997; Kosaka et al., 1998; Toida et al., 1998; Toida et al., 2000; Beck et al., 2001; Hayar et al., 2004a; Hayar et al., 2004b; Shipley et al., 2004; Hayar et al., 2005).

The PG cells are the smallest in size (5-20 μm) and the highest in number in the GL. Normally, they project their dendrites to a single glomerulus, but they have the capacity to extend their axons as far as 600 μm, enabling them to project to 5-6 glomeruli (Pinching and Powell, 1971a; Parrish-Aungst et al., 2007). Axonless PG cells also exist in the GL (Kosaka and Kosaka, 2011). The superficial SA (sSA) cells are smaller (8-12 μm) than ET cells, but slightly larger than

those of the PG cells. Although, traditionally, the so-called "short axon cells" were believed to project to a maximum of 1-2 glomeruli (Pinching and Powell, 1971a), a study by Aungst and colleagues (2003) suggests that SA cells can extend their axons so far as to include 20-30 glomeruli(Aungst et al., 2003). Among all of the JG cells, ET cells have the largest soma (10-15 μm) and although their primary dendrites are mostly confined to a single glomerulus, a few subpopulations have been proposed to be di-glomerular (Pinching and Powell, 1971a; Ennis and Hayar, 2008). The neurites of ET cells ramify in a larger volume of the glomerulus than those of PG cells.

OSNs form glutamatergic synapses with two types of excitatory principle OB neurons within a single glomerulus, mitral cells and ET (Berkowicz et al., 1994; Ennis et al., 1996). GABAergic SA and PG cells also receive direct excitatory input from OSN axons in the juxtaglomerular area. Additionally, PG cells can be directly activated by M/T cells and OSNs, resulting in the inhibition of M/T cells, OSNs, and neighbouring PG cells via GABA release (Murphy et al., 2005). Whereas these synaptic modulations take place within a single glomerulus, excitatory ET cells can act on distal glomeruli through a network of sSA cells.

Based on the current understanding of the synaptic relationships among OSNs, sSA cells, ET cells, PG cells, and M/T cells within the GL, Wachowiak and Shipley (2006) postulated 4 functional microcircuits in the glomerulus: (1) the OSN → M/T circuit (2) the OSN → PG circuit, (3) the OSN → ET → PG circuit, and (4) the OSN → ET → SA circuit (Wachowiak and Shipley, 2006).

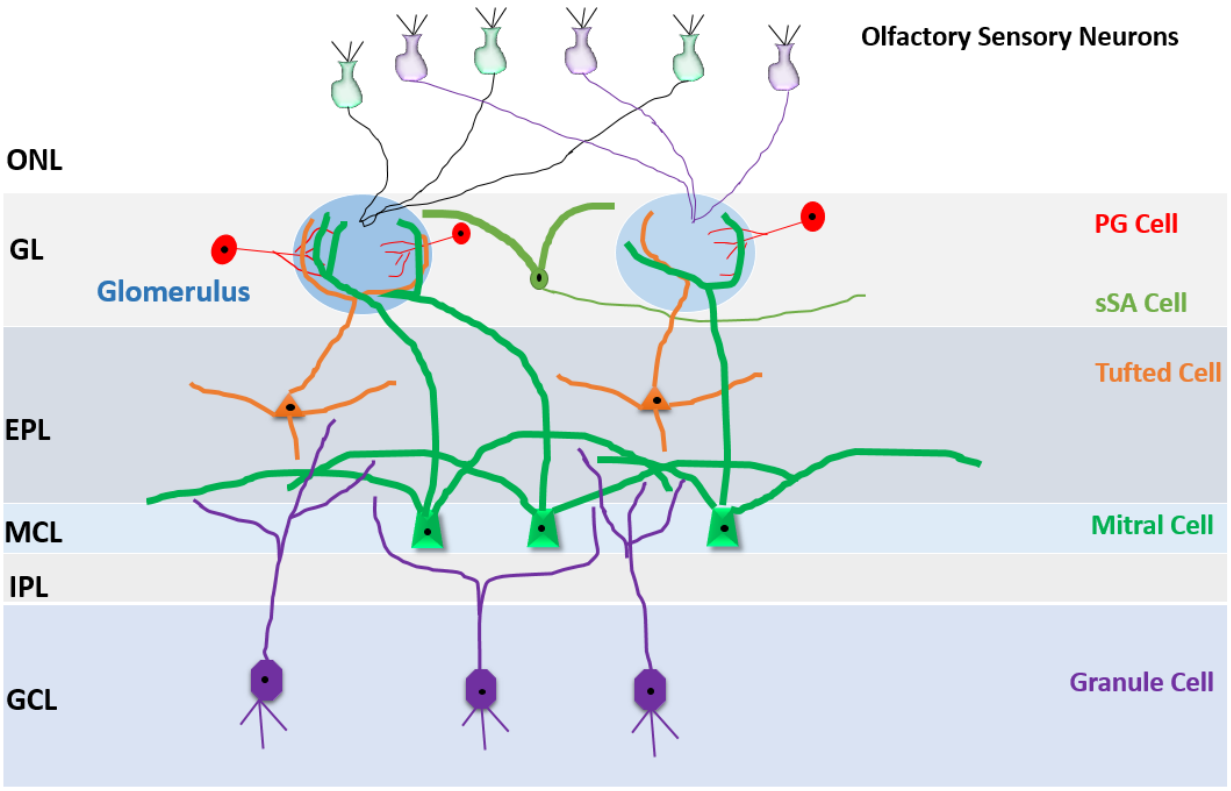

Figure 1.3 Laminar organization of the olfactory bulb

ONL-Olfactory Nerve Layer; **GL**-Glomerular layer; **EPL**-External plexiform layer; **MCL**-Mitral cell layer
IPL- Internal plexiform layer; **GCL**- Granule cell layer

1.2.1.3 The external plexiform layer

The second level of olfactory synaptic processing occurs in the EPL, which lies deep to the GL (Fig 1.3). Although the EPL has a lower cell density than the GL, the dendrites of M/T and GC cells in this layer form a very dense neuropil. The EPL is also enriched with a significant number of interneurons and different tufted cell and astrocyte subtypes (Schneider and Macrides, 1978; Macrides and Schneider, 1982; Bailey and Shipley, 1993; Kosaka et al., 1994; Mirich et al., 2002). Three types of multipolar neurons have been identified in the EPL: Van Gehuchten, SA, and parvalbumin-expressing interneurons (Gehuchten and Martin, 1891; Schneider and Macrides, 1978; Gall et al., 1986; Scott et al., 1987; Brinon et al., 1992; Kosaka et al., 1994; Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013). These GABAergic interneurons provide feedback inhibition to OB projection neurons through the activation of their AMPA/kainate receptors (Hamilton et al., 2005).

1.2.1.4 The mitral cell layer

In comparison with the other olfactory bulb layers, the MCL is the narrowest. It is situated directly below the EPL (Fig 1.3) and is mainly composed of mitral cell (MC) somata (25-30 μm diameters). Tufted cell somata also exist in the EPL, but are sparsely distributed. This contrasts with MC somata, which are located in close proximity with one another (Mori et al., 1983; Orona et al., 1983). This close proximity of MC somata increases their vulnerability to GC inhibition via reciprocal synapses (Nagayama et al., 2014). In terms of projections, the primary (apical) dendrites of both mitral and tufted cells extend to a single glomerulus. Thus, both of these OB projection neurons receive odor information exclusively from a single odorant receptor and therefore support the "single cell-single odorant receptor" rule. Interestingly, recent studies reveal that tufted cells

have shorter response latency and are more robust than MCs in detecting a wide range of odor concentrations (Fukunaga et al., 2012; Gire et al., 2012; Igarashi et al., 2012). Furthermore, it has been suggested that only tufted cells are directly activated by OSNs; whereas MCs receive strong OSN input via the dendrites of external tufted cells (De Saint Jan et al., 2009; Gire et al., 2012). However, it is widely believed that MCs also receive direct input from OSN. In support of this idea recent ultrastructural studies reveal OSN-to-MC direct synaptic contact (Kosaka et al., 2001; Najac et al., 2011). It is still under debate whether such limited synaptic contact can elicit spikes in MC (Gire et al., 2012).

Unlike their primary dendrites, the secondary dendrites of both mitral and tufted cells project to different subdivisions of the EPL. The secondary dendrites of tufted cells extend to the superficial/outer EPL, while those of MCs extend to the deep/inner EPL. As opposed to tufted cells, the lateral dendrites of MCs are much more elongated and thus subjected to more inhibition from GCs (Nagayama et al., 2004). Once the odor information is partially refined in the glomeruli, it is then extracted by the cell bodies of mitral and tufted cells in the EPL and MCL. This information is then horizontally propagated via secondary dendrites of the EPL and undergoes GABAergic lateral inhibition exerted by GCs in the EPL (Xiong and Chen, 2002). Some unique physiological properties that distinguish tufted cells from MCs are a low spike threshold, highly sensitive and plastic responses to sensory deprivation, a weak and narrow tuning range of lateral inhibition, high firing frequency, strong respiratory phase locking activity, and the ability to respond to a broad range of odorants (Schneider and Scott, 1983; Ezeh et al., 1993; Nagayama et al., 2004; Imamura et al., 2006; Griff et al., 2008; Fukunaga et al., 2012; Igarashi et al., 2012; Kikuta et al., 2013). Mitral and tufted cells also differ in terms of their axonal targets in other brain structures (Haberly and Price, 1977; Skeen and Hall, 1977; Scott et al., 1980; Scott, 1981;

Schneider and Scott, 1983). Although MC axons project predominately to the entire piriform cortex, tufted cell axons are restricted to the aPC and more rostral structures (Haberly and Price, 1977; Nagayama et al., 2010; Igarashi et al., 2012). It is to be noted that M/T cells are not morphologically well segregated and hence are considered as a single group of projection neurons in most olfactory research (Satou, 1990; Bargmann, 2006).

1.2.1.5 The internal plexiform layer

Immediately deep to the MCL is another thin layer called the IPL (Fig 1.3). This layer contains the axons of M/T cells; the dendrites of GCs; and axons arising from the LC (noradrenergic), the raphe nuclei (serotonergic), and the nucleus of the diagonal band (cholinergic) (Price and Powell, 1970a, b; Shipley et al., 1986; McLean and Shipley, 1987c, b; McLean et al., 1989).

1.2.1.6 The granule cell layer

The GCL is the innermost neuronal layer of the OB (Fig 1.3) and is mostly occupied by small, spiny, ovoid granule cell (GC) – one of the most abundant inhibitory interneurons in the OB (6–8 μm in diameter; (Golgi, 1875; Blanes, 1898; Price and Powell, 1970a)). Granule cells (GCs) send thick, long apical dendrites into the EPL and ramify extensively in that layer. Their basal dendrites bifurcate in the GCL (Price and Powell, 1970a; Orona et al., 1983). In 1983, Mori et al. classified a subclass of GCs near the MCL (Mori et al., 1983). These cells have short dendrites and project to the deep EPL. In addition to GCs, the GCL contains deep SA cells (dSA). The axons of dSA project to different layers of the OB, while Golgi studies show that GCs are axonless. Hence, the output of GCs exclusively relies on dendrodendritic synapses. Other than these two

major neurons, the GCL also accommodates Golgi cells, Cajal cells, and Blane cells (Schneider and Macrides, 1978; Shepherd et al., 2004; Eyre et al., 2008). One very important bulbar information processing function known as contrast enhancement occurs in the synaptic arrangement of MC-GC-MC microcircuits (Yokoi et al., 1995; Mori et al., 1999); however, the first level of contrast enhancement occurs in the glomerulus microcircuit. The synaptic arrangements of the OSN-ET-sSA is thought to mediate pattern normalization and initial contrast enhancement in the OB (Aungst et al., 2003; Wachowiak and Shipley, 2006).

1.2.1.7 The subependymal cell layer

The SEL is the deepest cell layer of the OB and contains considerably lower cell numbers than the other cell layers. This layer is a harbour for ependymal cells, glial cells, and the dendrites of the deepest GCs (Price and Powell, 1970a). Cells in this layer have the characteristics of progenitor cells and, hence, are a source of adult-born GCs and PG cells in the OB (Lois and Alvarez-Buylla, 1993; Luskin, 1993).

1.2.2 The piriform cortex

The word “piriform” is derived from the Latin word “pirium,” meaning “pear-shaped,” and it is for this appearance that the PC is named. The pear-shaped cortex is located on the ventrolateral aspect of the brain next to lateral olfactory tract (LOT) (Loscher and Ebert, 1996). The LOT is a conglomerate of myelinated M/T axon bundles of the M/T cells that convey odor information from the OB to the PC (Haberly and Price, 1977; Haberly, 1985). It is suggested that the LOT consists solely of two types of axon bundles: a thinner bundle and a thicker bundle (Price and Sprich, 1975; Bartolomei and Greer, 1998). The thinner bundle originates from tufted cells and projects to

multiple rostral olfactory cortices; the thicker bundle originates from mitral cells and projects throughout the entire PC (Nagayama et al., 2010; Igarashi et al., 2012). In stark contrast to other sensory cortices, the PC is only two synapses away from the external world and thereby receives odor information from the OB without any thalamic interventions. Being a phylogenetically ancient paleocortex and the largest recipient of bulbar projections, the PC has long been considered the "primary" olfactory cortex (Haberly and Bower, 1989; Wilson et al., 2006; Isaacson, 2010; Wilson and Sullivan, 2011). Unlike other primary cortical areas, which are typically six-layered, the cytoarchiteture of the PC reveals a trilaminar organization similar to that of the hippocampus. Morphological studies show that the PC is reciprocally and extensively connected to other higher order cortical structures, including the endo-piriform nucleus, anterior olfactory nucleus, olfactory tubercle, prefrontal cortex, entorhinal cortex, perirhinal cortex, and cortical amygdala (de Olmos et al., 1978; Luskin and Price, 1983b; Carmichael et al., 1994; Haberly, 1998; Johnson et al., 2000; Haberly, 2001; Chen et al., 2003; Cleland et al., 2003; Wilson et al., 2003; Neville and Haberly, 2004; Lundstrom et al., 2011; Hagiwara et al., 2012). Interestingly, the PC not only receives information from the OB and relays it to higher-order cortices, but it also influences bulbar output by modulating granule cell activity through pyramidal cell feedback (de Olmos et al., 1978; Haberly and Price, 1978b; Kay and Freeman, 1998; Boyd et al., 2012; Boyd et al., 2015). Such a distributed bidirectional link of the PC between the periphery and higher cortical networks that regulate cognition, emotion, memory and behavior highlights the importance of the PC in regulating many physiological and emotionally arousing events in mammals.

Early studies have described the PC as a non-homogeneous structure. Due to anatomical, physiological, and functional differences, it is commonly divided into two segments named for their anatomical relationship: the aPC and posterior piriform cortex (pPC) (Brodmann, 1909;

Cajal, 1911a; Rose, 1912; de Olmos et al., 1978; Haberly and Price, 1978b; Luskin and Price, 1983b; Litaudon et al., 1997; Chabaud et al., 2000; Mouly et al., 2001; Gottfried et al., 2002; Litaudon et al., 2003; Martin et al., 2004a; Zelano et al., 2005; Calu et al., 2007; Roesch et al., 2007). As opposed to the pPC, the aPC receives relatively more afferent inputs from the OB and fewer associational inputs. This suggests that the aPC decodes odor identity and the pPC is for content addressable memory e.g., odor object identification (Barkai et al., 1994; Johnson et al., 2000; Haberly, 2001; Litaudon et al., 2003; Gottfried et al., 2006; Kadohisa and Wilson, 2006; Rennaker et al., 2007; Barnes et al., 2008; Gottfried, 2010; Nagayama et al., 2010; Chapuis and Wilson, 2012; Hagiwara et al., 2012; Luna and Morozov, 2012).

In recent years, the PC has received significant attention as an ideal model system for studying how the brain recognizes, categorizes, and discriminates odor objects (Suzuki and Bekkers, 2006; Barnes et al., 2008; Poo and Isaacson, 2009; Stettler and Axel, 2009; Isaacson, 2010; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010a; Wilson, 2010; Wilson and Rennaker, 2010; Wilson and Sullivan, 2011; Wilson et al., 2014). This is because of its (1) comparatively simple anatomy; (2) high-level synthetic role in odor perception; (3) lack of thalamic relays from the periphery; (4) anatomical location; (5) laminar organization; (6) afferent, efferent, and auto-associative connectivities; and (7) accessibility for physiological and behavioral studies (Shepherd, 1970; Kauer, 1987, 1991). It is the largest and best studied sub-region of the olfactory cortex. A detailed anatomical description of the PC is provided by Neville and Haberly (Neville and Haberly, 2004). In brief: as mentioned earlier, the PC is a three-layered structure. From superficial to deep, these layers have been named layer I, II, and III; however, the first two layers – layers I and II – have been further subdivided into layers Ia, Ib, IIa, and IIb. Layer Ia contains the axonal fibres of M/T cells, horizontal interneurons (HZs), and neurogliaform cells

(NG). The neurons in this layer are thought to mediate dendritic feedforward inhibition to the apical dendrites of semilunar and superficial pyramidal cells in layers IIa and IIb, respectively. In contrast to this feedforward inhibition, the interneurons deep to this layer – multipolar cells, Chandelier cells, bitufted cells, fast-spiking interneurons, regular-spiking interneurons, and deep neurogliaform cells– provide feedback inhibition (Neville and Haberly, 2004; Luna and Schoppa, 2008; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2010a, b, 2012; Bekkers and Suzuki, 2013). Interestingly, the pyramidal cell-like semilunar cells of layer IIa lack basal dendrites and do not project back to the OB. Their main inputs are from M/T cells and, to a lesser extent, association fibres (Suzuki and Bekkers, 2006, 2011; Bekkers and Suzuki, 2013). Layer III contains deep pyramidal cell bodies and at least five types of interneurons (Young and Sun, 2009; Suzuki and Bekkers, 2010a, b; Bekkers and Suzuki, 2013). In all three layers, interneurons are uniformly distributed, and exert GABAergic inhibition – either feedforward or feedback – on principal PC neurons (Price, 1973; Haberly, 1983; Kapur et al., 1997; Ekstrand et al., 2001; Suzuki and Bekkers, 2007). M/T cells project to the PC in such a way that they create a diffuse map of the dissolved odorant (Wachowiak and Cohen, 2001) and, hence, odor representation in the PC is highly dispersed without any spatial preference (Illig and Haberly, 2003; Litaudon et al., 2003; Rennaker et al., 2007; Yoshida and Mori, 2007; Poo and Isaacson, 2009; Stettler and Axel, 2009; Ghosh et al., 2011; Miyamichi et al., 2011; Sosulski et al., 2011).

Two important characteristics of the PC that enable it to act as a context addressable memory device are (1) dense associative connectivity (Johnson et al., 2000; Haberly, 2001; Chapuis and Wilson, 2012) and (2) sparse coding resulting from global inhibition (Poo and Isaacson, 2009; Isaacson and Scanziani, 2011). Furthermore, the highly plastic nature of auto-associative fibers allow for the complete reconstruction of a piriform cortical odor engram in the

face of degraded input (Kanter and Haberly, 1990; Wilson, 2009). These features ensure the perceptual stability of an odor object in an ever-changing olfactory environment. It has been estimated that each pyramidal cell receives roughly 2000 recurrent inputs from other pyramidal cells (auto-associative connections) compared to 200 afferent inputs (Davison and Ehlers, 2011). It has also been shown that the pPC receives more associational connections than the aPC (Hagiwara et al., 2012). Sparse coding enhances overall computational power and is an energy efficient way to represent sensory stimuli in the cortex (Barlow, 1972; Attwell and Laughlin, 2001; Laughlin and Sejnowski, 2003; Lennie, 2003; Olshausen and Field, 2004; Shoham et al., 2006; Wolfe et al., 2010; Barth and Poulet, 2012). The sparse coding properties of the PC have been confirmed by many different techniques such as 2-deoxyglucose (Cattarelli et al., 1988), single-unit electrode arrays (Rennaker et al., 2007), voltage-dependent dye imaging (Litaudon et al., 1997), immediate early gene mapping (Illig and Haberly, 2003), optogenetics,(Choi et al., 2011) and optical imaging (Stettler and Axel, 2009; Mitsui et al., 2011). Quantitatively, as few as 300 cells (~0.5% of a piriform cortical odor engram) have been reported to be sufficient to induce learned olfactory behavior (Choi et al., 2011). Such sparse coding allows the PC to store numerous possible odor objects with a distinct pyramidal network for each individual object. The ultimate result of sparse coding is an extremely sensitive ability to discriminate odors –even very similar odors.

1.2.3 Centrifugal inputs to the OB and PC

A contributing factor to the remarkable plasticity of the olfactory system is its vulnerability to centrifugal modulation at its early stages of processing, such as at the OB and the PC. Both the OB and PC are innervated by major neuromodulators in the brain, serotonin, acetylcholine, and

the catecholamines: dopamine and noradrenaline. These cortical inputs originate from the brainstem, the midbrain and the basal forebrain – regions of the brain known to be involved in mood, attention, motivation, arousal, and learning. Once activated, these neuromodulators reach their neuronal targets via their widespread axonal projections and alter the efficacy of synaptic communication. Released neuromodulators act on their respective receptors situated on both excitatory and inhibitory neurons to initiate a series of intracellular cascades that contribute to synaptic change and subsequent learning. Slice physiology, *in vivo* recording, and behavioral experiments have advanced our understanding of how these neuromodulators mechanistically promote experience-dependent plasticity in multiple brain regions and govern how we adapt to our environment (Berridge and Waterhouse, 2003; Hasselmo, 2006; Robbins and Roberts, 2007; Sara, 2009; Fletcher and Chen, 2010; Meneses and Liy-Salmeron, 2012; Puig et al., 2014a, b; Mather et al., 2015). Similarly, neuromodulators also play a major role in odor learning from infancy to adulthood. A significant amount of work has shown a critical role for neuromodulators in inducing olfactory plasticity in both the OB and the PC to support odor-guided behavior (Fletcher and Chen, 2010). The following sections will individually address the role of each neuromodulator in olfactory learning.

1.2.3.1 Norepinephrine

NE is produced by dopamine β-hydroxylase and can be released either as a hormone into the blood or a neuromodulator into the brain. Although some of the brain's NE is produced by cells in the lateral tegmental field, the majority is produced by the LC (Jones and Moore, 1977; Smythies, 2005). Medium-sized NE-producing LC neurons are located within the dorsal wall of the rostral pons in the lateral floor of the fourth ventricle (Jones and Moore, 1977). Historically,

the LC-NE system was the first neuromodulatory system to be delineated both anatomically and neurochemically (Dahlstroem and Fuxe, 1964; Maeda, 2000). The LC is comprised of 1,500 neurons in the rat and their axonal projections spread to all areas of the brain except to the basal ganglia (Jones et al., 1977; Foote and Morrison, 1987). Its ubiquity in the brain sparked much of the early interest and speculation about its role in cognitive processing (Amaral and Sinnamon, 1977; van Dongen, 1981). As a result of this interest, a large body of information has been garnered within the last fifty years regarding the LC-NE system's role in different brain functions – arousal, attention, emotional state, motivation, learning and memory – in different brain regions – OB, PC, hippocampus, amygdala, prefrontal cortex – through manipulating synaptic efficacy (Harley, 1987; Wilson and Sullivan, 1994; Cahill and McGaugh, 1996; Berridge and Waterhouse, 2003; Harley, 2007; Robbins and Roberts, 2007; Sara, 2009).

LC efferent projections heterogeneously innervate all layers of both the OB (Fallon and Moore, 1978; Macrides et al., 1981; Shipley et al., 1985) and the PC (Fallon and Moore, 1978; Loughlin et al., 1982; Datiche and Cattarelli, 1996; Shipley and Ennis, 1996). Similar to other sensory modalities, the LC-NE system has been shown to influence different types of odor learning such as adult odor discrimination learning, early odor preference learning, habituation, associative learning, and non-associative learning (Doucette et al., 2007; Guerin et al., 2008; Mandairon et al., 2008b; Escanilla et al., 2010; Yuan and Harley, 2014; Yuan et al., 2014).

As previously discussed, numerous studies have suggested that NE-dependent modulation alters synaptic communication between neurons, gene transcription within individual cells, and many other processes that ultimately impact overall neural function and, consequently, behavior. Activity-dependent NE acts on the adrenoreceptors at their target sites to modulate signal processing of both principal neurons and interneurons. As both α- and β - adrenoreceptors are

present in the OB, NE-dependent plasticity likely occurs via these adrenoreceptors sub-types. More specifically, α_1, α_2, β_1, and β_2 adrenoreceptors exist in the OB. Both α_1 and α_2 adrenoreceptors have been found to be localized to MCs and GCs (McCune et al., 1993; Pieribone et al., 1994; Day et al., 1997; Winzer-Serhan et al., 1997a, b; Winzer-Serhan and Leslie, 1999; Hayar et al., 2001; Nai et al., 2010). Radiographic techniques have identified β_1-adrenoreceptors in the granule cell, internal plexiform, and glomerular layers and β_2-adrenoreceptors in the external plexiform layer (Woo and Leon, 1995). A later study by Yuan and colleagues demonstrated a β_1-adrenoceptor distribution in MCs, PGs, and – to a lesser extent – GCs (Yuan et al., 2003a). The details surrounding the role of the LC-NE system in mediating odor learning together with its cellular mechanisms will be discussed in later sections.

1.2.3.2 Serotonin (5-HT)

Serotonin, also known as 5-hydroxytryptamine or 5-HT, is a monoamine neurotransmitter that is produced near the midline of the brainstem in cell groups called the raphe nuclei. Like other neuromodulators, it is produced by a small number of neurons whose efferent fibers project throughout the brain. Also like other neuromodulators, serotonin can act as either a neuro-hormone or a neuromodulator. It has been widely studied in the peripheral system due to its importance in functions such as intestinal motility (Foxx-Orenstein et al., 1996), immune and inflammatory responses (Ahern, 2011), and nociception (Cervantes-Duran et al., 2013). In the brain, serotonin, with the exception of at the 5-HT_3 subtype, acts on GPCR to enhance or inhibit neurotransmitter release from target synapses. Through such synaptic changes, the serotonergic system has the capacity to modulate many brain functions, including: sensations related to environmental stimuli; nociception (Viguier et al., 2013); learning and memory (King et al., 2008; Meneses and Liy-

Salmeron, 2012); sleep (Monti and Jantos, 2008); mood (Young and Leyton, 2002; Meneses and Liy-Salmeron, 2012); stress and anxiety (Lowry et al., 2005); circadian rhythms (Morin, 1999); hormone secretion (Valverde et al., 2000); and feeding behavior (Magalhaes et al., 2010). Serotonin exerts its diverse action in different cell types via seven families of 5-HT receptors – 5-HT_1 through 5-HT_7 – including their distinct subtypes (Kitson, 2007). Serotonin also plays an important role in olfactory learning, as has been shown using paradigms such as early odor preference learning, adult odor learning, associative conditioning, and short term memory (McLean et al., 1993; Moriizumi et al., 1994; McLean et al., 1996; Langdon et al., 1997; Price et al., 1998; Marchetti et al., 2000; Yuan et al., 2003b).

Whereas the OB receives its serotonergic innervation from both the dorsal and median raphe nuclei (de Olmos et al., 1978; Macrides et al., 1981; Shipley and Adamek, 1984; McLean and Shipley, 1987a, c), the PC receives its serotonergic innervation from the dorsal raphe nuclei only (Azmitia and Segal, 1978; De Olmos and Heimer, 1980; Vertes, 1991; Datiche et al., 1995). Raphe projection patterns to the different layers of both of these structures (i.e., the OB and PC) are not homogeneous.

All five layers of the OB receive raphe fiber innervations, but these fibers project most densely to the GL; here, their primary target is PG cells (Halasz et al., 1978; McLean and Shipley, 1987b). The GL is also the primary recipient of thicker 5-HT fibers; thinner fibers primarily innervate the deeper layers of the OB (McLean and Shipley, 1987b; Gomez et al., 2005). Heterogeneous projections of raphe fibers can also be seen within the GL. For example, dorsal glomeruli are more heavily innervated than lateral glomeruli (Vertes, 1991; Shipley and Ennis, 1996; Gomez et al., 2005). In the PC, innervations were observed to be densest in both the rostral

part and in deeper layers compared with the caudal part and superficial layers, respectively (Datiche et al., 1995).

Although three 5-HT receptors subtypes (5-HT_{1A}, 5-$HT_{2A,}$ and 5-HT_{2C}) have been identified in the OB, only two (5-HT_{1A}, 5-HT_{2A}) are prominent. These two subtypes are present in the EPL, MCL, and– to a lesser extent – GCL (Pompeiano et al., 1992; McLean et al., 1995). As in the OB, three 5-HT receptors subtypes (5-HT_{1A}, 5-$HT_{2A,}$ and 5-HT_{2C}) have been identified in the PC; all three of these subtypes have been observed in layers I and II (Pompeiano et al., 1992, 1994).

Although the OB and PC receive heavier serotonergic innervations than they do NE and dopaminergic (DA) fibers, the role of serotonergic modulation in olfactory learning has historically received relatively less attention (Shipley and Ennis, 1996). Early studies by McLean and colleagues found that early odor preference learning could be prevented by either depleting 5-HT input to the bulb or by blocking the 5-HT_2receptor via subcutaneous injection of an antagonist drug for this receptor (McLean et al., 1993; McLean et al., 1996). Later studies have shown, however, that this preference learning can be rescued by increasing the dose of isoproterenol (Langdon et al., 1997). Additionally, it has been also shown that, on its own, a 5-$HT_{2A/2C}$receptor agonist is not sufficient to induce this learning (Price et al., 1998). Taken together, these results suggest that the 5-$HT_{2A/2C}$ receptors plays a supporting role to the β-adrenoreceptor in mediating early odor preference learning.

Multiple studies have demonstrated a role for 5-HT in adult odor learning through the global activation of its receptors. For example, an intraperitoneal injection of a $5HT_4$ antagonist impaired acquisition of an olfactory associative discrimination task in rats (Marchetti et al., 2000); co-injection of a $5HT_4$ agonist with an antagonist rescued the impairment on this associative

discrimination task (Marchetti et al., 2000); and $5HT_4$activation has been shown to enhance olfactory short-term memory in a social recognition task (Letty et al., 1997). Since intraperitoneal injections of 5-HT receptor agonists/antagonists have the capacity to cross the blood brain barrier and thus affect many brain areas, more specific investigations of 5-HT in the OB and PC are required before its role in olfactory learning can be clearly established. One such investigation does exist, however: depleting bulbar serotonergic fibers has been shown to cause glomerular atrophy and odor discrimination learning impairments (Moriizumi et al., 1994).

1.2.3.3 Acetylcholine

The central nervous system is heavily innervated by two cholinergic systems: one originating from the basal forebrain and the other from the upper brain stem. Through its widespread projections, the brain's cholinergic systems play a major role in several critical brain functions, including attention, learning and memory (Bear and Singer, 1986; Blokland, 1995; Weinberger and Bakin, 1998; Hasselmo, 1999; Himmelheber et al., 2000), cerebral blood flow (Biesold et al., 1989; Barbelivien et al., 1999; Sato et al., 2004), cortical activity (Detari et al., 1999; Lucas-Meunier et al., 2003), sleep wake cycles (Jones, 2005; Lee et al., 2005), cognitive function, and cortical plasticity (Arendt and Bigl, 1986; Bigl and Schliebs, 1998; McKinney and Jacksonville, 2005). Similar to its role in other sensory modalities, cholinergic modulation has also been implicated in several types of olfactory learning (Linster and Cleland, 2002).

Both the OB and PC receive cholinergic input from the horizontal limb of the diagonal band of Broca (HDB) (Shute and Lewis, 1967; Wenk et al., 1980; Macrides et al., 1981; Luskin and Price, 1982; Carson, 1984; Woolf et al., 1984; Zaborszky et al., 1986; Lysakowski et al., 1989; Senut et al., 1989; Wright and Fitzgerald, 2001); however, a small amount of the OB's cholinergic

input originates from the vertical limb of diagonal band of Broca (Carson, 1984; Shipley and Adamek, 1984). The distribution of HDB fibers are heterogeneous in the bulb with their heaviest innervations found in the GL and IPL (Ichikawa and Hirata, 1986; Kasa et al., 1995; Gomez et al., 2005). In those layers, HDB fibers primarily synapse with GC and PG cells (Nickell and Shipley, 1988; Le Jeune and Jourdan, 1993; Kasa et al., 1995). In the PC, layers II and III receive the densest HDB projections (Luskin and Price, 1982; Woolf et al., 1984; Lysakowski et al., 1989). Multiple receptor subtypes of acetylcholine (ACh), such as muscarinic (M_1, M_2, M_3, and M_4) and nicotinic ACh receptors, were identified in different layers (EPL, IPL, and GCL of the OB; layer I and II of the PC) and different cell types (PG and tufted cells) of the PC and OB (Hunt and Schmidt, 1978; Rotter et al., 1979; Spencer et al., 1986; Buckley et al., 1988; Zilles et al., 1989; Fonseca et al., 1991; Levey et al., 1991; Sahin et al., 1992; Hill et al., 1993; Seguela et al., 1993).

Studies involving the lesioning or pharmacological blockade of cholinergic modulation highlight the importance of the cholinergic system in multiple olfactory learning paradigms. For example, some olfactory learning paradigms that have been reported to be impaired due to cholinergic manipulation include habituation, investigation, social recognition (Soffie and Lamberty, 1988; Hunter and Murray, 1989; Perio et al., 1989; Paolini and McKenzie, 1993; Winslow and Camacho, 1995; Paolini and McKenzie, 1996; Miranda et al., 2009), associative conditioning (Roman et al., 1993; Levy et al., 1997a; Saar et al., 2001; Kroon and Carobrez, 2009), delayed match-to-sample (Ravel et al., 1992; Ravel et al., 1994), rule learning (De Rosa and Hasselmo, 2000; De Rosa et al., 2001), and perceptual learning (Fletcher and Wilson, 2002; Linster et al., 2002; Chaudhury et al., 2009). Computational studies of the OB and PC also support the view that circuit-level cholinergic modulation is a necessary component of olfactory information processing (Hasselmo, 1993; Hasselmo and Barkai, 1995; Linster and Gervais, 1996; Linster and

Hasselmo, 1997; Linster and Cleland, 2002; Mandairon et al., 2006). One interesting function of the cholinergic system in the OB is the modulation of olfactory information transformation between hemispheres via the anterior commissure (Nickell and Shipley, 1993).

1.2.3.4 Dopamine

The dopaminergic system is considered one of the key modulators in controlling movement, emotion, reward-seeking behavior, attention, motivation, and cognition (Nieoullon, 2002; Nieoullon and Coquerel, 2003; Bjorklund and Dunnett, 2007; Joshua et al., 2009; Stuber et al., 2012; Nieh et al., 2013; Schultz, 2013). Despite the extensive knowledge of the CNS dopaminergic system, little attention has been paid to the role of dopamine (DA) in the OB and PC. The OB is known to contain a large number of intrinsic dopaminergic PG cells, but extrinsic DA innervations to the OB were thought to be absent (Halasz et al., 1977; Wilson and Sullivan, 1994; Shipley and Ennis, 1996). However, a recent tracing study has shown dopaminergic projections from the substantia nigra pars compacta (SNc) to the MCL, EPL, and GCL, but not the GL (Hoglinger et al., 2015). In the PC, a clear gradient of dopamine fiber innervations along the rostro-caudal axis has been reported, albeit the origin of these fibers is unknown (Datiche and Cattarelli, 1996; Shipley and Ennis, 1996). However, Datiche and Cattarelli (1996) found ventral tegmental area (VTA) projections to the PC from three different nuclei, including parabrachial pigmented, paranigral, and inter-fascicular. The D_1 receptor has been shown to be involved during the consolidation phase of early odor preference learning (Weldon et al., 1991). Interestingly, direct bulbar manipulation of dopamine suggests that D2 but not D1 receptors significantly affect adult rats' odor discrimination performance (Wei et al., 2006; Escanilla et al., 2009). Meanwhile, slice physiology studies have revealed a mixed influence of DA on synaptic transmission in the

PC (Collins et al., 1985). Synaptic transmission between OSN and OB neurons has also been reported to be modulated via presynaptic D2 receptors (Berkowicz and Trombley, 2000).

1.3 Cortical Feedback to the Olfactory Bulb

The OB has been shown to be heavily innervated by centrifugal projections arising throughout the olfactory cortex (Price and Powell, 1970b; Davis et al., 1978; de Olmos et al., 1978; Haberly and Price, 1978a; Luskin and Price, 1983a; Reyher et al., 1988; De Carlos et al., 1989; Matsutani, 2010). Orthograde and retrograde labelling studies have traced heavier feedback projections from the olfactory cortex to the OB, as opposed to less heavier projections *vice versa* (Shipley and Adamek, 1984; Shipley and Ennis, 1996; Neville and Haberly, 2004). These projections originate from the deeper layers of PC: layer IIb and layer III, and terminate in GCs of the GCL of the OB (Nicoll, 1971; Matsutani, 2010). The OB also receives cortical feedback from the lateral entorhinal cortex and some amygdaloid cells (Shipley and Adamek, 1984; Shipley and Ennis, 1996).

Centrifugal or feedback projections from the higher cortical areas can substantially alter sensory information even at the first stage of processing (Kay and Laurent, 1999). The functional significance of such feedback projections in bulbar output received recent attention (Boyd et al., 2012; Markopoulos et al., 2012; Rothermel and Wachowiak, 2014). Selective activation of anterior olfactory nucleus (AON) axons via optogenetics elicits direct spikes in MCs (Markopoulos et al., 2012). Similar *in vivo* optogenetics' manipulations found that both spontaneous and odor evoked activity of MCs are suppressed by AON feedback projections (Markopoulos et al., 2012). Feedback projections from PC have also been shown to modulate diverse populations of bulbar interneurons (Boyd et al., 2012). However, the net effect of piriform cortical feedback on bulbar

output has been shown to be the augmentation of odor-evoked inhibition (Boyd et al., 2012). Furthermore, genetically-encoded calcium reporters (GCaMPs) were also used to study how neuromodulators, such as the cholinergic system, indirectly influences M/T cells output via AON (Rothermel and Wachowiak, 2014). Electrical stimulation of horizontal limb of the HDB significantly prolongs GCaMP3 fluorescence in AON axon terminals compared to control (Rothermel and Wachowiak, 2014). Blocking AON input by microinjecting muscimol eliminates HDB stimulation-evoked activity in the OB (Rothermel and Wachowiak, 2014), which suggest that basal forebrain nuclei, in addition to their well-known direct influence on OB (Macrides et al., 1981; Shipley and Adamek, 1984; Rothermel et al., 2013), can also modulate bulbar output via increased AON inputs to the OB. Together these results indicate the richness of cortical feedback in modulating odor-evoked activity in the first relay station of the olfactory system.

1.4 Learning-Induced Olfactory Plasticity

Different learning paradigms in different species have demonstrated long term plastic changes in the two main structures of the olfactory system: the OB and PC. Global, molecular, and structural changes have been observed in these structures following odor experience/conditioning. For example, associative learning in mice has been shown to alter neurotransmitter release patterns in the OB (Brennan et al., 1998). Also, associative conditioning has been shown to change the response patterns of M/T and glomerular cells in the OB (Coopersmith and Leon, 1984; Wilson and Leon, 1988a; Johnson et al., 1995; Buonviso et al., 1998; Kay and Laurent, 1999; Buonviso and Chaput, 2000; Yuan et al., 2002; Fletcher and Wilson, 2003; Salcedo et al., 2005; Woo et al., 2007; Doucette and Restrepo, 2008; Fletcher et al., 2009). Studies have shown learning-induced plastic changes in the inhibitory GC network. For example, both associative conditioning and

olfactory enrichment alter immediate early gene expression patterns in GCs (Woo et al., 1996; Funk and Amir, 2000; Montag-Sallaz and Buonviso, 2002; Mandairon et al., 2008a). Additionally, olfactory enrichment has been shown to promote neurogenesis in the OB (Rochefort et al., 2002) or reduce GC death (Woo et al., 2006), which ultimately affects olfactory learning and memory. Some of the physiological changes that result from learning include enhanced synaptic transmission between principal neurons of the OB and PC (Roman et al., 1987; Litaudon et al., 1997; Saar et al., 2002; Cohen et al., 2008), reduced after-hyperpolarization (Saar et al., 2002), increased inhibition of pyramidal cells in the PC (Brosh and Barkai, 2009), and structural modification of pyramidal cell dendritic spines (Knafo et al., 2001). Olfactory learning has also been shown to change the oscillation patterns of the OB, indicating global modification of the plasticity of the excitatory versus inhibitory network in the bulb (Freeman and Schneider, 1982; Ravel et al., 2003; Martin et al., 2004b; Beshel et al., 2007). Multi-site recording from the PC using voltage-sensitive dye has demonstrated significant enhancement in the activity of PC cells following conditioning (Litaudon et al., 1997). All of the research presented in this section supports the concept that learning-induced synaptic modification occurs both in the bulb and olfactory cortex.

1.5 Animal Models of Olfactory Learning

Research using both vertebrates and invertebrates has advanced our understanding of how sensory phenomena – like olfaction – occur across species. A wide variety of species have been employed in olfactory research, including, but not limited to: moths (Vogt and Riddiford, 1981), lobsters (Wachowiak and Ache, 1994), honey bees (Menzel, 2001), mice (Brennan and Keverne, 1997; Doucette et al., 2007), drosophila (McKenna et al., 1994; Vosshall et al., 2000), trout (Rhein

and Cagan, 1980), humans (Ben-Arie et al., 1994; Gottfried et al., 2006; Gottfried, 2010), rats (McLean and Shipley, 1987c; Lethbridge et al., 2012; Yuan et al., 2014; Grimes et al., 2015), turtles (Berkowicz and Trombley, 2000), rabbits (Charra et al., 2013), sheep (Burger et al., 2011), and zebrafish (Braubach et al., 2009). In particular, olfactory studies in rodents provide us with an ideal model system with which we can use to investigate many of the complex sensory phenomena that are necessary for life, namely: pattern separation, pattern completion, infant-mother attachment learning, and associative learning (Wilson and Sullivan, 1994; Sullivan et al., 2000a; Wilson and Stevenson, 2003a; Mandairon et al., 2006; Wilson et al., 2006; Wilson, 2009; Yuan and Harley, 2014). In the following sections, I will focus on two behavioral models that were used in respective projects.

1.5.1. Early Odor Preference Learning and the Critical Period

A sensitive period for odor learning is critical for mammalian survival: it is evolution's safeguard to ensuring that the young approach their caregiver (Leon, 1975; Galef and Kaner, 1980; Rosenblatt, 1983). Olfactory-based mother-infant attachment learning is not only necessary for an animal's survival, but it also affects their reproductive behavior, littermate contact, maternal behavior as an adult, and conspecific identification abilities (Leon and Moltz, 1971; Pager, 1974; Alberts, 1978; Galef and Kaner, 1980; Alberts and May, 1984; Coopersmith and Leon, 1984; Fillion and Blass, 1986; Woo and Leon, 1987; Moore et al., 1996b; Fleming et al., 1999; Shah et al., 2002). In addition, it has been suggested that childhood experience during this attachment period has a significant influence on the development of adult character traits and mental health (Melges and Bowlby, 1969; Glaser, 2000; Teicher et al., 2003).

Rat pups, during their first postnatal week, and even human infants, during their first day after birth, have shown a tendency to form associations with maternal odor through associative learning (Moriceau et al., 2006; Romantshik et al., 2007). Smotherman has shown that aversive odor conditioning can be induced in the rat fetus (Smotherman, 1982). Similarly, other researchers have shown that human fetuses learn the odor of amniotic fluid, as three-day-old humans will orient themselves toward their mother's amniotic fluid versus that of another mother (Hepper, 1987; Marlier et al., 1998; Schaal and Marlier, 1998; Schaal et al., 1998; Robinson and Mendez-Gallardo, 2010). Similarities in the olfactory components of amniotic fluid and colostrum have been found to initiate the neonate's first approaches to, and meals from, the nipple (Coureaud et al., 2002). Furthermore, it has been shown that the mother's scent has a soothing effect on the crying infant (Sullivan and Toubas, 1998). During this critical period, rat pups are limited to olfactory, gustatory, and somatosensory system functioning. Although many of the rat brain structures related to learning and memory formation are very immature and non-functional during this critical period (Thoman et al., 1968; Campbell and Coulter, 1976; Cowan et al., 1981; Rakic and Goldman-Rakic, 1982; Harris and Teyler, 1984; Wilson, 1984), neonates are still capable of learning (Caldwell and Werboff, 1962; Thoman et al., 1968; Johanson and Hall, 1982; Pedersen et al., 1982; Rudy and Cheatle, 1983; Sullivan et al., 1986b; Sullivan et al., 1986a). Regardless of the quality of the maternal stimuli, pups learn to approach the dam for nourishment, protection, and warmth (Sullivan et al., 2000a). That is, pups acquire a conditioned response (CR) to a novel odor (a conditioned stimulus, CS) that is paired with unconditioned stimuli (UCS). Such conditioning not only induces a variety of conditioned responses to the CS odor (Johanson and Hall, 1982; Sullivan and Hall, 1988; Wilson and Sullivan, 1994), but it can modify other adaptive

behaviors in the pup such as huddling, independent feeding (Sullivan et al., 1986b; Sullivan and Leon, 1986), and nipple attachment (Pedersen et al., 1982).

A variety of UCS that mimic maternal care have been employed to generate many types of CR in the neonate. Some examples of the UCS that have been used are: nesting environment (Galef and Kaner, 1980; Alberts and May, 1984), milk presentation (Johanson and Hall, 1979; Johanson and Teicher, 1980; Johanson and Hall, 1982), stroking/tactile stimulation (Pedersen et al., 1982; Sullivan and Leon, 1986; Sullivan and Hall, 1988; Weldon et al., 1991; Moore and Power, 1992; McLean et al., 1993), tail pinch (Sullivan et al., 1986b), the odor of maternal saliva (Sullivan et al., 1986b), mild foot shock (Camp and Rudy, 1988; Wilson and Sullivan, 1990), and intracranial brain stimulation (Wilson and Sullivan, 1990).

A noteworthy fact about the olfactory critical period is that it lacks some types of learning, including passive avoidance, fear conditioning, and inhibitory conditioning (Collier and Mast, 1979; Haroutunian and Campbell, 1979; Blozovski and Cudennec, 1980; Emerich et al., 1985; Sullivan et al., 1986a; Camp and Rudy, 1988; Myslivecek, 1997; Sullivan et al., 2000a). Moreover, this sensitive period of learning is unique in that even an aversive stimuli (e.g., a mild foot shock or tail pinch) can induce odor preference learning (Roth and Sullivan, 2003). This is adaptive as, during this sensitive period, pups are not only receiving licking, light grooming, and other appetitive stimuli from the mother, but are being stepped on, bitten, and roughly groomed by her as well (Roth and Sullivan, 2005; de Medeiros et al., 2009). It has been shown that a pup's inability to discriminate between aversive and appetitive stimuli disappears during the second postnatal week – that is, the critical period ends during the second postnatal week (Camp and Rudy, 1988; Sullivan et al., 2000a; Moriceau et al., 2006). For example, pups trained in pairing a CS odor with an UCS foot shock during the critical period will develop a preference for the CS odor; however,

pups trained in pairing this same CS odor with the same UCS foot shock after the critical period will develop an aversion to the CS odor.

The early odor preference learning model was first demonstrated by Leon and colleagues (Leon et al., 1977; Alberts and May, 1984; Coopersmith and Leon, 1984). Here, exposing the neonatal rat to peppermint odor for 3-4 hours each day from PND 1-19 induced a robust behavioral preference for this odor when pups were tested on PND 20. Around the same time, Caza and Spear (Caza and Spear, 1984) proposed that a mere 3 minute odor exposure per day was just as effective as a daily 3-4 hour exposure in inducing an odor preference in similarly aged rats. This trend was also observed in one-day-old human infants (Balogh and Porter, 1986). Sullivan and colleagues showed a similar type of odor preference learning in humans using classical conditioning (Sullivan et al., 1991b). In their experiment, one-day-old infants were found to preferentially orient themselves towards a previously novel odor, citrus, when subjected to classical conditioning by simultaneously pairing the odor with stroking. This same research group also showed that, in neonatal rats, pairing a 10 minute odor exposure with tactile stimulation during PND 1-18 induces a behavioral preference for the conditioned odor on PND19 (Sullivan and Leon, 1986). Later studies have shown that a single 10 minute pairing of an odor with stroking on PND 6 is sufficient to induce an early odor preference 24 hours following this training (i.e., on PND7) (Sullivan and Leon, 1987). Sullivan and colleagues have also shown that this conditioned response only appears when both odor presentation and tactile stimulation occur simultaneously or in a forward pairing (CS-UCS) (Sullivan and Leon, 1987; Sullivan et al., 1989a, b). CS-only, UCS-only, random CS-UCS pairing, and backward UCS-CS pairing were all unable to induce a significance preference to the trained odor (Galef and Kaner, 1980; Galef, 1982; Pedersen et al., 1982; Alberts and May, 1984; Sullivan et al., 1986a; Sullivan and Leon, 1986, 1987; Sullivan et al., 1989b, a). It was also

found that a conditioned response would appear if the pairing occurred before or around PND 10, otherwise stroking was unable to induce an odor preference for the trained odor (Woo and Leon, 1987). In the search for understanding the neurobiology of such a unique critical period in learning and memory, the rat pup early odor preference learning model quickly became popular (Yuan et al., 2014).

1.5.1.1 NE-mediated learning mechanisms

Several lines of evidence support the hypothesis that LC-mediated NE release plays a major role in critical period learning. An unusual surge of NE occurs immediately after birth and this surge has been hypothesized to provide a means by which early odor learning can take place – even in the absence of traditional UCS (Sulyok, 1989; Ronca et al., 2006). In fact, NE is abundant during the perinatal period (Herlenius and Lagercrantz, 2001) and is responsible for many events including postnatal learning (Leon, 1998) and independent respiration (Ronca and Alberts, 1995). Elevated NE levels were detected in both parturient females and their pups indicating a prominent role for NE in early life experience (Sperling et al., 1984). A similar observation has been reported in human infants. A positive correlation between umbilical cord blood NE levels and head turning towards trained odor was observed in human subjects (Varendi et al., 2002).

Nakamura and colleagues found that the reinforcing tactile stimulations (e.g., stroking, tail pinch, air puff) used for classical conditioning in early odor learning activate LC neurons as early as PND1 (Nakamura et al., 1987). Around this same period, many studies showed that interventions in the olfactory NE system altered pup odor learning (Marasco et al., 1979; Pedersen et al., 1982; Cornwell-Jones and Bollers, 1983). Importantly, it has been shown that the noradrenergic system is functionally present in the OB during the critical period (McLean and

Shipley, 1987a; Wilson and Leon, 1988b). The UCS elicits NE release from the LC to the OB for acquisition of the conditioned odor preference (Nakamura et al., 1987; Rangel and Leon, 1995). Subsequent studies conducted by several other laboratories support the notion that LC-mediated NE release is both necessary and sufficient for early odor preference learning (Sullivan et al., 1989a; Sullivan et al., 1991a; Sullivan et al., 1992; Sullivan et al., 1994; Sullivan et al., 2000b; Yuan et al., 2002). It has been shown that both pharmacological blockade of NE in the OB, and LC lesions prevent odor preference learning (Sullivan et al., 1989a; Sullivan et al., 1991a; Sullivan et al., 1994; Sullivan et al., 2000b). Alternatively, odor preference can be induced by direct NE infusions in the OB or LC stimulation paired with odor exposure (Sullivan et al., 1992; Sullivan et al., 2000b; Yuan et al., 2002). Odor preference conditioning is also achieved by pairing an odor with β-adrenoreceptor activation as an alternative UCS (Sullivan et al., 1989a; Langdon et al., 1997; Sullivan et al., 2000b; Yuan et al., 2003a; Harley et al., 2006; Lethbridge et al., 2012).

Many olfactory laboratories have been interested in elucidating the underlying physiology of heightened plasticity during the critical period. Several publications credit the neonatal properties of the LC as the major source of bulbar plasticity during this period. One such property is its lack of inhibitory noradrenergic autoreceptors during the first post-natal week (Nakamura et al., 1987; Nakamura and Sakaguchi, 1990; Winzer-Serhan and Leslie, 1999), which results in an exceptionally increased LC neuron response duration compared to that of adults (Nakamura et al., 1987). Another interesting property of the immature LC is that its neurons are sensitive to a wide range of stimuli and are more electrically coupled than the mature LC (Nakamura et al., 1987; Christie et al., 1989). This immature LC physiology increases the probability that the LC will remain active for an extended duration to a non-noxious UCS. NE levels were found to be significantly higher in the OB following odor plus tactile stimulation compared to odor or tactile

stimulation alone; furthermore, although a marked NE level increase was detected in the bulb during the first postnatal week by CS + UCS conditioning, this same increase was not observed for PND10 pups (Rangel and Leon, 1995). Another interesting neonatal characteristic of the LC-NE system is that it reduces M/T cell habituation to repetitive odor presentations during associative training. This reduced habituation increases the responsiveness of M/T cells to the CS odor (Wilson and Sullivan, 1992) and, therefore, pups are better able to make odor-UCS associations than adults. All of the aforementioned characteristics of the immature LC contribute to creating the conditions for excellent associative learning in the pup as compared to the adult.

This mammalian model of imprinting ends around PND 10. After that pups develop adequate motor abilities to explore their environment (Bolles and Woods, 1965) and gain the ability to exhibit passive avoidance, active avoidance and inhibitory conditioning (Collier and Mast, 1979; Blozovski and Cudennec, 1980; Camp and Rudy, 1988; Myslivecek, 1997; Sullivan et al., 2000a). With respect to inhibitory conditioning, the developmental emergence of the functional amygdala seems to be the reason for increased learning at this time (Sullivan and Wilson, 1993; Sullivan et al., 2000a). Receptor autoradiography and mRNA analysis have shown that although the LC alpha 2 autoreceptors are present in the neonate (Winzer-Serhan and Leslie, 1999), their activity remains muted until the PND 10 week (Kimura and Nakamura, 1987; Nakamura et al., 1987; Nakamura and Sakaguchi, 1990; Winzer-Serhan and Leslie, 1999). In addition to the developmental emergence of functional α_2 inhibitory noradrenergic autoreceptors, reduced excitatory α_1 function at older ages has been related to the older pups' inability to rapidly acquire odor preferences (Nakamura et al., 1987; Pieribone et al., 1994; Scheinin et al., 1994; Moriceau and Sullivan, 2004). Experimental designs that mimic LC activity during the post-

sensitive period such that it is similar to that in the critical period produce odor preference learning in older pups as can bulbar infusion of a β-adrenoceptor agonist (Moriceau and Sullivan, 2004).

Beside maturation of the LC-NE system, altered adrenoceptor function (Pandipati et al., 2010), reductions in NMDA receptor signaling (Poo and Isaacson 2007; Franks and Isaacson 2005), and increased levels of corticosterone (Moriceau et al., 2009a) have been hypothesized to contribute to the termination of the critical period.

1.5.1.1.1Olfactory Bulb

Pharmacological evidence suggests β-adrenoreceptors as one of the major pathways through which NE plays its critical role as an UCS in early odor preference learning. Both global and OB administrations of the β-adrenoreceptor antagonist propranolol prevent neonatal odor preference learning (Sullivan et al., 1989a; Sullivan et al., 2000b). Additionally, the Sullivan group was able to induce learning in pups by pairing an odor with the β-adrenoreceptor agonist isoproterenol (Sullivan et al., 2000b). Later studies by Harley and colleagues found that learning occurs when β_1-adrenoreceptors are activated, but not β_2 (Harley et al., 2006). Dose-response curves indicate that only 2 mg/kg of isoproterenol is effective in promoting learning; higher (6mg/kg) or lower (1 mg/kg) doses are unable to create preference memories (Sullivan et al., 1989a; Langdon et al., 1997; Yuan et al., 2003a). Further studies found that when both stroking and the optimal isoproterenol dose are used as the UCS and odor presentation as the CS, training does not lead to memory formation. However, pairing lower doses of isoproterenol and sub-threshold stroking did lead to odor preference memory (Sullivan et al., 1991a). This suggests an additive effect of isoproterenol and stroking on β-adrenoreceptor activation in the OB and that excessive NE activation can prevent early odor preference learning. Although β-adrenoreceptors

are considered to be one of the major players in early odor preference learning, recent studies also demonstrate a role for α-adrenoreceptors in this learning paradigm. For instance, Harley and colleagues were able to induce an odor preference memory in rat pups by pairing the α_1-adrenoreceptor agonist, phenylephrine, with an odor (Harley et al., 2006).

1.5.1.1.1.1 Representational changes

Early odor preference learning-induced long-term metabolic changes that have been reported in the OB are increased 2-deoxyglucose (2-DG) uptake (Coopersmith et al., 1986; Sullivan and Leon, 1986), c-fos activation (Guthrie et al., 1993; Johnson et al., 1995), and glycogen phosphorylase activation (Coopersmith and Leon, 1987). Sullivan has shown that only pairing odor with tactile stimulation results in increased focal 2-DG uptake in the bulb, whereas odor alone fails to do so (Sullivan and Leon, 1986; Sullivan and Hall, 1988). In addition, such enhanced 2-DG uptake did not accompany simple increases in respiration (Coopersmith and Leon, 1984; Coopersmith et al., 1986; Sullivan and Leon, 1986). More tellingly, enhanced focal uptake of 2-DG was specifically identified in the glomerular layer following odor preference learning compared to odor alone (Sullivan et al., 1991a; Johnson and Leon, 1996). Similarly, intrinsic optical recording from the glomerular layer showed an increased optical signal in trained pups compared to controls (Yuan et al., 2002). *In vivo* studies reveal olfactory nerve (ON)-evoked lasting increases in the MC excitatory responses following an early odor preference training protocol (Yuan et al., 2000). Neonatal odor preference learning also increased the number of JG surrounding odor-activated glomeruli and the glomerular size (Woo et al., 1987; Woo and Leon, 1991), again indicating learning-induced long term plastic modifications in the bulb.

1.5.1.1.1.2 Electrophysiology

A recent study by Pandipati and Schoppa characterized the age-dependent physiological effect of NE in rats (Pandipati and Schoppa, 2012). They discovered that α_2-adrenoreceptor-mediated MC disinhibition by GCs only pertains in pups within PND 13. This acute disinhibitory effect leads to potentiating effects on MC-GC synaptic transmission such as enhanced evoked γ frequency oscillations originating from the MC-GC network (Gire and Schoppa, 2008; Pandipati et al., 2010). However, such strong gamma frequency oscillation enhancement was not evident in older animals at PND 18-23 (Pandipati and Schoppa, 2012).

Both behavioral (as discussed in section-1.5.1.1) and electrophysiological evidence suggests that the modulatory role of NE in early odor preference learning is mostly mediated by β-adrenoreceptors. Our lab also proposed that NE via β-adrenoreceptors could potentially affect ON-MC synaptic transmission, which may lead to long-term potentiation of ON-MC synapses (Yuan et al., 2014). Mechanistically, NE via β-adrenoreceptors suppresses PG activity, thus disinhibiting MCs and enhancing MC responses to ON input (Yuan, 2009). On the same note, Lethbridge *et al* found that NE via β-adrenoreceptors can also increase MC firing responses to olfactory nerve stimulation (Lethbridge et al., 2012). Disinhibition of mitral cells via granule cells is another way NE exerts plasticity through β-adrenoreceptors (Wilson and Leon, 1988b; Wilson and Sullivan, 1992).

1.5.1.1.1.3 Intracellular signaling: cAMP/PKA/CREB model

cAMP-mediated signaling cascades in many species (e.g. *Aplysia* and *Drosophila*) have a critical role in the processes of learning and memory (Byers et al., 1981; Schacher et al., 1988; Ghirardi et al., 1992; Levin et al., 1992). Detailed intracellular events mediated by cAMP were

outlined by Frank and Greenberg (1994) (Frank and Greenberg, 1994). In short, neurotransmitter binding to the receptors triggers intracellular activation of adenylyl cyclase, which in turn elevates the amount of cAMP. Increased cAMP facilitates PKA translocation to the nucleus, which then triggers phosphorylation of cAMP response element-binding protein (CREB) (Meinkoth et al., 1990). Phosphorylated CREB (pCREB) links neural activity to gene transcription and contributes to cell-wide transcriptional modification. This unique characteristic of pCREB gave rise to the idea that it mediates the encoding of the memory. In fact, pCREB elevation has been implicated in synaptic modification across many models (Bito et al., 1996; Deisseroth et al., 1996; Impey et al., 1996; Moore et al., 1996a).

Although the cAMP-dependent intracellular signaling cascade (cAMP/PKA/CREB) for learning and memory is well established in *Aplysia* (Brunelli et al., 1976; Pittenger and Kandel, 2003) and *Drosophila* (Byers et al., 1981; Shotwell, 1983; Yin and Tully, 1996), direct evidence for such intracellular events in mammals is sparse (Alberini, 1999). McLean and colleagues were among the first investigators to describe the role of pCREB in mammalian associative learning (McLean et al., 1999). In particular, only learning effective training (pairing odor with tactile stimulation) increases pCREB levels significantly in MCs, odor or tactile stimulation alone do not (McLean et al., 1999). This highlights the convergent effects of odor-induced calcium/calmodulin signaling and the NE-cAMP cascade in producing enhanced pCREB and learning (Yuan et al 2003a). Odor-induced calcium/calmodulin enhances adenylate cyclase elevation of cAMP as first reported by Yovell *et al* in *Aplysia* (Yovell et al, 1992*)*. Interestingly, such an effect requires forward pairing (Abrams et al, 1998). Subsequent studies found that while the optimal isoproterenol (2 mg/kg) dose as the UCS similarly increased pCREB amounts in trained pups,

saline or higher dose of isoproterenol paired with odor failed to increase pCREB synthesis (Yuan et al., 2000).

β_1- adrenoreceptor and 5-HT_{2A} receptors co-localize in MCs and 5-HT depletion results in reduction of the cAMP levels normally observed following learning-induced UCS application (Yuan et al., 2003a). The causal role of cAMP in odor preference learning was demonstrated by blocking phosphodiesterases with cilomilast. Phosphodiesterases normally breakdown cAMP. The cilomilast manipulation converts a low ineffective UCS (1 mg/kg isoproterenol) into an effective one for learning (McLean et al., 2005). Furthermore, odor preference learning in 5-HT-depleted pups was rescued by pairing 2 mg/kg isoproterenol with cilomilast (McLean et al., 2009). Temporal pattern investigations of cAMP in the rat pup learning model suggest a pulsatile cAMP modulation in MCs, with a critical10 min cAMP peak following learning-inducing training (Cui et al., 2007).

The causal role of pCREB in the pup learning model was established by injecting a Herpes simplex virus expressing CREB (HSV-CREB) or a dominant-negative mutant CREB (HSV-mCREB) in both OBs (Yuan et al., 2003b). Bilateral infusion of HSV-mCREB prevented learning in pups that received stroking paired with odor or the learning dose (2 mg/kg) of isoproterenol paired with odor. However, learning was achieved in those pups by a supraoptimal dose of isoproterenol (4 mg/kg), indicating a higher level of β adrenoreceptor activation is necessary to recruit a sufficient amount of pCREB to generate learning with these gene manipulations. On the other hand, excessive pCREB expression via HSV-CREB infusion prevented pups learning that received either optimal (2 mg/kg) or higher (4 mg/kg) isoproterenol doses in the presence of odor. Interestingly, learning was restored in HSV-CREB-treated pups when a suboptimal (1 mg/kg) dose of isoproterenol was applied.

Further study established a causal role for PKA in this learning model. A series of experiments by Grimes *et al* (2012) suggested that similar to cAMP, PKA activation is maximal at 10 min following odor conditioning training. Furthermore, intrabulbar infusions of the PKA blocker Rp-cAMPs results in CREB phosphorylation blockage and prevents normal 24 h preference learning from occurring. Emergence of 24h odor preference memory following a PKA agonist Sp-cAMP infusion in the bulb together with novel odor presentation, suggested that direct PKA activation itself can act as an UCS in this pup learning model. Consistent with other literature suggesting that the cAMP/PKA/CREB cascade is selectively involved in learning and memory (Huang et al., 1994; Nguyen et al., 1994; Alberini et al., 1995; Bailey et al., 1996; Nguyen and Kandel, 1996, 1997), the foregoing data support such a model in early odor preference learning.

1.5.1.1.2 Anterior Piriform Cortex

Kucharski and colleagues were the first to demonstrate the PC's role in neonatal odor preference learning (Kucharski et al., 1986a; Kucharski and Hall, 1987). Hall and colleagues found that 6 day old pups show no sign of preference to an odor that was paired with milk, when tested with the odor to a naris occluded during training. However, robust preference was obtained when the unoccluded naris (trained naris) was used during testing. Interestingly, when anterior commissural connections were developed at 12 days (Schwob and Price, 1984), the untrained hemisphere can access memory acquired at 6 days from the learned hemisphere (Kucharski and Hall, 1987). Disrupting the anterior commissure retains lateralized memory in the spared hemisphere. Later studies from the Sullivan lab found increased c-fos activation in the aPC following odor preference learning (Roth et al., 2006). Consistent with these earlier findings, transient silencing of aPC using either lidocaine or muscimol prevents early odor preference

learning (Morrison et al., 2013). In addition, pharmacological blockade of NMDA and β-adrenoreceptors in aPC prevents odor preference learning in rat pups. Odor preference memory can be induced in rat pups by pairing odor with infusion of the β-adrenoreceptor agonist isoproterenol in the aPC (Morrison et al., 2013). These series of experiments arguably suggest that piriform cortical plasticity also contributes to early odor preference learning and memory.

1.5.1.1.2.1 Electrophysiology

Morrison *et al* (2013) has shown a significant augmentation of LOT long term potential (LTP) amplitude when theta burst induction was combined with isoproterenol bath application (Morrison et al., 2013). Isoproterenol reduces the paired pulse ratio of the LOT-evoked field excitatory post synaptic potential (EPSP) indicating increased presynaptic release. This acute effect of isoproterenol may lead to the observed LTP enhancement. *Ex vivo* recording found that both 3h and 24h after odor preference training LOT field EPSP enhancement is observed. While both pre- and post-synaptic potentiation was evident following 3h of training, only post-synaptic potentiation was observed following 24h of training. In addition, they also found that blocking NMDA receptors by D-APV application prevented LTP induction at the LOT synapse. Another remarkable finding in establishing aPC involvement in early odor preference learning came from a calcium imaging study (Fontaine et al., 2013). Calcium imaging of aPC pyramidal networks reveal a reduction of pyramidal cell firing thresholds within the memory window, leading to the hypothesis that learning increased the responsiveness of pyramidal cells to the LOT input. Altogether these data suggest that plastic changes at LOT-aPC synapses and global changes in the pyramidal network of aPC occur during early odor preference memory.

1.5.2 Adult go-no-go

In this learning model rodents are conditioned to distinguish between odors depending on the valence of the odor (e.g. positively reinforced or non-rewarded). Computer controlled olfactometers have been used to demonstrate a rodent's ability to detect and discriminate odors (Laing et al., 1989; Youngentob et al., 1991; Brown et al., 1996; Bodyak and Slotnick, 1999; Larson and Sieprawska, 2002). In olfactometers, rodents are allowed to either positively respond, which is called a "go" response following reinforced odor delivery/presentation, or refrain from entering the odor delivery port, which is referred to as "no-go" response following unrewarded odor delivery. Other than go/no-go tasks, rodents have been trained to go in a left or right direction for reward or they have been trained to dig for food. The digging method requires fewer trials, while the first two behavioral paradigms take significantly more trials to reach learning criteria.

1.5.2.1 General behavioral paradigm considerations

Animals usually are either food or water deprived. This deprivation keep rodents motivated to participate in the task and learn the discrimination. However, for habituation measures of odor detection or spontaneous odor discrimination, animals do not need to be deprived of food or water. Go-no-go odor discrimination training begins with shaping where rodents become familiarized with the procedure. For example, in the case of the digging method (Berger-Sweeney et al., 1998) rodents are initially trained to find the hidden food (visible, semi-visible and buried) in the absence of scent. A limited amount of time is usually assigned to finish the task and position of the baited food is randomized. After the shaping period, hidden foods are presented with an odor of interest. After several trials rodents are able to retrieve the food using odor as a cue. During probe trials the

percentage of choice accuracy, the latency to retrieve the hidden food and errors (digs in the unrewarded place) are usually recorded.

In principle odor discrimination training with a computer-controlled olfactometer is similar to other go-no-go tasks (Bodyak and Slotnick, 1999). Shaping or initial training is usually performed by using software e.g. the ALL-BEGIN program. This program automatically delivers a certain amount of water (~30 µl) from a reservoir following each lick in the water port and automatically advances to next stage after 20-30 water deliveries. At the next stage, reinforced odor (S^+) is introduced into the system. Each snout insertion briefly operates the odor channel and the duration of odor exposure is increased in subsequent trials. Next, a fixed amount of time is provided for rats to sample the odor stimulus and make a decision, either they can lick the water port (usually a minimum of 6 times) for a water reward or reject it by withholding their snout from the port. Once rodents are acquainted with this procedure, the next training is called rule learning. During rule learning rodents, for the first time, experience an unrewarded odor (S^-). This rule learning training in our system utilizes software called the IN-D2 program. S^- presentation is followed by no water delivery. Initially rodents might respond randomly to this new odor but following a few trials they stop responding to the S^-. Usual training in IN-D2 program consists of 10 S^+ and 10 S^- deliveries in a random fashion. The percentage of correct responses is calculated by the computer. Once a rodent reaches criterion for the correct response rate (~80%) in rule learning training, odor discrimination training for two new odors can be employed. Training is exactly the same as the rule learning phase except two new novel odors are introduced.

With IN-D2, the same port is used for water delivery and odor delivery. It is also possible to deliver water in one port and odor in another port using an OUT-D2 program. In this training

program, the investigator can train rodents to go either to the left or right from the odor delivery port using different odor stimuli.

Shaping/begin/rule learning training is not necessary for experiments using habituation to test odor detection, and spontaneous discrimination (Escanilla et al., 2010). Rodents are allowed to investigate certain odors for a fixed period (~ 50 sec). A fixed amount of odorant is placed onto filter paper in a specified place randomly chosen. The amount of time rodents spend investigating the odors is measured as a test of the rodent's ability to discriminate or to discern odor novelty. Habituation itself can also be assessed.

1.5.2.2 The roles of NE in adult odor learning

Despite a wealth of data supporting NE's critical role in neonatal odor learning (Morrison et al., 2013), few studies have been done to delineate its potential role in adult odor learning. However, the role of NE in innate odor learning has recently been reported in juvenile rats (Kabitzke et al. 2011).

1.5.2.2.1 Olfactory Bulb

Several studies found increased NE levels in the OB following novel odorant presentation, repeated odor delivery and even associative conditioning (Brennan et al., 1998; Veyrac et al., 2009). These initial findings potentially suggest a role for OB NE in adult odor learning.

1.5.2.2.1.1 Behavioral studies

Unlike neonatal odor learning, where pairing odor with increased NE levels in the OB induces a robust preference for the paired odor, the same training in anesthetized adult mice leads

to habituation to the paired odor (Shea et al., 2008). The role of NE in habituation needs further investigation to settle the seemingly contradictory results in this field. For example, localized blocking of adrenoreceptors in the bulb using either α or β receptor antagonists showed no effect on habituation to repeated odor exposure (Mandairon et al., 2008b; Escanilla et al., 2010). On the other hand, global impairment of NE release by pharmacological lesion of the LC results in an impairment in habituation, which could be restored by local NE infusion in the bulb (Guerin et al., 2008). In other studies it has been shown that NE is essential in reversing or preventing olfactory habituation (Smith et al., 2009).

Spontaneous odor discrimination and detection were found to be dependent on NE modulation. Studies where α adrenoreceptors were blocked in the bulb showed impaired spontaneous odor detection and discrimination in rats (Escanilla et al., 2010). Although in the case of a reward-motivated odor discrimination task, Mandairon *et al* (2008b) discovered that both α- and β- adrenoreceptor blockade in the bulb only slowed down discrimination learning (Mandairon et al 2008b), Doucette *et al* (2007) showed a similar adrenoreceptor blockade in the bulb prevented discrimination of very similar odors (Doucette et al., 2007). Contradictory findings may result from the different learning paradigms employed and the species used in these two studies.

1.5.2.2.1.2 Electrophysiological evidence

Numerous OB slice physiology studies highlight its potential role in odor learning (Fletcher and Chen, 2010). Originally it was thought that NE inhibited MC firing by acting on GCs (Bloom et al., 1964; Salmoiraghi et al., 1964; McLennan, 1971). Consistent with this hypothesis a recent study found a reduction of spontaneous MC firing following LC stimulation (Jiang et al., 1996). However, in the same experiments they found NE increased MC activity when the sensory neurons

are subject to peri-threshold stimulation, leading to the hypothesis that NE could enhance MC responses to weak odor input (Jiang et al., 1996). In other experiments it has been shown that NE can directly excite both MCs and GCs via α_1-adrenoreceptors (Mouly et al., 1995; Ciombor et al., 1999; Hayar et al., 2001; Araneda and Firestein, 2006; Nai et al., 2010). Interestingly, it has been shown that NE can also indirectly excite MCs via disinhibition. In this particular case Trombley and Shepherd (1992) showed that NE presynaptically inhibits MC mediated GC firing, thus preventing feedback inhibition of MCs by GCs (Trombley and Shepherd, 1992). All together these results suggest that NE function in the OB is diverse and complicated.

NE action may depend on receptors activated at various concentrations (Nai et al., 2009; Nai et al., 2010). For instance, at lower concentrations NE acts on α_2-adrenoreceptors and enhances MC excitation via a disinhibition mechanism (Nai et al., 2009; Nai et al., 2010; Pandipati et al., 2010). On the other hand, at higher concentrations NE excites GCs via α_1-adrenoreceptors, which in turn inhibit MCs by releasing GABA (Nai et al., 2009; Nai et al., 2010). NE also has been shown to exert longer term effects in the OB. When ON is stimulated in the presence of an NE agonist, gamma frequency oscillations in the OB enhance significantly, indicating a global impact of NE on OB circuitry (Gire and Schoppa, 2008; Pandipati et al., 2010). Furthermore, long lasting suppression of MCs to odor input was also observed when LC stimulation was paired with odor exposure (Shea et al., 2008). The MC response to odor input was not affected if both α and β receptor antagonists were applied during LC stimulation.

1.5.2.2.2 Anterior piriform cortex

Other than electrophysiological studies, the role of aPC NE in mediating adult odor learning is largely unknown. However, considering the anatomical position and rich projection of NE in aPC warrant elaborate investigation to delineate aPC NE role in odor guided behaviour.

1.5.2.2.2.1 NE cellular mechanisms

Similar to the OB, concentration-dependent differential effects of NE in PC have been reported. Although at higher concentration it reduces the cortical response to OB input, at lower concentrations NE enhances the overall cortical response to OB input either by increasing MC excitatory transmission or by increasing pyramidal cell excitability (Collins et al., 1985). Electrical stimulation of LC *in vivo* results in overall enhanced PC neuron firing or increased temporal precision in response to odors (Bouret and Sara, 2002). In addition to LOT-PC synapses, NE also modulates excitatory associative fibers within the PC. Hasselmo *et al* (1997) found a reduction in excitatory synaptic transmission between pyramidal cells following NE application, suggesting that this suppression might help to enhance the exogenous signal-to-noise ratio in the PC (Hasselmo et al., 1997).

1.6 Large Scale Neuronal Mapping Techniques

To uncover the mysteries of the nervous system, neuroscientists need modern techniques to trace the activity patterns of large numbers of neurons. A large literature suggests that the neural basis of behavior and cognition is the result of the day-to-day orchestration of neuronal network activity distributed widely throughout the brain (Marom and Shahaf, 2002; Marom and Eytan, 2005; Chiappalone et al., 2008). Thus, the first step to understanding behavior is to capture detailed

functional maps of neural circuits within the brain. Although activity-dependent changes in synaptic strength have been studied extensively at the single neuron level (Bliss and Lomo, 1973; Stanton and Sejnowski, 1989; Artola and Singer, 1993; Bliss and Collingridge, 1993; Mulder et al., 1997; Werk and Chapman, 2003; Malenka and Bear, 2004; Mapelli and D'Angelo, 2007), how synaptic plasticity is implemented at the network level to permit the storage and recall of information remains elusive (Marom and Shahaf, 2002; Marom and Eytan, 2005; Chiappalone et al., 2008). Therefore, the ability to monitor larger-scale neuronal activity or 'neuronal ensembles' is required. Large-scale neuronal mapping techniques have been used to address some of the fundamental questions (e.g. how brain represents and processes sensory information) that have baffled neuroscientists for many years. Techniques such as electroencephalography (EEG) (Singh et al., 2003; Waldert et al., 2008), magnetoencephalography (MEG) (Luo and Poeppel, 2007; van Dijk et al., 2008), functional imaging (positron emission tomography (PET) and functional magnetic resonance imaging (fMRI)) (Schacter and Wagner, 1999; Mayes and Montaldi, 2001; Sowell et al., 2004; Jasanoff, 2005; Mechelli et al., 2005; Smirnakis et al., 2005), two photon imaging (Mainen et al., 1999; Ohki et al., 2005) and multi-neuron recording (Wilson and McNaughton, 1993; Gothard et al., 1996; Barnes et al., 1997; Nicolelis et al., 1997b; Hoffman and McNaughton, 2002; Nicolelis et al., 2003; Doucette and Restrepo, 2008) have been used to capture the blue print of cognition since 1980. Though fMRI and PET are capable of recording from large areas of the brain containing millions of neurons in action, single-cell resolution with these methods is not yet possible. On the other hand, multielectrode recording, though providing the necessary single cell resolution, is limited by the number of neurons sampled and often requires a large number of animals before enough units are collected for statistical analysis. This task becomes more challenging for a brain region like the dentate gyrus where activity is sparse (Small

et al., 2004). Although EEG and MEG fall in between functional imaging and multi electrode recording, single cell resolution is still not possible. In addition to the aforementioned imaging techniques, calcium-sensitive and voltage-sensitive dye imaging (Baker et al., 2005; Djurisic and Zecevic, 2005) as well as fluorescence resonance energy transfer (FRET)-based systems (Chanda et al., 2005) can be implemented to trace behaviorally relevant neural circuitry at large scales. Furthermore, immediate early genes (IEG, e. g. Arc, c-fos, homer1a, zif268) can also be used as markers to visualize dynamic neuronal ensembles in the brain (Morgan et al., 1987; Koya et al., 2009). Despite the drawbacks of each of these imaging techniques, large scale brain activity mapping methods have accelerated our understanding of the neural underpinnings of cognition that results from interactions within and between distributed brain systems.

1.6.1 Tetrode recording

One of the large scale neuronal recording techniques that allows segregation of individual spikes from multi-unit recording is called tetrode recording. As the name implies it is made of four electrodes, each about 10-15 μm in diameter (Emondi et al., 2004). To obviate the spike resolution problem of traditional extracellular recording that arises with burst discharges and with closely packed neuronal cells groups, initially the stereotrode (McNaughton et al., 1983) and later the tetrode (O'Keefe and Reece, 1993; Wilson and McNaughton, 1993) recording techniques were developed. Some of the inherent limitations of tetrode recording include mechanical damage associated with the probe movements (Claverol-Tinture and Nadasdy, 2004; Bjornsson et al., 2006; Seymour and Kipke, 2007; Tsai et al., 2009; Kozai et al., 2010), and excluding neurons with low firing rates (Shoham et al., 2006; Buzsaki and Mizuseki, 2014; Schwindel et al., 2014), and low spike amplitudes (Schomburg et al., 2012). In the last decade significant progress has been

made in the field to meet the increased demand for better recording with substantially increased numbers of monitoring sites and less tissue damage (Nordhausen et al., 1996; Motta and Judy, 2005; Rennaker et al., 2005; Hofmann et al., 2006; Ludwig et al., 2006; McCreery et al., 2006; Musallam et al., 2007; Neves and Ruther, 2007; Bartels et al., 2008; Kipke et al., 2008; Neves et al., 2008; Ruther et al., 2011). This technical advancement now allows us to record discharge properties of larger numbers of well-isolated cells simultaneously at different times in behaving animals (Du et al., 2011; Kozai et al., 2012). Therefore it is possible to study the behavior of multiple cells in a variety of brain structures for weeks and even months in various species (Nicolelis et al., 1997b; Rousche and Normann, 1998; Pouzat et al., 2002; Csicsvari et al., 2003; Kipke et al., 2003; Bartho et al., 2004; Blanche et al., 2005; Suner et al., 2005; Broome et al., 2006; Jackson and Fetz, 2007; Fujisawa et al., 2008; Montgomery et al., 2008; Chestek et al., 2011; Du et al., 2011; Ruther et al., 2011; Agarwal et al., 2014; Lin et al., 2014). Interest in manipulating multiple neurons under investigation requires technological breakthroughs to, for example, combine optogenetic manipulations with larger scale neural recording electrodes. In a recent paper Buzsaki *et al* (2015) has extensively discussed how some of the technical difficulties in this field can be resolved to take full advantages of the available methods (Buzsaki et al., 2015).

1.6.2 Optical recording using intrinsic signals

It is a general phenomenon in biology that the functional state of a tissue influences its optical properties. One physiological basis of optical changes in a tissue is a wavelength-specific absorption of photons by oxygenated and deoxygenated haemoglobin (Villringer and Chance, 1997). The historical roots of activity-dependent changes of optical properties of nerve cells can be traced to as early as 1949 (Hill and Keynes, 1949). Nearly four decades ago Jöbsis described

the possibility of measuring blood and tissue oxygenation changes in the brain of a cat using near-infrared (NIR) light (Jobsis, 1977). Since that time changes in optical properties of neurons have been measured in bloodless brain slices (Lipton, 1973; MacVicar and Hochman, 1991), in intact cortical tissue (Jobsis, 1974; Harik et al., 1979; Grinvald et al., 1986), and in cell cultures (Stepnoski et al., 1991). Both animal (MacVicar and Hochman, 1991; Yuan et al., 2002) and human subjects (Haglund et al., 1992) have been used to map neuronal activity by capturing optical signals from surgically exposed areas of interest. However, recent technical advancements allow assessing brain activity even non-invasively (Maki et al., 1995; Hirth et al., 1996; Chance et al., 1997), and through the intact skull (Chance et al., 1993; Hoshi and Tamura, 1993; Kato et al., 1993; Villringer et al., 1993; Gratton et al., 1995). In fact, non-invasive optical imaging has been employed in adult human subjects (Chance et al., 1993; Hoshi and Tamura, 1993; Kato et al., 1993; Villringer et al., 1993; Gratton et al., 1995; Maki et al., 1995; Hirth et al., 1996; Chance et al., 1997) and it was possible to assess several types of brain activity including the response to auditory stimulation (Hoshi and Tamura, 1993), visual activation (Kato et al., 1993; Villringer et al., 1993; Gratton et al., 1995; Meek et al., 1995; Wenzel et al., 1996), motor activity (Maki et al., 1995; Hirth et al., 1996; Obrig et al., 1996) and the performance of cognitive tasks (Chance et al., 1993; Hoshi and Tamura, 1993; Villringer et al., 1993).

1.6.3 c-fos

Probably *c-fos* is one of the best studied IEGs that has been used to produce high-resolution functional maps of cellular activation in the CNS since the late 1980s (Greenberg and Ziff, 1984; Curran and Morgan, 1985; Dragunow et al., 1987; Morgan et al., 1987; Dragunow and Faull, 1989; Sheng and Greenberg, 1990; Morgan and Curran, 1991; Herrera and Robertson, 1996; Herdegen

and Leah, 1998; Montag-Sallaz and Buonviso, 2002). Now, localization of c-fos protein has been an effective tool in neuroscience to visualize patterns of neuronal activation in the brain and spinal cord for decades (Hyman et al., 1993; Sharp et al., 1993; Hughes and Dragunow, 1995; Chaudhuri, 1997; Chaudhuri et al., 2000). Although it was thought that c-fos induction is primarily associated with the functional activity of neurons (Sagar et al., 1988; Dragunow and Faull, 1989; Duncan et al., 1993), the absence of significant c-fos expression in regions with high levels of neuronal activity {e.g. visual cortex (Kaczmarek and Chaudhuri, 1997)} suggest that normal levels of neuronal activation are not sufficient to induce IEG expression. Consistent with this idea it has been shown that IEG activation is inversely correlated with the burst-intervals of action potentials (Fields et al., 1997). Different types of challenges (seizure, sound, water stress, intra-parenchymal injection of various substances, fear, odors, including convulsing agents, etc.) have been used to induce c-fos to map relevant functional neural circuitry in different cortical regions including visual cortex (Kaczmarek and Chaudhuri, 1997), auditory cortices (Campeau and Watson, 1997), amygdala (Dragunow et al., 1988; Cullinan et al., 1995), hippocampus (Hughes et al., 1992), thalamus (Gholami et al., 2006), cingulate cortex (Duncan et al., 1993), medial prefrontal cortex (Duncan et al., 1993), cerebellum (Carbo-Gas et al., 2014), limbic structures (Le Gal La Salle, 1988), neocortex (Simler et al., 1994), striatum (Szyndler et al., 2009) and piriform cortices (Dragunow and Robertson, 1987). Despite its widespread application, c-fos immunohistochemistry (IHC) is time consuming and labour and resource intensive (Deutch et al., 1991; Hughes et al., 1992; Smith and Day, 1993; Conde et al., 1995; Lin et al., 1998; Sebens et al., 1998; D'Hondt et al., 1999; Leman et al., 2000; Ishida et al., 2002; Cohen et al., 2003; Koya et al., 2009). However, a faster c-fos IHC protocol has been published by Sundquist and Nisenbaum (2005).

Several learning-related synaptic events such as changes in neurotropic factors, depolarization, release of neurotransmitters, elevation of intracellular/intranuclear Ca^{2+} and increase of Ca^{2+} influx, facilitate c-fos induction in cells (Greenberg and Ziff, 1984; Szekely et al., 1987; Morgan and Curran, 1989a, b; Doucet et al., 1990; Sheng and Greenberg, 1990; Sheng et al., 1990; Ghosh et al., 1994; Gaiddon et al., 1996). One of the reasons that c-fos has been used to map stimulus-driven functional circuitry is that c-fos mRNA and protein are very low under basal conditions (Hughes et al., 1992). However, c-fos mRNA can be induced by acute challenge within minutes and peaks between 30 and 60 min post challenge. c-fos protein level reaches its maximum between 1h and 3h, then gradually it decays from the nucleus by 4-6 h after the induction protocol (Sonnenberg et al., 1989; Chan et al., 1993; Imaki et al., 1993; Ding et al., 1994; Ikeda et al., 1994; Cullinan et al., 1995; Kovacs and Sawchenko, 1996). Recently the combination of c-fos immunohistochemistry with localization of a second antigen has provided an advanced c-fos mapping technique identifying neurochemically-specified groups of cells in the brain (Mikkelsen et al., 1994; Kovacs, 1998; Hoffman and Lyo, 2002). Indeed, these technological advancements permit the design of novel experiments to define the role of active neuronal ensembles in cognitive behaviors.

1.6.4 Cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH) of immediate early genes

Since Hebb postulated that learned associations occur within specific patterns of neurons (Hebb, 1949), which we now call neuronal ensembles, many *in vivo* electrophysiologists have provided evidence that the association between the conditioned stimulus and the unconditioned stimulus takes place in neuronal ensembles that are activated at the same time by both the stimuli

(Hebb, 1949; Schwindel and McNaughton, 2011). Later this ensemble hypothesis was adopted by many investigators and became the foundation for numerous learning and memory studies. Subsequent studies have characterized learning-induced changes in putative neuronal ensembles (Pennartz et al., 1994; Nicolelis et al., 1997a; Guzowski et al., 2004; Schwindel and McNaughton, 2011; Knierim and Zhang, 2012; Penner and Mizumori, 2012; Buzsaki and Moser, 2013).

In the last 25 years several IEG-based labelling methods have been used to map neuronal components of brain circuits associated with specific behaviors (Morgan and Curran, 1991; Lerea et al., 1992; Sgambato et al., 1997; Reijmers et al., 2007; Mattson et al., 2008; Garner and Mayford, 2012). For example, IEG methods have been employed in studies of addiction and withdrawal; learning and memory; pain; sensory processing; mating; feeding; maternal behaviors; circadian rhythm entrainment; and fear and stress (Guzowski et al., 2005). Conventional IEG techniques that either stain for protein levels (immunohistochemistry) or the mRNA of interest (*in situ* hybridization), permit one time visualization of the neuronal ensemble. To obviate these drawbacks, Guzowski and colleagues (1999) developed an IEG imaging technique to visualize the activity history of neural ensembles activated in two events separated by a fixed interval. Importantly, this methodological advance enables an investigator to map behaviorally relevant circuitry with reasonable temporal and good single cell resolution. The technique is termed "cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization" (catFISH). This method mostly takes advantage of the temporal dynamics of the IEGs *Arc*, *Homer1a*, or *Zif268*. This technique has been used to label behaviorally relevant neural circuitry in the olfactory system (Yuan and Harley, 2014), hippocampus (Guzowski et al., 1999; Guzowski et al., 2004; Czerniawski and Guzowski, 2014; Pevzner and Guzowski, 2014), amygdala (Barot et al., 2008; Orsini et al., 2013), and infra limbic cortex (Orsini et al., 2013) among other structures.

Furthermore, this technique has been employed to study different learning paradigms including odor associative learning (Yuan and Harley, 2014), extinction (Orsini et al., 2013), fear conditioning (Hashikawa et al., 2011; Czerniawski and Guzowski, 2014; Pevzner and Guzowski, 2014), conditioned taste aversion (Barot et al., 2008),and spatial navigation (Kubik et al., 2012). The catFISH technique has even been employed to image replay while an animal is at rest (Marrone et al., 2008) and to study the hippocampal function of rats that are susceptible to Posttraumatic Stress Disorder (PTSD)-like behaviors (Nalloor et al., 2014).

1.6.4.1 catFISH principles

The general principle for catFISH is to use one neuronal activity marker (e.g. *Arc* or *Homer1a*) to detect neurons activated during the first episode of a sensory experience and a different or the same neuronal marker to label neurons that are activated by a second sensory experience. Likelihood of double labelling is indicative of the same neurons being recruited during the two episodes. While other brain imaging techniques either offer cellular resolution (conventional IEG immunohistochemistry) or temporal resolution (PET or fMRI), catFISH provides both temporal and cellular resolution of the brain's responses to the external world. The drawback is that catFISH can only be applied when two events occur with a fixed time interval due to the constrained expression dynamics of the IEGs used. Although catFISH is not applicable for real time study, it can be employed to trace large numbers of neurons that are activated by two defined episodes across brain structures.

1.6.4.2 *Arc*

The immediate early gene *Arc* (***a***ctivity-**r**egulated **c**ytoskeleton-associated protein) is a commonly used activity marker in catFISH. This method exploits the time-dependent migration profile of the *Arc* mRNA from the nucleus to the cytoplasm of a neuron (Guzowski et al., 1999). As a result, it is possible to monitor neuronal ensembles activated at two times separated by a resting period (~20-30 min). This unique technical advantage of *Arc* catFISH permits us to address questions like how learning alters neural activity patterns to cope with an ever changing environment or whether the same neuronal ensemble that is recruited during learning also participates in the retrieval process (although the two events are temporally constrained). It can also be used to trace spatial activity maps of neuronal ensembles that encode specific contexts or cues. Following any supra-threshold neuronal activity, *Arc* mRNA transcription occurs within ~1-5 min and can be detected as bright transcription foci in the nucleus. Afterwards, *Arc* mRNA leaves the nucleus and diffuses to the cytoplasm. As a result within ~20-30 min of neural activity *Arc* mRNA emerges in the cytoplasm. Hence, *Arc* mRNA signals in the nucleus represents a behavioral epoch that takes place ~2-5 min before sacrifice and *Arc* mRNA in the cytoplasm indicates neural activity that occurs~20-30 min before sacrifice. However, neurons with both cytoplasmic and nuclear *Arc* mRNA are involved in both behavioral epochs. Thus by counting these three characteristic *Arc* expression patterns in a neuronal network, one can identify the two individual ensembles representing each event and the common cells activated by the two events. Several lines of evidence suggest that *Arc* is dynamically regulated in multiple brain regions (e.g. hippocampus, entorhinal cortex, amygdala, striatum) and it has been proven to be necessary for memory consolidation (Guzowski et al., 2001; Miyashita et al., 2008). In line with this, it has been reported that *Arc* is tightly coupled to neuronal activity associated with synaptic plasticity and memory

(Miyashita et al., 2009). In fact, *Arc* has been proposed to be involved in every form of synaptic plasticity (Lanahan and Worley, 1998; Guzowski, 2002; Plath et al., 2006; Bramham et al., 2008; Miyashita et al., 2009). For instance, genetic reduction of *Arc* protein expression in hippocampus leads to impairment in LTP maintenance and consolidation of hippocampus-dependent long term memory (Guzowski et al., 2000; Plath et al., 2006). Accumulation of *Arc* in inactive synapses facilitates surface GluA1 removal from the inactive synapse and is thus proposed to be involved in homeostasis and restabilization of active synapses (Rial Verde et al., 2006; Shepherd et al., 2006; Okuno et al., 2012). Furthermore, somatic background staining of *Arc* is significantly lower compared to other dynamically regulated IEG such as *Zif268*. All these advantages of *Arc* make *Arc* catFISH a powerful tool for the study of various cognitive functions such as perception, addiction, extinction, learning, and memory.

1.6.4.3 *Homer1a*

Arc catFISH is the first of the two catFISH methods initially proposed by Guzowski and his colleagues. In the second catFISH method, the activity history of neurons is readout by using two IEGs e.g. *Arc* and *Homer 1a* (Guzowski, 2002; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004; Kubik et al., 2007; Czerniawski and Guzowski, 2014; Pevzner and Guzowski, 2014). While *Arc* is expressed in the nucleus shortly following a sensory event, *Homer1a* emerges in the nucleus around 30 min following an event. Colocalization of *Arc* and *Homer1a* in the same cells suggests participation of the same neurons in both events. As cytoplasmic *Arc* expression is diffuse in nature, the nuclear foci signal of *Homer1a* offers a better readout for cells that are activated30 min before sacrifice. However, despite the fact that *Arc*/*Homer1a* catFISH eases the manual quantification process, *Arc* catFISH is less time consuming due to the need for processing

of only one marker. It is to be noted that although both catFISH techniques are excellent analytical tools to dissect the behaviorally relevant neural circuitry, the time consuming manual counting procedures of these techniques is still a major challenge to overcome. In addition, IEG catFISH techniques are not able to measure the rate coding properties of neurons.

1.6.4.4 *Zif268*

The inducible nature of IEG *zif268* allows investigators to use it as another activity marker in the brain. Similar to other IEGs, *Zif268* has been implicated in synaptic plasticity and is constitutively expressed in the neocortex, hippocampus, primary olfactory and entorhinal cortices, amygdaloid nuclei, nucleus accumbens, striatum, visual cortex and cerebellar cortex (Worley et al., 1991; Lanahan and Worley, 1998; Bozon et al., 2003; Davis et al., 2003). Studies suggest that *zif268* is tightly coupled to neural activity in the visual cortex where its protein is detectable after 2h of light stimulation and *zif268* mRNA appears within 30 min of activation (Worley et al., 1991; Kaminska et al., 1996). Using this differential time course of appearance and disappearance of the IEG *zif268* and its protein as an advantage, Chaudhuri *et al* (1997) developed a double labelling technique to visualize the neurons that are activated by two different visual experiences (Chaudhuri et al., 1997). Basically this technique combines immunocytochemical staining (ICC) and *in situ* hybridization (ISH). Since IEG mRNA and the protein's half-life varied substantially and the double ICC/ISH labelling mapping method is technically more challenging than catFISH, this technique has not been exploited as much as catFISH as a mapping technique (Morgan and Curran, 1991; Guzowski et al., 2001).

1.7 Objectives

This thesis explored the following questions

1. What is the role of the α_2-adrenoceptor in early odor preference learning? It has been shown that α_2-adrenoreceptors mediate the disinhibitory effects of NE on mitral cells (Trombley and Shepherd, 1992; Trombley, 1994; Pandipati et al., 2010) and promote long-term enhanced gamma-oscillations within the OB network (Pandipati et al., 2010). The α_2-adrenoceptor mediated effect is age-dependent and the window of $\alpha_2$function in the OB coincides with the critical period for early odor preference learning. However, whether α_2 activation plays a role in early odor preference learning, the molecular mechanisms underpinning its action and its synergistic effects with other adrenoreceptors have not been studied.
2. How does early odor preference learning influence odor representations in the OB and aPC? Previous research has shown both the OB and aPC are critical for early odor learning and they work in concert to promote learning plasticity (Yuan et al., 2014). However, whether and how odor learning modifies neuronal ensemble dynamics in the OB and aPC to support memory is unknown.
3. How does the aPC represent odors in adult rats and how do odor representations adapt to differential behavioral demands? Pattern separation and completion have been studied in both hippocampus and PC (Wilson, 2009; Rolls, 2013, 2015). Recent work from Wilson's lab has demonstrated a bi-directional plasticity of the aPC ensembles in odor discrimination learning using extracellular unit recording in anesthetized rats (Chapuis and Wilson, 2012). The *Arc* catFISH method employed as the advantage of *post-hoc* monitoring of large ensembles of neurons during behavior. Pattern separation,

completion and dynamics of the ensembles responding to the same stimulus over time can be visualized in a non-invasive manner.

4. What are the roles of NE in adult odor discrimination learning and odor representations? Previous research has suggested that NE is critical for olfactory learning and odor discrimination in adult rodents (Doucette et al., 2007; Mandairon et al., 2008b; Escanilla et al., 2010). Recently it has been shown that pharmacological blockade of adrenoreceptors in the OB impairs difficult odor discrimination learning and reduces synchronized firing of mitral cells to rewarded odors(Doucette et al., 2011).How NE manipulations in the PC influence odor learning and odor representations in the OB is not known. Furthermore, although NE manipulation in the OB has been implicated in odor learning, how such altered OB signaling influences PC odor ensemble representation has not been characterized.

Chapter-02 : Olfactory bulb α_2-adrenoceptor activation promotes rat pup odor preference learning via a cAMP-independent mechanism.(This chapter is a version of the manuscript published in Learning and Memory 19 (11): 499-502, 2012)

2.1 Introduction

Odor-preference learning in the week-old rat pup occurs when a novel odor (conditioned stimulus, CS) is paired with activation of the noradrenergic locus coeruleus. The locus coeruleus is activated by the range of stimuli that can induce odor-preference learning including stroking (Sullivan and Leon, 1986; McLean et al., 1993) and feeding (Johanson and Teicher, 1980; Kucharski and Hall, 1987), all of which serve as unconditioned stimuli (UCS). Even rough maternal handling mimicked by mild shocks will engage odor-preference learning (Camp and Rudy, 1988; Sullivan et al., 2000a). Odor-preference learning enables rat pups to locate the dam at a period when visual and auditory input is minimal. Odor paired with the activation of β-adrenoreceptors in the olfactory bulb is sufficient to induce odor learning, while a bulbar β-adrenoceptor antagonist prevents odor-preference learning (Sullivan et al., 2000b). Thus, the olfactory bulb appears to be the critical site for the CS–US pairing, and the likely location of the odor memory.

However, in addition to β-adrenoceptors, which induce odor learning via activation of the cAMP/PKA/CREB cascade (McLean et al., 1999; Yuan et al., 2003b; Yuan et al., 2003a; Cui et al., 2007; Grimes et al., 2012), there are bulbar α-adrenoreceptors likely to be engaged by norepinephrine (NE) release. Recently, studies of α_2-adrenoceptor activation in the olfactory bulb *in vitro* have revealed receptor effects that could promote odor learning (Nai et al., 2010; Pandipati

et al., 2010). In particular, the α_2-adrenoceptor agonist, clonidine, has been shown to decrease granule cell excitability (Nai et al., 2010), releasing the odor-encoding mitral cells from tonic inhibition, and to promote olfactory bulb synchrony at γ EEG frequencies (Pandipati et al., 2010). These studies predict a role for α_2-adrenoceptor activation in odor-preference learning.

The present experiments assess the role of bulbar α_2-adrenoceptors in rat pup odor preference learning.

2.2 Methods

In all experiments, drugs were infused into the olfactory bulbs on postnatal day (PND) 6. Day of birth was considered PND 0. Sprague-Dawley rat pups of both sexes were used and litters were culled to 12 pups on PND 1. Dams were maintained under a 12-h reverse light/dark cycle at 22°C in polycarbonate cages with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and followed the Canadian Council on Animal Care guidelines.

2.2.1 Odor Conditioning and Drug Infusion

Details of infusion methods have been reported previously (Lethbridge et al., 2012). Briefly, PND 5 rat pups were anesthetized via hypothermia and two customized guide cannulae (27-gauge, 2.5 mm apart, anchored by dental acrylic and extending ~1 mm beyond the acrylic) were implanted into the center of the olfactory bulb and fixed to the skull with dental acrylic. Infusion cannulae made from 30-gauge stainless-steel tubing were inserted into PE20 polypropylene tubing attached to a 10-μL micro syringe and placed in a multi-syringe pump.

In the first experiment, on PND 6, peppermint odor was paired with mild electrical shock. Animals received bilateral intra-bulbar infusions of saline or yohimbine (500 µM, 1 µL/bulb at 0.1 µL/min) and were randomly assigned to one of three groups: (1) saline + shock, (2) saline + shock + odor, or (3) yohimbine + shock + odor. The training chamber included a grid assembly floor connected to a shock generator (Muromachi Kikai Co.). The paired group received 11 presentations of a 30-sec odor stimulus delivered by sliding an odorized bedding tray (0.3 mL peppermint extract/500 mL clean bedding) under the grid for 30 sec, ending with a 1-sec shock (0.5 mA). The intertrial interval was 2 min.

2.2.2 Odor Preference Testing

On PND 7, pups were tested for odor-preference memory in a stainless-steel test box placed on top of two bedding boxes separated by a 2-cm neutral zone. One box contained peppermint bedding while the other box contained clean, unscented bedding. Each pup underwent five 1-min trials during which it was placed in the neutral zone of the test box and allowed to move freely. The amount of time spent over peppermint bedding and unscented bedding over five trials was calculated. Values reported are the percentages of time spent over peppermint bedding divided by total time spent over both beddings. One-way ANOVA and *post-hoc* Fisher tests were used to evaluate statistical significance with P set at <0.05. An intra-bulbar infusion of 4% methylene blue dye was followed by dissection of the olfactory bulbs to check cannulae position (Appendix-A). Pups with cannulae blockage during infusion, or misplaced cannulae, were excluded from analysis.

2.3 Results

Pairing peppermint odor with shock induced odor-preference learning, while the α_2-adrenoceptor antagonist yohimbine prevented odor-preference learning ($F_{(2,33)} = 12.18$, $P < 0.001$) (Fig. 2.1A). Saline + shock + odor pups spent significantly more time (56.05% ± 3.68, $n = 12$) over peppermint than either the saline + shock group (37.63% ± 4.04, $n = 12$) or the yohimbine + shock + odor group (33.86% ± 2.21, $n = 12$). Blocking α_2-adrenoreceptors locally in the olfactory bulb prevented preference learning that was induced by pairing odor with electrical shock.

We next asked whether α_2-adrenoceptor activation could act as an UCS for odor-preference learning. PND 6 rats were placed on peppermint bedding for 10 min and the α_2-adrenoceptor agonist, clonidine (5, 50, or 500 µM) or saline, was infused into the olfactory bulb bilaterally at the rate of 0.1 µL/min. To control for the potential effect of clonidine on α_1-adrenoreceptors at the higher concentration, a group of animals with co-infusion of prazosin (10 µM, α_1-adrenoceptor antagonist) and clonidine (500 µM) was included in the study. Clonidine dose-dependently induced odor-preference learning on PND 7 ($F_{(4,62)} = 4.77$, $P = 0.002$) (Fig. 2.1B). The 500 µM clonidine infusion group spent significantly more time on the peppermint side (63.89% ± 3.03, $n = 14$) than the saline group (43.82% ± 2.97, $n = 18$), the 5 µM clonidine group (51.19% ± 2.25, $n = 14$), or the 50 µM clonidine group (52.48% ± 4.49, $n = 16$). The coinfusion of 500 µM clonidine and 10 µM prazosin group (57.8% ± 5.97, $n = 5$) still showed a significant learning effect when compared with the saline group. This outcome suggests that clonidine-mediated α_2-adrenoceptor activation can act as an UCS for odor-preference learning.

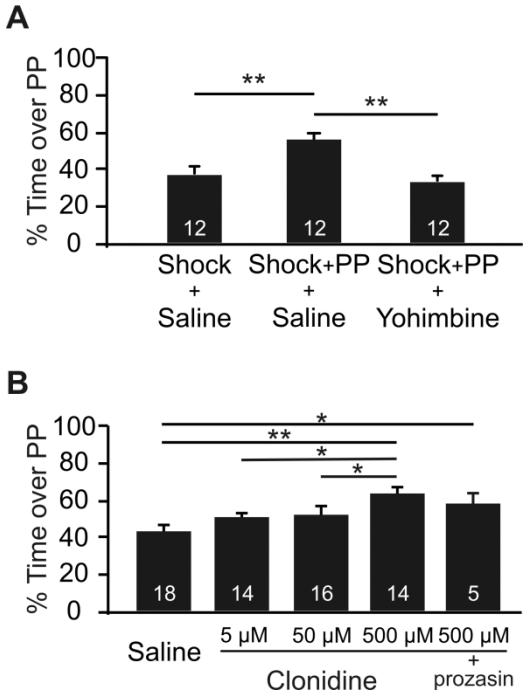

Figure 2.1 Olfactory bulb α_2-adrenoreceptors are critically involved in early odor-preference learning in rats.
(A) Bulbar infusion of the α_2-adrenoceptor antagonist yohimbine prevented odor preference learning induced by odor + shock pairing. (PP) Peppermint. **(*B*)** Clonidine bulbar infusion dose-dependently induced odor-preference learning. Bars show the percentages of time spent on the peppermint side in a two-choice test box in different experimental groups. (**) $P< 0.01$; (*) $P< 0.05$. Error bars, mean ± SEM.

We then sought to clarify the cellular mechanisms of α_2-adrenoceptor action during clonidine-induced learning. The evidence that clonidine reduces granule cell activity (Nai et al., 2010; Pandipati et al., 2010) predicts elevated mitral cell excitation during odor paired with clonidine. We performed pCREB immunohistochemistry as an index of mitral cell activation and to assess the role of CREB in the clonidine model. Unilateral bulbar infusions of either clonidine (500 μM) or the GABA-A receptor antagonist gabazine (100 μM, previously shown to induce odor-preference learning) (Lethbridge et al., 2012) were paired with odor. The remaining bulb was infused with saline as a control. Additional animals were given intrabulbar isoproterenol (50 μM) (Fig. 2.2A,B) or a systemic isoproterenol injection (2 mg/kg, data not shown) to confirm the β-adrenoceptor-associated increase in pCREB and cAMP patterns reported previously (Yuan et al., 2000; Yuan et al., 2003b; Yuan et al., 2003a; Cui et al., 2007). At 5–10 min following the end of training, animals were anesthetized with chloral hydrate and perfused transcardially with ice-cold saline followed by ice-cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4). Brains were removed and post-fixed for 1 h in fixative and then immersed in 20% sucrose overnight at 4°C. They were stored in sucrose until cutting. Brains were quick-frozen on dry ice and 30-μm coronal sections cut in a cryostat at −20°C. A pCREB antibody (1:100, Cell Signalling) was used to probe for CREB phosphorylation at Ser133. The antibody was dissolved in phosphate-buffered saline with 0.2% Triton-X-100, 0.02% sodium azide, and 2% normal goat serum and applied to sections overnight at 4°C in a humidified chamber. The next day, sections were incubated in a biotinylated secondary antibody (Vectastain Elite) followed by a diaminobenzidine tetrahydrochloride reaction. Sections were dehydrated and cover slipped with Permount.

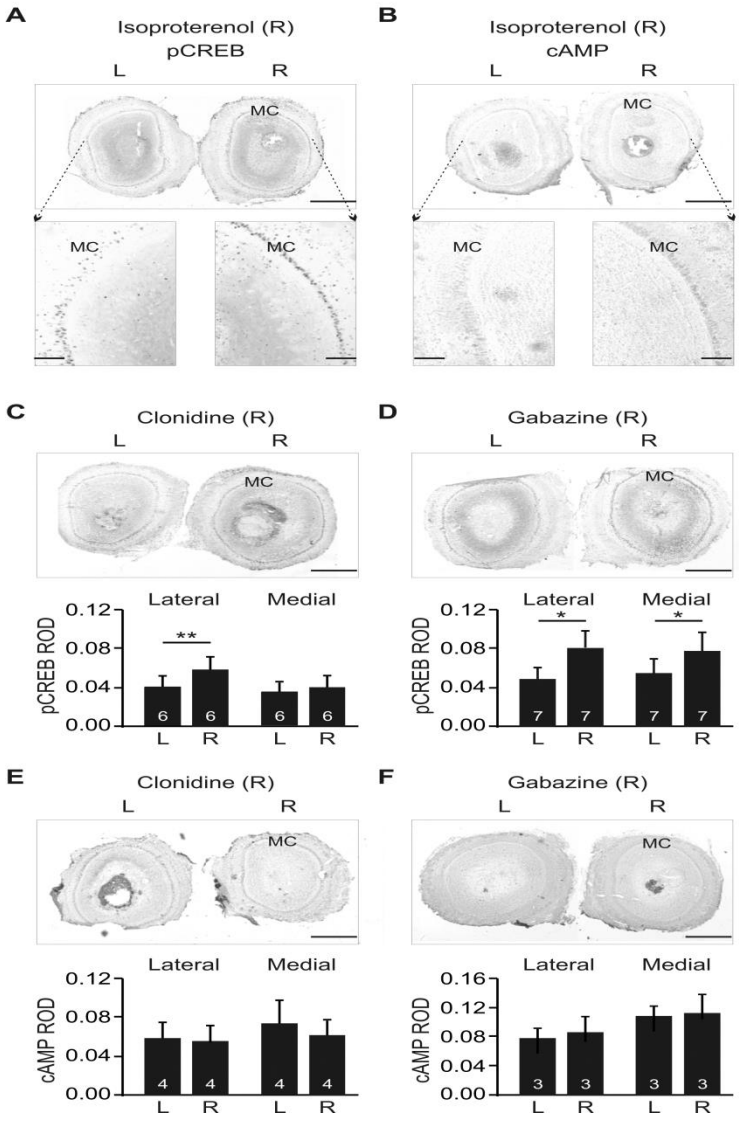

Figure 2.2 α_2-adrenoceptor activation increases pCREB expression in mitral cells via a cAMP independent pathway

(*A*) An example of mitral cell pCREB expression following bulbar infusion of the β-adrenoceptor agonist isoproterenol. (MC) Mitral cell layer. Scale bars, 500 µm and 100 µm. **(*B*)** Mitral cell cAMP activation in an alternate section from the same bulb induced by isoproterenol infusion. Scale bars, 500 µm and 100 µm. **(*C*)** Clonidine infusion increased mitral cell pCREB expression in the olfactory bulb. Clonidine was infused into the right olfactory bulb. Bars show the relative optical densities (RODs) of mitral cell pCREB in the lateral and medial regions of the two olfactory bulbs. (**) $P< 0.01$. **(*D*)** Gabazine infusion increased mitral cell pCREB expression in the olfactory bulb. Gabazine was infused into the right olfactory bulb. (*) $P< 0.05$. **(*E*)** Clonidine infusion did not change mitral cell cAMP expression in the olfactory bulb. **(*F*)** Gabazine infusion did not change mitral cell cAMP expression in the olfactory bulb. Error bars, mean ± SEM**.**

Staining for pCREB was analyzed using a Bioquant image analysis system. Images of sections were captured with a CCD camera connected to a Leitz microscope. For each section analyzed, the optical density (OD) of the olfactory nerve layer was used as a measure of background OD. Regions of interest (ROIs) were selected using a hand tracing tool. The relative OD of each ROI was obtained using the following formula: (OD of ROI–OD of background)/OD of background. Image analysis was conducted on every third to fourth section across the rostro-caudal extent of the olfactory bulb measuring the mitral cell layer in both the lateral and medial regions. The relative ODs (RODs) of the lateral and medial measurements were compared among groups and the mean ± SEM are reported for each ROI. Paired *t*-tests were used to evaluate differences ($P < 0.05$).

Unilateral clonidine infusion significantly increased mitral cell layer pCREB expression in the lateral (ROD clonidine: 0.059 ± 0.012 vs. saline: 0.041 ± 0.010, $n = 6$), but not medial (ROD clonidine: 0.040 ± 0.012 vs. saline: 0.036 ± 0.010, $n = 6$), regions of the olfactory bulb (Fig. 2.2C). Gabazine infusion increased mitral cell layer pCREB expression in both the lateral (gabazine: 0.082 ± 0.017 vs. saline: 0.050 ± 0.011, $n = 7$) and the medial (gabazine: 0.079 ± 0.019 vs. saline: 0.055 ± 0.015, $n = 7$) regions of the olfactory bulb (Fig. 2.2D). These results suggest that α_2-adrenoceptor-mediated disinhibition synergizes with odor input to activate pCREB in odor-encoding mitral cells in the peppermint presentation region (Lethbridge et al., 2012), while gabazine disinhibition is strong enough to directly activate mitral cell pCREB more globally.

The activation of pCREB by clonidine and gabazine learning doses paired with odor 5–10 min post-training is consistent with a role for an α_2-adrenoceptor-mediated disinhibition in learning and parallels the pCREB increases reported with an isoproterenol US (Yuan et al., 2000) and verified in examples for the present experiments (Fig. 2.2A).

Using alternate sections from a subset of the infused bulbs, we asked whether increases in cAMP occurred 5- to 10-min post-training as reported earlier for β-adrenoceptor-mediated learning (Fig. 2.2B; (Yuan et al., 2003a; Cui et al., 2007)). The procedures for cAMP staining and analysis were the same as those used for pCREB immunoctyochemistry except that a cAMP antibody (1/2000, Genscript) was used.

Neither unilateral clonidine nor gabazine infusion changed mitral cell cAMP expression in either the lateral (clonidine: 0.055 ± 0.016 vs. saline: 0.059 ± 0.016, $n = 6$; gabazine: 0.086 ± 0.022 vs. saline: 0.078 ± 0.012, $n = 4$) or the medial (clonidine: 0.061 ± 0.016 vs. saline: 0.074 ± 0.025, $n = 4$; gabazine: 0.113 ± 0.025 vs. saline: 0.108 ± 0.014, $n = 4$) regions of the olfactory bulb (Fig. 2.2E, F).

The optical density of pCREB and cAMP staining did not vary among conditions in the granule cell layer in contrast to what we found for the mitral cell layer (data not shown). This result and the observation that the pCREB-reactive nuclei in the mitral cell layer were, in general, equal to or larger than 10 μm in diameter (in contrast to ∼5–7 μm in the granule cell layer) (see Fig. 2.2A, lower panel) suggest that changes in pCREB optical density in the mitral cell layer are due to changes in mitral cell reactivity rather than to changes in granule cell pCREB. However, using antibodies to positively identify cell types should be considered in future studies to further strengthen this inference.

Finally, we probed the interaction between α_{2}- and β-adrenoceptor activation during early odor-preference learning. We asked whether clonidine infusion would enable learning in animals that receive subthreshold doses of isoproterenol during training. We first replicated the reported inverted U-curve effect of isoproterenol (Sullivan et al., 1991a) by giving PND 6 pups subcutaneous injections of saline or various doses of isoproterenol (1, 1.5, 2, 6 mg/kg, made in

saline). Thirty minutes after injection, pups were removed from the dam and individually placed on unscented clean bedding for a 10-min habituation period and then transferred to peppermint bedding for a 10-min odor exposure.

Odor-preference testing the next day showed that only the moderate dose of isoproterenol, 2 mg/kg, induced learning ($F_{(4,43)} = 2.94$, $P = 0.031$) (Fig. 2.3A). Post-hoc tests showed significant differences between the 2 mg/kg group (56.64% ± 4.48, $n = 9$) and all lower dose groups: saline (34.61% ± 3.73, $n = 13$), 1 mg/kg isoproterenol (35.81% ± 4.53, $n = 9$), and 1.5 mg/kg isoproterenol (40.24% ± 9.10, $n = 8$).

We next tested whether coapplication of clonidine infused bilaterally as described earlier would left-shift the isoproterenol dose curve. A saline infusion-only group was included as a negative control. Co-application of the previously suboptimal 50 μM clonidine enabled odor-preference learning when combined with the previously suboptimal 1.5 mg/kg dose of isoproterenol ($F_{(5,46)} = 2.78$, $P = 0.028$) (Fig. 2.3B). The 1.5 mg/kg group (53.77% ± 3.61, $n = 9$) spent significantly more time over the peppermint bedding than the 2 mg/kg group (31.75% ± 3.69, $n = 7$) and the saline infusion group (38.84% ± 2.09, $n = 13$), which did not differ. These results reveal additive effects of α_2- and β-adrenoceptor activation in the formation of early odor-preference learning.

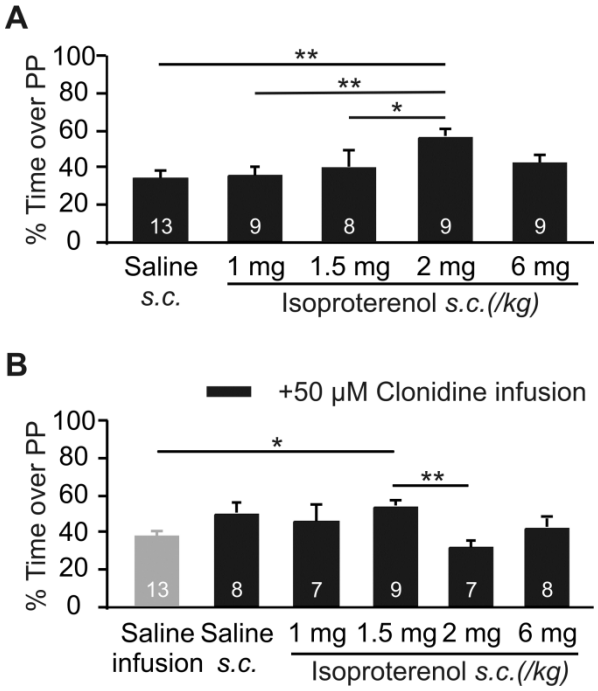

Figure 2.3 α_2-adrenoceptor coactivation enables odor learning with suboptimal doses of isoproterenol

(*A*) Isoproterenol dose dependently induced early odor-preference learning in an inverted U-curve fashion. **(*B*)** Addition of subthreshold 50 µM clonidine enabled odor-preference learning with a subthreshold 1.5-mg/kg dose of isoproterenol. (**) $P < 0.01$; (*) $P < 0.05$. Error bars, mean ± SEM.

2.4 Conclusion and Discussion

Taken together, our pattern of results is consistent with a critical role for the α_2-adrenoceptor in early odor-preference learning and supports the prediction from recent *in vitro* studies demonstrating an α_2-adrenoceptor-mediated disinhibition of mitral cells from granule cells (Pandipati et al., 2010), which would enhance mitral cell excitation and facilitate recruitment of NMDA-mediated plasticity. These effects together with β-adrenoceptor-mediated effects on mitral cells (Hayar et al., 2001; Yuan et al., 2003a; Yuan, 2009; Lethbridge et al., 2012), as well as behavioral evidence (Harley et al., 2006) that systemic α_1-adrenoceptor activation serves as a US for odor-preference in rat pups, argue that US-associated NE release in the olfactory bulb acts through multiple adrenoreceptors to promote optimal plasticity-inducing activation of the odor-encoding mitral cells. Memory for the conditioning odor is likely to be represented as a stronger, sharper, and more synchronized mitral cell output from the olfactory bulb. An increased output synchrony has recently been shown to indicate encoded reward (Doucette et al., 2011). γ frequency enhancement by the α_2-adrenoceptor may confer the reward signature in odor preference learning.

It is not known at this point how the varying forms of adrenoceptor plasticity promotion interact intracellularly. Although the role of disinhibition is well understood, the specific route to CREB phosphorylation in the absence of a cAMP increase in mitral cells remains to be elucidated. The ability to combine subthreshold α_2- and subthreshold β-adrenoceptor activation to induce odor learning suggests a converging intracellular interaction. It will be interesting to examine cAMP changes in the additive model in future experiments.

The amount of NE released as a function of the US is also of considerable interest. Milk infusion (Kucharski and Hall, 1987) and mild shock (Moriceau et al., 2009b) produce longer-lasting memories than those induced by stroking or isoproterenol. We predict that the

concentration of NE released in the vicinity of the bulbar adrenoreceptors determines memory duration by acting through multiple concentration-sensitive receptor subtypes. The inverted U curve associated with β-adrenoceptor activation is well characterized (Sullivan et al., 1991a; Langdon et al., 1997; Yuan et al., 2000). Additionally, at the granule cell-to-mitral cell synapse, NE has differing effects depending on both concentration and developmental stage (Nai et al., 2009; Pandipati et al., 2010). Under natural conditions, NE concentrations likely will favor complex interactions of excitation and inhibition, fine-tuning odor encoding at more than one level.

Finally, whether early odor-preference memory is restricted to the olfactory bulb after initial encoding is unknown. Some evidence suggests stronger memories come to be shared with downstream sites such as the piriform cortex (Kucharski and Hall, 1987)). For this to occur, changes in output synchrony such as those associated with α_2-adrenoceptor activation may be even more important than changes in the strength of olfactory nerve-to-mitral cell firing (Lethbridge et al., 2012).

Chapter-03: Visualizing the Engram: Learning Stabilizes Odor Representations in the Olfactory Network (This chapter is a version of the manuscript published in The Journal of Neuroscience 34(46): 15394-15401, 2014)

3.1 Introduction

The rat pup odor preference learning model is highly attractive as a tractable model of mammalian associative learning. The rodent pup readily acquires preferences for odors paired with maternal care signals to support maternal recognition (Logan et al., 2012). The conditioned stimulus in this associative model is typically a novel odor, whereas the unconditioned stimulus is provided by norepinephrine (NE) release from the locus coeruleus acting through an ensemble of noradrenergic receptors, the best studied of which is the β-adrenoceptor (Yuan et al., 2014). This NE release can be induced by tactile stimulation with a brush to mimic maternal care (Rangel and Leon, 1995). A single trial in which pups on peppermint-scented bedding are stimulated creates a preference for peppermint lasting 24 h, whereas multiple trials spaced over days creates more enduring memories (Fontaine et al., 2013).

Cellular events critical for learning have been identified in both the olfactory bulb (OB) and aPC. Mechanisms for learning include activation of NMDA receptors (NMDARs; (Lethbridge et al., 2012; Morrison et al., 2013)), L-type calcium channels (Jerome et al., 2012), metabotropic glutamatergic receptors (Rumsey et al., 2001), adrenergic receptors (Sullivan et al., 2000b; Harley et al., 2006; Shakhawat et al., 2012; Morrison et al., 2013), and disinhibition (Lethbridge et al., 2012). Intracellular changes critical for learning in the OB include a temporally specific cAMP transient (Cui et al., 2007), activation of protein kinase A (Grimes et al., 2012), phosphorylation of CREB (McLean et al., 1999), and an insertion of AMPA receptors (AMPARs; (Cui et al., 2007).

Changes that relate to long-term memory expression are fewer in number. Visualization methods have shown an increase in intrinsic optical signaling (Yuan et al., 2002), an increase in AMPARs at the glomerular level (Cui et al., 2011), and an increase in network strength in the aPC (Fontaine et al., 2013). Electrophysiological methods have shown potentiation of the olfactory nerve to mitral cell synapse in the OB (Yuan and Harley, 2012) and of the lateral olfactory tract mitral cell output to an aPC pyramidal cell synapse (Fontaine et al., 2013; Morrison et al., 2013).

Maintained increases in AMPAR strength, which have been hard to demonstrate with memory in other systems, have been clearly seen in this model (Fontaine et al., 2013). The commissural connections are not mature in the 1-week-old rat pup, and thus odor input is lateralized both in the OB and piriform cortex (Kucharski et al., 1986b; Kucharski and Hall, 1987; Fontaine et al., 2013). Taking advantage of this within-animal control, AMPAR changes congruent with memory duration were readily revealed (Fontaine et al., 2013).

In the present study, catFISH of *Arc* mRNA was used to identify odor ensemble representations in the OB and aPC of rat pups that had undergone odor preference training with one naris occluded. The outcomes support current views of cortical representations in mammalian brain and suggest stability of cell participation in representations is the signature feature of learning and memory.

3.2 Materials and Methods

3.2.1 Animals

All experiments with animals were approved by the Animal Care Committee of Memorial University of Newfoundland in compliance with the guidelines of the Canadian Council on Animal Care. Sprague Dawley rat pups of both sexes were used in this study. Dams with pups were housed in a vivarium that was temperature controlled and on a 12 h light/dark cycle. The date of birth for the pups was designated postnatal day 0 (PND0).

3.2.2 Early odor preference training

The early odor preference training protocol with single naris occlusion has been established previously (Yuan and Harley, 2012; Fontaine et al., 2013). Rat pups were assigned to one of two conditions: odor paired with stroking (O/S^+) or odor only (O/S^-). Four-day behavioral training was performed from PND3 to PND6. During training, all pups received left naris occlusion for each session. Nose plugs were constructed from polyethelene-20 tubing (Yuan and Harley, 2012; Fontaine et al., 2013). Pups were given a sterile 2% xylocaine gel application on the left naris 5 min before plug insertion. Pups were left to rest for 5 min before subsequently being given either O/S^+ or O/S^- training. During training, pups were placed on peppermint-scented bedding (0.3 ml of peppermint for 500 ml volume of bedding). Pups in the O/S^+ group were simultaneously stroked with a paint brush (30 s stroking interleaved with 30 s rest) for 10 min. Pups in the O/S^- group were placed on peppermint bedding for 10 min without being stroked. Nose plugs were removed immediately after the training, and pups were returned to the dams.

3.2.3 Tissue collection

On PND7, pups were placed into covered plastic jars with charcoal-filtered clean air flow for 1.5 h before being given two 5 min odor deliveries separated by 20 min: either 2× peppermint or peppermint followed by vanillin or 2× vanillin (Fig. 3.1*A*). For odor delivery, pups were moved to an adjacent covered jar with peppermint or vanillin bedding at the bottom (0.3 ml of odor extract mixed with 500 ml of normal bedding) and then switched back to the clean-air jar in the 20 min interval. A naive group was used initially to test odor input specificity. Pups in this group were exposed to two different odors without prior training. For this latter experiment (Fig. 3.1), 1% peppermint or vanillin odor diluted in mineral oil was delivered through the air-delivery system (Knosys olfactometer) for the 5 min odor periods (Shakhawat et al., 2014a).

After the second odor exposure, rats were decapitated, and their brains were flash-frozen in 2-methyl-butane immersed in an ethanol/dry ice slurry. Brains were preserved in a −80°C freezer until being sectioned at 20 μm in a cryostat set at −20°C. Sections of right hemispheres of the animals in the input specificity study and both hemispheres of pups from all other groups were mounted onto 2% 3-aminopropyltriethoxysilane-treated slides (Snowcoat; Leica) using OCT compound (Tissue-Tek; Sakura Fintek USA). Each block usually contained four to six brains from a particular experiment so that these brains were processed together. Five to six slides taken evenly through the rostral to caudal range of the OB and the aPC were used for fluorescent *in situ* hybridization and stored at −20°C.

3.2.4 Fluorescence *in situ* hybridization

The fluorescence *in situ* hybridization protocol used was established previously (Guzowski and Worley, 2001; Shakhawat et al., 2014a). Briefly, *Arc* full-length DNA plasmid was digested using EcoRI (Invitrogen) and run against a DNA ladder to confirm yield and base pair accuracy (~2.5 kb) (Appendix-B). Digoxegenin-labeled riboprobes were synthesized from the digested DNA template using a Maxiscript transcription kit (Ambion). *Arc* antisense riboprobe yields were confirmed using 1% agarose gel electrophoresis. Slides were brought to room temperature, fixed with 4% paraformaldehyde, bathed with acetic anhydride and methanol/acetone (Thermo Fisher Scientific), and treated with prehybridization buffer followed by hybridization buffer (Sigma-Aldrich) and *Arc* riboprobe. Hybridization occurred overnight in a 56°C oven. The next day, after a series of sodium citrate washes, any remaining single-stranded RNA was cleaved using Rnase A (Sigma-Aldrich) at 37°C. Endogenous peroxidases were quenched with H_2O_2, and slides were blocked with 5% sheep serum (Sigma-Aldrich) and incubated with anti-digoxegenin–horseradish peroxidase (Roche) for 2 h. After a series of Tris-buffered saline washes, the Cy3 fluorescent marker (PerkinElmer) was applied to visualize *Arc* mRNA, and nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; 1:2000; Sigma-Aldrich). Finally, slides were covered with Vectashield antifade medium (Vector Laboratories) and sealed with clear nail polish after cover-slipping. Slides were kept at 4°C before confocal microscopy scanning.

3.2.5 Confocal image acquisition

Using an FV1000 confocal microscope (Olympus), optical *z*-sections were taken from both the OB and the aPC. Images of mitral cell layers were taken at 40× with two standardized areas (~0.06 mm^2 each) in the dorsolateral quadrant and two areas in the ventromedial quadrant of the OB (Fig. 3.2*A*). Images of pyramidal cell layers (II/III) of the aPC were taken at 20×. Two standardized-sized areas (~0.3 mm^2 each; one in lateral and one in medial aPC; Fig. 3.4*A*) were scanned. The *z*-stacks (1.0 μm thickness) throughout each section (20 μm) of the OB and the aPC were acquired from three to four slides spread evenly over the rostral to caudal range. Photomultiplier tube assignments, confocal aperture size, and contrast remained constant for each slide. The average counts of the two areas were used for final counts for the dorsolateral and ventromedial OB and for the aPC.

3.2.6 Image analysis

Off-line image analysis was performed using ImageJ software. The total numbers of DAPI cells were assessed using the ImageJ automatic cell-counting application for the aPC and the manual counting option for the OB. Foci, cytoplasmic, and double labeling of *Arc*-positive (Arc^+) cells were counted manually. Labeling of cells as foci, cytoplasmic, and double was achieved by checking multiple optical sections (20% midrange of the *z*-stack) that comprised each individual cell (Miyashita et al., 2009). Counting was performed by an individual blind to all experimental training conditions.

3.2.7 Statistics

OriginPro 9.0 software was used to analyze all data sets. Data were reported as the mean ± SEM. Two-sample paired *t* tests were used for statistical comparisons for all experiments except for the input specificity experiment in Figure 3.1 and the comparison of occluded hemispheres across groups, in which a two-sample unpaired *t* test was used. Differences between groups were considered significant when *p* values were <0.05.

3.3 Results

The immediate-early gene *Arc* has been established as a marker to index plasticity-related neuronal activation in multiple brain areas, including the olfactory cortex (Guzowski et al., 2005; Shakhawat et al., 2014a). Although previous research using Northern blots suggested *Arc* was not expressed early in development in the forebrain (Lyford et al., 1995), the more sensitive *in situ* hybridization technique readily reveals the presence of *Arc* mRNA in our study. *Arc* transcription first appears in the neuronal nucleus within 5 min of neuronal activity. Thirty minutes later, initial *Arc* mRNA has trans-located to the cytoplasm, and a second event can initiate new transcription of nuclear *Arc* (Guzowski et al., 2005). Therefore, *Arc* permits discrimination of two separate odor events through analysis of compartmentalized expression (Fig. 3.1*A*). In the present experiments, we were also able to use *Arc* to examine granule cells, although it is not normally often expressed in inhibitory interneurons (Vazdarjanova et al., 2006; McCurry et al., 2010) and did not occur here in the juxtaglomerular neurons.

Two sets of experiments were included in this study. First, naive rat pups were used to test whether *Arc* can serve as an input-specific activity marker in the OB. Second, rat pups underwent either odor paired with stroking (O/S^+) or odor-only (O/S^-) training and were given 2× peppermint or vanillin before brain extractions (Fig. 3.1*A*).

3.3.1 Odor input specificity in the OB indexed by *Arc* mRNA

To test the odor input specificity of *Arc* activation, naive pups were exposed to two 5 min episodes of odor: either peppermint on both occasions separated by a 25 min interval (Fig. 3.1*A*, top, PP-PP) or peppermint followed by vanillin 25 min later (Fig. 3.1*A*, top, PP-VA). Animals were killed immediately after the second episode and processed for *Arc* catFISH. Cells that expressed *Arc* in the cytoplasm were only active during the first odor episode (peppermint) whereas cells that expressed *Arc* only in the nuclei were active only during the second odor episode (peppermint or vanillin), and cells expressing *Arc* in both the nuclei and cytoplasm were activated by both odor episodes (see example cells in Fig. 3.1*A*, bottom).

Peppermint activated both mitral cells and granule cells in the OB, especially the dorsolateral and ventromedial regions that were previously shown as "hot spots" for peppermint (Johnson and Leon, 1996); Fig. 3.1*B1*). Arc^+ cells in the mitral cell layer were counted in the dorsolateral region of the OB. On average, novel peppermint activated ~7.5% of the cells in the mitral cell layer of the dorsolateral OB, whereas novel vanillin activated ~6.4% of the cells in the same region. Comparing the overlap ratio (OLR; the proportion of cells with double staining relative to the total number of Arc^+ cells) of the cell ensembles activated by two odor events, we demonstrated that repeated peppermint exposure was associated with significantly greater overlap (32.43 ± 1.64%, $n = 4$) than peppermint followed by vanillin exposure (18.73 ± 2.79%, $n = 4$, $t = 4.23$, $p = 0.006$; Fig. 3.1*B2*, *B3*). This experiment suggests that *Arc* mRNA can be used as a marker for input-specific representations of odors in the OB. The same odor is more likely to initiate *Arc* transcription twice in the same cells.

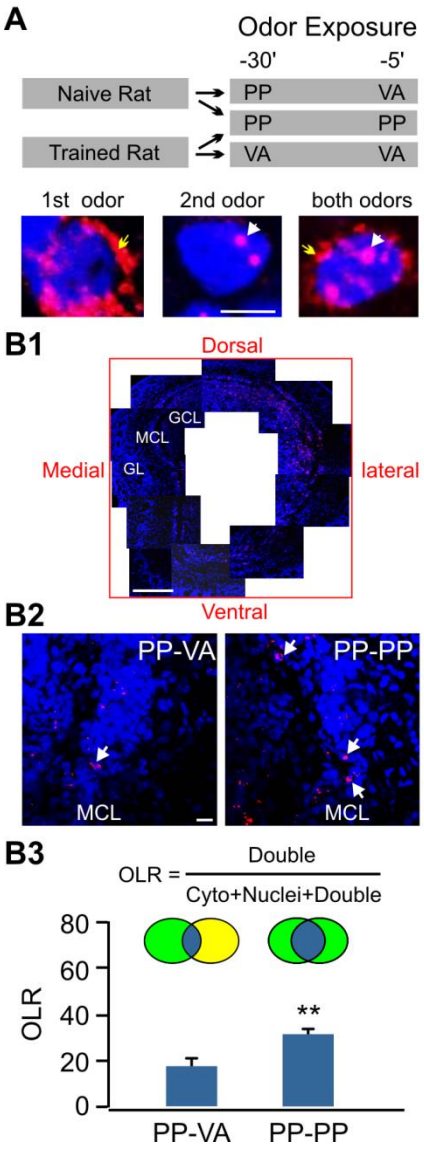

Figure 3.1 Arc mRNA visualization reveals odor input-specific activation of mitral cell ensembles in the OB.

A, Schematic of tissue collection protocols in naive and trained rat pups (top) and example images for Arc^{+} cells (bottom). Blue indicates nuclei staining by DAPI. Red indicates *Arc* staining. White arrows indicate *Arc* staining in nuclei. Yellow arrows indicate *Arc* cytoplasm staining. Scale bar, 10 μm. ***B1,*** Example image of *Arc* expression in the OB of a naive rats exposed to 2× peppermint. GL, Glomerular layer; MCL, mitral cell layer; GCL, granule cell layer. Scale bar, 500 μm. ***B2,*** Example images of dorsolateral OB *Arc* expression in a naive rat pup to two odor episodes. White arrows indicate Arc^{+} double cells in the MCL. Scale bars, 20 μm. ***B3,*** OLRs of the cell ensembles of the two odor episodes. $^{**}p < 0.01$, PP, Peppermint; VA, vanillin.

3.3.2 Odor preference training leads to more stable odor representation in the mitral cell layer of the OB

We next trained rat pups in a multiday (P3–P6) peppermint O/S^+ conditioning with a single naris occluded. The ensembles of neurons responding to peppermint in the OB after training were assessed by *Arc* mRNA expressions induced by two peppermint episodes (Figs. 3.1*A*, 2, PP-PP). The trained OB was compared with the occluded side to achieve an intra-animal control. We have shown that single naris occlusion during multiday training leads to lateralized learning and synaptic changes that are confined to the spared olfactory hemisphere (Yuan and Harley, 2012; Fontaine et al., 2013). O/S^- pups were used as controls to test for any nonspecific effects of repeated odor exposure training.

In the dorsolateral region, the OLR of mitral cell ensembles in the spared OB in the O/S^+ rats was significantly greater (49.01 ± 0.79%) than in the occluded bulb (24.56 ± 1.48%, n = 4, t = 24.84, p = 1.43E^{-4}; Fig. 3.2*B1*, *B2*). After associative learning, mitral cells are activated more reliably by peppermint odor, and the same cell is likely to respond to both episodes of peppermint. Interestingly, the total number of Arc^+ cells activated by two odor events did not change in the spared bulb (11.58 ± 1.39%) compared with the occluded one (12.05 ± 1.72%, n = 4, t = 0.276, p = 0.80; Fig. 3.2*B3*). However, double-stained Arc^+ cells were significantly increased after O/S^+ learning (5.67 ± 0.69% in the spared bulb vs 3.01 ± 0.57% in the occluded bulb; n = 4, t = 4.29, p = 0.02; Fig. 3.2*B3*). The percentage of single-stained Arc^+ cells responding to either episode of peppermint showed a trend toward decreasing in the spared OB but did not reach statistical significance (5.91 ± 0.72% in the spared bulb vs 9.04 ± 1.19% in the occluded bulb; n = 4, t = 2.75, p = 0.07). The increase in double cells that are likely strongly activated by

peppermint suggests odor preference learning in rat pups results in the potentiation of previously weakly activated cells.

The OLR for O/S$^-$ rats was not different between the two bulbs (spared, 33.65 ± 0.93%; occluded, 34.22 ± 2.42%; n = 3, t = 0.17,p = 0.88; Fig. 3.2*C1*,*C2*), suggesting no effect of odor exposure itself on initial odor ensemble representation. Consistently, no differences were observed in the numbers of cells expressing *Arc* in any compartment (Fig. 3.2*C3*).

Peppermint representation in the ventromedial OB revealed the same trends. In the O/S$^+$ pups, the OLR of mitral cell ensembles was greater in the spared OB (45.07 ± 3.59%) than in the occluded bulb (24.40 ± 2.22%; n = 4, t = 5.49, p = 0.01; Fig. 3.2*D1*, *D2*). Consistent with the dorsolateral region, the double-stained *Arc*$^+$ cells increased after O/S$^+$ learning (6.26 ± 1.52% in the spared bulb vs 2.57 ± 0.34% in the occluded bulb; n = 4, t = 3.07, p = 0.05; Fig. 3.2*D3*), whereas the total *Arc*$^+$ cells and single-stained *Arc*$^+$ cells were not different in the two bulbs (Fig. 2*D3*). In O/S$^-$ pups, neither the OLR of cell ensembles (Fig. 3.2*E1*, *E2*) nor the numbers of *Arc*$^+$ cells (Fig. 3.2*E3*) are different in the ventromedial OB.

An unexpected outcome was a significant reduction in the OLR of the peppermint representation in the occluded OB in the O/S$^+$ group compared with that in the O/S$^-$ group (t = 3.60, p = 0.02, unpaired t test). This may relate to a backward conditioning effect when the naris plug was removed and residual peppermint odor remained on the pup. Such an effect might be expected to reduce the stability of peppermint encoding.

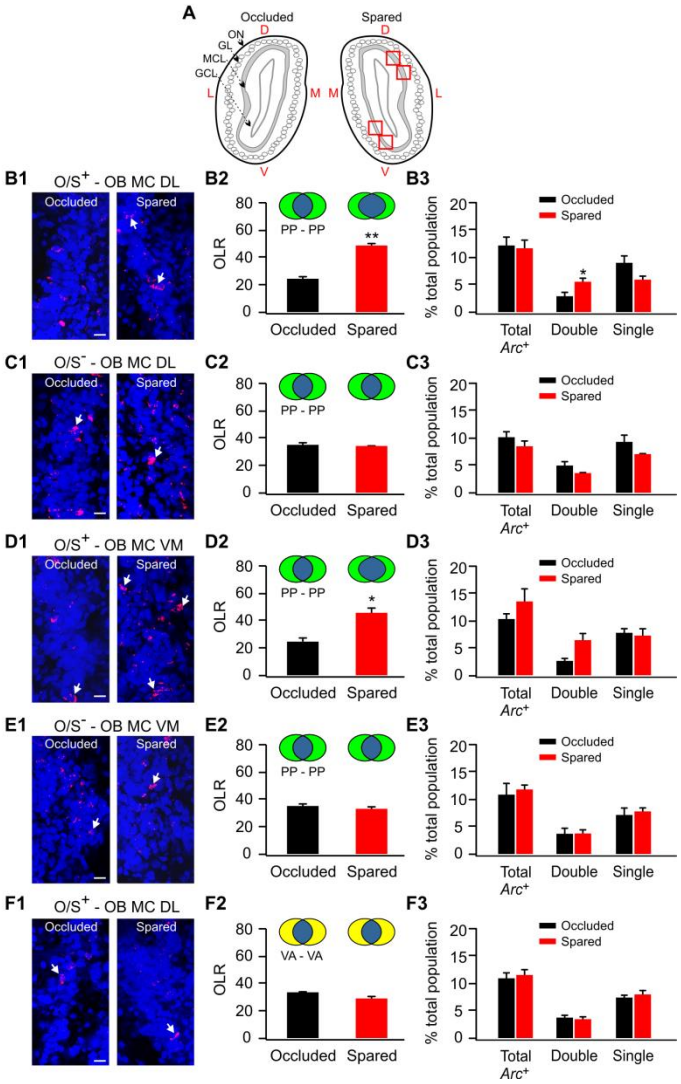

Figure 3.2 Early odor preference learning stabilizes the mitral cell ensemble to the conditioned odor in the OB.

A, Schematic of OB anatomy and *Arc* sampling regions (red rectangles). D, Dorsal; V, ventral; L, lateral; M, medial; ON, olfactory nerve; GL, glomerular layer; MCL, mitral cell layer; GCL, granule cell layer.*B1–B3*, O/S$^+$ training leads to increased overlap of mitral cell ensembles in the dorsolateral olfactory bulb responding to 2× peppermint exposures. ***B1,*** Example images of the mitral cell layer in the occluded and spared olfactory bulbs from the same animal. ***B2,*** OLR of mitral cell ensembles responding to 2× peppermint exposures. ***B3,*** Percentage of *Arc*$^+$ cells over the total population indexed by DAPI staining.***C1–C3***, O/S$^-$ training does not change the OLR of mitral cell ensembles in the dorsolateral OB responding to 2× peppermint exposures. ***D1–D3,*** O/S$^+$ training leads to increased overlap of mitral cell ensembles in the ventromedial OB responding to 2× peppermint exposures. *E1–E3*, O/S$^-$ training does not change the OLR of mitral cell ensembles in the ventromedial OB responding to 2× peppermint exposures. *F1–F3*, O/S$^+$ training with peppermint does not change the OLR of mitral cell ensembles in the dorsolateral OB responding to 2× vanillin exposures. MC, Mitral cell; DL, dorsolateral; VM, ventraomedial; PP, peppermint; VA, vanillin. Arrows indicate double-stained *Arc*$^+$ cells. Scale bars, 20 μm. **p*< 0.05; ***p*< 0.01.

3.3.3 Mitral cell ensemble stabilization is specific to the conditioned odor

In another set of experiments, we examined dorsolateral OB Arc^+ mitral cell ensembles to vanillin after O/S^+ training with peppermint (Fig. 3.2*F*). The OLR (29.48 ± 1.71% in the spared bulb vs 33.14 ± 0.85% in the occluded bulb; $n = 5$, $t = 2.19$, $p = 0.10$; Fig. 3.2*F1*,*F2*) and the pattern of *Arc* expression (Fig. 3.2*F3*) were not different between the spared and occluded bulbs. This demonstrates that odor learning is input specific in the OB such that only the representation of the conditioned odor is altered.

3.3.4 Odor preference training also results in a more stable odor representation in the underlying granule cells of the OB

We next compared the granule cell ensembles in the OB granule cell layer after O/S^+ training. The areas of interest were taken from the same rectangle regions where we measured cell ensembles in the mitral cell layers. Granule cell ensembles in the dorsolateral region showed greater OLR in the spared OB (48.07 ± 2.99) compared with the occluded OB (24.40 ± 2.43; $n = 4$, $t = 4.56$, $p = 0.02$; Fig. 3.3*A1*, *A2*). The total Arc^+ cells (7.25 ± 0.32% in the spared bulb vs 8.31 ± 1.60% in the occluded bulb; $n = 4$,$t = 0.60$, $p = 0.59$) and double-stained Arc^+ cells (7.25 ± 0.32% in the spared bulb vs 8.31 ± 1.60% in the occluded bulb; $n = 4$, $t = 0.60$, $p = 0.59$; Fig. 3.3*A3*) were not different in the two OBs. However, the single-stained Arc^+ cells showed a trend of decreased numbers in the spared OB (3.76 ± 0.21) compared with the occluded OB (6.20 ± 1.01; $n = 4$, $t = 2.67$, $p = 0.076$; Fig. 3.3*A3*). There were no differences in either OLR or Arc^+ cell numbers in the ventromedial region of the OB (Fig. 3.3*B1–B3*). Changes in granule cell ensembles are also training odor specific. Neither the OLR nor numbers of Arc^+ cells were different in the spared and occluded OB in the dorsolateral regions to the control odor vanillin (Fig. 3.3*C1–C3*).

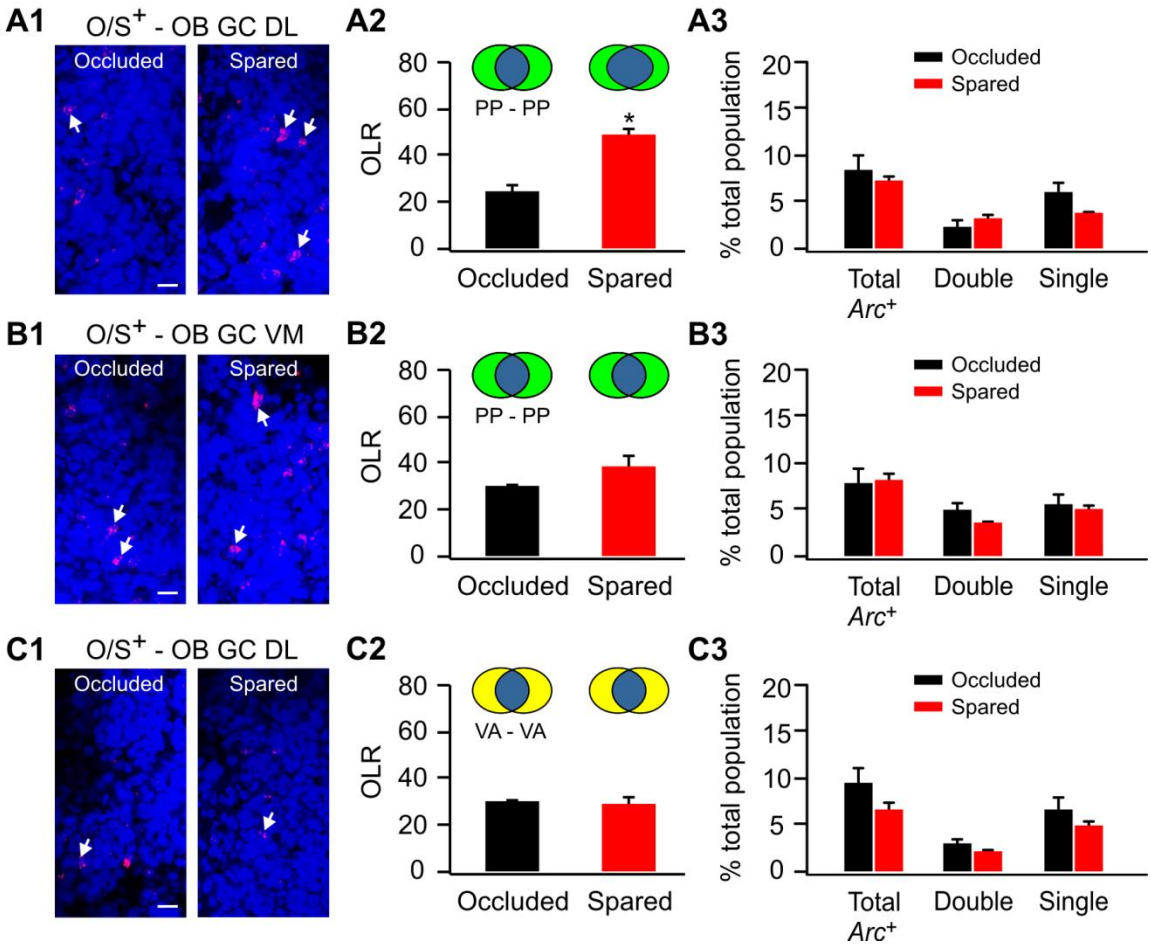

Figure 3.3 Early odor preference learning stabilizes the granule cell ensemble to the conditioned odor in the OB.

A1–A3, O/S$^+$ training leads to increased overlap of granule cell ensembles in the dorsolateral olfactory bulb responding to 2× peppermint exposures. *A1*, Example images of the granule cell layer in the occluded and spared olfactory bulbs from the same animal. *A2*, OLR of granule cell ensembles responding to 2× peppermint exposures. *A3*, Percentage of *Arc*$^+$ cells over the total population indexed by DAPI staining.*B1–B3*, O/S$^+$ training does not change the OLR of granule cell ensembles at the ventromedial OB responding to 2× peppermint exposures. *C1–C3*, O/S$^+$ training with peppermint does not change the OLR of granule cell ensembles at the dorsolateral OB responding to 2× vanillin exposures. GC, Granule cell; DL, dorsolateral; VM, ventromedial; PP, peppermint; VA, vanillin. Arrows indicate double-stained *Arc*$^+$ cells. Scale bars, 20 μm. *p< 0.05.

3.3.5 A more stable odor map in the aPC

We have previously shown that the OB and the aPC are both involved in, and support, early odor preference learning (Lethbridge et al., 2012; Yuan and Harley, 2012; Fontaine et al., 2013; Morrison et al., 2013). We examined pyramidal cell ensemble changes in the aPC after early odor preference learning from the same animals as in the OB experiments. Single-odor exposure activates ~1% pyramidal cells in the aPC. Similar to mitral cell ensembles in the OB, the stability of the odor representation as indexed by the OLR of pyramidal ensembles in the spared aPC (35.74 ± 2.38%) was significantly greater than that in the occluded one (18.44 ± 2.62%; $n = 4$, $t = 7.84$, $p = 0.004$; Fig. 3.4*B1*,*B2*). The increase in the overlap ratio was caused by an increased number of double-stained Arc^+ pyramidal cells (0.75 ± 0.12% in the spared hemisphere vs 0.45 ± 0.12% in the occluded side; $n = 4$, $t = 3.45$, $p = 0.04$), whereas the total number of Arc^+ cells to two odor events and the single-stained Arc^+ cells were not different in two hemispheres (Fig. 3.4*B3*). Odor experience alone did not alter either the overlap of the two peppermint ensembles (Fig. 3.4*C1*, *C2*) or the numbers of Arc^+ cells activated (Fig. 3.4*C3*).

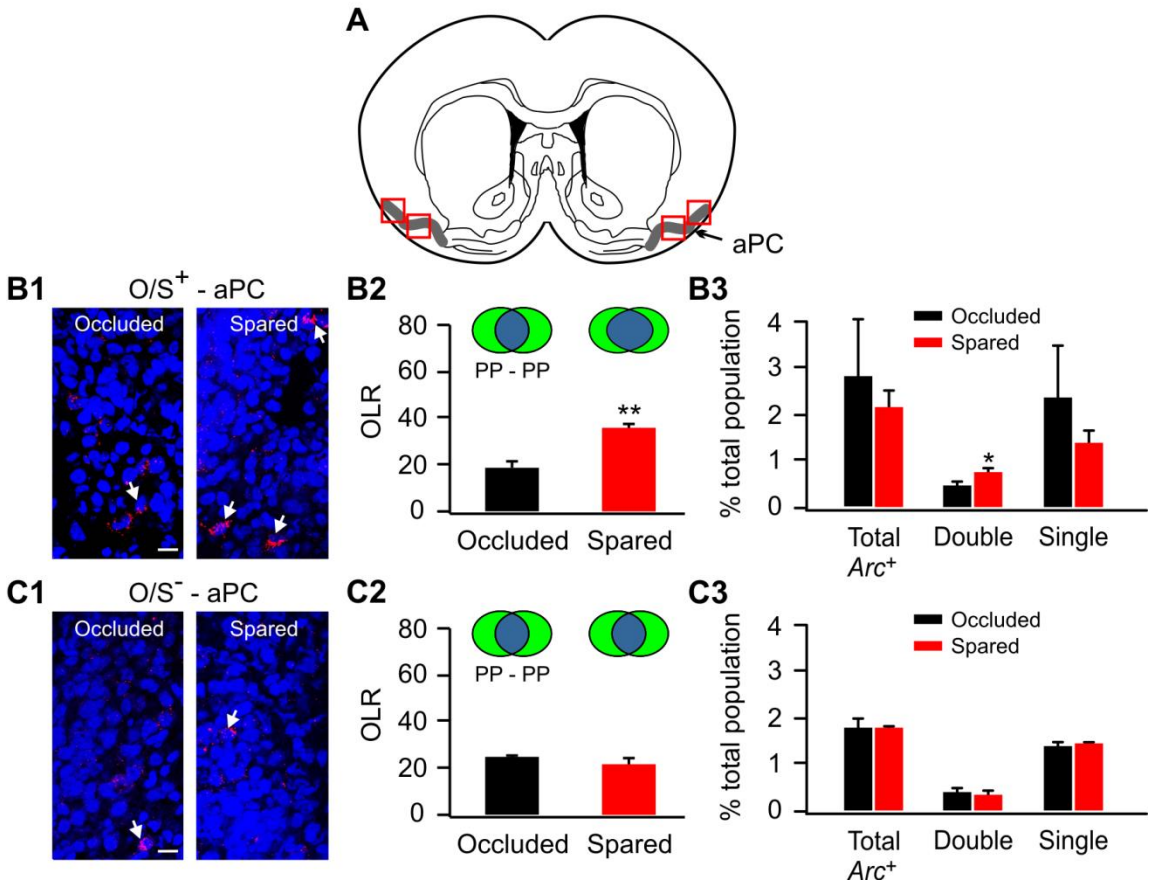

Figure 3.4 Early odor preference learning stabilizes the odor map for the conditioned odor in the aPC.

(A) Schematic of aPC and *Arc* sampling regions (indicated by red rectangles). **(B1-B3)** O/S^+ training leads to increased overlap of pyramidal cell ensembles in the aPC responding to two times peppermint exposures. **B1**, example images of pyramidal cell layer in the occluded and spared olfactory bulbs from the same animal. **B2**, OLR of pyramidal cell ensembles responding to two times peppermint exposures. **B3**, percentage of Arc^+ cells over the total population indexed by DAPI staining. **(C1-C3)** O/S^- training does not change OLR of pyramidal cell ensembles in the aPC responding to two times peppermint exposures. PP: peppermint Arrows indicate double stained Arc^+ cells. Scale bars: 20 μm. * $p < 0.05$; ** $p < 0.01$.

3.4 Discussion

3.4.1 The nature of representations

Cortical representations are known to be both sparse, reflecting a dynamic balance of excitatory and inhibitory inputs, and variable (Shadlen and Newsome, 1998; Olshausen and Field, 2004). These characteristics are thought to account for the large storage capacity of mammalian brain and reflect the dynamic aspects of its operation. Although representation in the OB itself is more like that of sensory cortices in having a spatial organization such that we are able to target representational regions, the aPC behaves like the general associative cortical model (Johnson et al., 2000). Compared with adult aPC (Shakhawat et al., 2014a), the odor ensembles in rat pup aPC were significantly smaller (~3% vs ~1%). We suggest this difference relates directly to the maturation of lateral olfactory tract input to the piriform cortex, which is about one-third of the adult value at this age (Sarma et al., 2011). Earlier estimates of piriform ensemble size have been substantially larger (Poo and Isaacson, 2009; Stettler and Axel, 2009), but this is likely a function of probing ensembles in the anesthetized versus awake state (Kato et al., 2012). The present values derive from ensemble measurements in awake animals.

Rat pups have similar numbers of piriform pyramidal cells as the adult (Sarma et al., 2011), and so dividing total piriform stereological counts (Capurso et al., 1997; Duffell et al., 2000) in half provides an estimate of ~150,000 cells available in each hemisphere to participate in aPC representations. Thus, a 1% representation (~1500 cells) is well above the calculated threshold of 500 piriform pyramidal cells required to reliably drive odor preference behavior (Choi et al., 2011) and identical to the percentage of Kenyon cells estimated to underlie odor ensembles in the mushroom body of the insect (Campbell et al., 2013). It would be interesting if ensemble size was conserved in nervous system evolution.

A curious aspect of the present data is the finding of an ~15–20% overlap among unrelated odors, which is substantially larger than a 0.5% overlap that would be predicted from the size of each odor's representation (~7%) by random draw with replacement. We suggest that this overlap reflects the existence of an active subset of cortical neurons that are primed to participate in any representation in a given time window (Yassin et al., 2010; Luczak and Maclean, 2012; Mizuseki and Buzsaki, 2013; Klinshov et al., 2014). Such primed subsets require a reconfiguration of our normal thinking about distributed random neural networks.

The first conclusion that can be made about granule cell participation in odor ensembles from these data is that it appears to be odor specific, arguing for different mitral cell/granule cell ensembles for different odors. Johnson and Leon (1996), using 2-deoxyglucose (2-DG), showed that peppermint activates two hot spots in the glomerular layer of the OB, one in the dorsolateral region and one in the ventromedial region. Early preference learning predominately enhanced 2-DG activation in the dorsolateral glomerular region (Johnson and Leon, 1996) and phosphorylated CREB in the dorsolateral mitral cell layer (McLean et al., 1999), consistent with the more prominent change in the *Arc* expression of mitral and granule cells in dorsolateral region. In 19-d-old rats, c-Fos granule cells significantly decrease with odor learning (Woo et al., 1996), as do Arc^{+} pyramidal cells in piriform cortex of adult rats (Shakhawat et al., 2014a). The lack of a decrease here in either area is likely related to age.

The second conclusion is that, like mitral cells, the granule cell representation of an odor increases its stability after the pairing with stroking reward. This parallel change in the granule cell and mitral cell ensembles is consistent with the idea that changes in excitation in any cortical system will be accompanied by balanced inhibition (Isaacson and Scanziani, 2011; Saar et al., 2012; Xue et al., 2014). Mitral cells driving granule cells provides the most parsimonious account

of these effects, and if that is the case, it again underlines a highly selective relationship among mitral and granule cells. Consistent with such selectivity, electrical coupling between mitral cells and nearby underlying granule cells has been reported in rat pups (Paternostro et al., 1995). Feedback effects from aPC that drive granule cell inhibition for individual odors has also been demonstrated (Restrepo et al., 2009; Boyd et al., 2012) and is another possible source of support for the parallel stability increases observed in the granule cell ensembles.

Overall, the striking feature of the learning-related changes in odor representation observed in these experiments is the increase in the stability of ensemble representation from ~25% to 49% in the OB and from ~18% to ~35% in the aPC. The level of overlap after our odor reward pairings in the OB is similar to what has been observed using *Arc* to identify representations of repeated strong visual input in secondary visual cortex (50% overlap (Rudinskiy et al., 2012). This similarity of overlap levels in sensory stimuli for rewarded odor and for strong visual stimulation is consistent with data showing odor learning modifies OB responses to be similar to responses to a higher concentration of odorant (Abraham et al., 2014). Recent modeling work on cortical system representations argues that the stability parameter in population vectors is critical for adaptive behavior (Montijn et al., 2014). These changes in the responses to simple odorants were not able to be previously characterized using electrophysiological methods to probe representations (Chapuis and Wilson, 2012).

3.4.2 Generality of the rat pup model

There are many parallels between the rat pup odor preference model and adult odor associative learning models. Adult aPC ensembles also show the stabilization effect of learning and memory, but the number of neurons participating in an ensemble becomes somewhat sparser

than before learning (Shakhawat et al., 2014a), whereas that number did not change in the rat pup. In neither model does enlargement of the rewarded representation occur; this is consistent with data suggesting enlargement of sensory representations does not account for long-term memory even when it is seen (Reed et al., 2011). However, multiple groups have reported enlarged OB glomerular representations with learning in both rodent pups and adults (Woo et al., 1987; Johnson and Leon, 1996; Abraham et al., 2014). We have also described such a glomerular effect using intrinsic optical imaging in the odor preference learning model (Yuan et al., 2002), and as mentioned earlier, these effects are similar to those of increasing concentrations of the odorant (Abraham et al., 2014). Both enhanced glomerular input and increased stability of principal neuronal network representations should serve to create a stronger and more discriminable experiential input.

The machinery for NE to act as an unconditioned stimulus in the rat pup (Yuan et al., 2014) remains in the OB of older rats, and recent data suggest that blocking both α- and β-adrenoreceptors in the adult OB prevents discrimination of similar odors (Doucette et al., 2007; Mandairon et al., 2008b). Whereas NE via β-adrenoceptor activation also mediates early odor learning in rat pup aPC (Morrison et al., 2013), as in rat pup OB, the role of NE projections and the function of NE in the aPC in adult rat odor learning requires future investigation. NMDARs and L-type calcium channels are critical in the OB as calcium sources mediating plasticity (Jerome et al., 2012; Lethbridge et al., 2012), and they are likely involved in aPC plasticity and aPC-mediated learning (Morrison et al., 2013), as both NMDARs and L-type calcium channels are critical mediators for *Arc* activation in the hippocampus (Bateup et al., 2013).

The cellular and intracellular supports of rat pup learning and memory (see Introduction) are also those implicated in invertebrate and vertebrate associative learning and appear central for learning and memory in mammalian brain across the life span.

3.4.3 *Arc* and plasticity

Arc here identifies the neurons participating in responding to odors, with the advantage of capturing the ensembles to the same odor twice. Neurons either alter their firing rate or increase their firing reliability to an odor after learning (Doucette et al., 2011). Increases in neuronal firing reliability translate into a tighter overlap in the condition in which an animal receives the same odor twice. Our data from both rat pups and adults (Shakhawat et al., 2014a) suggest that the probability that weakly activated cells transcribe *Arc* twice is lower before conditioning than after.

Arc is also part of the plasticity story. Others have suggested that CREB and/or immediate-early genes like *Arc* identify neurons that are primed to participate in memory ensembles (Han et al., 2007; Yiu et al., 2014). *Arc* has recently been shown to promote thin spine production as sites for connectivity strengthening while homeostatically downregulating weaker connections (Peebles et al., 2010). *Arc*-negative mice show neither depression nor potentiation as a function of visual experience, whereas with olfactory experience, both potentiation and depression operate and are hypothesized to modulate the aPC ensemble changes across pups and adults (Saar et al., 2012; Yuan et al., 2014). Thin spine growth, as reported in hippocampus, may contribute to lasting ensemble strengthening in aPC, as well as among OB granule cells.

Chapter-04: Arc Visualization of odor objects reveals experience-dependent ensemble sharpening, separation, and merging in anterior piriform cortex in adult rat (This chapter is a version of the manuscript published in The Journal of Neuroscience 34(31): 10206-10, 2014)

4.1 Introduction

In the brain, the activity of ensembles of neurons represents features of the external world. However, how experience modifies neuronal activity patterns to influence our perceptions and memories remains elusive. The aPC is a prototypical ensemble-encoding network in the mammalian brain. In the adult rodent aPC, spatially organized inputs from the olfactory bulb activate layer II/III pyramidal neurons throughout the cortex to create "odor objects" lacking spatial order (Wilson and Sullivan, 2011). Odor experience readily modifies pyramidal cell properties in the aPC (Chapuis and Wilson, 2012; Saar et al., 2012; Morrison et al., 2013). Hence, it provides us with a model system for studying plasticity processes in an associative cortex and, here, using immediate early gene activation techniques permits us to visualize experience-dependent remodeling of perceptual objects.

In the present experiments, we use cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH) to visualize activation of the immediate early gene *Arc* and directly assess ensemble encoding of odors in aPC. Our results provide images of ensemble pattern reorganizations in appetitive learning paradigms that support, or require, such changes in the odor objects for behavioral success. All odor discriminations required only a few days of training before *Arc* visualization. The reward-contingent changes in representations are not seen in animals given random odor and reward associations over the same time intervals.

4.2 Materials and Methods

4.2.1 Animals

Sprague Dawley rats (8–10 weeks old) of both sexes (n = 54 total) were assigned randomly to groups. Rats were housed in polycarbonate cages (at least two same-sex rats per cage) on a 12 h light/dark cycle with food and water *ad libitum* except during behavioral experiments. Rats were adapted to 1 h of water access daily for 4–5 d before behavioral training. During conditioning, rats were given 25 ml water daily. All procedures were approved by the Memorial University Institutional Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

4.2.2 Odorants

Odorants (Sigma-Aldrich) were diluted with mineral oil to specific concentrations. Concentrations were chosen as recommended for mice (Bodyak and Slotnick, 1999). Odorants (10 ml) were freshly prepared for each experiment. Odorants used were 2% by volume of peppermint, 1% of vanillin, 2% of peppermint plus vanillin (50:50 mixture), 2% of amyl acetate, 2% of 1-heptanol plus 1-octanol (a 53:47 mixture), and 2% 1-heptanol plus 1-octanol (a 55:45 mixture). The latter two odors were used previously by Doucette et al. (2007), whereas the other odors have all been used in early odor preference learning (Yuan et al., 2002; Mukherjee et al., 2014).

4.2.3 Behavioral Apparatus

All behavior training was conducted in a Knosys olfactometer. Discrimination training methods were as described previously (Slotnick and Restrepo, 2005). Polyvinyl carbonate bottles

were used for each odor. The C-flex tubing used by the control pinch valves was changed for each new odor.

4.2.4 Olfactometer rule learning

Initially, rats were trained in the IN-BEGIN program for 3 d. Odor sampling and water delivery were given through the same port. For the first 20–30 trials, snout insertion breaking the light beam activated water delivery, and 30 μl of water were delivered for each lick. Subsequently, odor delivery on the spout signaled availability of water. The separation between odor delivery and water availability increased from 0.05 to 1 s over trials. Positive odors (S^+) were introduced for 2.5 s. The subject could respond by either licking a minimum of six times for water reward or rejecting the odor. A 5 s intertrial interval was used. Rats underwent 100 trials/d and rapidly acquired this behavior.

4.2.5 Odor discrimination training and testing

Odor discrimination was conducted using the IN-D2 software and consisted of introducing a negative odor (S^-) in addition to the S^+. Intertrial intervals were fixed at 6 s, during which rats were unable to initiate trials. To initiate a trial, rats were required to leave the port for 1 s. If the response criterion was met, reward was given after S^+ delivery or withheld after S^-. After training blocks of 20 trials (10 S^+ and 10 S^- odors randomly delivered), rats refrained from licking in response to the S^- odor. Rats in the random groups completed the same number of trials as the associative groups, but water was delivered randomly and they were not required to discriminate between the two odors.

Performance was evaluated in each block of 20 trials. The equation (*n* positive responses to S^+ + *n* negative responses to S^-)/20 × 100 was used to determine the percentage of correct responses. Rats reaching ≥85% correct responses over three blocks were considered successful learners (Belnoue et al., 2011). Two to 3 d were typically required to achieve this criterion.

Two untrained control groups were also examined. A group used only in Experiment 1 consisted of naive rats exposed to the peppermint and vanillin in the same manner as experimental rats before they were killed. In addition, a group of caged rats receiving daily water similar to that received by the trained rats and exposed to clean charcoal-filtered air for 1.5 h before being killed were used to estimate the background "noise" level of *Arc* expression. Background *Arc*-expression (Arc^+) was very low (0.13 ± 0.03% of cells, $n = 5$); therefore, the subtraction of the noise *Arc* level was omitted in our experimental calculations.

4.2.6 Brain collection and dissection

Rats were killed 24 h after discrimination training. Individual rats were put in a covered plastic jar connected to the olfactometer air delivery channel. Rats were exposed to clean charcoal-filtered air for 1.5 h before odors were delivered via C-flex tubing from the olfactometer for a 5 min period. Two 5 min odor deliveries were interleaved by 20 min. Rats were quickly anesthetized by isoflurane and decapitated, and brains were rapidly removed (~2 min) and flash frozen in 2-methylbutane immersed in ethanol/dry ice slurry. Brains were preserved in a −80°C freezer until cryosectioned for *in situ* hybridization.

4.2.7 Tissue processing

Right hemispheres were used during tissue sectioning. Each block usually contained four to six hemisections to include all the behavioral groups from a particular experiment. OCT medium (Tissue-Tek) was used to mold brains together in the same block. Coronal tissue sections (20 μm) were collected every 200 μm on 2% 3-aminopropyltriethoxysilane-treated slides (Snowcoat; Leica) using a cryostat set at −20°C. Five to six slides (taken evenly through the rostral-to-caudal range of the aPC) were taken for fluorescent *in situ* hybridization and stored at −20°C.

4.2.8 Fluorescence *in situ* hybridization

The fluorescent *in situ* hybridization method used was described previously (Guzowski and Worley, 2001). In short, digoxigenin-conjugated full-length *Arc* riboprobes were extracted using a commercial transcript kit (Ambion). The yield and integrity of the riboprobes were ensured by purifying on a mini quick-spin RNA column (Roche Diagnostics), and 2 μl of probe was subjected to gel electrophoresis analysis before use (Appendix-B). Slides were removed from the freezer and thawed for 10–15 min at room temperature before fixing in 4% paraformaldehyde. After fixation, slides were bathed in acetic anhydride and then treated in a 1:1 methanol/acetone (−20°C) solution. Prehybridization buffer was applied to the slides, which were then incubated for 60 min in a humid chamber. Thereafter, slides were incubated overnight with 100 ng of *Arc* probe in a hybridization oven at 56°C. All solutions used for first-day *in situ* hybridization were made in Diethylpyrocarbonate (DEPC, OmniPur)-treated water (0.1%). The next day, slides were washed in a series of Saline-Sodium Citrate (SSC) buffers, treated with RNase A at 37°C, submerged in 2% H_2O_2/SSC buffer solution, blocked with normal sheep serum, and incubated with anti-

digoxigenin–horseradish peroxidase antibody (Roche Diagnostics) overnight at 4°C. The following day, slides were labeled with Cy3 (1:50) using a tyramide signal amplification labeling kit (PerkinElmer Life and Analytical Sciences). Subsequently, cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; 1:2000; Sigma-Aldrich). Finally, sections were coated by applying Vectashield antifade medium (Vector Laboratories). Slides were cover slipped and sealed with clear nail polish.

4.2.9 Image acquisition

Image stacks were collected using an Olympus Fluoview FV1000 confocal microscope as described previously (Guzowski and Worley, 2001). Briefly, images of pyramidal cell layers (II/III) were taken at 20× with photomultiplier tube assignments, confocal aperture size, and contrast remaining constant for each slide. Two standardized-sized areas (~0.8 mm^2 each; one in lateral and one in medial aPC) were scanned. *Z*-stacks (1.0 μm optical thickness) throughout the thickness (20 μm) of each section of lateral and medial aPC were acquired from three to four slides spread evenly over the rostral-to-caudal range. The average count of the lateral and medial regions was used for the final count.

4.2.10 Image analysis

Offline image analysis was performed using NIH ImageJ software. The total numbers of DAPI cells were assessed using the NIH ImageJ automatic cell counting application. Foci, cytoplasmic, and double labeling of *Arc* were counted manually. Labeling of cells as foci, cytoplasmic, and double was achieved by checking multiple optical sections (20% mid-range of the *Z*-stack) that comprised each individual cell (Miyashita et al., 2009). Counting was performed

by the same individual throughout the experiment to maintain consistency. In a subset of animals, a second individual blind to conditions performed counts for comparison after work with a standardized set for visual training. Observations were highly consistent across the two observers (Appendix-F).

4.2.11 Statistics

OriginPro 9.0 software was used to analyze all datasets. Data were reported as mean ± SEM. Two-sample, two-tailed Student's *t* tests were used for statistical comparisons. Differences between groups were considered significant when *p* values were <0.05.

4.3 Results

Arc mRNA appears first in the nucleus within 5 min of neuronal activity that engages its transcription. Twenty five minutes later, initial *Arc* mRNA has translocated to the cytoplasm and a second event can initiate new transcription of nuclear *Arc* (Guzowski et al., 2005). The *in situ* hybridization methodology permits comparison of two separate odor events.

4.3.1 Odor input specificity of Arc catFISH

We initially exposed naive rats to two 5 min episodes of odor, either peppermint followed 25 min later by vanillin or peppermint on both occasions (Fig. 4.1a1, top). Animals were killed immediately after the second episode and processed for *Arc* catFISH. Cells that expressed *Arc* in the cytoplasm only were active during the first odor episode (peppermint), whereas cells that expressed *Arc* only in the nuclei were active during the second odor episode, and cells expressing *Arc* in both nuclei and cytoplasm were activated by both odor episodes (Fig. 4.1*a1*,

bottom). Comparing the overlap ratio (the proportion of cells with double staining relative to the total number of Arc^+ cells) demonstrates that repeated peppermint exposure was associated with significantly greater overlap (25.68 ± 2.11%, $n = 7$) than peppermint/vanillin exposure (17.85 ± 2.84%, $n = 7$, $t = 2.21$, $p = 0.047$; Fig. 4.1*a2*). In any given exposure, the total number of cells that were Arc^+ was ∼5% of the total neurons. This proportion is consistent with previous estimates of aPC representations of odor encoding (Poo and Isaacson, 2009; Stettler and Axel, 2009) and typical of the sparse encoding of cortical structures generally (Olshausen and Field, 2004).

4.3.2 Sharpening of the odor map by positive associative training

To assess the representation of odor memories in aPC, we water deprived rats and trained them in a go–no-go discrimination task in which a positive odor stimulus (S^+) was paired with water reward and a negative odor stimulus (S^-) was unrewarded. Control rats received random rewards with exposure to either odor. A correct response was defined as licking only in the presence of the rewarded odor or not licking in the presence of the unrewarded odor.

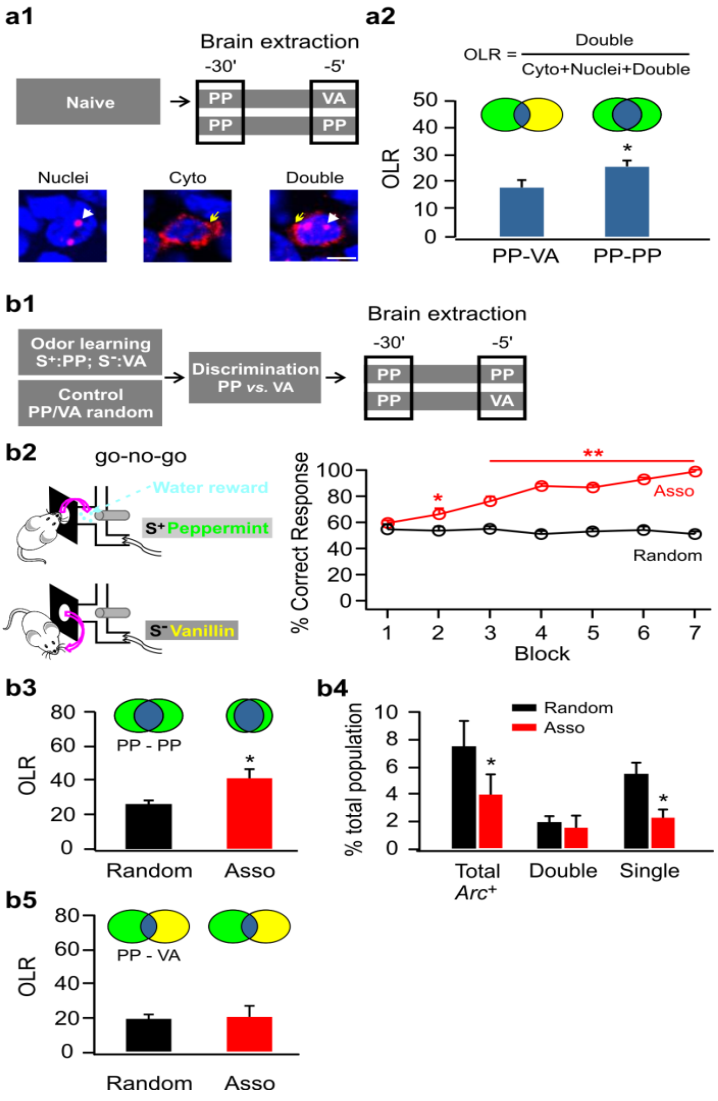

Figure 4.1 Contrast enhancements after odor associative learning

a1, Schematic of brain extraction protocol in naive rats (top) and example images for *Arc*$^+$ cells (bottom). Blue indicates nuclei staining by DAPI. Red indicates *Arc* staining. White arrows indicate *Arc* staining in nuclei. Yellow arrows indicate *Arc* cytoplasm staining. Scale bar, 10 μm. ***a2,*** Overlap ratios (OLRs) of the cell ensembles of the two odor episodes. Cyto, cytoplasmic; PP, peppermint; VA, vanillin.***b1,*** Schematic of odor associative training and brain extraction protocol.***b2,*** Go–no-go behavioral paradigm (left) and percentage correct responses in the associative (Asso) group and the random group (right). ***b3,*** OLRs of the cell ensembles representing two peppermint episodes.***b4,*** Percentage *Arc*$^+$ cells over the number of total cells measured by DAPI staining. ***b5,*** OLRs of the cell ensembles representing two different odor episodes (peppermint and vanillin).*p< 0.05, **p< 0.01.

In the first discrimination experiment, rats were trained with peppermint as S^+ and vanillin as S^- (Fig. 4.1*b1*). Rats quickly learned within the first three blocks (20 trials each; randomized 10 S^+/10 S^-) to lick at the water port only in the presence of peppermint (n = 10, t = 5.07, p = 8.01E^{-5} compared with the random group). Twenty-four hours after the seventh block when discrimination was nearly perfect (98 ± 1.53%, t = 18.67, p = 3.15E^{-13}; Fig. 4.1*b2*), a subset of rats were given two episodes of peppermint exposure and killed for catFISH. The overlap ratio of cell ensembles in the S^+ associative rats was significantly greater (41.01 ± 5.67%) than in the random group (25.58 ± 3.15%, n = 5, t = 2.38, p = 0.045; Fig. 4.1*b3*). The overlap ratio of the random rats was not different from naive rats (25.68 ± 2.11%, n = 7; Fig. 4.1*a2*), suggesting no effect of random pairings on initial ensembles. After associative learning, pyramidal cells are activated more reliably by peppermint odor and the same cell is likely to respond to both episodes of peppermint. The total *Arc*$^+$ cells were fewer in the associative group (3.94 ± 0.56%, n = 5) relative to those in the random group (7.48 ± 1.08%, n = 5, t = 2.91, p = 0.020; Fig. 4.1*b4*). The reduction of total *Arc*$^+$ cells was attributable to a reduction in the cells responding to only one episode (2.40 ± 0.56% in the associative group vs 5.52 ± 0.77% in the random group, n = 5, t = 3.29, p = 0.011), whereas the percentage of double-stained cells responding to both episodes of peppermint were similar in the two groups (1.54 ± 0.24% in the associative group vs 1.96 ± 0.44% in the random group, n = 5, t = 0.83, p = 0.431; Fig. 4.1*b4*). The reduction in single episode activated cells suggests that the S^+ odor representation in the associative group had become sharper with a larger proportion of more reliably activated cells. However, when comparing peppermint and vanillin representations after training, there were no differences in ensemble overlap between discriminating (21.10 ± 5.94%) and random (20.10 ± 2.10%, n = 5, t= 0.159, p = 0.877) groups

(Fig. 4.1*b5*). This suggests that the strengthened peppermint representation was related to the acquisition of discriminative behavior, but decorrelation between the two ensembles did not occur.

Peppermint odor was originally selected because it has been used widely in rat pup odor preference learning. Vanillin was chosen as being distinct from peppermint spatially in the olfactory bulb (http://gara.bio.uci.edu/). Consistent with the change in odor representations from spatial patterns in the olfactory bulb, to sparse random networks in the aPC (Wilson and Sullivan, 2011), there was no clustering of Arc^+ neurons for either odor in the aPC.

4.3.3 Odor mixture associative training leads to merging of odor ensembles

In our second experiment, we examined Arc^+ ensembles after training with a mixture of peppermint and vanillin combined as the S^+, whereas amyl acetate served as the S^- (Fig. 4.2*a*). After successful discriminative performance, rats were able to respond positively to single component peppermint (99 ± 1%, $n = 5$, $t = 17.01$, $p = 1.45E^{-7}$ compared with control) or vanillin (95 ± 1.58%, $n = 5$, $t = 12.68$, $p = 1.41E^{-6}$ compared with control; Fig. 4.2*b1*). Arc^+ responses to peppermint and vanillin individually revealed that the overlap ratio between the two different component ensembles was significantly greater in the associative learning group (44.31 ± 4.78%) than the random group (23.81 ± 5.31%, $n = 5$, $t = 2.87$, $p = 0.010$; Fig. 4.2*b2*). This demonstrates that the aPC directly supports merging of the ensemble patterns when they have been rewarded as part of a mixture.

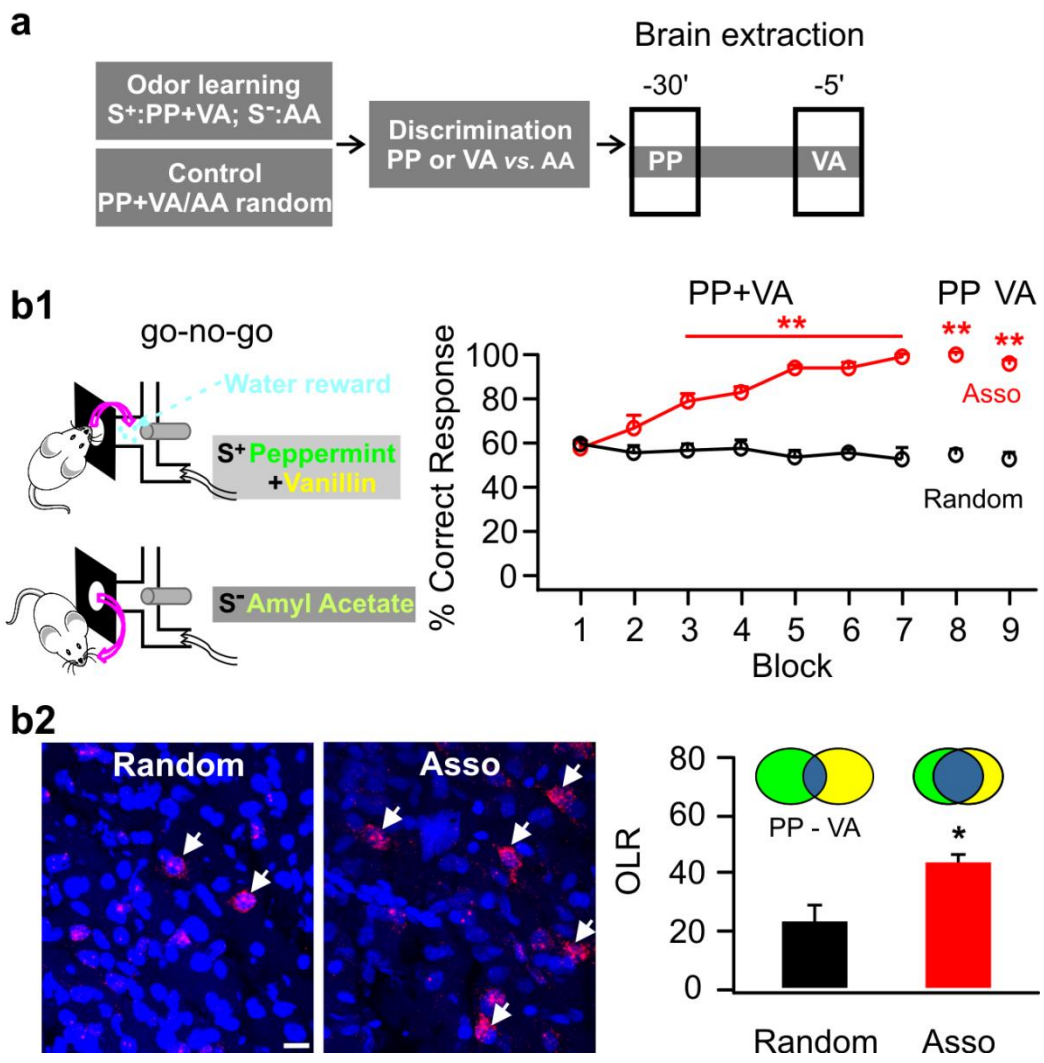

Figure 4.2 Odor mixture associative learning merges neuronal ensembles of odor components

a, Schematic of odor mixture associative training and brain extraction protocol. ***b1,*** Go–no-go behavioral paradigm (left) and percentage correct responses in the associative (Asso) group and the random group (right). ***b2,*** Representative images of *Arc*$^{+}$ cells in the aPC (left) and overlap ratios (OLRs) of the cell ensembles representing peppermint (PP) and vanillin (VA; right). Arrows indicate double-stained *Arc*$^{+}$ cells. Scale bar, 20 μm. **p*< 0.05, ***p*< 0.01.

4.3.4 Similar odor discrimination training leads to pattern separation

In our final experiment, we examined ensemble overlap ratios in rats required to perform challenging odor discrimination problem using two very similar odor mixtures (1-heptanol and 1-octanol; S^+, 53%/47%; S^-, 55%/45%; Fig. 4.3*a*). Chapuis and Wilson (2012) found that, with simple odor discrimination, decorrelation of ensembles was not observed in electrophysiological sampling, but with challenging discriminations, decorrelation occurred. Rats experienced difficulty in discriminating these odor mixtures and were unable to discriminate after eight blocks of training (Fig. 4.3*b1*), when rats in the easier discrimination task had performed nearly perfectly (Fig. 4.1*b2*). Continued training eventually led to successful discrimination in the associative group (98 ± 1.22% vs random group: 43 ± 2% at the 16th block, $n = 5$, $t = 23.45$, $p = 1.16E^{-8}$; Fig. 4.3*b1*). A significant decrease in the Arc^+ overlap between these odor pairs occurred in the associatively trained group (12.54 ± 1.01%, $n = 5$) relative to the random condition (23.95 ± 0.82%, $n = 5$, $t = 8.75$, $p = 2.28E^{-5}$; Fig. 4.3*b2*). Easy and difficult discriminations both induce remodeling of naive ensemble representations, but only the difficult discrimination leads to the reduced overlap of ensemble activity characteristic of pattern separation and likely necessary for its successful behavioral solution.

Unexpectedly, the difficult and easy odor discriminations demonstrated a similar degree of ensemble overlap among rats receiving random odor plus reward (easy odor pair in Fig. 4.1*b5*: 20.10 ± 2.10% vs difficult odor pair in Fig. 4.3*b2*: 23.95 ± 0.82%, $n = 5$, $t = 1.71$, $p = 0.125$), suggesting that the degree of initial overlap of Arc^+ cell ensembles does not predict behavioral discrimination ability.

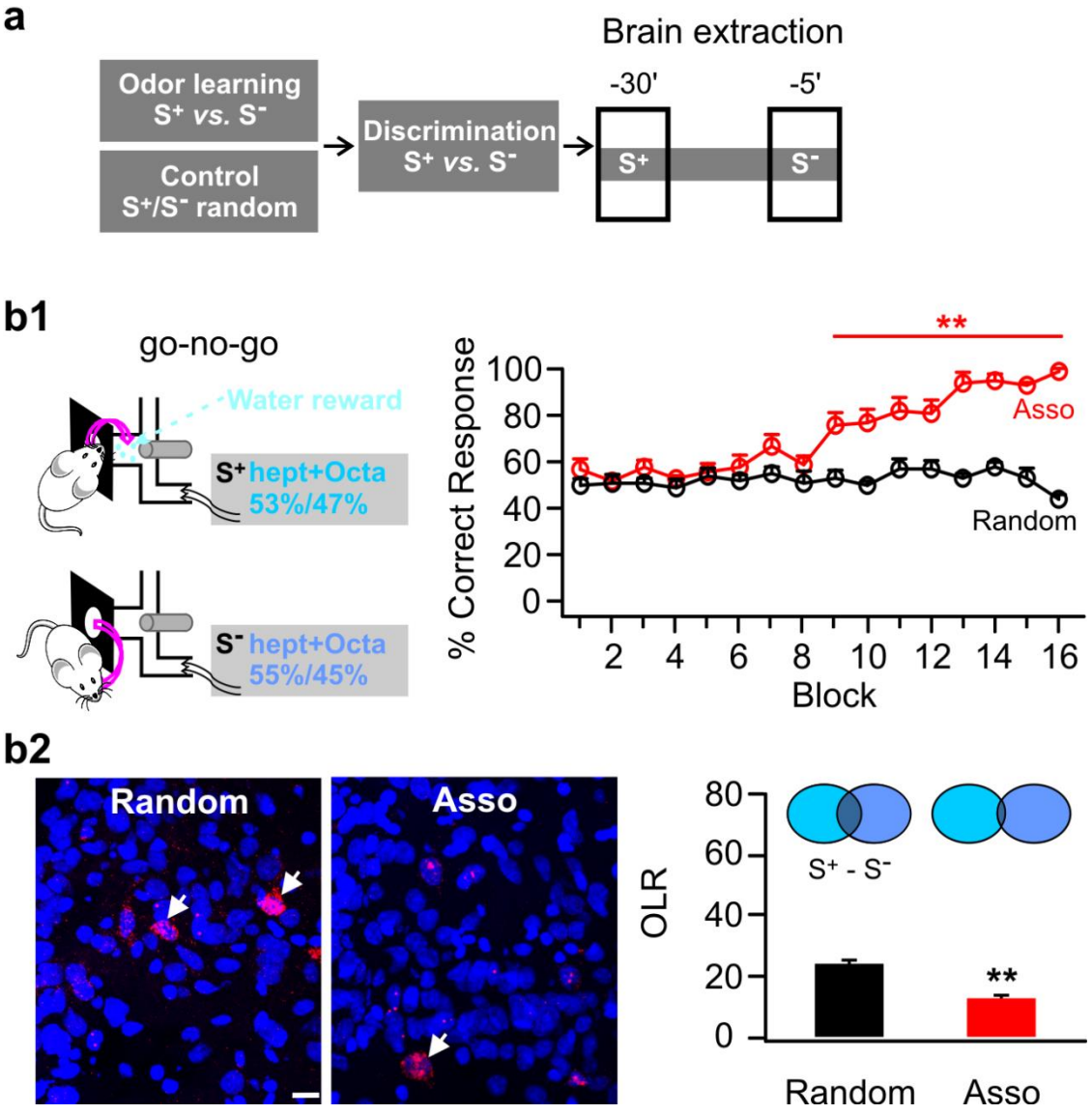

Figure 4.3 Similar odor discrimination learning promotes pattern separation
a, Schematic of similar odor discrimination training and brain extraction protocol. ***b1,*** Go–no-go behavioral paradigm (left) and percentage correct responses in the associative (Asso) group and the random group (right). ***b2,*** Representative images of *Arc*$^{+}$ cells in the aPC (left) and overlap ratios (OLRs) of the cell ensembles representing S^{+} (1-heptanol plus 1-octanol, 53%/47% mixture) and S^{-} (1-heptanol plus 1-octanol, 55%/45% mixture; right). Arrows indicate double-stained *Arc*$^{+}$ cells. Hept+Octa, 1-heptanol plus 1-octanol; Scale bar, 20 μm. ***p*< 0.01

4.4 Discussion

Wilson and Sullivan (2011) have proposed that the aPC generates odor objects. Direct visualization of those objects here as indexed by neuronal transcription of the immediate early gene *Arc* is consistent with the sparse ensemble characteristics seen previously in the aPC (Poo and Isaacson, 2009; Stettler and Axel, 2009). Here, in the adult rat, such representations appear rapidly modifiable (within the few days required for successful behavior). We have visualized three forms of aPC representational plasticity: (1) an increase in consistent ensemble participation together with a reduction in ensemble size for an S^+; (2) an increase in ensemble overlap for components when odor mixtures signal reward; and (3) a decrease in ensemble overlap when a discrimination among highly similar odor mixtures is required, the mechanistic definition of pattern separation. These outcomes are supported by observations from electrophysiological population sampling (Chapuis and Wilson, 2012). Chapuis and Wilson demonstrated that cell response profiles were decorrelated for a series of odors in anesthetized rats after training in challenging odor discriminations. Decorrelation was not seen with simple discriminations, consistent with the present observations. After training with odors signaling similar outcomes, the correlations among cell response profiles increased, similar to the increased overlap seen here in Experiment 2. The data are consistent with Chapuis and Wilson's proposal that pattern completion and pattern separation both occur in the aPC.

However, the present experiment did not directly assess pattern completion. Although it is possible to suggest that training on peppermint plus vanillin and then successfully solving the go–no-go task to either peppermint or vanillin alone is pattern completion, it is more parsimonious to suggest that this is an example of each component changing to be more similar to the mixture (Linster and Smith, 1997). It is clear that there is increased overlap when both components are

associated simultaneously with reward. Similarly, there is decreased overlap when components are differentially associated with reward and no reward. These results contrast with the recent report in *Drosophila* in which ensemble odor representation in the mushroom bodies predict behavioral discrimination performance but are not altered by discrimination training (Campbell et al., 2013). In the present study, the representation of peppermint did not differ in naive rats from those given random odor and reward experience, but with systematically paired odor and reward, peppermint representations were invariably modified.

A feature not predicted from the Chapuis and Wilson experiments was the finding of a smaller but more reliable representation of the S^+ after reward pairing. Electrophysiological testing does not permit the documentation of spatial sharpening for rewarded stimuli revealed by *Arc*. Previous work with c-*Fos* supports this characterization because animals well trained in odor discriminations have smaller aPC c-*Fos* representations (Roullet et al., 2005). However, only *Arc* methodology permits the assessment of the increased reliability of the representation because it allows a given odor to be compared with itself. The present study does not address changes that may occur when an odor is systemically unrewarded. There was a trend in the data for such odors to have larger representations, but this did not reach significance and will require additional experimentation. It would also be of interest to know whether punishment and non-reward differ in their impact on aPC ensembles.

There are a number of possible mechanisms to support the changes observed here. Increases in the strength of connections through LTP-like changes with concomitant increases in lateral inhibition (Brosh and Barkai, 2009; Saar et al., 2012) or even LTD-like changes of weak cells could account for the increased reliability of cell participation, as well as the smaller ensembles, characteristic of associative representations (Gdalyahu et al., 2012). Changes in

overlap of two odor representations could also relate to Hebbian mechanisms supporting reward-congruent and -incongruent activation patterns. In rat pups, we have shown both LTP and norepinephrine-mediated enhancement of connectivity in aPC (Morrison et al., 2013), but whether a norepinephrine effect occurs in the present paradigm is unknown.

The present data demonstrate the ability of sparse random cortical networks in the adult mammalian brain to be rapidly tuned by consequential environmental feedback to optimize perceptual representations. We suggest that the suite of changes seen here in ensemble representations with discrimination training contribute to the neuronal substrate of perceptual expertise.

Chapter-05: *Arc*-expressing neuronal ensembles supporting pattern separation require adrenergic activity in anterior piriform cortex: an exploration of neural constraints on learning (This chapter is a version of the manuscript published in The Journal of Neuroscience 35(41): 14070-14075, 2015)

5.1 Introduction

In rodents, OB receives massive adrenergic input from LC (McLean et al., 1989). NE release in the OB is critical for associative learning in rat pups (Wilson and Sullivan, 1994; Yuan et al., 2014). In adult rats, increases in OB NE are associated with improved signal to noise ratios (de Almeida et al., 2015), lower thresholds for odor discriminations (Escanilla et al., 2010), and appear necessary for learning similar odor discriminations (Doucette et al., 2007; Mandairon et al., 2008b).

OB mitral cells demonstrate sparse coding and temporally dynamic firing in awake rodents (Rinberg et al., 2006; Wachowiak et al., 2013). Even at this early stage, OB processing is shaped by experience and context. Mitral cell firing patterns diverge for rewarded and unrewarded odors in mice undergoing discrimination training (Doucette and Restrepo, 2008). Mitral cells synchronize firing for rewarded odors and adrenergic blockade disrupts this synchrony as well as similar odor discrimination (Doucette et al., 2011). Synchronized mitral cell firing increases the likelihood of driving piriform target neurons (Franks and Isaacson, 2006).

PC receives direct projections from OB *via* the lateral olfactory tract and is proposed as a critical site for integrating odor features into odor objects (Wilson and Sullivan, 2011). PC pyramidal cells exhibit sparse and diffuse coding to odor input (Stettler and Axel, 2009; Poo and Isaacson, 2011; Shakhawat et al., 2014a). Additionally, PC pyramidal cells project back to the OB

and shape mitral cell responses to odors (Boyd et al., 2015; Otazu et al., 2015). PC itself receives extensive NE input from the LC (Shipley and Ennis, 1996). In rat pups, aPC odor-NE pairings are sufficient to induce odor preference learning (Morrison et al., 2013). In adult rat, PC LC-NE appears to sharpen odor representations (Bouret and Sara, 2002).

How altered OB signaling following NE neuromodulation influences cortical processing and *vice versa,* how cortical changes feedback to influence odor ensemble representation in the OB, has not been characterized experimentally. Here we examine OB or aPC ensemble representation in adult rats following a similar odor discrimination task, with adrenoceptor blockade in aPC, or OB respectively. We find changes in odor encoding index learning success and aPC adrenergic blockade prevents learning of a similar odor discrimination.

5.2 Materials and Methods

5.2.1 Subjects

Sixty Sprague-Dawley rats (Charles River), 8-10 weeks old, of both sexes, were subjects. Rats were housed under a 12 h light/dark cycle with *ad libitum* dry food and water, except during training. Water deprivation was implemented 4-5 days before training began with either *ad libitum* water 1 h/day or a total volume of 25 ml/day. Water deprivation was maintained during training. Procedures were consistent with Canadian Council on Animal Care guidelines, and approved by the Memorial University Institutional Animal Care Committee.

5.2.2 Odorants

Odorant solution (10 ml, in mineral oil) was freshly prepared for each experiment. Odors were 1% of orange *vs*. 2% of peppermint, or 0.001% of 1-Heptanol *vs.* 0.001% 1-Heptanol+1-Octanol (50:50 mixture; Sigma-Aldrich) (Escanilla et al., 2010; Shakhawat et al., 2014a).

5.2.3 Go/no-go odor discrimination training and drug infusions

Odor discrimination training was performed in a custom-built computer controlled four-channel Knosys olfactometer.

5.2.3.1 Initial rule learning

Orange odor (S^+) was introduced with water reward (30 µl drop/lick). Each trial lasted 2.5 sec. Rats were allowed 0.5 s to sample the odor and 2.0 sec to make a decision. Rats either licked the water port for a minimum of 6 times for reward or rejected the odor by removing their snouts from the port. Inter-trial intervals were 5 seconds. Initially rats underwent ~100 reinforcement trials per day for 3 days.

Rats were then exposed to the same S^+ odor while a negative peppermint odor (S^-) not paired with water reward was introduced. Blocks were 20 trials in which 10 S^+ and 10 S^- odors were randomly delivered. Rats completed 5-10 blocks per day until criterion was reached within a block on a given day. The percentages of correct responses to both odors were calculated by software (BBC Basic), and converted to percentages (correct S^+ response # + correct S^- response #)/20 x100. Discrimination learning was defined as ≥ 80% correct responses in one block. All rats learned to discriminate between the two odors within 3-4 blocks (Appendix-C). Following rule

learning, *ad libitum* water was reinstated. The next day, all rats underwent OB or aPC cannulation surgery and had approximately one-week recovery.

5.2.3.2 Cannula implantation

Guide cannulae were custom-made by anchoring two stainless steel tubes (23-gauge) to a dental acrylic base.

Rats were anesthetized with a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and secured in a stereotaxic apparatus. Two holes were drilled +8.0 mm anterior and ~1.5 mm bilateral relative to bregma for OB or +2.5 mm anterior, ~4.0 mm bilateral for aPC. Guide cannulae were inserted ventral to the skull surface; 2.0 mm for OB; 6.0 mm for aPC. Guide cannulae were attached by dental cement to two skull screws.

5.2.3.3 Similar odor discrimination training and drug infusion

After recovery, rats were infused with vehicle or a mixture of adrenoreceptor antagonists before each training session. For the antagonist mixture, the non-selective α-adrenoreceptor antagonist phentolamine hydrochloride (Sigma Aldrich, 10 mM) and the β-adrenoreceptor antagonist alprenolol hydrochloride (Tocris, 120 mM) were dissolved in sterile saline (Mandairon et al., 2008b). Three microliters of drug or vehicle were infused bilaterally at a rate of 1.0 µl/min via a multi-syringe pump twenty minutes prior to training.

Training on the similar odor discrimination problem (1-Heptanol *vs.* 1-Heptanol+1-Octanol 50:50 mixture) followed the procedures described for orange *vs.* peppermint. Rats were trained over 3-4 days until criterion was achieved in a block or for a fixed number of blocks (10) if criterion was not achieved.

5.2.4 Tissue Collection

Twenty four hours following similar odor discrimination training, rats were placed in a sealed container, ventilated with a continuous flow of charcoal-filtered air for 1.5-2 hrs, followed by two 5-min episodes of odor delivery (either S^+ twice or S^+ followed by S^-) in the same container. The two episodes were separated by 20 min. Immediately after the 2nd episode, rats were killed under isoflurane anesthesia and brains collected and flash frozen in 2-methylbutane immersed in an ethanol/dry ice-slurry. Brains were kept at -80ºC.

During sectioning, the right hemispheres of 4-6 rats were arranged side-by-side in a custom-made plastic box filled with OCT medium in a cryostat at -20^{0}C to form a frozen block. Saline and drug groups were matched in each block. Coronal sections (20 µm) were collected on 3-aminopropyltriethoxysilane (2%) treated slides. Five to six representative slides over the rostral-to-caudal range of the OB and aPC were chosen for fluorescent *in situ* hybridization and stored at -20^{0}C.

5.2.5 Fluorescence *in situ* hybridization

Full-length *Arc* riboprobes conjugated to digoxigenin were extracted using a commercial transcript kit (Ambion). The purity and integrity of synthesized riboprobes were ensured by a mini quick spin RNA column (Roche Diagnostics). Two µL of the probe was tested via gel electrophoresis before use (Appendix-B).

Brain slides were thawed for 10-15 min at room temperature. They were quickly fixed in 4% paraformaldehyde. The slides were bathed in acetic anhydride and treated in 1:1 methanol/acetone solution at -20^{0}C. The slides were then incubated for 60 min in prehybridization buffer in a humid chamber. Next, the slides were incubated overnight with 100 ng of *Arc* probe in

a hybridization oven at 56^0C. All solutions were made in DEPC (Sigma) treated water (0.1%). The next day, slides were washed in a series of saline-sodium citrate buffer. They were then treated with RNase A at 37^0C, submerged in 2% H_2O_2/ SSC buffer solution, blocked with normal sheep serum, followed by incubation with anti-digoxigenin-horseradish peroxidase antibody (Roche Diagnostics) overnight at 4^0C. The slides were labeled with Cy3 (1:50) using a tyramide signal amplification (TSA) labeling kit (Perkin Elmer) and cell nuclei counterstained with DAPI (4'-6-diamidino-2-phenylindole: Sigma-Aldrich). Finally, sections were coated with Vectashield antifade medium, coverslipped and sealed with nail polish.

5.2.6 Image acquisition and analysis

Tissue damage from cannulation prevented examination of the cannulated structure, but here we address the influence on the projection structure. All slides were scanned in a Fluoview FV1000 confocal microscope (Olympus Canada) (see (Shakhawat et al., 2014a). Images of aPC were taken at 20X magnification (one medial and one lateral region, Figure 5.1A) and images of OB at 40X (two dorsolateral and two ventromedial regions, Figure 5.2A). The photomultiplier tube assignments, confocal aperture size, and contrast remained constant for each slide. The z-stacks (optical thickness: 1.0µm) throughout the thickness of the section (20 µm) were acquired from 3-4 slides for each animal.

Image J software was used for counting cells in the scanned images. Cell labeling (foci, cytoplasmic, double, and DAPI) was done manually and was achieved by checking the multiple optical sections (20% mid-range of z-stack) that comprised each cell (Shakhawat et al., 2014a). Average cell counts of all regions in the OB or aPC from all slides in the same animals were reported.

5.2.7 Statistics

OriginPro 9.0 software was used to analyze all data sets. Student *t test*s between the saline and drug infused groups were used for statistical comparisons. Data are presented as mean ± sem.

5.3 Results

5.3.1 OB adrenoceptor blockade impairs similar odor discrimination learning, reliability of rewarded odor representations, and pattern separation in aPC

We first tested whether blocking OB adrenoreceptors has any effect on similar odor discrimination learning. β- and α-adrenoceptor blockade slowed discrimination learning of highly similar odor pairs. The saline-infused group learnt to discriminate the S^+ and S^- odors in 6 blocks (84 ± 1.25% success rate) while the drug group was unable to discriminate after 6 blocks (49 ± 1.25%, n = 4; t = 19.80, p = $1.08E^{-6}$, Figure 5.1B1, see also Figure 5.1C1 and 5.1D1). However, extended training eventually led to successful discrimination in the drug group. By 14 blocks of training, the drug group showed significant discrimination (88 ± 2.55%) and was no different from the saline group (92 ± 4.06%, n = 5; t = 0.83, p = 0.43, Figure 5.1C1).

To investigate the effect of OB adrenergic blockade on odor representations in the aPC, we looked at *Arc* expression in rats that were exposed either to the S^+ odor twice or to the S^+ followed by the S^-. *Arc* mRNA is expressed in the nucleus of the cell shortly (~5 min) following an odor stimulation and is translocated into the cytoplasm ~20-30 min later. Therefore, nucleus and cytoplasm double labeled Arc^+ cells following repeated exposures to the same odor indicate those that are reliably activated by the odor, while double labeled Arc^+ cells following two different odor episodes are likely those activated by both odors and index the correlation of the two odors (Shakhawat et al., 2014a).

Overlap ratio (OLR) defined by the percentage of double *Arc*$^+$ cells over total *Arc*$^+$ cells was used to measure the reliability of odor representations to the rewarded odor in the aPC following two consecutive S^+ episodes. At the 6th block, when the saline-infused animals learnt to discriminate between S^+ and S^-, whereas the drug group did not, there was a significant difference in the *Arc*$^+$ cell patterns between the two groups. The OLR in the saline group was significantly larger (37.56 ± 1.57%; n = 4) than that in the drug group (22.16 ± 0.26%; t = 9.70, p = 6.87E^{-5}, Figure 5.1B2&3). This replicates the finding of increased stability with reward seen previously (Shakhawat et al., 2014a) and suggests a less stable representation of the S^+ in the aPC occurs due to OB adrenergic blockade and is associated with failure to discriminate. The smaller OLR in the drug group was due to fewer double *Arc*$^+$ cells in the aPC (0.83 ± 0.39%) relative to the saline group (1.85 ± 0.39%, n = 4; t = 2.63, p = 0.04, Figure 5.1B4).

With 17 blocks of extensive training, the drug group was discriminating between S^+ and S^- (88 ± 1.22% *vs.* 92 ± 3% in the saline group, n = 5, t = 1.23, p = 0.25, Figure 5.1C1), and the OLR in the drug group was larger (45.72 ± 0.88) and not different from that in the saline group (43.07 ± 2.78%, n = 5, t = 0.91, p = 0.39, Figure 5.1C2&3). The distribution of *Arc*$^+$ cells (total, double, single) was similar in both groups (Figure 5.1C4). OB adrenergic blockade compromised the natural course of enhanced stability of aPC representations with training and made discrimination of similar odors more challenging.

Pattern separation in the aPC was also examined in rats that underwent 6 blocks of training (Figure 5.1D1). *Arc* expression was visualized following exposure to S^+ then S^-. Here, OLR indexes the overlap between two different odor representations. The OLR was significantly smaller in the saline group (16.78 ± 1.39%, n = 5) than in the drug group (28.56 ± 0.96%; t = 6.97, p = 1.16E^{-4}, Figure 5.1D2&3), suggesting pattern separation in the saline, but not the drug, group.

Correspondingly, there were more double Arc^+ cells in the drug group (3.61 ± 0.50%) than in the saline group (1.76 ± 0.21%, n = 5, t = 3.39, p = 0.009, Figure 5.1D4). Inability to discriminate similar odors after 6 blocks of training was accompanied by lack of pattern separation, and lack of rewarded odor stability, in the drug group aPC odor ensembles.

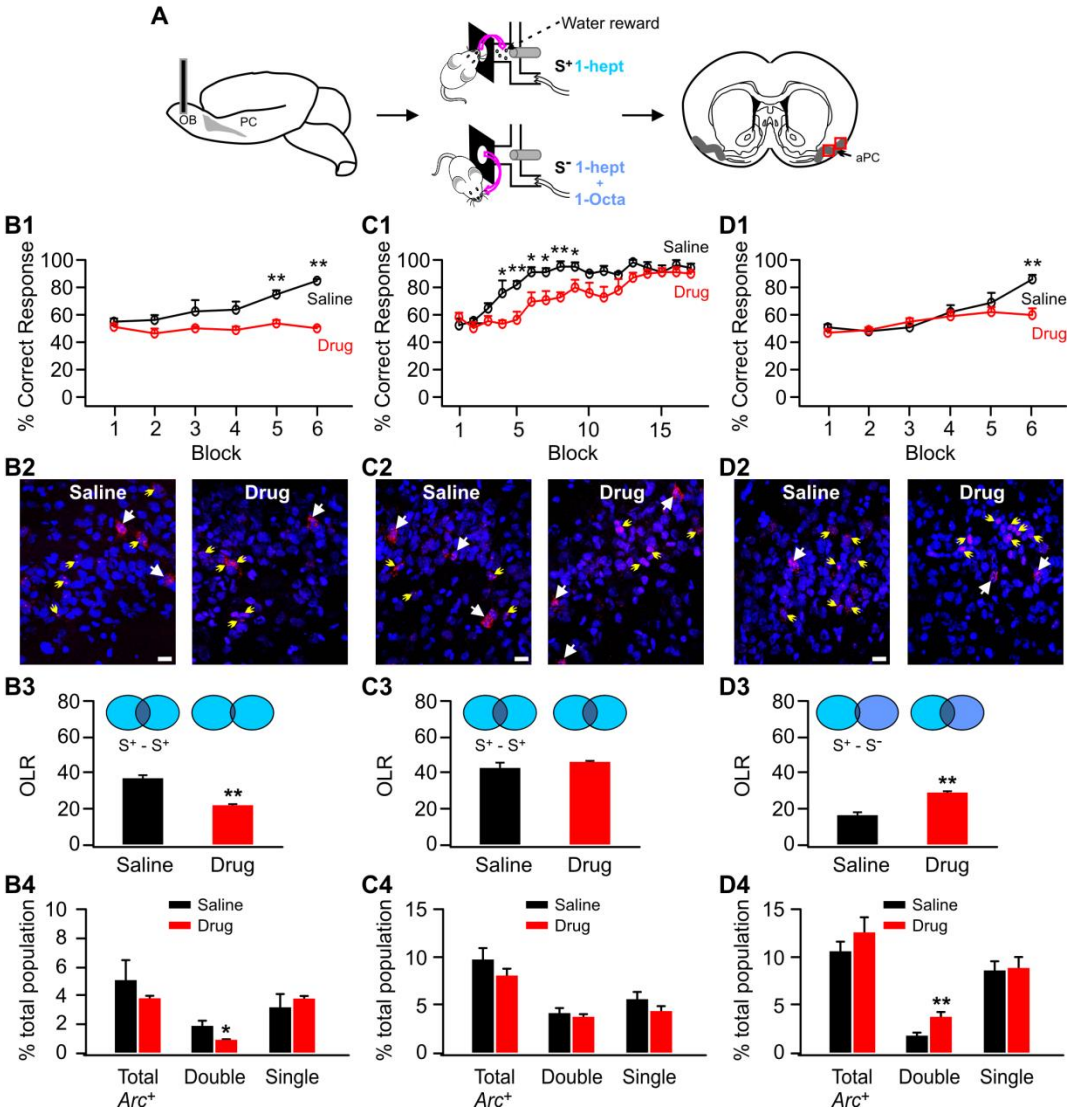

Figure 5.1 OB adrenoceptor blockade slows down similar odor discrimination learning and odor representation and pattern separation in the aPC

(A) Schematic of experimental procedures. **(B1-B4)** Impaired odor discrimination by adrenoceptor blockade is accompanied by reduced reliability of pyramidal cell activity. B1: Correct responses in the drug and saline groups following 6 blocks of training. B2: Example images of *Arc*$^+$ cells in aPC. Blue indicates DAPI staining of nuclei. Red indicates *Arc* signals. White and yellow arrows indicate double- and single-stained *Arc*$^+$ cells respectively. Bar, 20 μm. B3: Overlap ratio (OLR) of the two ensembles to the same reward odor (S^+) in the drug and saline groups. B4: Distribution of *Arc*$^+$ cells including total, double and single-stained cells. * $p < 0.05$; ** $p < 0.01$. **(C1-C4)** Prolonged training leads to odor discrimination in adrenoceptor-blocked group and restores reliability of pyramidal cell activity. **(D1-D4)** Impaired odor discrimination by adrenoceptor blockade is accompanied by reduced pattern separation between rewarded (S^+) and unrewarded (S^-) ensembles.

5.3.2 APC adrenoceptor blockade prevents similar odor discrimination, and impairs reliability of odor representations in the OB

The role of aPC adrenoreceptors in odor discrimination learning has not been studied. Here we infused adrenoceptor blockers into aPC before training and infused rats were unable to discriminate the similar odors despite extensive training (Figure 5.2B1 and 5.2C1). At the 17^{th} block, the drug group is unable to discriminate (55 ± 4.2%, n = 5, t = 9.07, p = $1.75E^{-5}$, Figure 5.2C1), while the saline group is highly successful (94 ± 1%) having reached discrimination criterion at 6 blocks. This is the first evidence that aPC adrenoreceptors are critical for similar odor discrimination learning. We also tested a subset of these rats for their memory of the earlier orange and peppermint discrimination. Recall and discrimination of these distinct odors was not affected by aPC adrenoceptor blockade (Appendix-E).

Arc visualization in the OB following odor discrimination training with aPC adrenoceptor blockade revealed differences in both mitral and granule cell ensemble representations to the rewarded odor. The OLR of mitral cell ensembles to the rewarded odor in the saline group (35.69 ± 3.72%, n = 4) was larger than in the drug group (19.58 ± 1.55%; t = 4.00, p = 0.007, Figure 5.2 B2&3). Double Arc^+ cells were fewer in the drug group (1.26 ± 0.11%) than in the saline group (3.00 ± 0.36%, n = 4, t = 4.60, p = 0.004, Figure 5.2B4). Similarly, the OLR of granule cell ensembles to the rewarded odor was larger in the saline group (38.72 ± 2.96%) than the drug group (17.36 ± 0.55%, n = 4; t = 7.08, p = $3.97E^{-4}$, Figure 5.2B2&5). This was again due to fewer double Arc^+ cells in the drug group (0.94 ± 0.13% drug group *vs.* 2.96 ± 0.40 saline group, n = 4, t = 4.86, p = 0.003, Figure 5.2B6). Together, aPC adrenergic disruption during similar odor discrimination training impairs the reliability of neuronal representations to the rewarded odor in the OB.

Finally, we tested whether aPC adrenergic blockade affects pattern separation of the OB S^+ and S^- ensembles. This was performed after 17 blocks of training (Figure 5.2C1). There were no differences in the OLR of the two ensembles in the two groups (16.84 ± 1.63 in the saline group *vs.* 12.58 ± 2.41% in the drug group; n = 4, t = 1.46, p = 0.18, Figure 5.2C2&3). There was also no difference in the distribution pattern of *Arc*$^+$ neurons (Figure 5.2C4).

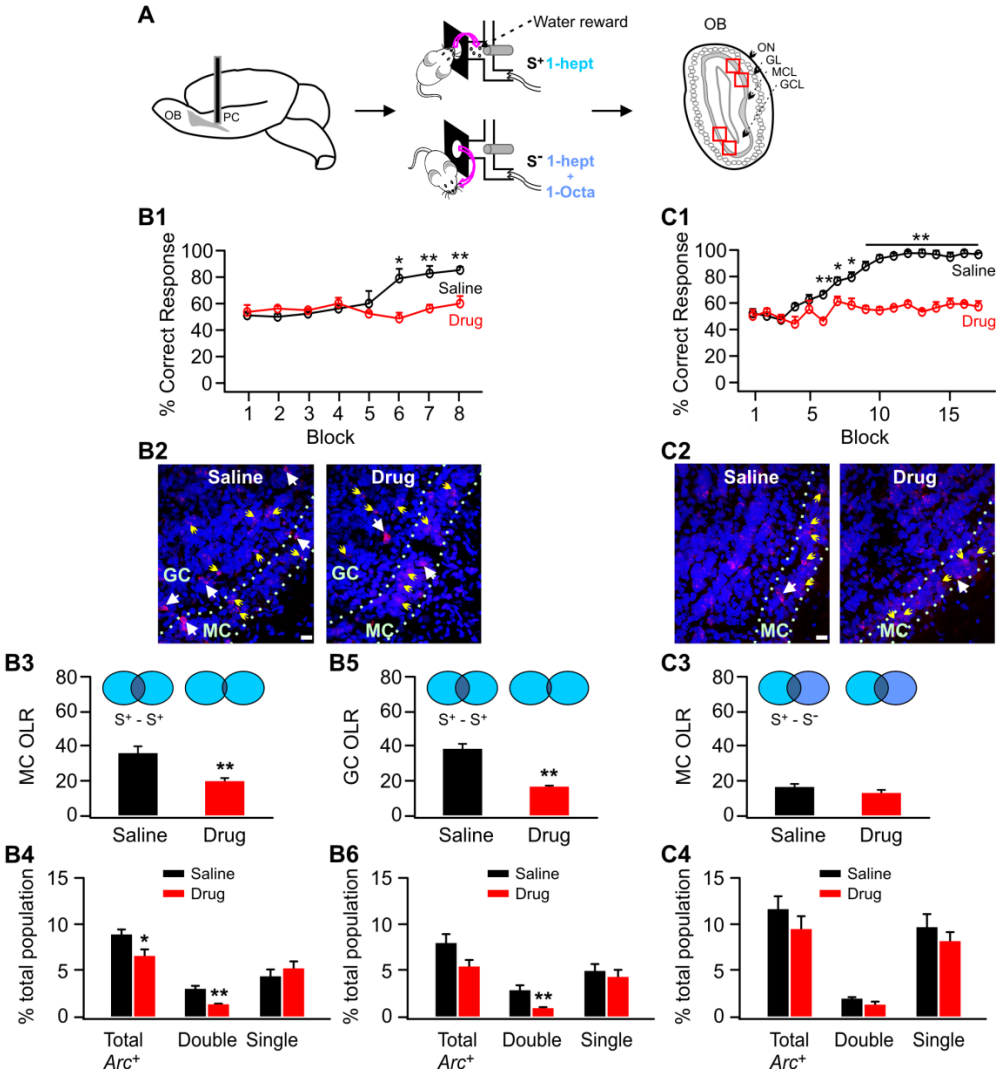

Figure 5.2 aPC adrenoceptor blockade prevents similar odor discrimination learning and changes in OB odor representations

(A) Schematic of experimental procedures. **(B1-B6)** Impaired odor discrimination by adrenoceptor-blockade is accompanied by reduced reliability of mitral and granule cell representations. **B1:** Correct responses in the drug and saline group with 8 training blocks. **B2:** Example of *Arc*$^+$ cells in the OB. Blue indicates DAPI staining of nuclei. Red indicates *Arc* signals. White and yellow arrows indicate double- and single-stained *Arc*$^+$ cells respectively. GC, granule cell layer, MC, mitral cell layer. Bar, 20 μm.**B3:** OLR of two mitral cell ensembles to the same reward odor (S^+) in the drug and saline groups. **B4:** Distribution of *Arc*$^+$ mitral cells including total, double and single-stained cells. **B5:** OLR of two granule cell ensembles to the same reward odor (S^+) in the drug and saline groups. B6: Distribution of *Arc*$^+$ granule cells including total, double and single-stained cells. * $p< 0.05$; ** $p < 0.01$. **(C1-C4)** No difference in pattern separation between rewarded (S^+) and unrewarded (S^-) mitral cell ensembles in the OBs of the drug and saline groups.

5.4 Discussion

5.4.1 Blockade of OB adrenoreceptors slows similar odor discrimination and the stabilization of reward odor encoding in aPC

Rats with OB blockade of adrenoreceptors did not learn the similar discrimination in 6 blocks of training, but did reach criterion after 14 blocks. After 6 blocks the rewarded odor representation in aPC for saline-infused rats revealed increased stability relative to that in OB-NE blockade rats (OLR 38% *vs.* 22%). When the drug group reached criterion there was no longer a difference in OLR from the saline group; both showed increased stability. Increased stability of cortical motor (Peters et al., 2014; Cao et al., 2015) and sensory (Shakhawat et al., 2014a; Shakhawat et al., 2014b; Poort et al., 2015) representations with learning appears to be a general feature of learning-induced network change. Ensemble sizes were similar to previous reports for aPC (~4-5%; Shakhawat et al., 2014a).

Critically for this discrimination task, pattern separation in aPC for the saline-infused group (OLR S^+/S^- 17%) was significantly greater after 6 blocks than for the drug infused group (29%). Pattern separation in aPC ensembles has also been reported following similar discrimination learning (Chapuis and Wilson, 2012; Shakhawat et al., 2014a).

5.4.2 Blockade of adrenoreceptors in aPC prevents similar odor discrimination and stabilization of reward odor encoding in OB

Adrenergic antagonists in aPC prevented successful discrimination learning even after 17 blocks of training. At 6 blocks, saline-infused rats reached criterion and had greater overlap in rewarded odor ensembles for both mitral cells (~36%) and granule cells (~39%). This is similar to OB changes in odor ensemble encoding with preference training in the rat pup (Shakhawat et al.,

2014b). Ensemble size (7.5%) was also similar for pups and adults. A parsimonious explanation of the ensemble changes is that activated mitral cells recruit their associated granule cells.

Pattern separation in OB *Arc*+ ensembles was not seen during this task. Whether the OB contributes to pattern separation is still under debate (Sahay et al., 2011).

5.4.3 Neural constraints on similar odor discrimination learning

Learning increases in the OLR of rewarded odors (Shakhawat et al., 2014a; Shakhawat et al., 2014b), but not unrewarded odors (Shakhawat et al., 2014b) reveals the importance of reduced variability in encoding survival-relevant odors.

The present data suggest aPC-NE is required for difficult odor discrimination learning. NE enhances signal to noise ratio of the afferent inputs to aPC (Hasselmo et al., 1997). The data also imply that feedback from the learned aPC representation is necessary to the development of stability in the OB ensemble. Odor/reward associations are slowed when centrifugal feedback to the bulb is transected (Kiselycznyk et al., 2006). Blockade of OB NE does not prevent stability, or pattern separation, changes in aPC ensembles, but slows their appearance. This result is consistent with evidence that OB NE facilitates similar odor discriminations (Doucette et al., 2007; Mandairon et al., 2008b).

The obligatory role of aPC NE for the acquisition of similar odor discriminations was unexpected. Pattern separation in aPC is likely fundamental to successful learning. Pattern separation requires the dissociation of ensembles either through enhancement of inhibitory processes or a weakening of connections. The long training required for separation of similar odor representations might relate to competing demands for strengthening rewarded representations and weakening overlap to facilitate discrimination. Simple discrimination only requires a

strengthening of connections (Shakhawat et al., 2014a). NE facilitates inhibition and supports LTD and LTP, in the latter case the implicated receptors differ (Kirkwood et al., 1999). Selective antagonism in aPC may help reveal the mechanisms implicated in odor pattern separation.

Another requirement for discriminating similar odors is neural space. Odor objects activate similar-sized ensembles. With less overlap more neurons are needed for odor differentiation. This is consistent with human evidence reporting more discriminable episodic memories in individuals with larger relevant neural space (Chadwick et al., 2014).

Chapter-06: Discussion

The major goal of this research was to delineate the role of NE in odor learning. Thus, this dissertation explored how NE via multiple adrenoreceptors modulates early odor preference learning in rat pups and odor discrimination learning in adult rats. In pups, NE-mediated intracellular cascades facilitate the plastic changes necessary for odor memory. In adult rats, this research work discovered NE-mediated network plasticity in the olfactory system following learning. Below are the summaries of all findings, starting from Chapter 2 to Chapter 5.

6.1 Major Contributions to the Field

6.1.1 NE acts as a UCS in early odor preference learning via multiple adrenoreceptors

Previous research revealed that β_1-adrenoreceptors are one of the major contributors to neonatal odor learning (Yuan et al., 2003a, b; Harley et al., 2006). α_1-adrenoreceptors also play a role in early odor preference learning (Harley et al., 2006). Additionally, α_2-adrenoreceptors are expressed in the OB (McCune et al., 1993; Pieribone et al., 1994; Day et al., 1997; Winzer-Serhan et al., 1997a,b; Winzer-Serhan and Leslie, 1999; Hayar et al., 2001; Nai et al., 2010), and have been shown to be critically involved in NE-mediated long-term plasticity of mitral cell networks in the OB (Pandipati et al., 2010). In this work (Chapter 2), an α_2-adrenoreceptor antagonist, yohimbine, was directly infused into the OB (Shakhawat et al., 2012). Odor, when paired with mild shock, paradoxically results in odor preference in rat pups (Sullivan et al., 2000a). Yohimbine infusion prevented odor preference learning to the conditioned odor using the shock + odor paradigm (Fig-2.1).

The next obvious question was whether activating α_2-adrenoreceptors by a local bulbar infusion can itself serve as an UCS to induce odor preference memory. Infusion of clonidine, an α_2-adrenoreceptor agonist, when paired with the conditioned odor, induced a significant preference for that odor (Fig 2.1). To circumvent the potential effects of clonidine on α_1-adrenoreceptors, an antagonist of α_1-adrenoreceptors (prazosin) was co-infused with clonidine into the bulb. This cocktail infusion was also able to induce learning in rat pups, supporting our hypothesis that α_2-adrenoreceptor activation can also act as an UCS for odor preference learning Fig 2.1).

Since past physiological evidence suggests that NE via α_2-adrenoreceptors indirectly excites mitral cells via disinhibition (Nai et al., 2010; Pandipati et al., 2010), we studied the effect of clonidine on mitral cell intracellular signaling, especially on pCREB and cAMP, two players critically involved in β-adrenoceptor mediated learning plasticity (Shakhawat et al., 2012). To compare to other disinhibitory effects a $GABA_A$ receptor antagonist gabazine, was infused in another cohort. Either clonidine or gabazine infusion increased pCREB expression in the mitral cells in the drug-infused bulb compared to the saline infused bulb (Fig 2.2). Whereas clonidine infusion increased pCREB expression in the lateral domain (peppermint hot spot; Lethbridge et al. 2012), gabazine treatment increased pCREB expression both in the lateral and medial domains of the olfactory bulb (Fig 2.2). These results suggest that odor input together with α_2-adrenoreceptor mediated disinhibition act conjointly to enhance mitral cell activity and induce pCREB synthesis in the peppermint representation region, while gabazine-mediated disinhibition elicited a global pCREB expression change in the MCL (Fig 2.2).

β_1-adrenoreceptor mediated cAMP increase has been a prominent intracellular mechanism underpinning early odor preference memory (Yuan et al., 2014). In this experiment we also tested the molecular signalling underlying α_2-adrenoreceptor mediated learning. First, neither clonidine

nor gabazine application in the bulb changed the cAMP level compared to the saline-treated bulb, excluding the possibility that the calcium-enhanced adenylate cyclase pathway activates cAMP in this learning paradigm (Fig 2.2). This indicates that α_2-adrenoreceptor mediated learning occurs via a cAMP-independent pathway. It should be noted that α_2-agonist clonidine can also act on I1-imidazoline receptor, which has been shown to be expressed in the OB (Friedrich et al., 2008). Thus one might speculate this as one of the reason behind the unexpected cAMP independent learning observed here. Another interesting discovery of this experiment was that neither pCREB nor cAMP levels changed in the GC layer (Fig 2.2).

Finally we asked whether β and α_2-adrenoreceptors act concurrently to induce learning. We found that co-application of a previously suboptimal dose of both clonidine (50μM) and isoproterenol (1.0 mg/kg) leads to odor memory formation (Fig 2.3). In addition, co-application of 500 μM clonidine enabled odor preference learning with a saline s.c. injection (59.47% ± 3.20, *n* = 11) or a 1 mg/kg isoproterenol injection (66.37% ± 5.06, *n* = 11). The latter group differed significantly from no learning saline infusion group (38.84% ± 2.10, *n* = 13), the 2 mg/kg group (45.34% ± 8.93, *n* = 9) and the 6 mg/kg group (43.22% ± 7.06, *n* = 8), while the saline s.c. + 500 μM clonidine infusion group differed from the saline infusion control group and the 6 mg/kg no learning control group (Appendix-G). These results are consistent with the hypothesis that NE facilitates early odor preference memory formation via multiple adrenoreceptors and those adrenoreceptors have an additive effect to enable odor preference learning.

6.1.2 Odor preference learning results in stable odor representations in both the OB and aPC

One of the major goals of this dissertation was to explore the modulatory role of NE on odor representations following learning. In order to do that, first we studied how associative learning changes odor representations in both the OB and aPC. A notion that network ensembles stabilize following learning was proposed by Hebb in 1949. A myriad of data have shown that odor learning modifies odor representations in the OB and aPC similar to other sensory modalities (Woo et al., 1987; Woo and Leon, 1991; Roth and Sullivan, 2005; Roth et al., 2006; Jones et al., 2008; Busto et al., 2009; Fletcher, 2012; Kass et al., 2013). In line with these studies, our lab has also described learning-induced physiological changes in the OB and aPC in the rat pup learning model (Yuan et al., 2002; Yuan and Harley, 2012; Fontaine et al., 2013; Morrison et al., 2013). However, those earlier imaging and electrophysiological techniques have limited capability to capture large ensemble activity with single cell resolution. In this study, *Arc* catFISH was used to monitor learning-induced spatiotemporal activity patterns of sparsely distributed neurons with single cell resolution (Shakhawat et al., 2014a,b). Due to the lack of mature anterior commissural projections in one week-old rat pups, odor learning can be confined to one olfactory bulb hemisphere through single naris occlusion during training, permitting the learned vs unlearned bulb to be tested within the same animal (Kucharski et al., 1986a; Kucharski and Hall, 1987; Yuan and Harley, 2012). Previous work from our lab using *ex vivo* calcium imaging suggests an enhanced odor representation in the aPC following learning – the threshold for pyramidal cells to respond with action-potential dependent calcium transients was lowered in the learned hemisphere (Fontaine et al., 2013). This suggests early odor learning may strengthen previously weakly

responsive cells through synaptic potentiation so that those cells are recruited more reliably to the conditioned odor input.

Here using *Arc* catFISH we were able to demonstrate that learning increased the likelihood of reliably recruiting more similar ensembles to the rewarded odor (Shakhawat et al., 2014a,b). The overlap between two ensembles activated by the rewarded odor increased from ~25% to ~49% in the OB (Fig 3.2) and from ~18% to ~40% in the aPC (Fig 3.4). An increased number of repeatedly activated neurons to the rewarded odor resulted in a more stable learning-induced odor representation. Interestingly, the overall size of the odor representation remained unchanged following learning (Fig 3.2 & 3.3). Whereas ~7-8% of mitral cells were shown to be responsive for odors in the OB, sparser, i.e. 1%, odor representation was observed in the aPC (Fig 3.2, 3.3 & 3.4). Generally interneurons do not express *Arc* (Vazdarjanova et al., 2006; McCurry et al., 2010), but surprisingly granule cells of the OB do recruit *Arc* (Fig 3.3). In granule cells, we found that learning did not change the overall sparse activity pattern (~5%) of granule cells in the OB (Fig 3.3). However, similar to principle cells of the OB and aPC, the stability of the granule cell network responsive to the rewarded odor increased significantly following learning (up to 50% overlap of the two ensembles responding to the rewarded odor) (Fig 3.3). Altogether, with odor associative learning, variable odor representations became more stable and thus the precision for and likelihood of memory recall may have been enhanced (Shakhawat et al., 2014a,b).

6.1.3 Activity-dependent ensemble modification in aPC following odor discrimination learning in adult rats

A variety of theoretical and computational models propose that the PC possesses characteristics of associative cortices (Haberly, 1985; Ambros-Ingerson et al., 1990; Hasselmo et al., 1990; Granger and Lynch, 1991; Haberly, 2001; Linster et al., 2009), thus it becomes a plausible model system to study activity-dependent synaptic plasticity in general (Gottfried, 2010; Wilson and Sullivan, 2011; Yuan and Harley, 2014). Using *Arc* catFISH, we visualized three forms of aPC representational plasticity: adult odor discrimination learning (1) creates a stable odor engram for the rewarded odor; (2) enhances pattern separation between highly similar conditioned odor pairs when discrimination is required; and (3) reconstructs an odor engram from the fragmented input of the odor mixture that signals reward. In general, consistent with other network studies, we also found robust and sparse odor representation in the aPC of the adult rats (Shakhawat et al., 2014a,b).

6.1.3.1 Successful odor discrimination in adult rats sharpens the ensemble representation for the rewarded odor.

Arc imaging revealed that odor discrimination learning resulted in the creation of a stable odor engram in the aPC, similar to what we observed in the neonate aPC, following early odor associative learning (Shakhawat et al., 2014a,b). Ensemble overlap to the rewarded odor increased from 25% to 40% after learning (Fig 4.1). Further mechanistic investigation showed that the stable engram arose from a reduction in weakly or randomly activated cells, leading to sharper, and a more reliable network representation of the rewarded odor. Furthermore, unlike neonates, there

was a significant reduction of the odor representation size (from 5% to 2.5% to the rewarded odor) in adult rats (Fig 3.4 & 4.1).

6.1.3.2 Successful odor discrimination de-correlates neuronal ensembles representing highly similar odors in the aPC.

Although dissimilar odors did not show enhanced spatial segregation in their odor representations in aPC for successful discrimination (Fig 4.1), more challenging similar odor discrimination learning promoted pattern separation in the aPC (Fig 4.3). Discrimination of a highly similar odor pair required significantly more training, but evolved with disambiguated odor representations (less overlapping) for rewarded and unrewarded odors in the aPC (Fig 4.3).

6.1.3.3 Reward learning with a two-odor mixture increases the similarity of the two odor representations

Following associative training of an odor mixture with the water reward, the degree of the overlap between the two components of the odor mixture significantly increased (from ~ 20% to ~ 45%) in the trained rat, suggesting that the odor representations of the two odorants became highly similar after conditioning (Fig 4.2).

6.1.4 Adrenergic modulations in the OB and aPC underlie highly similar odor discrimination learning in adult rats.

Adrenergic blockade in the aPC prevented the discrimination of highly similar odors (Fig 5.2). This suggests for the first time that NE in the aPC is vital in similar odor discrimination learning. The same intervention in the OB slows similar odor discrimination learning (Fig 5.1). *Arc* ensemble visualization demonstrated that aPC ensemble stability was reduced and pattern separation was impaired when the OB was subjected to adrenergic blockade (Fig-5.1). However, although impairment in ensemble stability was observed in the OB, pattern separation was not seen in the OB whether or not adrenergic receptors were blocked in the aPC (Fig 5.2).

6.2 Our findings in the neurobiology of learning and memory

6.2.1 Role of α_2-adrenoreceptors in learning and memory

Although β-adrenoreceptors have been extensively studied as a primary mediator for early odor preference learning, current literature suggests multiple types of adrenoreceptors are involved in this learning model (Hayar et al., 2001; Yuan et al., 2003a; Harley et al., 2006; Yuan, 2009; Lethbridge et al., 2012). The diffuse nature of noradrenergic fiber innervations in the OB and the possibility of volume transmission of NE (Agnati et al., 1995; Umbriaco et al., 1995)increases the likelihood of the involvement of multiple adrenoreceptors expressed in different layers of the OB during odor preference learning. We have shown that α_2-adrenoreceptors together with β-adrenoreceptors act in concert to enable odor preference learning (Fig 2.3). The α_2-adrenoreceptor is not only involved in odor learning, it has also been shown to be crucially involved in amygdala-dependent fear memory creation in chicks (Gibbs and Summers, 2003).

6.2.2 Role of adrenoreceptors in adult odor discrimination learning

Though the role of OB adrenoreceptors in adult rodent odor discrimination learning has been characterized previously (Doucette et al., 2007; Escanilla et al., 2010), we are the first to demonstrate the essential role of adrenoreceptors in the aPC for adult rat odor discrimination learning (Fig 5.2). Doucette *et al* (2007) reported that pharmacological blockade of both β- and α-adrenoreceptors in the mouse OB impairs similar odor discrimination learning. Impairment in discrimination learning is only observed when both types of adrenoreceptors are blocked. Easy odor discrimination learning remains unaffected following adrenoreceptor blockade in the OB. One year later Mandairon *et al* (2008b) reported that reward-motivated discrimination learning slows down when both adrenoreceptor antagonists are applied in the OB. Though the odor pairs employed in the above two studies are different, all the odors are perceptually similar and hence difficult to discriminate. However, the behavioral paradigms used by these two studies were different. Whereas Doucette *et al* (2007) used an olfactometer to train-water deprived mice in the discrimination task, Mandairon *et al* (2008b) used a food digging paradigm to train-food deprived rats to discriminate two odors. Using a similar digging paradigm in mice, a recent study suggested that, as opposed to odor associative learning, bulbar blockage of adrenoreceptors is required for odor perceptual learning (Vinera et al., 2015). We have used an olfactometer and go-no-go discrimination learning, similar to Doucette *et al* (2007), to test the role of NE in odor discrimination learning. Our results suggest that bulbar adrenoreceptor blockade only partially impacts similar odor discrimination learning, whereas aPC adrenoreceptors are necessary for the similar odor discrimination since adrenoceptor blockade in the aPC completely prevented discrimination of highly similar odors (Fig 5.2).

6.2.3 Sparse coding and discriminability of sensory stimuli

Sparse distributed coding has several beneficial features that assists the brain in storing nearly unlimited information (Willshaw et al., 1969; Marr, 1971; Field, 1987; McClelland et al., 1995; Norman and O'Reilly, 2003; Waydo et al., 2006; Babadi and Sompolinsky, 2014; Wixted et al., 2014). It is an energy efficient way to code information (Levy and Baxter, 1996), and eases the subsequent readout of complex data for further processing (Olshausen and Field, 2004). Obviously it not only increases the capacity of the brain to store numerous associative memories (Brunel et al., 2004), but can speed up the learning process as well (Schweighofer et al., 2001). For that idea, it is usually assumed that sparse coding reduces the probability of overwriting previously stored information (Willshaw et al., 1969; Olshausen and Field, 2004). Different faculties of the brain have been shown to utilize sparse coding ubiquitously to encode various forms of sensory information (Young and Yamane, 1992; Rolls and Tovee, 1995; Vinje and Gallant, 2000; Brecht and Sakmann, 2002; Laurent, 2002; Perez-Orive et al., 2002; Vinje and Gallant, 2002; DeWeese et al., 2003; Theunissen, 2003; Yuan and Harley, 2014). Sparse coding is preserved across phyla (Young and Yamane, 1992; Rolls and Tovee, 1995; Vinje and Gallant, 2000; Brecht and Sakmann, 2002; Laurent, 2002; Perez-Orive et al., 2002; Vinje and Gallant, 2002; DeWeese et al., 2003; Theunissen, 2003; Yuan and Harley, 2014). Sparse coding is not only limited to sensory coding, but is also applicable in the motor system (Hahnloser et al., 2002; Beloozerova et al., 2003; Brecht et al., 2004). Despite the numerous advantages of sparse coding, certain trade-offs, such as a more limited capacity for generalization, associated with sparse coding should also be considered (Spanne and Jorntell, 2015).

Sparse and distributed coding has also been reported in the OB (Assisi et al., 2007; Luo et al., 2010; Olsen et al., 2010; Koulakov and Rinberg, 2011; Yu et al., 2013) and PC (Stettler and

Axel, 2009; Isaacson, 2010; Davison and Ehlers, 2011; Wilson and Sullivan, 2011). These coding properties optimize the OB and aPC capacity to represent odors in confined networks (Shadlen and Newsome, 1998; Olshausen and Field, 2004) and it is particularly advantageous in the face of network degradation (Slotnick and Bisulco, 2003; Slotnick et al., 2004; Bracey et al., 2013). Specifically, significant experimental evidence has accumulated which suggests that mitral cells contribute to odor identification processes through sparse (Fantana et al., 2008), spatially distributed (Johnson et al., 1999), and multidimensional (Johnson and Leon, 2007) glomerular activity, which was found to be preserved across species and individuals (Soucy et al., 2009). Consistent with these studies, *Arc* catFISH revealed that mitral cell and granule cell representations are sparse and widely distributed in the OB (Shakhawat et al., 2014b). We found that only ~7–8% of the mitral cells (Fig 3.2) and ~5% of granule cells responded to the peppermint odor in rat pups (Fig 3.3). aPC odor representation is sparser than the OB (Fig 3.4). Only ~1% of pyramidal cells in the aPC responded to odor in pups (Fig 3.4) and ~3-5% in adult rats (Fig 4.1). The smaller representation size in pups may be attributed to the immaturity of mitral cell axons at this age (Sarma et al., 2011) such that fewer inputs are active than in the adult for any given representation. As discussed, sparser representation of sensory information has been observed in different species, including humans. For example, Waydo *et al* (2006) shows that only 1% of hippocampal neurons participate in semantic memory representations in humans.

6.2.4 Emergence of a more stable odor representation following learning

It has been a challenge in neuroscience for a long time to prove that the same neuronal network that is involved during information encoding is also engaged during the retrieval process. The reason behind the failure to study engram dynamics using traditional scientific methods is, in

large part, due to the elusive nature of the memory representation in the brain. Lashley's three decades of work leads to the conclusion that memory is sparse, widely distributed and dynamic in nature (Lashley, 1950). Only in recent years have improvements in modern technologies allowed us to visualize the engram, and even to manipulate it (e.g. erasing a memory) (Boyden et al., 2005; Liu et al., 2012; Ramirez et al., 2013; Nabavi et al., 2014).

The prevailing view of memory formation at the neuronal network level is that plasticity mechanisms allow the formation of stable neuronal ensembles by strengthening connections between populations of neurons that are involved in encoding (Bliss and Collingridge, 1993; LeDoux, 2000; Josselyn et al., 2015), although this idea has recently been challenged (Ryan et al., 2015). Once memory is formed, the likelihood of the same population of neurons participating in both memory retrieval and encoding is significantly increased (Reijmers et al., 2007; Denny et al., 2014). In fact the term "memory engram" originally referred to the hypothetically encoded information stored in the brain, which must participate in recall (Semon, 1904; Josselyn, 2010). Recent findings in the hippocampus support this idea by showing memory engram cells that are involved in memory encoding are both necessary (Tanaka et al., 2014) and sufficient (Liu et al., 2012; Ramirez et al., 2013; Redondo et al., 2014) for recall of the learning event (contextual fear memory) in the future. Although these findings show that memory encoding neurons are also involved in retrieval, they fail to describe what percentage of initially activated cells is finally incorporated into the engram. Not necessarily all the activated cells during the initial encoding will be part of the final engram for that particular memory. Along with this idea, recently Denny *et al* (2014) have shown that only a very small percentage of neurons (DG and CA3) that are involved in encoding are reactivated during memory expression. This suggests that a small percentage of cells involved in the initial encoding may be required for successful memory recall.

It would be interesting to test the overlap between the encoding and the memory recall ensembles in our model; however, this is not feasible due to the limitation of *Arc* temporal dynamics. In this body of research, instead, we asked whether adaptive learning facilitates stable engram formation. We have shown that early odor preference learning creates more stable rewarded odor representations in both the OB and aPC (Shakhawat et al., 2014b). We took a simple approach and asked how many cells are repeatedly activated by the rewarded odor. The percentage of the cells that are repeatedly activated by a rewarded odor may potentially be the cells that drive learned behavior. We found an estimated ~300 aPC neurons are repeatedly activated for the rewarded odor, which is within the limit on which olfactory decision are based (Choi et al., 2011; Miura et al., 2012). Furthermore, we have shown that the likelihood of reactivating the same neurons to the rewarded odor increases following odor associative training (Shakhawat et al., 2014 a,b). Principle neurons that are activated twice by the rewarded odor peppermint were found to be ~ 49% and ~40% of the total activated cells (cells activated once + cells activated twice) in the OB and aPC respectively (Fig 3.1, 3.2 &3.4). This overlap ratio is significantly higher than the control group (~25%; Fig 3.1, 3.2 &3.4). Similar to the principle cells of the OB, the likelihood of the same granule cells being activated to the rewarded peppermint odor increases from ~25% to ~50% following learning (Fig 3.3). Increased stability of the odor representation was found to be preserved in adult rats as well (Shakhawat et al., 2014a). Following adult odor discrimination learning, the stable component of the rewarded odor representation in the aPC is significantly higher (~40%) than that in the control group (~25%) (Fig 4.1-4.3).

In subsequent experiments we have shown that noradrenergic modulation is required for highly similar odor discrimination learning (Shakhawat et al., 2015). Less stability of the rewarded odor representation is seen when the adrenoreceptors are blocked either in the OB (~20% vs. ~40%

in the saline group) or the aPC (~20% vs. ~35%) (Fig 5.1-5.2). A similar trend is also observed in granule cell odor representations (~17% in the drug group vs. ~38% in the saline group). Thus our data support the view that plasticity induced by learning results in a more stable representation of the memory (Shakhawat et al., 2014a,b).

6.2.5 Representational variability indexed by Arc

Odor ensemble representation in the OB and aPC is found to be highly variable, which holds true for both pups and adult rats (Shakhawat et al., 2014a,b). The overlap between the neuronal ensembles activated by the same odor (in this case peppermint) varies from ~18% to ~30%. Following learning, the overlap increases up to ~ 40% to ~50%. What accounts for such a big representational variability to a single odor even after learning? The rationale behind this high variability in *Arc* readout has recently been extensively discussed (Yuan and Harley, 2014). Odor representation in the OB is state-dependent and is subjected to multiple top-down cortical feedback inputs. It has been shown that neuromodulatory input (Rinberg et al., 2006; Mandairon and Linster, 2009; Doucette et al., 2011), context (Kay and Laurent, 1999; Doucette and Restrepo, 2008; Restrepo et al., 2009), and other cortical top-down modulations (Chapuis et al., 2013; Rothermel and Wachowiak, 2014) may substantially influence odor representation even at the level of the OB. For example, Mandairon *et al* (2014) have recently shown that even visual information is encoded in the OB. Furthermore, this variability may also be the result of the variability in the odor environment (Babadi and Sompolinsky, 2014). Similarly, non-olfactory activity during discrimination tasks (participation in the odor sampling, when/where to lick, and receiving reward) has been shown to influence piriform cortical activity (Schoenbaum and Eichenbaum, 1995; Zinyuk et al., 2001). Another possibility for representational variability is that memory retrieval

initiates subsequent changes in the already consolidated engram for reconsolidation to occur (Dudai, 2000; Nader, 2003). Such remodeling of the memory engram may add extra variation in the learned odor representation for the second odor exposure.

One of the major reasons behind the high variability in representation might be related to the *Arc* readout itself. Although *Arc* has been shown to be promoted by glutamatergic excitatory synaptic input (Cole et al., 1989), it may not be expressed in cells that fire spontaneously (Rinberg et al., 2006) or are activated via muscarinic inputs (Padmanabhan and Urban, 2010; Angelo and Margrie, 2011). Hence *Arc* readout may vary from the real time odor representation in the bulb and aPC. Despite the fact that animals are very fast in odor coding and perception (Uchida and Mainen, 2003; Wesson et al., 2008), the odor-evoked activity in the OB and aPC itself is dynamic and continues to emerge from first sniff to perception or olfactory decision making (Friedrich and Laurent, 2001; Rennaker et al., 2007; Schaefer and Margrie, 2007; Patterson et al., 2013). For example, it has been recently shown that initial odor representations which arise from first single sniff may vary from olfactory after-images (Broome et al., 2006; Patterson et al., 2013). *Arc* readout may not necessarily capture all representational variability at different time points of odor evoked activity pattern in the OB and aPC and this may induce additional variation in the odor representation.

6.3 Limitation of Arc catFISH

Despite the numerous advantages of *Arc* catFISH as a technique to visualize spatiotemporal representations of sensory information at the single cell level (Yuan and Harley, 2014), it is unable to capture sensory representations that evolve through rate coding (McAdams and Maunsell, 1999). Our data reveal that simple odor representations in the aPC are not spatially segregated,

though rats are able to discriminate that odor pair (Chapter 3). Pyramidal cells in aPC might disambiguate the simple odor pair using rate coding. Recent studies demonstrate pattern separation in the OB (Doucette et al., 2011; Gschwend et al., 2015), but it was not seen by the *Arc* catfish method used in this thesis (Shakhawat et al., 2015).

One of the challenges in *Arc* catFISH is the time consuming, manual counting methods used for analysis. To circumvent this problem a machine readable automated counting 3D-catFISH technique has been proposed (Chawla et al., 2004). Another limitation of this technique is the temporal constraint (~30 min) to visualize activity patterns for two consecutive events. This technical drawback limits our ability to monitor the activity pattern of cell assemblies for extended time periods at multiple intervals. The requirement to sum over a 5 min interval also precludes second to second temporal resolution.

6.4 Arc catFISH – advantages of this technique for studying activity-dependent system level synaptic plasticity in the olfactory system

Visualizing hippocampal ensembles activated by context-A twice shows ~70% overlap between two representations in naive animals (Guzowski et al., 2001; Marrone et al., 2014). In line with this idea, 50% ensemble- overlap was reported in the extrastriate visual cortex when the same visual stimulus is repeatedly presented to a naïve mouse (Rudinskiy et al., 2012). Interestingly, for odor stimulation, a single odor such as peppermint only results in ~30% overlap between the two aPC ensembles when given repeatedly (Shakhawat et al., 2014a). Such variability in the odor representation captured by *Arc* catFISH in naive animals leaves room to test how learning promotes pattern stability, separation, and completion in this system.

Another advantage of this technique is that it can be employed to study ensemble dynamics in pups. Currently available tetrode recording, or *in vivo* calcium imaging, though applicable in adult animals for studying the neural circuitry underlying learning (Ziv et al., 2013), is not suitable for such investigation in pups.

6.5 Synthetic vs elemental perception of odors

A point of general interest in olfactory physiology is whether odor representation in the OB and PC is analytic (elemental) or synthetic (configural) (Wilson and Stevenson, 2003b; Gottfried et al., 2006; Kadohisa and Wilson, 2006). Whether the olfactory system perceives a given odor in an analytic or synthetic format is still unclear and often confusing (Kay et al., 2005). The question of where that computation occurs, whether it is in the OB or in the olfactory cortex, remains to be explored. Recent studies in newborn rabbits indicate that the ratio of the components in an odor mixture is the determinant factor of how the olfactory system (mainly the bulb and posterior piriform cortex) perceives an odor (elemental vs. synthetic) (Schneider et al., 2015). However, a different study in the OB suggests that the spatial activity pattern that emerges due to an odorant mixture is not always a good marker for specifying component recognition in the mixtures (Grossman et al., 2008). Although different computation/theoretical models have been proposed to resolve these issues in this field (Olsson, 1994; Linster and Cleland, 2004), clear important links between the ensemble activity and the behavioral outcome are still missing (Migliore et al., 2010).

We have shown that when a mixture of vanillin and peppermint (50:50) is used as the rewarded odor, trained rats respond to both components just as to the mixture (Fig 4.2), suggesting a unified perception of this mixture and its components, similar to what has been proposed by Linster and Smith (1997). After learning, significantly more overlap (~44%) is observed between

the two odor ensembles activated by peppermint and vanillin alone (Fig 4.2). This supports the idea that associative training of the odor mixture with a reward leads to the merging of the component ensembles in the aPC. Therefore, the odor processing in the aPC is synthetic in nature. It would be interesting to look at whether the ensemble overlap between the component and the mixture also increases with learning. If true, this would index pattern completion.

6.6 Future challenges to meet

6.6.1 Role of adrenoreceptors in odor preference learning

Although we have shown that α_2-adrenoreceptors, in addition to β-adrenoceptors, are involved in early odor preference learning, further investigation is necessary to segregate their role in the different steps of learning. Whether they involved during encoding, consolidation, and/or memory expression requires more systematic investigation. It would be interesting to test whether mice with homozygous deletion of dopamine-β-hydroxylase (DBH; Sanders et al., 2006), which lack NE, have difficulties in early odor preference learning and whether α- or β-adrenoceptor activation can rescue the learning deficiency. Interestingly, Thomas and Palmiter (1997) have shown that $Dbh^{-/-}$ mice have deficits in active-avoidance learning. Furthermore, using the same transgenic mice, Zhang *et al* (2005) have also assessed the critical role of adrenergic signaling in contextual and spatial memory retrieval in the hippocampus. An early odor preference learning model has been recently established in mice (Roth et al., 2013). The mouse model will enable behavioral studies with genetic manipulations and open new avenues for molecular dissections of the underlying learning circuitry.

Another interesting question is the interaction between different modulators during learning. As we have found that multiple adrenoreceptors act synergistically to induce odor

learning in pups, it is plausible to speculate that NE might interact with other neuromodulators, such as ACh, in the olfactory system. A recent computational model proposed that NE and ACh together can enhance the signal-to-noise ratio and may facilitate synchronization among mitral cells (Li et al., 2015). Although this computational model suggests that NE plays a role in the regulation of cholinergic function, behavioral evidence for such a claim is still missing and warrants further investigation.

6.6.2 Causality of CREB in neonate odor preference learning

Increased pCREB expression in the MC following the pairing of the α_2-adrenoceptor agonist clonidine and a novel odor suggests that CREB is a common factor that different signalling pathways converge on. A causal role for CREB in β-adrenoceptor-mediated early odor preference learning has been shown by McLean's lab using OB infusion of a Herpes simplex virus that carries either CREB or mutant CREB genes (Yuan et al., 2003b). Mutant CREB prevents normal learning induced by pairing an optimal dose of isoproterenol with an odor. The causal role of CREB in α_2-adrenoceptor mediated learning can be tested similarly using CREB knock-in or knock-out mice. Han *et al* (2007) showed that microinjecting $CREB^{WT}$ in the lateral amygdala of the CREB-deficient mice rescues fear memory that would otherwise be impaired in this mutant strain. Results from this study leads to the conclusion that cells that over express CREB are more likely to be recruited by fear learning compared to other cells in the region. Similarly we could also test this hypothesis in our model. The question would be whether early odor preference memory preferentially recruits cells that overexpress CREB. Tests could be done by checking the preferential recruitment of *Arc* in the cells that overexpress $CREB^{WT}$ (Han et al., 2007) following either training or testing.

6.6.3 Furthering our understanding of olfactory circuit dynamics using Arc catFISH

Odor-selective excitation and inhibition between mitral cells and granule cells suggested by this leads to the question of whether PG would show similar changes to mitral and granule cells following learning assuming they could express *Arc*. Schoppa and Westbrook (2001) discovered a synchronized oscillation among mitral cells that project to the same glomerulus. According to a recent computational model, periglomerular cells and granule cells differentially influence mitral cells' spiking (Arruda et al., 2013). Thus, *Arc* catFISH readout of PG cell activity following learning would help us to shed light on whether ensembles of PG cell activity are synchronous with and/or coupled to MC and GC activity.

Genetic deletion of NMDA/NR1 subunits and optogenetic inhibition of aPC pyramidal neurons, similar to that recently proposed in the striatum (Land et al., 2014), will help us to unravel the underlying synaptic mechanisms involved in pattern separation, completion and increased stability of odor representations in the aPC following odor learning. Similar manipulation in the hippocampus impairs pattern separation and completion (Gilbert et al., 2001; Nakazawa et al., 2002; Gold and Kesner, 2005; McHugh et al., 2007; Willshaw et al., 2015). Neurotoxin lesion in DG results in impaired pattern separation, leading to the idea that the DG is involved in detecting subtle differences among similar objects (Gilbert et al., 2001). Similarly control manipulation of aPC (sodium channel blocker administration or optogenetic inhibition of aPC neurons) together with *Arc* catFISH will ascertain aPC role in all those sensory phenomena.

Odors may be perceived differentially in the two hemispheres, and a few studies suggest that the right hemisphere is dominant in odor perception in humans (Zucco and Tressoldi, 1989; Jones-Gotman and Zatorre, 1993; Levy et al., 1997b). On the other hand recent studies reveal a transient asymmetry in piriform cortical oscillation during odor discrimination learning, with a

transient bias to the left hemisphere (Cohen et al., 2015). This dispute in the current literature could be resolved by looking at the ensemble activity of the two hemispheres using *Arc* catFISH.

It has been shown that Alzheimer patients have impaired pattern separation and completion ability (Ally et al., 2013; Wesnes et al., 2014). Olfactory performance (odor habituation and identification), particularly performance associated with piriform cortical function, has been shown to be impaired in both human Alzheimer patients (Li et al., 2010) and in an animal model of Alzheimer's (Wesson et al., 2010; Wesson et al., 2011). Human amyloid β precursor protein expression in the piriform cortex abnormally elevates the local field potential in Tg257 mice (Wesson et al., 2011). *Arc* catFISH could be employed in the alzheimer disease (AD) mouse model to test whether their circuit dynamics are disrupted along with their ability to discriminate odors.

6.6.4 The role of norepinephrine in adult odor learning

We have shown that adrenoceptor blockade in either the OB or the aPC impairs similar odor discrimination learning and odor representation in the connected projection area (for example, aPC representation is affected when the adrenoreceptors are blocked in the OB) (Fig 5.1-5.2). However, direct visualization of the drug target region is elusive due to tissue damage. One way to visualize the area being directly manipulated is to optogenetically control the activity of the LC during odor guided behavior. However, results of such experimentation may not necessarily help us to discern the region-specific role of the noradrenergic system because of the global impact that will occur if the LC is activated optogenetically. LC optogenetic stimulation can be combined with local adrenoceptor blockade to dissect more region-specific roles of the LC-NE in odor learning.

6.6.5 Exploring neighbouring areas of the olfactory system

The olfactory tubercle (OT) critically mediates odor valence learning (Gadziola et al., 2015). A recent study using c-fos mapping in the OT, discovered two distinct sub-regions that are differentially activated by aversive and appetitive odors following odor associative training (Murata et al., 2015). Whereas the anteromedial domain of the OT is preferentially involved in approaching behavior, the lateral domain is activated during aversive behavior (Murata et al., 2015). These suggest the OT, like the aPC, also undergoes activity-dependent changes to support corresponding odor-guided behavior. *Arc* catFISH could be employed to study how ensemble stability in each sub-region of the OT changes following learning. A comprehensive comparison between the PC and the OT could be performed to define their differential roles in odor learning.

6.6.6 Remote odor memory

Our preliminary data suggest that rats can still remember the rewarded vs unrewarded odor after 30 days of training. Future experiments could be designed to look at the rewarded odor representations 30 days after learning. Will it still be in the aPC, or will it redistribute to other cortical areas similar to what has been proposed in hippocampal-dependent learning and memory (Frankland et al., 2006; Goshen et al., 2011)? It has been shown that 30 days following fear conditioning in context A, similar conditioned responses occurs for both context A and a novel context B. This phenomenon is termed “memory generalization”. Consistent with this behavioral generalization, significant overlap between ensembles activated by context A and context B was also observed 30 days following fear conditioning (Denny et al., 2014). *Arc* catFISH would be able to tell us whether ensemble representation changes in the case of remote memory.

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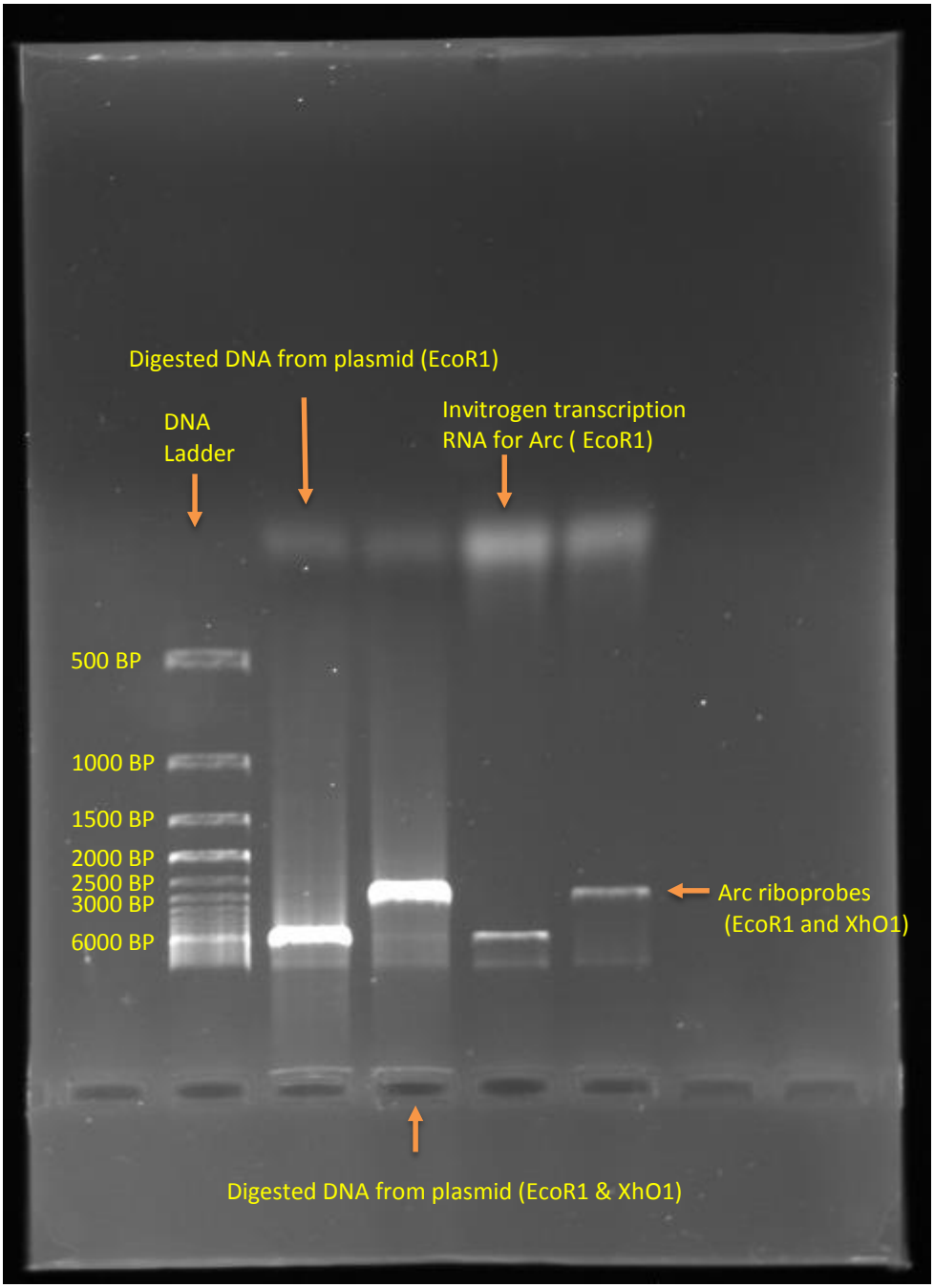
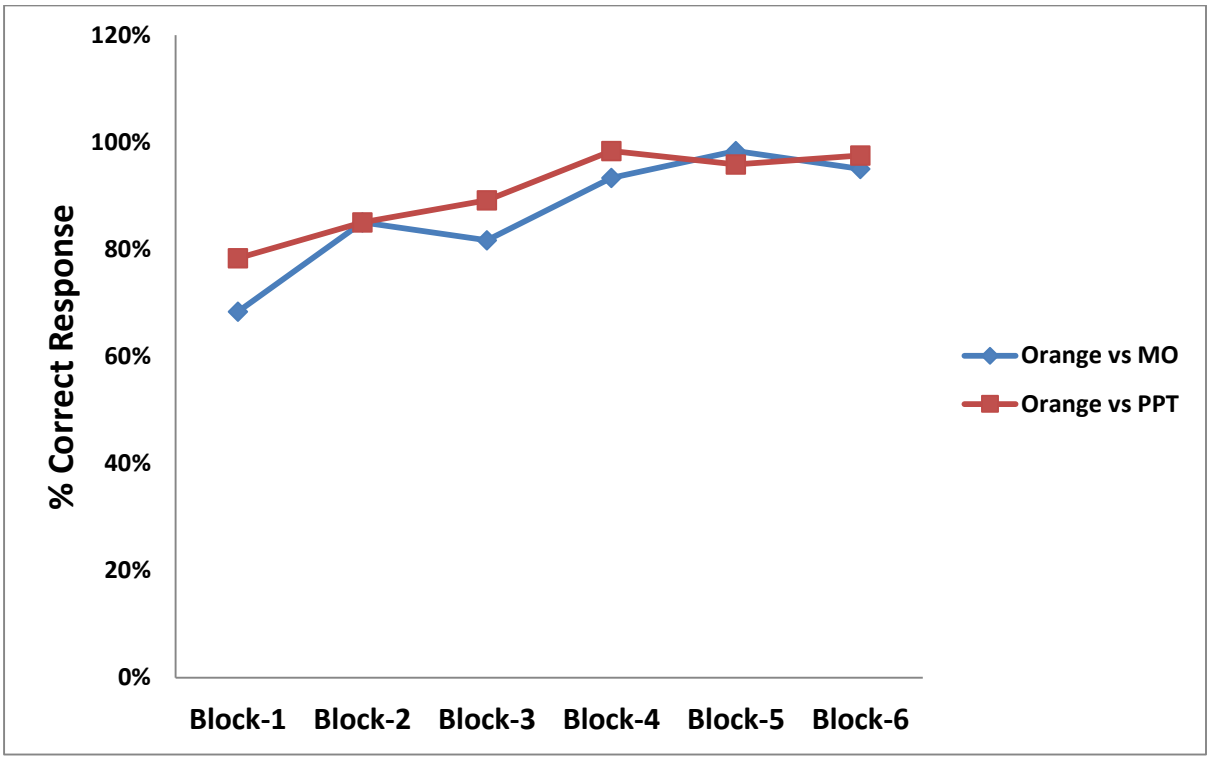
Appendix-A: Cannula placement verification

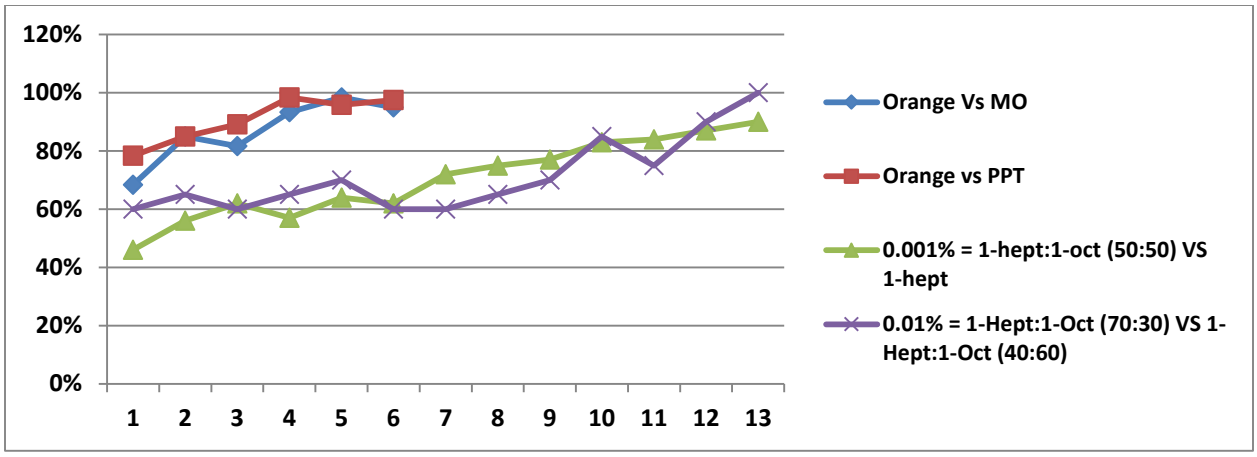
Description: An intra-bulbar infusion of 4% methylene blue dye targeting centre of the bulb. Coronal and horizontal view indicate successful targeting of cannulae position.



Description: 2 µl of riboprobe were run in 1% agarose gel (containing ethidium bromide of 0.5 µg/ml) to check the integrity of the *Arc* riboprobes used for catFISH. The gel were run at 200 V in 0.5-1 X TBE for 20 min.



Description: Rule learning training were performed before similar odor discrimination task. In this case water deprived rats were subject to orange vs ppt discrimination task before the surgery was performed on experimental rats. Another separate group of rats were allowed to discrimnate orange vs mineral oil (MO) which shows a learning curve very much similar to orange vs ppt.



Description: A pilot study was performed to find an odor pair that is difficult to discriminate. Compare to easy discriminable odor pair (e.g. Orange vs ppt & Orange vs MO) other two odor pair { e.g. 0.001% 1-hept: 1-Oct (50:50) vs 1- Hept and 0.01% 1-hept: 1-Oct (70:30) vs 0.01% 1 hept: 1-Oct (40:60) } require extra number of trials to reach the learning threshold.

Appendix-E: Memory recall (Orange vs. Peppermint) after saline/drug infusion

Memory Recall Testing when drug or saline in aPC	
	% Correct response rate in first block
Ex-11-SOD-Rat-02-Saline	85
Ex-11-SOD-Rat-06-Saline	80
Ex-12-SOD-Rat-01-Saline	95
Avg	86.67

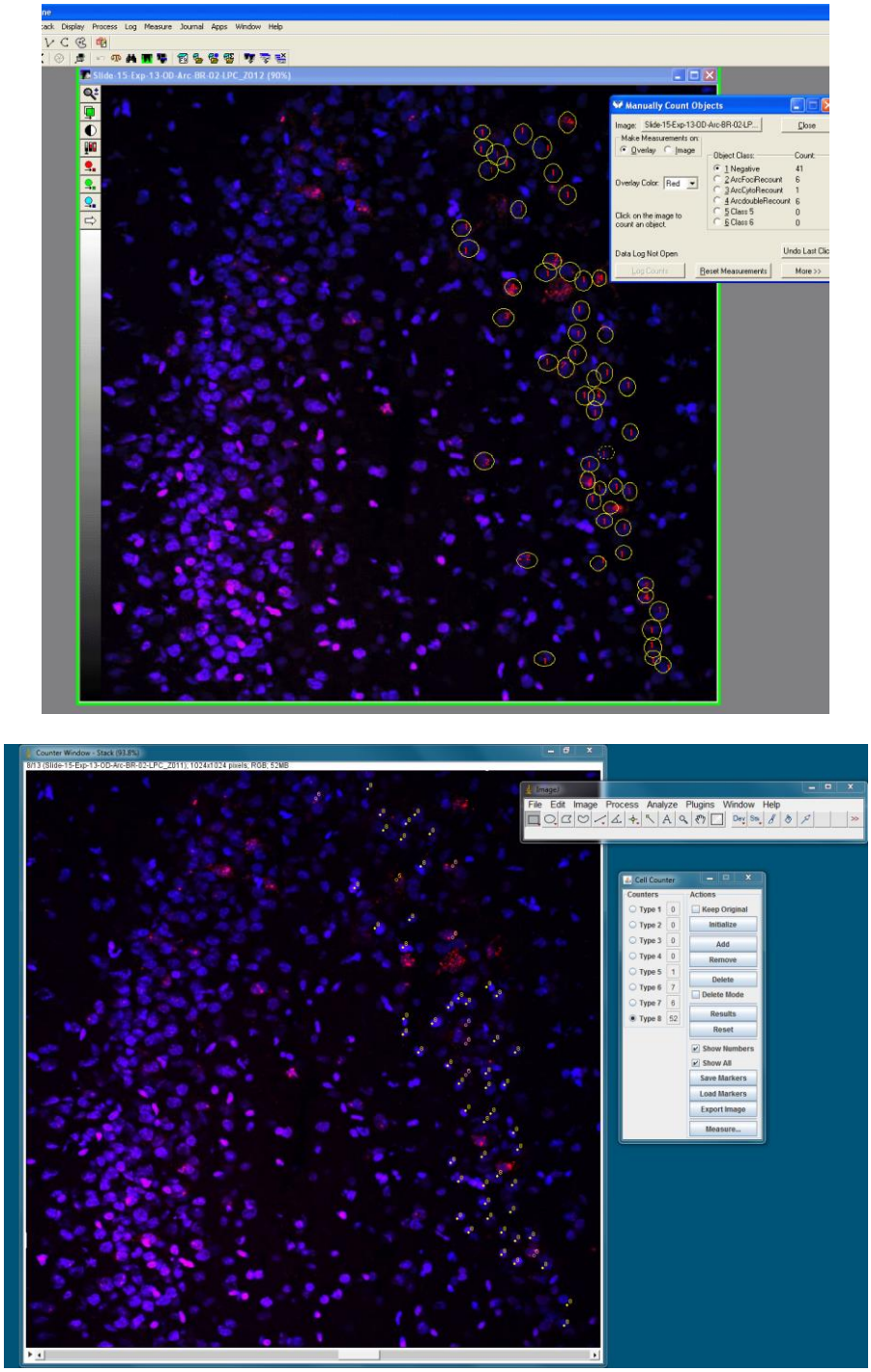
	% Correct response rate in first block
Ex-12-SOD-Rat-02-Drug	90
Ex-12-SOD-Rat-03-Drug	90
Ex-12-SOD-Rat-04-Drug	80
Avg	86.67

Memory Recall Testing when drug or saline in OB	
	% Correct response rate in first block
Ex-08-SOD-Rat-01-Saline	100
Ex-08-SOD-Rat-03-Saline	80
Ex-08-SOD-Rat-05-Saline	100
Avg	93.33

	% Correct response rate in first block
Ex-08-SOD-Rat-06-Drug	90
Ex-08-SOD-Rat-09-Drug	95
Ex-08-SOD-Rat-10-Drug	90
Avg	91.67

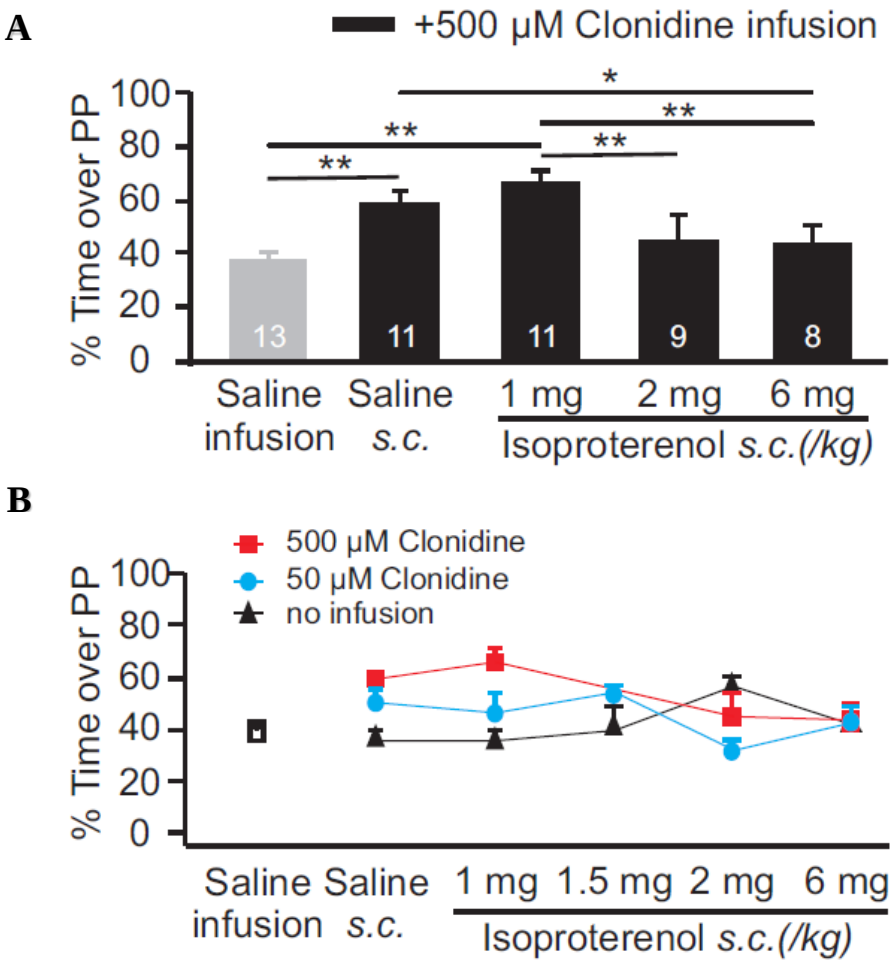
Description: One block of testing was performed to test the memory recall following saline or drug infusion. Table indicate successful memory recall (~ 86%-93% correct response rate) despite any treatment (Saline /Drug) in the OB/ aPC.

Appendix-F: Comparison between Dr. Ali Gheidi and Amin Shakhawat's counting respectively



Description: To ensure counting methodoly, an experimental blind person (Dr. Gheidi) perform counting on an *Arc* catFISH slide. Counting results were very much similar to what was found originally by the experimenter (Amin Shakhawat).

Appendix-G: α2-adrenoceptor co-activation enables odor learning with suboptimal doses of isoproterenol



Description: (A) Addition of 500 µM clonidine enabled odor preference learning in the 1 mg/kg isoproterenol group and the saline s.c. injected group **p<0.01. *p<0.05. Error bars, mean±SEM. (B) Combined dose curves including 50 µM clonidine, 500 µM clonidine and no infusion group. Note the shifts in the effective doses of isoproterenol when clonidine was co-applied.