MAKING MEMORY: NORADRENERGIC AND SEROTONERGIC INTERACTION LEADING TO cAMP RESPONSE ELEMENT BINDING PROTEIN ACTIVATION VIA cAMP MEDIATED 2nd MESSENGER SIGNALLING IN NEONATE RAT ODOR PREFERENCE LEARNING

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Making Memory: Noradrenergic and Serotonergic Interaction Leading to cAMP Response Element Binding Protein Activation via cAMP Mediated 2nd Messenger Signalling in Neonate Rat Odor Preference Learning

by

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A thesis submitted to the
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Ph.D.

Faculty of Medicine
Memorial University of Newfoundland

October, 2002

St. John's Newfoundland Canada

Abstract

Early odor preference learning offers a unique paradigm for the study of natural mammalian learning. Week-old rat pups form an approach response to an odor that is paired with a tactile stimulus such as stroking. In this associative conditioning paradigm, norepinephrine (NE) input to the olfactory bulb from the locus coeruleus serves as the unconditioned stimulus (UCS) while olfactory nerve stimulation by odor input serves as the conditioned stimulus (CS). β-adrenoceptors are critically implicated in NE-mediated UCS effects. The β-adrenoceptor agonist, isoproterenol, can substitute for stroking to induce early odor preference learning. Activation of β-adrenoceptors in the olfactory bulb is both necessary and sufficient to induce early odor preference learning. The effects of isoproterenol exhibit an inverted-U curve; while a moderate dose of isoproterenol is effective in inducing odor preference learning when paired with an odor, both higher, or lower doses of isoproterenol fail to induce learning. Serotonin (5-HT) depletion shifts the isoproterenol UCS curve to the right. In an earlier model for odor preference learning, Sullivan and Wilson proposed that early odor preference learning results from the NE disinhibition of mitral cells from granule cells via β-adrenoceptors. This strengthens the mitral to granule cell synapses and increases mitral cell inhibition during memory retrieval.

In the present thesis, I propose a new model for early odor preference learning. I

suggest that a cAMP cascade activated by the NE UCS, likely via β_1-adrenoceptors, directly modulates the olfactory nerve to mitral cell connections and results in CREB transcriptional activation in the mitral cell which underpins long-term memory formation. The new model is based on the following evidence: effective CS and UCS pairing enhances phosphorylated CREB (pCREB) expression in mitral cells of the olfactory bulb and potentiates the olfactory nerve evoked field potentials of mitral cells (Chapter 2). Odor preference learning produces long-lasting increases in blood flow at the level of the olfactory nerve to mitral cell synapses which are observed during memory retrieval, and which support the hypothesis of a stronger odor input signal during memory. This is demonstrated by intrinsic optical imaging showing an enhanced response at the level of glomeruli 24 hrs after odor conditioning (Chapter 3). β_1-adrenoceptors and 5-HT_{2A} receptors co-localize in mitral/tufted cells. 5-HT depletion decreases NE-induced elevations of cAMP in mitral cells. 5-HT appears to promote NE-induced learning through convergence on the cAMP cascade in mitral cells (Chapter 4). CREB plays a causal role in early odor preference learning. Manipulations of CREB levels by a viral vector injection directly into the olfactory bulb change the likelihood of learning and this is reflected in alterations in pCREB (Chapter 5).

Acknowledgement

I wish to thank my supervisor Dr. John McLean, who has provided me the opportunity to participate in the Neuroscience program at Faculty of Medicine, Memorial University of Newfoundland, and to complete my Ph.D thesis work in his lab; who has opened a door for me toward a scientific world; who has guided me patiently and persistently; who has pulled me through frustrations and celebrated with me every small success I have achieved. I came to Canada with limited English language skill and dreams for a scientific career; without him, I could not have achieved what I have dreamed.

I wish to thank my co-supervisor Dr. Carolyn Harley, whose spirit and scientific creativity have always inspired me; who has shared her intelligence and ideas with me; who has encouraged me to face the challenges; who has guided me, little by little, to pursue the exciting projects for my thesis and to explore the real meaning of doing Science.

I wish to thank Andrea Darby-King, who has not only provided me her expertise and assistance throughout my program but shared my feelings like a sister; who has taught me every technique in the beginning of my research and helped me throughout my projects; who has shared laughs and tears with me and held my hands whenever I needed.

I wish to thank Dr. Thomas Knopfel, who has provided me an opportunity and resources to learn optical imaging and work on an exciting project; whose love for Science and dedication to research have inspired me.

I wish to thank my parents Shen Yuan and Suming Zhan, who have not only nurtured me but taught me the very first lessons in my life; who have given me my spirit and encouraged me to pursue my dreams; whose endless love has always accompanied me.

I wish to thank Daniel Palomo, who has given me his love and faith; who has supported me through my thesis writing; who has shared with me the excitement of doing Science and, the meaning of life.

I wish to thank CIHR for having provided me the financial support throughout my program, and all the faculty members and students in the Neuroscience program who have given me help and support. Special thanks to Dr. Richard Neuman for the correction and valuable suggestions to my thesis.

Co-authorship Statement

I, Qi Yuan, hold a principle author status for all the manuscript chapters (Chapter 2-5) in my thesis. However, each manuscript is co-authored by my supervisors and co-workers, whose contributions have greatly facilitated the development of my hypotheses in the manuscripts, the practical aspects of my experiments and the manuscript writing as described below.

Chapter 2, titled "Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve-Evoked Potentials in Normal and 5-HT-Depleted Olfactory Bulbs in Rat Pups Only at Doses That Produce Odor Preference Learning", is co-authored by Carolyn W. Harley, Jamie C. Bruce, Andrea Darby-King, and John H. McLean. As the principle author, I participated in the experimental designs and accomplished all the experimental work in Experiment 1 and a portion of the work in Experiment 2 (olfactory nerve evoked field potential (ON-EFP) recordings from 10 pups in normal rat pup groups). I accomplished the first draft writing. Carolyn W. Harley and John H. McLean contributed greatly to the design of the research proposal in this paper, and the completion and improvement of the writing. Jamie C. Bruce performed most of the physiological recordings from olfactory bulb 5-HT depleted pups. Andrea Darby-King provided substantial technical supports in both experiments and participated in data analysis, especially in Experiment 2. Mark Power and Steve Milway also provided technical

assistance in this work.

Chapter 3, titled "Optical Imaging of Odor Preference Memory in the Rat Olfactory Bulb" is co-authored by Carolyn W. Harley, John H. McLean and Thomas Knopfel. As the principle author for this work, I accomplished all the actual experimental work and data analysis, participated in the initial design of the research proposal and actual experimental designs. I co-wrote the manuscript with Thomas Knopfel, whose dedication and assistance enabled the accomplishment of the project described in this paper. He provided substantial technical assistance and participated in the actual experiments. Carolyn W. Harley and John H. McLean participated in the initial design of this work and contributed substantially to the correction and improvement of the manuscript.

Chapter 4, titled "Mitral Cell β_1 and 5-HT_{2A} Receptor Co-localization and cAMP Co-regulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb" is co-authored by Carolyn W. Harley and John H. McLean. As the principle author, I participated in the development of model hypothesis and actual experiment designs, performed most of the experimental work and finished the first draft of the manuscript. Carolyn W. Harley contributed substantially to the improvement of the manuscript, and the initiation of the proposal for a new model for the neonate rat odor preference learning from this work and previous ones. John H. McLean contributed greatly to the

experimental designs and partial experimental work, and also assisted in writing and improvement of the manuscript. Andrea Darby-King assisted in final graph making. Mary Primmer and Dr. Reza Tabrizchi provided technical assistance in this work.

Chapter 5, titled "Early Odor Preference Learning in the Rat: Bidirectional Effects of CREB and Mutant CREB Support a Causal Role for CREB" is co-authored by Carolyn W. Harley, Andrea Darby-King, Rachael L. Neve, and John H. McLean. As the principal author, I participated in the experimental design and accomplished most of the experimental work. I finished the first draft of the manuscript. Andrea Darby-King provided technical support. Carolyn W. Harley and John H. McLean participated in the experimental design and writing. John H. McLean also assisted in the actual experimental work. Rachael Neve kindly provided the viral vectors used in this study and made valuable suggestions for the manuscript. Eric Nestler completed the HSV-CREB and HSV-mCREB amplicon constructions. Dr. Jun Chen provided technical assistance in this work.

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Abbreviations

2-DG	2-deoxyglucose
5-HT	5-hydroxytryptamine;serotonin
5,7-dHT	5,7-dihydroxytryptamine
6-OHDA	6-hydroxydopamine
AA	amyl acetate
AC	adenylyl cyclase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid
ANOVA	analysis of variance
AOB	accessory olfactory bulb
AP-5	amino-5-phosphonopentanic acid
APV	D-2-amino-5-phosphonovaleric acid
ATF	activating transcription factor
BCA	bicinchoninic acid
bZip	leucine-zipper domain
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer binding protein
CF	centrifugal fiber
CNS	central nervous system
CREB	cAMP response element binding protein
CREM	cAMP response element modulater
CS	conditioned stimulus
DA	dopamine
DAB	diaminobenzidine dihydrochloride
DCG-IV	(2S, 2'R, 3'R)-2-(2'3'-dicarboxycycloproplyl) glycine
DGG	γ-D-glutamylglycine
DNQX	6,7-dinitroquinoxaline-2,3-dione
DOI	2,5-dimethoxy-4-iodoamphetamine hydrochloride

EFP	evoked field potential
EPL	external plexiform layer
fMRI	functional magnetic resonance imaging
GABA	gamma-amino butyric acid
$GABA_AR$	$GABA_A$ receptor
GC	granule cell
GCL	granule cell layer
GL	glomerular layer
Glu	glutamate
GluR	glutamate receptor
HSV	herpes simplex virus
ICER	inducible cAMP early repressor
IEG	immediate early gene
iGluR	ionotropic glutamate receptor
IPL	internal plexiform layer
LOT	lateral olfactory tract
Iso	isoproterenol
LTD	long-term depression
LTF	long-term facilitation
LTP	long-term potentiation
LTS	long-term sensitization
MCL	mitral cell layer
mCREB	mutant CREB
mGluR	metabotropic glutamate receptors
MC	mitral cell
MOB	main olfactory bulb
NE	norepinephrine
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
OB	olfactory bulb

ON	olfactory nerve
ORN	olfactory receptor neuron
O/O	odor only
O/S	odor+stroking
pCREB	phosphorylated CREB
PDE4	phosphodiesterase4
PKA	protein kinase A
PND	postnatal day
PP	peppermint
PP1	protein phosphatase 1
ROD	relative optical density
STF	short-term facilitation
UCS	unconditioned stimulus
VN	vomeronasal nerve

Chapter 1 Introduction

This introduction reviews, first, our understanding of the early odor preference learning literature up to the beginning of the experiments that comprise the thesis. The next sections review two additional topics of particular relevance for the present set of experiments: (1) the newer methodology of optical imaging and (2) the role of CREB as a putative "universal" memory molecule. The final section outlines the experiments undertaken here.

1.1 Odor preference learning in neonate rats

New born rat pups can not open their eyes until postnatal day (PND)10-12. During this early period of their life, they depend heavily on smell to locate and attach to the mother's nipples. In order to survive, a new born has to develop an attachment to its mother, regardless of the quality of care-giving (Sullivan et al., 2000a). The first postnatal week, therefore, has special meaning in the life of rats: they rely exclusively on olfactory and somatosensory stimuli to develop their initial relationship with their environment; their immature central nervous system (CNS) determines their potential for plasticity, both behaviourally and cellularly.

An approach response to an odor cue can be easily obtained by classical

conditioning in neonatal rats. This approach response in rat pups is quite stereotyped. Multiple stimuli, even aversive ones, such as foot shock and tail pinch, can produce a conditioned approach response to an odour (Camp and Rudy, 1988; Wilson and Sullivan, 1994; Sullivan et al., 2000a). The approach response can be tested 24 hr later by either Y-maze or two-odor testing protocols. This kind of learning is called odor preference learning. The classical conditioning paradigm of odor preference learning enables us to explore the neural substrates underlying learning associated changes. It provides information not only about the mechanisms of synaptic plasticity in the olfactory system but also about the mechanisms of learning and memory in general.

1.1.1 Behavioural paradigm

In 1986, Sullivan and Leon reported that early olfactory learning produced by pairing a novel odor with a reinforcing tactile stimulus from PND 1 to 18, led to an odor preference and enhanced 2-deoxyglucose (2-DG) uptake in specific areas of olfactory bulb glomeruli to subsequent presentations of that odor. In this paradigm, the tactile stimulus serves as an unconditioned stimulus (UCS); the odor itself serves as a conditioned stimulus (CS). The same changes were reported later by the same group after one-trial olfactory training (Sullivan and Leon, 1987). In this simplified paradigm, PND 6 rat pups are simultaneously subjected to a 10 min odor exposure and to a reinforcing tactile stimulation, stroking of their bodies using a brush. The control groups receive

either the odor only (CS only), or the stroking only (UCS only), or neither of these stimuli (naive). The next day, when the pups are assessed by a two odor choice test, the odor+stroking conditioned group demonstrates a preference for the conditioning odor, while the other groups fail to show approach responses.

Different strategies and stimuli have been explored since the initial work by Sullivan and her colleagues. A diversity of stimuli, including stroking, milk, tail pinch, the odor of maternal saliva, high humidity, mild foot shock, heat and intracranial brain stimulation have been used as UCSs (Wilson and Sullivan, 1994). An interesting characteristic of this early olfactory learning paradigm is that there is a sensitive period, during which various brain structures involved in emotion and learning are still immature and undergoing maturation. This sensitive period ends around postnatal day 10. During this period, rat pups have a high potential for acquiring approach behaviour, even when an aversive stimulus such as foot shock is used. After postnatal day 10, pups start to develop avoidance responses. Interestingly, young pups (<PND10) and older ones (>PND10) have similar responses to foot shock (vocalization, vigorous physical response etc.), although they show striking differences in the learned behaviour. Furthermore, the amygdala, a brain structure critically involved in the emotional aspect of learning and memory, does not demonstrate a change after shock-induced odor preference in PND8 pups as measured by 2-DG (Sullivan et al., 2000a), suggesting learning may occur primarily in the olfactory bulb itself during this sensitive period. This hypothesis is

reinforced by the observation of metabolic and electrophysiological changes in the olfactory bulb which will be discussed in 1.1.2.1.1.2.

1.1.2 Neural substrates for odor preference learning

1.1.2.1 Neuroanatomical structures and functions

1.1.2.1.1 Main olfactory bulb

In odor preference conditioning, as mentioned, learning associated changes have mainly been observed in the main olfactory bulb (MOB) (Wilson and Sullivan, 1994). The MOB is a simple cortical structure which receives direct sensory input from olfactory receptor neurons in the olfactory epithelium. The clear laminar structure and limited synaptic circuitry make it an excellent model system to study learning associated synaptic plasticity (Figure 1.1).

1.1.2.1.1.1 Basic structure

The sensory organ of the main olfactory system is the olfactory epithelium. The olfactory receptor neurons (ORNs) transmit the information of volatile compounds of odorants to the olfactory bulb via the olfactory nerve (ON). Located deep to the olfactory

nerve layer are distinctive spherical structures called glomeruli. Glomeruli are where the axons of ORNs synapse onto the apical dendrites of olfactory output neurons – mitral/tufted cells. The secondary dendrites of mitral cells form dendrodendritic synapses with the dendrites of inhibitory neurons -- granule cells in the external plexiform layer (EPL, Figure 1.1A). In rats, the ON forms glutamatergic synapses with the apical dendrites of mitral cells (Ennis et al., 1996; Aroniadou Anderjaska et al., 1997), the excitation of which is regulated by the GABAergic granule cells. The interaction between mitral cells and granule cells is through dendrodendritic synapses. The mitral cell releases glutamate onto granule cell dendrites upon activation; whereas granule cell dendrites, in turn, release gamma-amino butyric acid (GABA) back onto mitral cell dendrites to prevent their further excitation (Figure 1.1B). The chemosensory information is extensively processed and refined within the MOB before it is sent to the olfactory cortex.

The axons of mitral and deep tufted cells of the MOB project via the lateral olfactory tract (LOT) to the olfactory cortex (Schoenfeld et al., 1985; Scott, 1986). In rodents, the olfactory cortex includes the anterior olfactory nucleus; the piriform cortex; cortical and medial nuclei of the amygdala; the olfactory tubercle and the transitional entorhinal cortex. Olfactory information is also relayed to the thalamus and the orbitofrontal cortex via secondary and tertiary connections. The projection to the amygdala is importantly implicated in the mediation of the emotional and motivational aspects of smell and smell behaviour. Afferent projections to the orbitofrontal cortex via

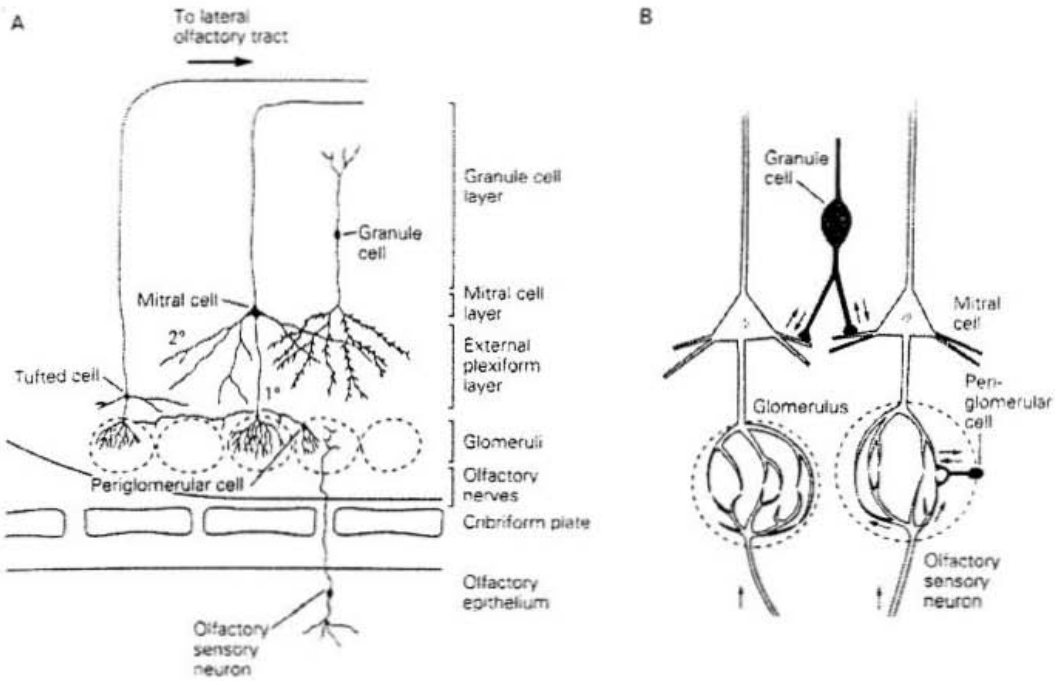

Figure 1.1 The basic structure of the main olfactory bulb (A) and the synaptic circuitry within the olfactory bulb (B). Adapted from Kandel et al. The Principles of Neuroscience. pp 631.

the thalamus are thought to be responsible for the perception and discrimination of the odors (Kandel et al. 2000, pp 633). At the present time no extrabulbar structures have been directly implicated in mediating early odor preference learning.

In addition to the editing of chemosensory information within the olfactory bulb through inhibitory interneurons, the multiple inputs to the olfactory bulb from the olfactory cortex as well as from the basal forebrain (horizontal limb of the diagonal band) and midbrain (locus coeruleus and raphe nuclei) also play modulating roles in olfactory bulb function. The same odor input can take on different behavioural significance depending on the physiological state of the animal (Kandel et al. 2000, pp 633). The activities of some of these systems play important roles in early olfactory conditioned learning which will be discussed in 1.1.2.2..

1.1.2.1.1.2 Changes at the bulbar level following odor preference learning

Neural changes occur at the level of the olfactory bulb following odor preference training. Rat pups that undergo odor conditioning show enhanced focal 2-DG uptake in the glomerular layer when subsequently exposed to the same odor (peppermint) compared to control pups (Coopersmith et al., 1986; Coopersmith and Leon, 1986; Sullivan and Leon, 1986). The increased 2-DG uptake is limited to the 2-DG foci in the midlateral region of the olfactory bulb, but not the ventrolateral 2-DG foci. The enhanced focal 2-

DG uptake may be related to training associated structural changes. For example, increased numbers of juxtaglomerular cells around the glomeruli and increased dendritic processes within glomeruli are also seen following odor conditioning (Woo et al., 1987; Woo and Leon, 1991). Furthermore, early odor preference training increases the density of Fos-immunopositive cells in midlateral glomerular regions underlying the high uptake 2-DG foci seen upon subsequent exposure to the learned odor (Johnson et al., 1995). These results suggest specific regions in the olfactory bulb that encode the conditioned odor are modified following the learning of that odor.

The olfactory responses to odors can be measured by single-unit recording of mitral/tufted cells. Wilson et al reported that granule cell suppression of mitral cell responses to a conditioned odor is increased by conditioning (Wilson et al., 1987; Wilson and Leon, 1988). The increase occurs in the region of the olfactory bulb that responds to the odor. The inhibition that is increased after conditioning may occur via lateral inhibition. The increased strength of lateral inhibition may refine the pattern of mitral cell activity induced by the learned odor (Brennan and Keverne, 1997).

The functional and structural changes in the olfactory bulb associated with odor preference learning occur only during the critical period; the changes cannot be induced after the second postnatal week although preferences can still be established (Woo et al., 1987). The olfactory bulb undergoes extensive growth after birth, the maturation of which

depends greatly on sensory input, which is analogous to the development of other systems, such as binocularity and orientation column formation in the visual system. In this sensitive developmental period, enriched odor environments enhance olfactory neuron survival and synapse formation, whereas unilateral naris occlusion leads to decreased surviving neurons in the deprived olfactory bulb (Meisami and Safari, 1981; Najbauer and Leon, 1995; McLean et al., 2001). Neonatal pups in this period thus have a high potential for neural, as well as behavioural, plasticity.

1.1.2.1.2 Other brain regions involved in odor preference learning

The MOB appears to be the major brain substrate for the acquisition of early conditioned odor preferences and for the processing of odor information during odor learning in general. However, a variety of other brain structures play roles in various aspects of odor learning in both neonate and adult rats (Brennan and Keverne, 1997). These will be briefly reviewed below.

The amgydala appears to be involved in neonate rat odor learning. Bilateral electrolytic lesions of the amygdala on PND5, mainly the cortical nucleus of the amygdala, disrupt the conditioned odor approach responses obtained on PND6, although rat pups still show conditioned behavioural activation (Sullivan and Wilson, 1993). However, the loss of the conditioned odor approach response can be compensated by

overtraining. With sufficient overtraining, pups can acquire both conditioned activation and an odor approach response, suggesting that the amygdala is not essential for this early odor preference learning, although it can facilitate the acquisition of an odor preference.

Interestingly, the amygdala is involved in a differential response to odor-shock conditioning as reported by Sullivan (2000). As mentioned in 1.1.1, there is a critical period for neonate odor preference learning. Odor-shock conditioning induces an approach response in rat pups before postnatal day 10, but results in odor aversion response after this critical period. Amygdala 2-DG uptake after odor-shock conditioning is enhanced in PND12 pups that develop an aversion response; but is not changed in PND8 pups that form an approach response following odor-shock. The olfactory projections to the amygdala are present near birth. Why the amygdala is not critically involved in the early odor-shock induced approach response is not clear, however, it seems to be necessary for the acquisition of an odor aversion in older rats when the same training protocol is used.

In addition to the amygdala, increases in 2-DG uptake have been found in both the anterior olfactory nucleus (Cierpial and Hall, 1988; Hamrick et al., 1993) and piriform cortex (Cierpial and Hall, 1988) following odor learning in rats. Long-term potentiation (LTP), a synaptic phenomena which has been suggested to be closely associated with long-term memory formation, can be induced in the piriform cortex in adult rats (Roman

et al., 1993).

Mediodorsal thalamus and hippocampal areas appear to play roles in adult odor learning. In an odor reversal learning model, water-deprived rats are trained on a go, no-go two-odor olfactory discrimination task to respond to one odor (S+) with water as a reward and to suppress responding to the other odor (S-). The rats are then tested for their ability to reverse the stimulus association. Lesions of mediodorsal thalamus disrupt odor reversal learning in rats (Slotnick and Risser, 1990), but odor discrimination is well preserved. The mediodorsal thalamus projects to the orbitofrontal cortex where olfactory information converges with gustatory and visual inputs and forms associations with rewards (Rolls and Baylis, 1994). In contrast, the parahippocampal areas are more involved with transient olfactory memory (Otto and Eichenbaum, 1992). Disruption of the olfactory input to the hippocampus does not affect odor reversal learning (Slotnick and Risser, 1990). The hippocampus is implicated in supporting the representations of relations among odor stimuli such as the formation of the spatial memory for odor cues (Eichenbaum, 1998). Interestingly, lesions to the above structures, as well as lesions to the amygdala, do not seem to disrupt a previously learned olfactory discrimination (Slotnick and Risser, 1990; Staubli et al., 1995; Brennan and Keverne, 1997).

1.1.2.2 Neurotransmitters implicated in early olfactory learning

As mentioned, the olfactory bulb receives a strong input from neuromodulatory systems: noradrenergic input from the locus coeruleus, serotonergic input from the raphe, and cholinergic input from the horizontal limb of the nucleus of the diagonal band. There are also many intrinsic dopaminergic neurons in the glomerular layer of the olfactory bulb. Different transmitters including NE, 5-HT, dopamine (DA), GABA and glutamate have been shown to play important roles in mediating odor preference learning which will be discussed in the following sections.

1.1.2.2.1 Norepinephrine

Noradrenergic neurons are located in the locus coeruleus. The locus coeruleus projects diffusely throughout the cortex, cerebellum and spinal cord (Kandel et al. 2000, pp 283). In mammals, ascending noradrenergic neurons project directly to the MOB and accessary olfactory bulb (AOB), which is present to a significant extent at birth (McLean and Shipley, 1991). NE terminals have the highest degree of laminar specificity in the MOB of any brain structure (McLean et al., 1989). Most NE fibers terminate in deep layers of the MOB, including the internal plexiform layer (IPL) and the granule cell layer (GCL) and to a lesser degree in the EPL, but it is sparse in the glomerular layer (GL). The ascending NE system is involved with attention and other complex cognitive functions

(Hasselmo, 1995). The role of NE in early olfactory learning has been extensively investigated.

1.1.2.2.1.1 Behavioural aspect

In rat pups, the pairing of a novel odor with tactile stimulation or stroking, which mimics maternal care, produces a conditioned odor preference for that odor. In this model, odor serves as the CS and stimulates the glutamatergic ON via olfactory receptor activation, while the tactile stimulus used as the UCS has been shown to activate the locus coeruleus (Nakamura et al., 1987) to release NE in the olfactory bulb (Rangel and Leon, 1995). The convergence of CS and UCS in the olfactory bulb is hypothesized to result in the structural, biological and physiological changes reported both at the level of glomeruli and the output neurons (as discussed in 1.1.2.1.1.2.).

Associative odor learning in neonate pups requires an intact NE input to the bulb . Bilateral lesions of the locus coeruleus by 6-hydroxydopamine (6-OHDA) significantly impair olfactory learning in rat pups (Sullivan et al., 1994). Systemic (Sullivan et al., 1994) or local bulbar (Sullivan et al., 1992) injection of the β-adrenoceptor antagonist propranolol, or timolol, prior to training blocks odor preference learning in a dose-dependent manner. This effect is not due to the loss of odor sensitivity or discrimination (Doty et al., 1988).

Stimulation of the NE input to the olfactory bulb is sufficient to induce an odor preference (Sullivan et al., 2000b). Putative stimulation of the locus coeruleus by idazoxan or acetylcholine produce an odor preference which could be blocked by a pretraining injection of propranolol. Association of odor with either systemic (Sullivan et al., 1989b; Sullivan et al., 1991) or intrabulbar (Sullivan et al., 2000b) injection of the β-adrenoceptor agonist isoproterenol can substitute for stroking to induce an odor preference in rat pups. Systemic injection of isoproterenol also results in the same enhanced 2-DG uptake within the olfactory bulb as that seen with the stroking+odor conditioning (Sullivan et al., 1991). β-adrenoceptor agonist and antagonist studies together suggest β-adrenoceptor activation is both necessary and sufficient for early odor preference learning. The evidence that intrabulbar β-adrenoceptor activation alone paired with odor is sufficient to induce an odor approach response also suggests that the critical CS-UCS seems to converge in the olfactory bulb during early odor preference learning (Sullivan et al., 2000b).

NE is not only involved in the acquisition, but also in the consolidation of odor preference memories in rat pups. Post-training injection of propranolol up to 1 hr after training blocks learned odor memory acquired through odor-milk association. NE does not appear to be necessary for expression of the learned response; propranolol injection immediately before testing (24 hr after training) does not affect memory (Sullivan and

Wilson, 1992).

While β-adrenoceptors have been extensively studied, other adrenoceptor subtypes have not been directly assessed in early odor preference learning.

1.1.2.2.1.2 β-adrenoceptor activation and the inverted U-curve for early odor preference learning

Interestingly, although the association of an odor with either stroking or isoproterenol results in an odor approach response, the effect is dose-dependent. The dose-response curve for this effect has an inverted U-shape function. Only an optimal activation of β-adrenoceptor produces learning (Sullivan et al., 1989b; Sullivan et al., 1991; Langdon et al., 1997). A moderate dose of isoproterenol, 2 mg/kg, when paired with an odor, can substitute for stroking to induce an approach response; whereas higher doses, 4 mg/kg and 6 mg/kg, or a low dose, 1 mg/kg, of isoproterenol fail to produce odor learning. These outcomes have suggested that the balance between inhibition and disinhibition of output cells of the olfactory bulb may be critical as will be discussed in 1.1.3.1.3 and Chapter 2. Additionally, a critical window for calcium and cyclic adenosine monophosphate (cAMP) coactivation of intracellular phosphorylation pathways may occur with a moderate level of β-adrenoceptors stimulation as will be discussed later in Chapter 2.

The effect of isoproterenol is additive to stroking in supporting early odor learning (Sullivan et al., 1991). An odor paired with a moderate level of either of these stimuli produces learning. However, a combination of 2 mg/kg isoproterenol stimulation with a normal magnitude of stroking does not produce learning. Combination of suboptimal levels of both of these stimuli results in learning. These outcomes suggests that β-adrenoceptor activation and the stroking UCS share a common substrate for inducing early odor learning.

1.1.2.2.1.3 Physiological effects of NE implicated in early odor preference learning

Early olfactory learning depends on MOB noradrenergic neurotransmission as discussed earlier. Studies of NE actions in the MOB are controversial (Jiang et al., 1996). In early work, NE was found to inhibit mitral cell discharge in rabbits and cats (Salmoiraghi et al., 1964; McLennan, 1971), although no pharmacological studies on receptor subtypes were done. More recently, NE was reported to disinhibit mitral cells in the adult turtle olfactory bulb slice preparation (Jahr and Nicoll, 1982). Trombley et al demonstrated the same disinhibition effect on cultured mitral cells from the rat MOB (Trombley, 1992; Trombley and Shepherd, 1992). This disinhibitory effect is mediated by an α-, but not a β-adrenoceptor, presynaptic mechanism (Trombley, 1994). NE application results in the disinhibition of mitral cells from granule cells through inhibition

of presynaptic calcium influx in the mitral cells. In vivo Jiang et al (1996) reported that locus coeruleus activation increased mitral cell responses to weak olfactory bulb stimulation, which is consistent with the disinhibition hypothesis. More importantly, in present concern, an *in vivo* study in rat pups reported activation of β-adrenoceptors in the olfactory bulb decreased granule cell inhibition (Wilson and Leon, 1988). By examining the effects of the β-adrenoceptor agonist, isoproterenol, and the antagonist, propranolol, on paired-pulse inhibition at the granule cell/mitral cell reciprocal synapse, Wilson and Leon (1988) found activation of β-adrenoceptors caused disinhibition of mitral cells, while its blockade resulted in increased inhibition. The conflicting results may be attributable to methodological differences (Jiang et al., 1996). Differences in drug concentration, duration and sites of action in different preparations may all influence the actual NE effect observed. More work is needed on the pharmacology and mechanism of NE modulation of inhibition in the rat pup. The in vitro work suggests there is NE mediated disinhibition via α-adrenoceptors, but the role of α-adrenoceptors in early olfactory learning is unknown. α-adrenoceptors disinhibition has been implicated in another form of olfactory learning (Brennan and Keverne, 1997).

Early odor preference learning in the MOB is blocked by β-adrenoceptor antagonists. Norepinephrine has been proposed to promote learning-dependent behavioural and neural changes through β-adrenoceptors (Wilson and Sullivan, 1994). The results of Wilson and Leon in the rat pup support a β-adrenoceptor-mediated

disinhibition as possibly playing a critical role in early odor preference learning. Based on the physiological results together with the behavioural results and bulbar changes following behaviour, Sullivan and Wilson (1994) proposed that during associative odor preference learning, the UCS activates NE input to the olfactory bulb, resulting in disinhibition of mitral cells via a β-adrenoceptor effect. This UCS disinhibition facilitates the excitation of mitral cells and could produce an enhancement of the CS excitatory effect on mitral cells. However, in the Sullivan and Wilson model, the critical learning related change is hypothesized to be a strengthening of the mitral cell glutamatergic input to the granule cell such that stronger inhibition of mitral cells is seen with retrieval of the odor memory. This parallels other odor learning models as will be discussed in 1.1.3.1.

The discrepancy between the α-, and β-adrenoceptor mediated disinhibition again, may result from different experimental preparations (Brennan and Keverne, 1997). However, the presence of multiple receptor types for NE in the olfactory bulb suggests their roles in odor preference learning might be diverse and merit further investigation. For example, besides a possible inhibitory role, another mechanism of NE action might be to directly enhance mitral cell excitability (Jiang et al., 1996; Hayar et al., 2001) by blocking spike accommodation (Jiang et al., 1996). In addition, β-adrenoceptor mediated activation of the cAMP cascade has also been hypothesized to be critically involved in early preference learning as will be discussed in 1.1.3.2.1 and Chapter 2, 4, and 5.

1.1.2.2.2 Serotonin

Another olfactory bulb modulating system is the 5-HT input from the dorsal and median raphe nuclei (McLean and Shipley, 1987). Serotonergic fibers are extensively distributed in the olfactory bulb, however, only 5-$HT_{2A/2C}$ receptor subtypes have been examined in early odor preference learning. 5-HT_{2A} receptor mRNA (McLean et al., 1995) and protein (Hamada et al., 1998) are localized in mitral and tufted cells, and to a lesser extent, in periglomerular and granule cells (Hamada et al., 1998).

Earlier work from McLean's lab suggested that 5-HT acting through 5-$HT_{2A/2C}$ receptors promotes noradrenergic-induced plasticity (McLean et al., 1999). Although NE input appears to act as the critical UCS for odor preference learning, the effect depends on an intact serotonergic input. 5-HT_2 activation itself does not induce learning, but depletion of olfactory bulb 5-HT fibers by 5,7-dihydroxytryptamine (5,7-dHT) injection into the anterior olfactory nucleus on PND2 prevents the acquisition of odor preference induced by a normally effective dose of isoproterenol (McLean et al., 1993). Sufficient β-adrenoceptor activation using a higher dose of isoproterenol can overcome the requirement for 5-HT input during acquisition (Langdon et al., 1997) and an increase of 5-HT_2 receptor activation using the receptor agonist 2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) can promote learning when a subthreshold isoproterenol is given (Price et al., 1998).

These results led us to the hypothesis that noradrenergic and serotonergic inputs may act synergistically on the same cells to promote a cAMP/protein kinase A (PKA) phosphorylation cascade which has been critically implicated in other memory system such as in Aplysia and Drosophila models (Silva et al., 1998). β_1-adrenoceptors stimulate adenyl cyclase induced cAMP production via a G-protein (Prisco et al., 1993; McLean et al., 1999). In contrast, 5-HT receptor activation by itself does not increase cAMP expression (Morin et al., 1992; Rovescalli et al., 1993), which is consistent with the behavioural failure to induce learning by 5-HT activation alone. However, 5-HT_2 activation can enhance isoproterenol-induced intracellular cAMP expression through other second messenger systems (Rovescalli et al., 1993). Therefore, 5-HT could play a permissive role in conditioned odor learning induced by norepinephrine; 5-HT appears to set the stage for an effective noradrenergic action.

1.1.2.2.3 Other transmitters

1.1.2.2.3.1 Dopamine

There is a large population of DA neurons in the olfactory bulb glomerular layer: the cells surround the glomeruli and are known as periglomerular cells (Wilson and Sullivan, 1994). The role of DA in the odor preference learning has been investigated in one study. Systemic injection of the D_1 antagonist SKF 83566 immediately after

odor+stroking blocks odor preference formation. However, the effects of pretraining injections of SKF 83566 are blocked by post-training injections of the DA agonist apomorphine (Weldon et al., 1991). Thus, the role of DA in the odor preference learning may be limited to the post-training consolidation phase. However, more experiments are needed to clarify the role and mechanism of dopamine in early odor preference learning.

1.1.2.2.3.2 GABA

The granule cell GABAergic interneurons may play an important role in the olfactory circuit by providing modulation to the output neurons, mitral cells, during odor preference learning as suggested in the previous section. Granule cells inhibit mitral cells by releasing GABA onto mitral cells through dendrodendritic synapses between these two cell types. Blockage of GABA activation in the olfactory bulb by a pretraining injection of picrotoxin, which blocks $GABA_A$ receptor, disrupts early odor preference learning (Wilson and Sullivan, 1994).

Work with GABAergic antagonists in older pups suggests a possibly complex relationship between level of inhibition and learning. A study of GABAergic control of olfactory learning in 14-day-old rats (Okutani et al., 1999) showed that a low dose of bicuculline, a $GABA_A$ receptor antagonist, when infused into the olfactory bulb, induces an odor preference; whereas a high dose induces an odor aversion. A moderate dose of

bicuculline, however, is ineffective in inducing odor learning of either type. This result, argues for a critical balance of inhibition vs. disinhibition in initiating odor preference learning.

Since NE has also been proposed to induce odor learning through the promotion of disinhibition of mitral cells from granule cell interneurons, NE and GABA regulating pathways may share common local mechanisms (Brennan and Keverne, 1997).

1.1.2.2.3.3 Glutamate

The role of glutamate and glutamatergic receptors have been extensively studied in long-term synaptic plasticity such as LTP formation in the hippocampus. There are two classes of receptors: ionotropic glutamate receptors (iGluR) that are linked directly to ion channels (N-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA) receptors and kainate receptors) and metabotropic glutamate receptors (mGluRs) that are linked to G-proteins (6533).

The glutamatergic olfactory nerve synaptic input has both an AMPA receptor mediated component and an NMDA component (Aroniadou Anderjaska et al., 1997). Mitral/tufted cells are also glutamatergic neurons, which release glutamate onto GluRs on granule cells. However, the role of glutamate and glutamatergic receptors in olfactory

learning has only been extensively studied in the AOB pheromonal learning system. The pheromonal learning paradigm in mice is known as the Bruce effect, in which exposure of newly mated female mice to males, different from those that they had been mated with, causes pregnancy failure (Kaba et al., 1989).

In parallel with the effect of bicuculline, AOB infusion of iGluR antagonists cause disinhibition of mitral cells and can influence pheromonal learning. However, only the non-selective antagonist γ-D-glutamylglycine (DGG) or a combination of the selective NMDA antagonist D-2-amino-5-phosponovaleric acid (APV) and the selective AMPA antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) disrupt pheromonal memory formation, while blockade of either receptor alone, only results in pregnancy failure without disrupting memory formation (Brennan and Keverne, 1997).

In early olfactory preference learning dependent on the MOB, systemic injection of amino-5-phosphonopentanoic acid (AP5) for the first 18 days blocks the acquisition of an odor preference. Pre-training APV injection suppresses both the behavioural preference and enhanced olfactory bulb response to the learned odor (Lincoln, et al., 1988). However, NMDA antagonists may disrupt normal bulb function (Wilson and Sullivan, 1994; Brennan and Keverne, 1997), therefore more work should be done to clarify the role of the NMDA/AMPA receptors in olfactory preference learning.

Among the many subtypes of mGluRs, the role of mGluR2 is most studied in the AOB. mGluR2 are located in the dendritic spines of granule cells in AOB (Hayashi et al., 1993). By using the specific mGluR2 agonist (2S,2'R,3'R)-2-(2'3'-dicarboxycyclopropyl) glycine (DCG-IV), it was reported that DCG-IV markedly reduced the GABA-mediated inhibitory current from granule cell activation in slices of rat AOB (Hayashi et al., 1993). Kaba et al (1994) investigated whether AOB infusions of DCG-IV could promote pheromonal memory formation by reducing mitral cell inhibition from granule cells. Their results showed that DCG-IV infusions into AOB result in memory formation for male odors without mating. Figure 1.2 summaries the neural circuitry of the mGluR2 in the AOB.

mGluR2 also occurs in the main olfactory bulb (Petralia et al., 1996). One recent study showed that DCG-IV infusion into the MOBs of 1-week-old rat pups, induced an odor preference for the conditioned odor, peppermint (Rumsey et al., 2001). In both cases, DCG-IV acts as a UCS. These results suggest mGluR mediated disinhibition of mitral cells is an additional mechanism for the induction of early olfactory preference learning.

1.1.3 Candidate mechanisms underlying odor preference learning

Research on mechanisms of the neurobiology of learning and memory has focused

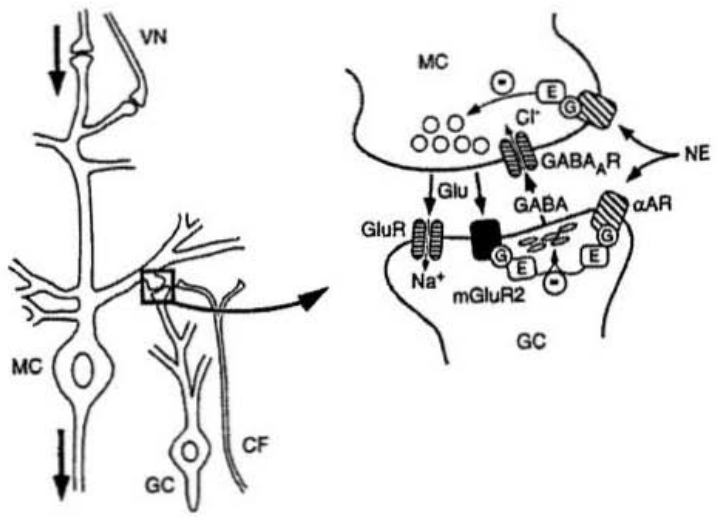

Figure 1.2 Neural circuitry and neurotransmitters between the mitral and granule cells in the AOB. Abbreviations: αAR, α-adrenergic receptor; CF, centrifugal fiber of norepinephrine projection from locus ceruleus; GABA, gamma-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; GC, granule cell; Glu, glutamate; GluR, glutamate receptor; mGluR, metabotropic glutamate receptor; MC, mitral cell; NE, norepinephrine; VN, vomeronasal nerve. Adapted from Kaba et al. Science. 265(5169):262-4 (1994)

on two levels: intercellular synaptic plasticity and intracellular signaling pathways. In terms of intercellular plasticity, in early odor preference learning, neuronal circuits between olfactory bulb output neurons and interneurons, especially the interaction between mitral cells and granule cells via dendrodendritic synapses has been studied extensively. A disinhibition model has been proposed in both pheromonal learning in the AOB and odor preference learning in the MOB.

In terms of intracellular signalling, long-term memory formation requires gene expression and new protein synthesis. cAMP response element binding protein (CREB) and CRE-mediated gene expression pathways have been critically implicated in long-term memory formation in animals as diverse as Aplysia, Drosophila, rats, and mice (Silva et al., 1998). A role for Ca^{2+}/cAMP-mediated phosphorylation signalling and protein phosphatase-mediated dephosphorylation has been suggested in a variety of olfactory learning paradigms (Brennan and Keverne, 1997).

1.1.3.1 Intercellular synaptic plasticity

This section briefly revisits the evidence for a role of disinhibition in olfactory learning. Reduction of inhibitory neurotransmission by manipulations of either NE, or GABA or $mGluR_2$ appears to be a common feature for the induction of different types of olfactory memory mechanisms, such as pheromonal memory formation in mice and odor

preference learning in rat pups, both of which have been characterized by an initial disinhibition of mitral cells from granule cell inhibition during the acquisition of the memory, followed by an enhanced inhibitory gain of the mitral – granule cell reciprocal synapses during the retrieval of the memory (Brennan and Keverne, 1997).

1.1.3.1.1 The AOB disinhibition model

In the AOB, the association of a mating male's pheromones with increased levels of NE after mating is thought to result in a long-lasting increase in the inhibitory gain of the mitral/granule cell synapses (Kaba et al., 1994; Brennan and Keverne, 1997). During subsequent pheromonal exposure, the mitral cells responding to the mating male's pheromones would be subject to greater self-inhibition and thus prevent the signal of the mating male's pheromones from being transmitted to activate central neuroendocrine mechanisms that cause pregnancy failure. The disinhibition effect of NE in AOB pheromonal learning system is mediated by α-adrenoceptor activation (Kaba et al., 1994).

1.1.3.1.2 The MOB disinhibition model

In the MOB, β-adrenoceptor activation induces a disinhibitory effect on granule cell inhibition during LOT (Wilson and Leon, 1988) stimulation. Norepinephrine has been proposed to promote learning and learning-dependent changes in the MOB by

disinhibiting mitral cells (Wilson and Sullivan, 1994). Odor learning induced changes include an increase in inhibitory neurotransmission upon odor re-exposure (Figure 1.3). Single-unit recording shows increased depression of mitral cell activation to the conditioned odor (Wilson et al., 1985; Wilson et al., 1987). The increased inhibition during odor re-exposure may be due to increased lateral inhibition, sharpening the mitral cell signal induced by the learned odor (Brennan and Keverne, 1997).

1.1.3.1.3 Disinhibition and the inverted U-curve

A simple disinhibiton model would not explain all the findings in early olfactory learning, such as the inverted U dose dependency curve observed with the β-adrenoceptor agonist isoproterenol, or the behavioural consequences resulting from different doses of bicuculline infused into the olfactory bulbs (Okutani et al., 1999). There may be a requirement for a critical balance between inhibition and disinhibition for learning to take place. Increased reciprocal inhibition induced by an increased intensity of mitral cell activation may override increased excitability resulting from granule cell disinhibition.

1.1.3.2 Intracellular signalling

Ca^{2+} and cAMP-dependent signalling cascades have been implicated in long-term memory formation in a variety of species in numerous studies which will be discussed in

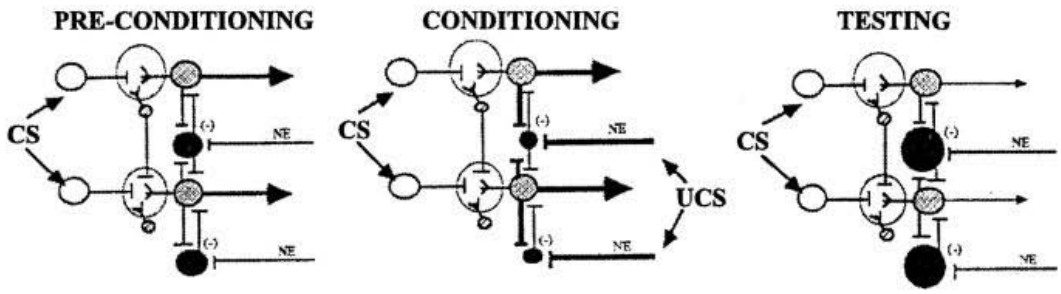

Figure 1.3 The disinhibition model for early odor preference learning proposed by Wilson and Sullivan. Adapted from Wilson and Sullivan. Behavioral and Neural Biology 61, 1-18 (1994)

1.3. Below is the hypothesis for the convergence of Ca^{2+} and cAMP-dependent signalling cascades in inducing the CREB pathway in early odor preference learning.

1.1.3.2.1 The cAMP/PKA/CREB hypothesis of early odor preference learning

McLean et al. (1999) proposed an intracellular model for odor preference learning in which a calcium signal initiated by the odor input via the olfactory nerve and a cAMP signal initiated by NE are required synergistically in mitral cells to elevate phosphorylated CREB (pCREB) and produce olfactory learning. The proposed model fits a Hebbian mechanism in that simultaneous activation in both the pre- and postsynaptic cells results in a long-lasting synaptic facilitation (Hebb,1949; Kandel et al. 2000, pp1260). While the disinhibition model of NE in odor preference learning predicts that granule cells are the critical substrates for learning, McLean et al (1999) hypothesized that mitral cells are the postsynaptic substrate for odor preference learning. The NE effect results from β-adrenoceptor activation of cAMP cascade in mitral cells. The association of Ca^{2+} entry through NMDA channel activation by odor input and cAMP signalling results in an enhanced olfactory nerve EPSP in the postsynaptic mitral cells and may promote a selectively enhanced mitral cell response to ON input during the memory phase.

1.2 A new strategy to study odor preference learning – optical imaging

The development of advanced new techniques has enriched our knowledge of olfactory coding and information processing within the olfactory bulb. The topography of the olfactory bulb has been explored with various techniques that permit visualization of the activity of olfactory neurons from metabolic mapping of 2-DG (Coopersmith and Leon, 1984; Johnson and Leon, 1996; Johnson et al., 1998) or immediate-early gene c-Fos expression (Matsuda et al., 1990; Guthrie et al., 1993; Guthrie and Gall, 1995), to recent utilization of optical imaging (Katz and Rubin, 1999; Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001), and functional magnetic resonance imaging (fMRI) (Yang et al., 1998; Xu et al., 2000). Among these techniques, optical imaging provides high spatial (intrinsic imaging) and temporal resolution (calcium imaging) (Galizia et al., 1999; Katz and Rubin, 1999). This method permits fast, noninvasive in vivo visualization of neuronal activity and repeated measurements from the same animal. Its utilization has been extensively explored in the visual system, the barrel cortex, and recently, in the olfactory system. It provides a new methodology to explore neuronal changes following odor learning.

1.2.1 Olfactory encoding

Olfactory encoding is particularly amenable to optical imaging because the initial

encoding of odor information takes place in the glomeruli, which is immediately below the surface of the bulb. An odorant is first detected by olfactory receptor neurons located in the olfactory epithelium. In the mammalian olfactory epithelium, each sensory neuron expresses only one olfactory receptor gene. In rats, a large multigene family codes for more than 1000 olfactory receptors. Neurons expressing an olfactory receptor are confined to one of the four olfactory receptor expression zones in the epithelium, where they are scattered throughout the zone with neurons expressing other olfactory receptors (Dudai, 1999; Kandel et al. 2000, pp630). The receptor neurons transmit odor information via unmyelinated axons to the glomeruli of the olfactory bulb (approximately 1000 glomeruli in mouse olfactory bulb, 2000 in rat bulb. Dudai, 1999). In the bulb, axons of receptor neurons expressing the same olfactory receptor converge onto a few glomeruli. Glomeruli are considered as the functional units of the olfactory bulb. Information about different odorants are coded in the glomeruli, in the sense that one odorant is coded by a combinational activation pattern in glomeruli: 1) each glomerulus recognizes one component (molecular feature) of the odorant; and 2) the pattern of the presentation of an odorant in a group of glomeruli determines odor coding. Therefore, the first step in central olfactory processing involves transformation of a chemical code (molecular features of an odorant deciphered by its binding to the olfactory receptors) into a distributed place code (Dudai, 1999).

1.2.2 Intrinsic signal imaging

Intrinsic signal imaging is a powerful method of analysing activity-dependent patterns of neuronal populations in the brain. Since Rubin and Katz (1999) first employed this technique to explore olfactory encoding in the rat, numerous studies have endeavoured to decipher odor coding in the olfactory bulb. Intrinsic signals are due to activity-dependent neuronal changes, which are reflected as optical changes of the imaged tissue. Activity-dependent changes involve hemodynamic changes such as a change in blood volume, oxygenation of hemoglobin, or light scattering changes caused by the local movement of ions and released transmitters (Dudai, 1999). Intrinsic imaging has an excellent spatial resolution (<50μm) which allows the measurement of neuronal changes at the level of glomeruli on the dorsal surface of the olfactory bulb.

Optical imaging of intrinsic signals in the rat olfactory bulb has revealed detailed spatial patterns of glomerular activation representing different odorants, the concentration dependence of glomerular activation, and the molecular receptive range of specific glomeruli (Dudai, 1999; Katz and Rubin, 1999; Uchida et al., 2000). The functional representation of odorant molecules revealed by intrinsic imaging conforms to a series of basic principles: 1) bilateral symmetry of odor representations; 2) local clustering of glomeruli activation; and, 3) local variability of odor presentations between animals (Katz and Rubin, 1999; Uchida et al., 2000; Belluscio and Katz, 2001).

Intrinsic optical imaging has its limits. One shortcoming is that only odor-induced activity changes on the surface of the olfactory bulb are revealed, not the cellular source of the activity (Dudai, 1999). The odor representation patterns reported from intrinsic imaging studies are postulated to occur in glomeruli based on the location, size, and shape of the active regions. However, a combination of optical imaging with voltage-sensitive dyes or electrophysiological measurement would help identify the cellular source of the neuronal activity. Another limit of intrinsic imaging is its relatively slow temporal resolution (seconds after odor application). In contrast, calcium imaging, has better temporal resolution.

1.2.3 Implications of optical techniques for the study of odor learning

Dudai, in his review paper (1999), predicted that the power of intrinsic optical imaging in the olfactory bulb is not only in illuminating the functional architecture of the bulb, but it is a promising technology to visualize the olfactory brain in action. As mentioned previously, one characteristic of intrinsic imaging is its non-invasiveness. Activity-dependent changes can be visualized through thinned bone or intact dura and repeated imaging can be applied to the same animal over a protracted period. Therefore, it would be especially beneficial for exploring experience-dependent modification of odor representation in the olfactory bulb, which may underlie odor learning and memory. Recording brain activity in vivo during learning is fundamental to understanding how

memories are formed (Faber et al., 1999).

Activity-dependent changes with odor experience have been reported in honeybee antennal lobes (an equivalent structure to the olfactory bulb in mammals). Faber et al.(1999) trained individual bees to discriminate a rewarded odor from an unrewarded odor. The rewarded odor (CS) is paired with sucrose solution (UCS), applied to the proboscis. Acquisition of the odor-reward association leads to an increase of the representation of the rewarded odor and different activity patterns representing rewarded and unrewarded stimuli. This is promising for similar research in the mammalian olfactory bulb. The present thesis takes advantage of this methodology to explore odor encoding following memory formation in early odor preference learning.

1.3 CREB, synaptic plasticity and memory

Learning and memory are strongly associated with synaptic plasticity in the CNS. Long-term memory formation requires new protein synthesis. CREB- dependent intracellular pathways are thought to be pivotal in mediating the transition from short-term memory, which lasts only 1-2 hours, to long-term memory, which lasts days, or even a lifetime. In a variety of species from Aplysia, Drosophila to mice and rats, CREB-dependent transcription appears to be a crucial component underpinning long-term memory formation (Silva et al., 1998). CREB levels appear to be delicately regulated in

memory systems; disrupting normal CREB functioning impairs long-term memory.

1.3.1 CREB and transcription

CREB is a member of a large family of transcriptional factors that bind to promoter cAMP responsive element (CRE) sites (Silva et al., 1998; Walton and Dragunow, 2000). The CREB transcriptional family of proteins consists of three functional domains: a leucine-zipper domain (bZIP) that mediates dimerization, a DNA-binding domain, and the transcriptional activation domain. Based on the differentiation of bZIP, members of the CREB family can be divided into three groups: activator CREB, repressor cAMP response element modulator (CREM), and activating transcription factor (ATF). CREB protein has three alternatively spliced isoforms, α, β and Δ. The repressor CREM gene consists of at least four different factors: CREM α, β, γ and inducible cAMP early repressor (ICER).

CREB has been implicated in the transcriptional control of numerous genes, such as immediate early genes c-Fos, c-jun, Egr-1, Bcl-2 (Walton and Dragunow, 2000). Many of these genes are expressed rapidly in response to an elevation of intracellular cAMP or Ca^{2+} levels. The transcriptional control of CREB on gene expression has been shown to be involved in a variety of biophysiological phenomena such as neuronal survival (Walton and Dragunow, 2000), learning and memory (Silva et al., 1998), drug addiction

(Lane-Ladd et al., 1997) and tumorogenesis.(Xie et al., 1997).

1.3.1.1 CREB phosphorylation and transcriptional activation

The transcriptional activation of CREB is crucially dependent on phosphorylation of Ser 133 by various protein kinases such as PKA, Ca^{2+}/CaM kinases, ribosomal S6 kinase 2, or mitogen-activated protein-kinase activated protein kinase 2 (MAPKK2) (Silva et al., 1998). The phosphorylation of CREB by different kinase pathways may be a mechanism for the convergence of these pathways to regulate downstream gene expression (Sheng et al., 1991; Silva et al., 1998).

CREB phosophorylation has been postulated as an initial step in the transcriptional control of synaptic plasticity underlying learning and memory (McLean et al., 1999). In invertebrates such as Aplysia, multiple pulses of 5-HT result in an increase in cAMP in the sensory neuron. This in turn activates the catalytic subunits of PKA to translocate into the nucleus, where they phosphorylate CREB and thus activate the transcription of IEGs. IEG activation may turn on the transcription of late response genes such as cytoskeletal proteins, adhesion molecules and neurotrophins/receptors which might encode proteins necessary for long-term synaptic plasticity (Frank and Greenberg, 1994).

CREB activation in the mammalian hippocampus appears to be more complicated than that observed in the invertebrate CNS. Studies from Bito's lab (Bito et al., 1996; Deisseroth et al., 1996) have focused on the cellular processes that regulate the phosphorylation state of CREB in hippocampal neurons. When pCREB was monitored at the single-cell level with an antibody specific for the Ser-133 phosphorylation site, they reported two important occurrences. First, NMDA- dependent synaptic stimulation, but not action potential firing, results in CREB phosphorylation, suggesting that CREB phosphorylation is a specific synaptic signalling marker engaged by both NMDA receptors and L-type calcium channels (Deisseroth et al., 1996; Mermelstein et al., 2000). Second, phosphorylation of CREB is necessary, but not sufficient, for the stimulation of CRE-mediated gene expression in the hippocampus; only sustained phosphorylation of CREB by a prolonged stimulus (5Hz for 180s instead of 18s) induced gene expression such as c-Fos and ss-14 (Bito et al., 1996). Furthermore, they provided strong evidence for the involvement of the Ca^{2+}/CaM pathway in inducing CREB phosphorylation in hippocampal neurons. CaM kinase IV (CaMKIV) is expressed in the nucleus at a time consistent with the appearance of pCREB (Bito et al., 1996) Both pCREB-CREB binding protein (CBP) formation and CaMKIV were blocked by the CaMK inhibitor KN-93, but a PKA inhibitor, KT5720, failed to block CREB phosphorylation (Bito et al., 1996; Deisseroth et al., 1996).

In contrast, Impey et al. (1996) argued for a critical role of PKA in activating

CREB-dependent transcription in hippocampal slices from transgenic mice, CRE-LacZ. PKA inhibitors blocked CREB-dependent transcription in hippocampal slices from the CRE-lacZ mice (Impey et al., 1996). PKA facilitates the MAPK (ERK) pathway phosphorylation of CREB (Impey et al., 1998a). The controversial outcomes may be explained by the different kinetics of protein kinases (Impey et al., 1998a). CaMKIV may mediate an early phase of CREB phosphorylation, while the sustained or late phase of CREB phosphorylation may require PKA/MAPK co-activation. Alternatively, different protein kinase pathway interactions may exist. For example, CaMKIV can also activate MAP kinases (Enslen et al., 1996). It is likely that CaMKIV and MAPK-dependent pathways co-operate to induce pCREB mediated gene activation (Silva et al., 1998).

1.3.1.2 Transcriptional repression

Two processes are responsible for regulating CREB phosphorylation and CRE-mediated gene expression.

First, dephosphorylation of pCREB at Ser133 is important for the inactivation of CREB. The protein phosphatases, calcineurin and protein phosphatase-1 (PP1), are thought to be involved in dephosphorylation of CREB (Blitzer et al., 1995; Liu and Graybiel, 1996; Blitzer et al., 1998; Winder et al., 1998). Treatment with FK506, a calcineurin inhibitor, enhances the duration of CREB phosphorylation and therefore

induces c-Fos and ss-14 expression by a previous ineffective brief stimulus (18s) in hippocampal cultures (Bito et al., 1996). One role of PKA is thought to be to "gate" the Ca^{2+}/CaMK phosphorylation pathway by suppressing phosphatase calcineurin via inhibitor-1 (Blitzer et al., 1998; Winder et al., 1998).

Second, CREB repressors such as CREM α, β, γ and ICER block the activation of CREB. CREM α, β and γ do not have the transcriptional domain, but compete with CREB to bind CRE sites (Foulkes et al., 1991). CREB repressors can be upregulated by CREB activation or CREB mutation (Silva et al., 1998). The ratio of CREB activator to repressor appears to be important in regulating memory formation (Silva et al., 1998).

1.3.2 The role of CREB in memory

Rapid progress has been made in understanding the molecular mechanisms of learning and memory by advanced genetic, pharmacological, and eletrophysiological techniques. For example, synaptic plasticity such as the phenomena of LTP was reported decades ago (Bliss et al., 1973). A brief high-frequency train of stimuli (a tetanus) to any of the three major synaptic pathways of the hippocampus increases the amplitude of the excitatory postsynaptic potentials in the target hippocampal neurons. Results from different studies all suggest that the intracellular cAMP-regulated CREB pathway plays a key role in these forms of LTP and in the long-term memory formation.

1.3.2.1 Long-term facilitation in Aplysia

The early studies on the invertebrate mollusk Aplysia have been invaluable for our understanding of the basic forms of associative learning. Aplysia withdraws its gill and siphon when a noxious stimulus is applied to its tail. A single stimulus produces a short-term sensitization (mins) to a subsequent stimulus, while repeated stimulation leads to long-term (hours to days) sensitization. The facilitation of the synapse between the sensory and the motor neurons is thought to be critical in mediating behavioural sensitization. The neurotransmitter serotonin, released from interneurons after stimulation of the Aplysia tail, leads to an enhanced synaptic transmission between the sensory and the motor neurons (Montarolo et al., 1986).

This Aplysia model for learning can be replicated by a reduced preparation in vitro. When a single Aplysia sensory neuron is co-cultured with a motor neuron, the two cells form a synapse. Multiple applications of 5-HT lead to both a long-term facilitation (LTF) of synaptic function and a long-term sensitization (LTS) behaviourally. Serotonin application results in the activation of adenylyl cyclase, which in turn, activates the cAMP second messenger system. cAMP activation of PKA leads to the subsequent translocation of the catalytic subunits of PKA to the nucleus to active CREB-dependent transcription of genes, which eventually leads to the growth of new synaptic connections (Frank and Greenberg, 1994; Silva et al., 1998). Direct injection of cAMP into the sensory neuron

triggers both short- and long-term facilitation (Schacher et al., 1988), which can be blocked by PKA inhibitors (Ghirardi et al., 1992) .

The first study to suggest that CREB is required for this LTS formation was reported by Dash et al (1990). Oligonucleotides with CRE sequences injected into cultured sensory neurons selectively blocked LTF, but not short-term facilitation (STF). A more recent study also showed that induction of LTF triggers CREB activation and CRE-mediated transcription by using a lacZ reporter gene transferred into individual Aplysia sensory neurons (Kaang et al., 1993)

The repeated pulses of 5-HT initiate a gene activation cascade that leads ultimately to the growth of new synaptic connections (Martin et al., 1997). Several genes have been identified in this process, including apCREB1, apCREB2, apCCAAT/enhancer binding protein (C/EBP), and the cell adhesion molecule apCAM. ApCREB2 represses ApCREB1-mediated transcription (Bartsch et al., 1995). Opposing forms of CREB (activator CREB1a vs. repressors CREB1b and CREB2) produce opposite effects on long-term facilitation (Bartsch et al., 1995; Bartsch et al., 1998). The injection of antibodies to ApCREB1 or antisense oligonucleotides to ApCREB1 into the sensory neurons selectively blocked long-term facilitation. On the other hand, injection of phosphorylated CREB-1 into the cell body (Barco et al., 2002) or injection of an antiserum against a CREB repressor (ApCREB2) (Bartsch et al., 1995) gives rise to long-

term memory by one pulse of serotonin, which normally only results in short-term facilitation in normal conditions. With five pulses of 5-HT, PKA recruits MAP kinase and both translocate to the nucleus, where they activate ApCREB1 and de-repress ApCREB2, leading to the induction of a set of immediate-early genes which are associated with late genes that are responsible for the growth of new synaptic connections (Martin et al., 1997).

1.3.2.2 cAMP and Drosophila

The importance of cAMP signalling and the CREB pathway for memory formation has been demonstrated in the fruitfly, Drosophila. Drosophila form robust and reliable olfactory discrimination memories. When Drosophila are exposed to two odors, if one is paired with an electric shock, they learn to avoid the paired odor in a T-maze test (Tully, 1991).

The involvement of the cAMP pathway has been shown in Drosophila olfactory learning by genetic manipulations. Four mutants that showed abnormal levels of cAMP also exhibited learning deficits: 1) *Dunce*, which lacks phosphodiesterase, an enzyme that degrades cAMP and therefore has a high level of cAMP; 2) *Rutabaga*, which is defective in adenylyl cyclase and therefore has a low level of cAMP; 3) *Amnesiac* which lacks a peptide transmitter acting on adenylyl cyclase, and, 4) PKA-R1 which is defective in

PKA (Kandel et al. 2000, pp1257). Furthermore, when Drain et al.(1991) generated a transgene that blocked the catalytic subunit of PKA under an heat-sensitive inducible promoter, they found that even transient blockade of PKA interfered with the fly's ability to learn and form short-term olfactory memories. These outcomes from Drosophila mutants suggest an important role for cAMP/PKA involvement in Drosophila olfactory learning.

Long-term associative memory in Drosophila requires CREB pathway activation and new protein synthesis. As in Aplysia, Drosophila has both a CREB activator and a CREB-2 repressor. Overexpression of the CREB activator enhances long-term memory, whereas over-expression of the repressor selectively blocks long-term memory without disrupting short-term memory (Yin et al., 1994; 1995).

1.3.2.3 CREB in transgenic mice

The role of CREB in synaptic plasticity and memory formation has been greatly advanced by neurogenetic manipulations in mice. The role of cAMP/PKA signalling in LTP and long-term memory was demonstrated by using a transgenic approach to reduce PKA activity in the hippocampus by using R(AB), a dominant negative form of the RI_{∂} regulatory subunit of PKA (Brandon et al., 1995). Recordings from slices of R(AB) mice showed impaired Late-LTP (L-LTP, lasts for at least 24 hours) but not Early-LTP (E-

LTP, lasts only for 1-2 hours) induced in the Schaffer collateral pathway. In parallel, when R(AB) mice were tested for their memory function, they exhibited normal short-term memory, but deficient long-term memory for contextual fear conditioning. These results suggest that L-LTP and long-term memory require cAMP/PKA second-messenger pathways in the hippocampus.

In $CREB^{\alpha\Delta-}$ mice, a neomycin resistance gene insertion causes the loss of the two main CREB isoforms α and Δ. However, CREB β and CREM isoforms showed enhanced expression levels in $CREB^{\alpha\Delta-}$ mice (Hummler et al., 1994). The issue of whether the $CREB^{\alpha\Delta-}$ mutation affected memory was tested in three behavioural tasks, each thought to be dependent upon hippocampal function (Silva et al., 1998): contextual fear conditioning, the Morris water maze, and the social transmission of food preferences. $CREB^{\alpha\Delta-}$ mice showed severe contextual memory deficits when tested 24 hr, but not 30 min, after training (Bourtchuladze et al., 1994). Also, the $CREB^{\alpha\Delta-}$ mice in the social transmission of food preference task showed impaired long-term, but not short term memory (Kogan et al., 1997). $CREB^{\alpha\Delta-}$ mice demonstrated profound spatial learning and memory deficits in the Morris water maze test, but not in a visible platform version of the water maze, which does not depend on hippocampal function (Silva et al., 1998). Importantly, these behavioural deficits did not appear to result from the more generalized impact of the mutation on CNS development (Bourtchuladze et al., 1994).

Unfortunately, different isoforms of CREB as well as CREM up-regulation and the potential developmental and general impact of such mutations have made the interpretation of behavioural results complicated. Restricted and regulated expression of a constitutively active form of CREB, has been constructed in hippocampal CA1 neurons of VP16-CREB mice (Barco et al., 2002). The induction of the VP16-CREB transgene lowers the threshold for eliciting Late-LTP in the Schaffer collateral pathway. Synaptic tagging and capture have been outlined as a novel property of hippocampal LTP (Frey and Morris, 1998; Barco et al., 2002). The induction of LTP is associated with the setting of a "synaptic tag" at activated synapses, whose role is to sequester plasticity-related proteins that then serve to stabilize temporary synaptic changes and so extend their persistence (Frey and Morris, 1998). Pharmacological and two-pathway experiments suggest a model in which VP16-CREB activates the transcription of CRE-driven genes and leads to a cell-wide distribution of proteins that prime the synapses for subsequent synapse-specific capture of Late-LTP by a weak stimulus. This result argues that activation of a CRE-driven pathway may be sufficient for consolidation of LTP (Barco et al., 2002).

1.3.2.4 CREB studies in rats

The design of CREB antisense oligonucleotides enables the acute modulation of CREB levels in specific brain areas in rats. Intrahippocampal infusion of these

oligonucleotides prior to training does not disrupt short-term spatial memory, but does affect long-term memory for the water maze tested two days after training (Guzowski and McGaugh, 1997). This is consistent with other studies showing CREB pathway involvement in long-term memory. Furthermore, the same study shows the critical period of CREB function is shortly after training, since infusion of the oligonucleotides one day after training does not affect memory two days after training. In another study (Lamprecht et al., 1997), the neural mechanisms of a conditioned taste aversion were investigated in amygdala. Local injection of CREB oligodeoxynucleotide antisense into the rat amygdala several hours before conditioned taste aversion training transiently reduces the level of CREB protein during training and impairs memory when tested 3-5 days later. CREB antisense in the amygdala has no effect on retrieval of a conditioned taste aversion memory once it has been acquired and does not affect short-term memory.

Long-term memory formation requires de novo protein synthesis and CREB may be one of the transcription factors which is required for the new protein synthesis. It therefore is proposed as a molecular switch for the formation of long-term memory. From invertebrates (e.g., Aplysia and Drosophila) to mammals, spaced training (repeated training trials presented with optimal rest intervals) is more effective than massed training (the same training protocal presented with no or shorter rest intervals) in producing long-term memory. Massed fear conditioning in rats produces no or weak long-term memory. However, increasing CREB levels, specifically in the basolateral amygdala via viral

vector- mediated gene transfer, significantly increases long-term memory after massed fear training (Josselyn et al., 2002). In contrast, overexpression of CREB does not alter short-term memory produced by massed training or long-term memory produced by spaced training.

As mentioned before, the levels of CREB are controlled at both the activation level (phosphorylation vs. dephosphorylation) and the transcription level (activator vs. repressor). At the first level, it has been reported that it takes 3-8 min for synaptic activation to trigger maximal CREB phosphorylation (Moore et al., 1996). Additionally, the longer intervals between training trials may result in optimal inactivation of phosphatases (e.g. calcineurin), which may produce longer CREB phosphorylation (Bito et al., 1996; Liu and Graybiel, 1996; Silva et al., 1998). The duration, but not necessarily the amount of CREB phosphorylation, is critical in producing CRE-mediated gene expression (Bito et al., 1996). At the second level, massed training may result in excessive activation of CREB repressors, reducing the ratio of activator/repressor activation. Application of additional CREB activator into the amygdala by virus vector injection therefore enhanced learning from massed training by enhancing activator/repressor ratio (Josselyn et al., 2002). Alternatively, activators can assume the role of repressors on occasion. An excess of activator may overwhelm the upstream kinases and result in excessive nonphosphorylated CREB which can act as a repressor of C/EBP- induced transcription (Vallejo et al., 1995; Silva et al., 1998).

Phosphorylation of CREB is thought to be important in processes underlying long-term memory. Overexpression of mutant CREB, with a single point mutation at Ser133, does not facilitate long-term memory (Josselyn et al., 2002). Moreover, CREB is phosphorylated in the CA1 pyramidal cells following electrical stimuli that induce LTP and after training in hippocampal-dependent tasks (Impey et al., 1998b). Increased pCREB is also present in the olfactory bulb shortly after conditioned odor preference training (McLean et al., 1999). CREB can be phosphorylated by various cascades, such as PKA, CaMKIV, MAPK, all of which have been implicated in late-LTP. Therefore, CREB is a strong candidate for the activation of CRE-driven gene expression observed during memory formation (Barco et al., 2002).

1.4 Rationale and hypotheses for the present thesis

McLean et al (1999) hypothesized an intracellular model for early odor preference learning. They proposed that NE and 5-HT interact in mitral cells of the olfactory bulb to elevate cAMP levels, which in turn, synergise with a calcium signal initiated by the odor input to activate the CREB phosphorylation pathway crucially implicated in memory formation. In addition to suggesting that mitral cells are the primary target of CS-UCS interaction, the model implies that the strengthening of the odor input to mitral cell connection and a subsequent increase in mitral cell response to the CS is a critical aspect of the memory representation. The work in this thesis evaluates and refines this model.

As previously reviewed, pairing odor with stroking or β-adrenoceptor activation induces early odor preference learning. McLean et al (1999) had demonstrated that stroking-induced preference learning was associated with increased levels of pCREB in mitral cells. In first series of experiments (Chapter 2), we hypothesize that NE and 5-HT act synergistically to increase CREB phosphorylation during odor conditioning and that their interaction potentiates olfactory nerve input to mitral cells. In this set of experiments, we ask if significant pCREB increases are also associated with isoproterenol-induced learning and whether pCREB follows the inverted U pattern seen with learning (Sullivan et al., 1991) or is simply dose-dependently related to β-adrenoceptor activation. Electrophysiological indices of odor nerve input before and after injection of isoproterenol in both normal pups and olfactory bulb 5-HT depleted pups are also evaluated.

The second series of experiments focuses on identifying changes in the response to odor input that occur during memory retrieval using intrinsic optical imaging. These experiments test the hypothesis that a strengthening of the CS-mitral cell input in the glomerular layer characterizes the memory representation. Previous work on the early odor preference model (Wilson and Sullivan, 1994) and in the accessory olfactory bulb (Brennan and Keverne, 1997) suggested instead that increased inhibition of mitral cells was the primary associate of the memory representation. The second series of experiments (Chapter 3) uses intrinsic optical imaging to examine odor-induced

activation at the glomerular level at the time of retrieval to further evaluate this issue.

In the third series of experiments (Chapter 4), the hypothesis that NE and 5-HT interact synergistically to produce an optimal cAMP level in the mitral cells of the olfactory bulb in early odor preference learning is examined. The changes in cAMP associated with stroking and isoproterenol-induced learning are examined with particular attention to the effects of 5-HT depletion. In earlier experiments it had been shown that prior depletion of 5-HT in the olfactory bulb prevents learning (McLean et al., 1993) unless a higher, normally ineffective dose of isoproterenol is used (Langdon et al., 1997). In these 5-HT depletion experiments the $5HT_{2A/2C}$ receptor was identified as playing a critical role, both by using DOI as an agonist to restore normal learning (Price et al., 1998) and ritanserin as an antagonist to prevent normal learning (McLean et al., 1996). Experiments in rat neocortical slices had demonstrated that cAMP activation by isoproterenol is enhanced by activation of the $5HT_{2A/2C}$ receptor and that in the presence of a $5HT_{2A/2C}$ antagonist there is reduced production of cAMP induced by isoproterenol (Morin et al., 1992). These results paralleled the 5-HT effects seen in the rat pup with the same drugs. Thus, we ask here, if cAMP levels relate to the behavioural effects of stroking and isoproterenol in normal learning and whether 5-HT depletion alters cAMP levels in the olfactory bulb and, specifically, in the mitral cells as predicted by our hypothesis.

The final set of experiments (Chapter 5) evaluates the causality of CREB's role in early odor preference learning by directly manipulating CREB using an HSV viral vector. Although previous work (McLean et al., 1999) demonstrated CREB phosphorylation following odor preference learning, the evidence for a role for pCREB was correlational rather than causal. We hypothesize in this study that increased CREB substrate would shift the isoproterenol dose-dependent learning curve to the left, and mutant CREB substrate would interefere with normal learning. The relation of the effects of CREB in early odor preference learning to pCREB levels is also assessed.

Chapter 2. Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve Evoked Potentials in Normal and Bulbar 5HT-Depleted Rat Pups only at Doses that Produce Odor Preference Learning

(Published in Learning & Memory 7:413-421, 2000)

2.1 Introduction

The neonate rat forms an odor preference to odors that are paired with either tactile stimulation (stroking) or 2 mg/kg of the β-adrenoceptor agonist isoproterenol (Sullivan et al., 1989b; Sullivan and Wilson, 1991; Langdon et al., 1997). In this early olfactory learning paradigm, stroking has been shown to activate the locus coeruleus (Nakamura et al., 1987), which releases norepinephrine in the main olfactory bulb, and engages β-adrenoceptors (Sullivan and Wilson, 1994; Woo et al., 1996). Intrabulbar infusion of the β-adrenoceptor antagonist, propranolol, prevents the development of the conditioned odor preference (Sullivan et al., 1989b). Co-activation of the glutamatergic olfactory nerve input (Berkowicz et al., 1994; Ennis et al., 1996) and β-adrenoceptors is hypothesized to be critical for triggering the long-term change in olfactory bulb processing which mediates conditioned odor preference learning (McLean et al., 1999). The pairing of odor with the β-adrenoceptor agonist exhibits an inverted U-curve with

both a lower dose (1 mg/kg) and a higher dose (4 mg/kg) of isoproterenol being ineffective relative to the moderate dose (2 mg/kg) that is optimal for learning (Sullivan et al., 1989b; Langdon et al., 1997). Selective serotonin (5-HT) fiber depletion in the olfactory bulb of rat pups shifts the isoproterenol inverted U-curve such that a higher dose (6 mg/kg) is now required for learning while the normal optimal moderate dose (2 mg/kg) is ineffective (Langdon et al., 1997).

Electrophysiological recordings carried out in the olfactory bulb of rat pups that have undergone conditioned odor preference training have revealed a significant decrease in the ratio of excitation to inhibition in single unit mitral cell activity recorded in the olfactory bulb (Wilson and Sullivan, 1991). However, it was not possible in the unit recording studies to know if the mitral cells encoding the learned odor were selectively sampled. The increased inhibition might reflect increased lateral inhibition concomitant with stronger signalling in the conditioned odor pathway. Disinhibition of mitral cells from granular cell GABAergic effects at dendrodendritic granule cell-mitral cell synapses has been suggested to play a critical role in conditioned olfactory learning (Jahr and Nicoll, 1982; Okutani et al., 1999). Such an effect might be expected to potentiate, rather than inhibit olfactory nerve (ON) throughput during acquisition. The occurrence of disinhibition is supported by evidence that norepinephrine (NE) applied to the external plexiform layer (EPL) decreases inhibitory postsynaptic potentials recorded in the granular cell layer by stimulating olfactory nerves (Jahr and Nicoll, 1982). Paired pulse

inhibition of the mitral cells is also suppressed by injections of NE or isoproterenol (Jahr and Nicoll, 1982; Wilson and Leon, 1988).

In another direction, extensive research has been carried out to identify the molecular components of synaptic plasticity underlying memory formation. Emerging from these studies, cAMP response element binding protein (CREB) has been identified as an important modulator of memory formation (Silva et al., 1998). Its activation is required to initiate the cellular events underlying long-term memory formation in a variety of species (Yin and Tully, 1996; Abel and Kandel, 1998; Bartsch et al., 1998; Silva et al., 1998). CREB phosphorylation at ser 133 by different protein kinases has been implicated as the initial step of CRE-related gene transcription (Walton and Dragunow, 2000). Down regulation of phosphorylated CREB (pCREB) or of related protein kinases impairs long-term memory formation (Silva et al., 1998). Electrophysiological evidence in hippocampal cultures (Bito et al., 1996; Deisseroth et al., 1998; Mermelstein et al., 2000) suggests CREB phosphorylation responds to specific synaptic signals engaging both NMDA receptors and L-type calcium channels. Increased pCREB activation has been shown to occur in the mitral cells of rat pup olfactory bulbs that are pre-treated with effective pairings of odor and stroking (McLean et al., 1999).

In the present study, we asked, first, whether the pairing of odor with a behaviourally effective dose of isoproterenol (2 mg/kg) could produce the same pCREB

increase seen previously with stroking. Further, we examined whether a higher dose of isoproterenol (6mg/kg), which cannot induce preference learning in normal rat pups, when paired with odor, could still increase pCREB. Then, the importance of pCREB in the mediation of serotonergic modulation of NE-induced odor preference in rat pups was examined by using selective serotonergic depletion of the olfactory bulb. If pCREB is specifically involved in learning, a higher dose of isoproterenol (6 mg/kg) should now be required to enhance pCREB expression. This would parallel the previous behavioural model in which selective 5-HT depletion of olfactory bulbs shifted the effective dose of isoproterenol, so that a higher dose (6 mg/kg) was required to induce odor preference learning.

Second, to illuminate possible changes in synaptic transmission in olfactory circuitry, which may trigger the subcellular signal transduction underlying associative learning, or functionally support such learning, we asked whether behaviourally optimal doses of isoproterenol altered ON-evoked potentials. Normal rat pups, and pups with bulbar 5-HT depletion, were again tested.

If pCREB is critical for odor preference learning, and odor preference learning alters the response to ON input, we expected that only behaviourally optimal doses of isoproterenol would selectively change both pCREB expression and the ON-evoked potential in rat pups with either normal or 5-HT depleted olfactory bulbs.

2.2 Experiment 1. Increased pCREB expressions following manipulation of NE and 5-HT inputs to the olfactory bulb correlate with odor preference learning in neonate rats.

To test the hypothesis that pairing odor with a behaviourally effective dose of the β-adrenoceptor agonist isoproterenol triggers phosphorylation of CREB (a postulated signalling substrate for learning), we subcutaneously injected isoproterenol into normal rat pups 40 min before conditioned pairing with odor (**Experiment 1a**). Isoproterenol can completely substitute for stroking as an unconditioned stimulus during odor conditioning (Sullivan et al., 1989b). This effect is consistent with the observed activation of locus coeruleus neurons, the source of NE in neonate rat olfactory bulbs, by somatosensory stimulation (Nakamura et al., 1987). Since a selective pCREB increase was observed in olfactory conditioned preference learning (increased pCREB in the bulbs of odor conditioned pups, but not in the bulbs of the pups that were trained by odor or stroke alone, McLean et al., 1999), we hypothesized that the intracellular cAMP second messenger system, activated by NE input to the β-receptor, works synergistically with the Ca^{2+} entry triggered by glutamatergic olfactory input, to influence CREB phosphorylation and the downstream gene transcription which are necessary for long-term memory formation. In the present study, direct activation of the β-receptor by isoproterenol should have the same effect as the tactile stimulation in odor preference learning. In addition, given the observation that 5-HT normally promotes the efficacy of β-adrenoceptor agonist

isoproterenol as an unconditioned stimulus during olfactory learning, we predicted that a higher dose of isoproterenol would be required in 5-HT depleted animals to induce a comparable increase in pCREB to that observed in normal animals (**Experiment 1b**).

2.2.1 Methods

In **Experiment 1a**, a total of 95 Sprague-Dawley rats of both sexes from 8 litters were used. In **Experiment 1b**, 33 rat pups from 6 litters were used. Three groups were included in each experiment: a saline group, a 2 mg/kg isoproterenol group and a 6 mg/kg isoproterenol group. Litters were culled to 12 pups/litter on PND1 (the day of birth is considered PND0). The dams were maintained under a 12hr light-dark cycle, with *ad libitum* access to food and water. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

2.2.1.1 Odor conditioning and drug injection

The procedure for conditioning has been described previously (Langdon et al., 1997). Briefly, on PND6, saline or isoproterenol 2 mg/kg or 6 mg/kg (Research Biochemicals) was injected subcutaneously into normal pups (**Experiment 1a**) or bulbar 5-HT depleted pups (**Experiment 1b**) 40 min prior to odor exposure. The treated pups were removed from the dam 30 min after injection and put on fresh wood bedding. Ten

minutes later, pups were placed on peppermint-scented bedding (0.3 ml peppermint/500 ml bedding) for a period of 10 min. Following training, one normal pup from each treatment condition was sacrificed at various intervals (10min, 1hr, 2hr, **experiment 1a**), whereas 5-HT depleted pups were sacrificed only at 10 min after odor conditioning (**experiment 1b**). After sacrifice, both olfactory bulbs were removed quickly from the skull, immediately frozen on dry ice and subsequently stored at -70°C in microcentrifuge tubes. Other treated littermates were used for odour preference testing the next day (PND7).

2.2.1.2 Preference testing

A stainless steel test box (30×20×18cm) with a polypropylene mesh screen inside was placed on two trays, which were separated by a 2 cm neutral zone. One tray contained fresh bedding; the other contained peppermint-scented bedding. Each pup was removed from the dam and placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint bedding or normal bedding was recorded for five 1 min trials. A timer was started when a pup moved its nose and one paw into one side of the test box. The percentage time the pup spent on peppermint bedding over the five minute period was calculated. One-way analysis of variances (ANOVA) were used to compare different treatment groups, and post hoc tests were performed using the Tukey-Kramer test.

2.2.1.3 5-HT depletion

The procedure for 5-HT depletion of the olfactory bulb has been described elsewhere (McLean et al., 1993). Briefly, PND1 pups were removed from the dams, pretreated with 10 mg/kg desipramine by intraperitoneal injection and placed on fresh bedding. Forty five min later, after being anaesthetized by hypothermia on ice, the pups were placed in a modified stereotaxic instrument, and 150 nl of 5,7-dihydroxytryptamine (5,7-dHT) in Ringer's solution plus 0.02% ascorbic acid were injected bilaterally into the anterior olfactory nucleus. The pups were returned to the dams after recovery. Immunocytochemistry was performed on the brains of some of the animals to confirm depletion of 5-HT. This procedure has been shown to produce greater than 80% 5-HT fiber depletion in the olfactory bulb and is specific for the serotonergic fibers (McLean et al., 1993).

2.2.1.4 Protein determination and Western blot analysis

pCREB protein expression was determined by Western blot using previously published methods (McLean et al., 1999). Briefly, each pair of olfactory bulbs were placed in microcentrifuge tubes and ground in 100 μl lysis buffer containing 0.1% SDS, 1% NP-40, 20 mM PMSF, 10% glycine, and 1.37 mM sodium chloride with 1 μl/ml leupeptin, 2 mM PMSF, 8.9 U/ml aprotinin, and 1 mM sodium orthovanadate. The

homogenate was placed on a rotator for 30 min and then centrifuged at 13,500 rpm for 15 min at 4°C. The clear lysate supernatant was stored in 50 μl aliquots at -70°C. A bicinchoninic acid (BCA) protein assay kit was used to determine the protein concentration from each pair of olfactory bulbs.

After protein determination, 20 μl of prepared samples were boiled, cooled on ice and loaded into each lane of a SDS-PAGE gel for each blot. In each sample, 4 μl of 5× sample buffer (0.25 M Tris-HCL, 10% SDS, 50% glycerol, 0.025% bromophenol blue, and 0.5 M dithiothreitol added prior to use) and sufficient water were added to volumes of lysate that contained equal amounts of protein. 10 μl of colour coded molecular standard (Bio-Rad) was loaded into a separate lane for each blot. Following sample loading, each gel apparatus was attached to a Bio-Rad power supply set to 100 mV for 10 min, then the voltage was reset to 150 mV until all the samples were loaded completely. The gel running buffer contained 25 mM Tris, 250 mM glycine, and 3.5 mM SDS (pH 8.3). Protein transfer to nitrocellulose paper (Hybond ECL, Amersham) was performed at 0.2 A for 1 hr in transfer solution (25 mM tris, 192 mM glycine, and 20% methanol). After transfer, the nitrocellulose blots were processed for detection of pCREB. Briefly, after 3×5 min rinses in PBS contains 0.05% Tween-20 (PBST), the blots were blocked for non-specific proteins using 5% milk in PBST for 1 hr. Following 3×5 min rinses in PBST, the blots were treated with a rabbit polyclonal pCREB antibody (1/1,500, Upstate Biotechnology) in PBST overnight at 4°C. The specificity and sensitivity of this antibody

has been shown before (McLean et al., 1999). After 3×5 min rinses in PBST and incubation in anti-rabbit IgG conjugated to horseradish peroxidase for 1 hr, the blots were rinsed and visualized by ECL chemiluminescence (Amersham). Then, the blots papers were immersed in Poncean S solution to determine if equal amounts of protein were loaded.

The analysis of Western blots was carried out using a ChemiImager (Alpha Innotech Corp.). The average optical density (AVG = integrated density value/area) was recorded in the defined region of the pCREB bands. The background integrated optical density was automatically subtracted from each defined area. One way repeated measure ANOVAs were used to compare different treatment groups at various intervals.

2.2.2 Results

Experiment 1a Figure 2.1 shows the change of pCREB in the olfactory bulbs produced by pairing two doses of the β-adrenoceptor agonist isoproterenol with odor, and the odor preference results in normal rat pups. Behavioural results showed that 2 mg/kg isoproterenol induced significant odor preference learning compared to either the saline group ($p<0.01$) or the 6 mg/kg isoproterenol group ($p<0.05$)(Fig.2.1A). Correspondingly, the olfactory bulbs of pups that were trained by pairing odor with 2 mg/kg isoproterenol injection showed increased pCREB expression 10 min after training compared to pups

from other treatment groups (Fig.2.1B & 2.1C). Statistical analysis revealed a significant treatment effect. ($F_{2,21}$=3.87, p=0.046). In the post hoc Tukey-Kramer tests, the 2 mg/kg isoproterenol group (AVG=58.0) showed significantly higher pCREB ($p<0.05$) than the saline group (AVG=31.2), whereas there was no significant difference between saline and 6 mg/kg isoproterenol (AVG=36.3). Analysis of Western blots from longer time durations (1 hr and 2 hr) after conditioning did not show any significant difference among the various treatment groups (Fig.2.1B).

Experiment 1b Odor preference tests showed that only the 6 mg/kg isoproterenol group exhibited a significant increase in odor preference when compared to either the saline or the 2mg/kg isoproterenol groups ($p<0.01$, 2.2A) in bulbar 5-HT depleted pups. The same pCREB measurements on bulbar 5-HT depleted animals were only examined at the10 min interval after training for Western blots because we had not shown any difference of pCREB expression at longer time intervals in **experiment 1a**. In the present experiment, we found the 6 mg/kg isoproterenol group, but not the 2 mg/kg group, showed significantly increased pCREB (Fig.2.2B & 2.2C). Following a one-way ANOVA analysis ($F_{2,15}$=5.61, p=0.015), Tukey-Kramer tests revealed a significant difference ($p<0.05$) between the 6 mg/kg isoproterenol group (AVG=65.1) and the saline group (AVG=34.6). No significant difference was found between the saline and the 2 mg/kg isoproterenol group (AVG=42.3) (Fig.2.2B).

2.2.3 Discussion

Enhanced pCREB expression has been shown in neonate rat olfactory bulbs following pairing of odour with stroking (McLean et al., 1999), a procedure demonstrated to induce reliable preference learning (Sullivan et al., 1989a&b; Sullivan et al., 1991; McLean et al. 1992). It is hypothesized that the observed increases in pCREB induced by stroking were the result of tactile stimulation enhancing NE release from the locus coeruleus and activating β-adrenoceptors in the olfactory bulb concomitant with glutamatergic receptor activation by odor input. Here we demonstrate that exogenous injection of 2 mg/kg isoproterenol, a dose that completely substitutes for stroking in odor preference learning, when paired with peppermint odor, increased pCREB expression in the olfactory bulbs of normal pups.

In contrast, a higher dose of isoproterenol, 6 mg/kg was required to increase pCREB, as well as to induce odor preference, in bulbar 5-HT depleted animals. These results corroborate our previous work suggesting that 5-HT acting through 5-HT_2 receptors normally promotes noradrenergic-induced plasticity in the olfactory bulb (McLean et al., 1999). In the mammalian model, 5-HT receptor activation does not by itself increase cAMP (Morin et al., 1992), but 5-HT_2 activation potentiates isoproterenol or adenylate cyclase induced cytoplasmic cAMP levels through the phosphatidyl inositol system (Rovescalli et al., 1993). The localized increases of pCREB in mitral cells in the

previous study (McLean et al., 1999) suggest that the 5-HT and NE interaction might occur in mitral cells.

Although a late phase of pCREB was observed in another hippocampal LTP plasticity paradigm (Schulz et al., 1999), we failed to show a 2nd peak of pCREB during the later time. It is possible that the high level of stimulation used in the previous hippocampal LTP paradigm may have led to seizure activity, thus recruiting the second peak of pCREB activation. Double peaks have not yet been reported in a natural learning paradigm.

Our previous study showed that an effective conditioning pairing, odor plus stroking, or in the present experiment, odor plus 2 mg/kg isoproterenol, but not odor alone or stroking alone, enhances pCREB expression. These data suggest both a glutamate-initiated calcium signal (Bozza and Kauer, 1998) triggered by odor input, and a cAMP/PKA signal initiated by β-receptor activation are required to significantly elevate pCREB. The striking result in the present experiment, however, is that odor plus 6 mg/kg of isoproterenol is ineffective in producing either odor preference learning or enhanced pCREB expression. This outcome argues that a critical window for calcium and PKA co-activation of phosphorylation events has been exceeded by pairing odor and the 6 mg/kg dose of isoproterenol in the normal rat pup. The argument that 6 mg/kg isoproterenol might be producing pharmacological effects that directly interfere with odor learning is

countered by the effectiveness of this dose in bulbar 5-HT depleted pups. The notion of critical windows for intracellular plasticity cascades is not novel. Long-term depression and long-term potentiation occur variously as a function of specific levels of intracellular calcium (Foehring and Lorenzon, 1999; Yang et al., 1999). Competition at the level of CREB factors leading to a failure of plasticity (Ptashne, 1988) has been described, but it has not been suggested previously that a narrow band window exists for the events triggering CREB phosphorylation itself. The present data demonstrate a strong correlation between effective conditioned stimulus plus unconditioned stimulus pairing and pCREB. Experiments are under way to probe a causal role for pCREB in this learning model.

2.3 Experiment 2. Increased ON-evoked synaptic potentials following manipulation of NE and 5-HT inputs to the olfactory bulb correlate with the requirements for conditioned odor preference learning in neonate rats.

We have established that a β-adrenoceptor agonist, isoproterenol, can completely substitute for tactile stimulation as the unconditioned stimulus in olfactory preference learning, while bulbar serotonin appears to facilitate this noradrenergic action (Langdon et al., 1997). In experiment 1, we hypothesized, and further showed, that the interaction of NE and 5-HT to produce odor preference learning might act through the cAMP 2nd message system to induce CREB phosphorylation, which in turn, would trigger transcriptional activation of downstream proteins. Here we examined the

electrophysiological changes induced by these modulating neurotransmitter interactions in the olfactory bulb. We hypothesize that the electrophysiological changes we observed reflect critical changes underlying odor preference learning.

2.3.1 Method

A total of 65 Sprague-Dawley rats of both sexes were used in this study. Thirty three rat pups had their left olfactory bulbs depleted of 5-HT on PND1 or 2 prior to electrophysiological recording on PND5-10 (see 5-HT depletion method in experiment 1). In **Experiment 2a**, normal pups were divided into four groups: a saline group, a 2 mg/kg isoproterenol group, a 6 mg/kg isoproterenol group and a 20 mg/kg propranolol group. In **Experiment 2b**, three groups as above (the propranolol group was excluded) were included using bulbar 5-HT depleted pups.

2.3.1.1 Surgery

On PND 5-10, pups were anaesthetized with a 2.25 g/kg intraperitoneal injection of 20% urethane. Each pup was placed in a modified stereotaxic apparatus using pressure exerted by the reverse side of normal ear bars to hold the head. The body was supported in a polymer mould through which water warmed to 37°C was continually pumped to maintain the body temperature of the pup. The nasal bone overlying the left olfactory bulb

was removed using a dental drill.

2.3.1.2 Electrophysiology

A bipolar twisted Teflon coated stainless electrode (MS303, Plastic One) was placed on the rostrolateral surface of the exposed olfactory bulb to stimulate the ON. The stimuli consisted of three square bipolar 40V pulses of 0.2 ms duration, 10 sec apart. Extracellular field potentials were recorded at varying depths with a saline filled glass electrode with a tip diameter around 50 μm. After a depth profile, the recording electrode was usually placed in the EPL, approximately 200-300 μm deep to the dorsal surface, to maximize the ON-evoked field potential (EFP). Either saline, or 2 mg/kg or 6 mg/kg isoproterenol, or 20 mg/kg propranolol in 50 μl volume was subcutaneously injected into the pup. Starting from time zero (the time of injection), three recordings with a 10 sec interval were taken every 10 min for a total time of 80-90 min using a Labmaster A-D board. Asyst software was used to deliver the stimulation and collect and store the EFPs. Kruskal-Wallis nonparametric ANOVA tests were performed to compare the EFP areas of each group at every time interval.

2.3.2 Results

A characteristic waveform of a field potential in the EPL of normal olfactory bulb

stimulated by olfactory nerve stimulation is shown in Fig.2.3. The field potentials recorded 200-300 μm below the surface were long lasting (>50 msec) and displayed N_1 (kainate/AMPA receptor mediated) and N_2 (NMDA receptor mediated) components comparable to those described *in vitro* by Aroniadou-Anderjaska et al (Aroniadou Anderjaska et al., 1997). Bulbar 5-HT depletion did not appear to alter the ON-evoked potential (Fig.2.3)

In **Experiment 2a**, 2 mg/kg isoproterenol produced long-lasting increases in olfactory nerve EFP area that were evident beginning 30 min after injection and most prominent at 60 min (Fig.2.4A & 2.4B). ON-EFPs showed little change following injections of saline, 6 mg/kg isoproterenol or 20 mg/kg propranolol. At 60 min, the 2 mg/kg isoproterenol group showed significantly increased percentage EFP area from 0 min ($F_{3,28}$=5.897, p=0.003) compared to either the saline group ($p<0.01$), or the 6 mg/kg group ($p<0.05$) or the propranolol group ($p<0.05$). Waveform changes suggest both N1 and N2 components (Aroniadou Anderjaska et al., 1997) contributed to the increase of EFP area. N1 and N2 change ratios were separately estimated in the 2 mg/kg isoproterenol group by selecting a time point and assessing increases at that time point relative to the same point during the initial measurement (0 minutes). The average effect of 2 mg/kg isoproterenol on ON-evoked potentials at 0 min and 60 min is illustrated in Fig.2.4C.

In **Experiment 2b**, 2 mg/kg isoproterenol was ineffective in 5-HT-depleted bulbs, as was saline, in producing increases in the ON-EFP area (Fig.2.5A). In contrast, 6 mg/kg isoproterenol produced a significant increase in EFP area at 60 min ($F_{2,30}$=4.317, p=0.025) when compared to the saline group ($p<0.05$, Fig.2.5B).

2.3.3 Discussion

Isoproterenol at a dose, 2 mg/kg, which normally produces an effective conditioned odor preference in the 5-10 day old rat pup, and which also increases pCREB expression in olfactory bulbs after conditioned odor preference training (as shown in **Experiment 1**), potentiates the ON-EFP in urethane-anesthetized pups of the same age. This effect was specific for the 2 mg/kg dose of isoproterenol. The 6 mg/kg dose of isoproterenol, which does not produce effective odor preference learning in the rat pup, did not potentiate the ON-EFP. However, in the olfactory bulb of rat pups depleted of bulbar 5-HT, 6 mg/kg of isoproterenol, but not 2 mg/kg isoproterenol, was required to potentiate the ON-EFP. This change in the effective potentiating dose of isoproterenol with 5-HT depletion parallels the result produced by bulbar 5-HT depletion on the acquisition of early conditioned odor preference learning and the activation of pCREB in the olfactory bulbs. These results also suggest that during early conditioned odor preference acquisition the glutamatergic ON input is potentiated.

Potentiation of the N_1 component of the ON glutamatergic input by its pairing with an effective β-adrenergic activation, for example, enhancement of ON depolarization (Kawai et al., 1999), enhancement of postsynaptic glutamate responses (Segal, 1982), possibly through increases in membrane resistance, or increased synaptic glutamate through decreased reuptake (Hansson and Ronnback, 1991). Potentiation of the N_2 component of ON synaptic input may be related to those factors and also to enhanced phosphorylation of NMDA channels related to cAMP elevation and to disinhibition of the mitral cells as the result of β-adrenergic suppression of granule cell feedback (Wilson and Leon, 1988).

An important result of Experiment 2 was the failure of the 6 mg/kg dose of isoproterenol to increase the ON-EFP in normal rat pups. This outcome suggests that the electrophysiological potentiation of ON input is in some way dose dependent. While dose-dependency has not been described for the direct actions of β-receptor activation, it has been suggested that NE alters mitral cell excitability primarily by indirect actions (Jahr and Nicoll, 1982). NE decreases GABA release from granular cells resulting in less inhibition from granule cells to mitral cells, whereas glutamate released from mitral cells enhances GABA release from granular cells and increases feedback mitral cell inhibition. An increase in the glutamate released onto granular cells that in turn enhances GABA release at the higher dose of isoproterenol may overcome a net disinhibition produced by a lower isoproterenol dose. Calcium influx through NMDA receptors also directly evokes

GABA release in olfactory bulb granular cells (Halabisky et al., 2000). The balancing of inhibition and disinhibition in olfactory bulb circuitry may explain the failure to induce a potentiated ON-EFP response using a higher dose (6 mg/kg) of isoproterenol in normal olfactory bulbs.

The effectiveness of 6 mg/kg isoproterenol in increasing the ON-EFP in bulbar 5-HT-depleted rat pups suggests that phosphorylation cascades are critical for the electrophysiological effects. If potentiated responses depend on phosphorylation of ion channels, then the failure to recruit intracellular phosphorylation would impair the production of electrophysiological potentiation as well as lead to the failure in CREB phosphorylation.

The present data suggest the ON input mediating a specific odor is strengthened by preference training during acquisition and, likely, more enduringly (Hebb 1949). Combined with the observed pCREB increase in Experiment 1 and earlier evidence that pairing of stroking with peppermint odor selectively induces pCREB increases, the electrophysiological data support the hypothesis that an enhancement of the odor representation is part of odor preference learning.

2.4 General discussion

We have investigated the 5-HT and NE interactions underlying olfactory preference learning from behavior to synaptic transmission to post synaptic biological signal transduction. Among numerous lines of evidence, we are one of the few research groups to look at changes in pCREB during natural learning. In the present study, we have also tried to illuminate the nature of the electrophysiological changes in the olfactory circuitry that accompany acquisition of odor preference in the neonate rat by pairing an electrical odor input (stimulation of olfactory nerve) with behaviorally effective doses of isoproterenol. This procedure mimics the biological components underlying natural learning. We found a remarkably robust correlation in which a behavioral effective dose (2 mg/kg), but not a higher ineffective dose (6 mg/kg), of isoproterenol potentiated the EPSP induced by glutamatergic olfactory nerve input, and selectively enhanced CREB phosphorylation in neonate rat olfactory bulbs. A bulbar depletion of 5-HT shifted the dose-dependent effect of NE so that a higher dose (6 mg/kg) of isoproterenol was required to overcome 5-HT deficiency in the olfactory bulbs for both the electrophysiological change and the increased CREB phosphorylation triggered by behaviorally effective pairing.

There are at least two ways in which the inverted U curve for isoproterenol might be understood. (1) The factors that determine the failure to produce electrophysiological

potentiation to olfactory nerve input are the critical factors in understanding the inverted U curve for CREB phosphorylation. The failure to phosphorylate CREB is a simple consequence of the failure of potentiation. (2) there are two parallel actions of ineffective pairings of odor input and β-receptor activation: one that influences the electrophysiology of the bulb; and one that influences the ability of intracellular cascades to promote CREB phosphorylation. The parallel mechanism hypothesis is less parsimonious and seems less likely.

In attempting to understand the failure of electrophysiological potentiation to occur, we have entertained two hypotheses. The failure to produce odor preference learning may relate to an imbalance in mitral cell inhibition/disinhibition accompanying ineffective doses of isoproterenol. Alternatively, since the known effects of isoproterenol are mediated via G-protein activation and recruitment of adenylate cyclase, another possible node for the failure to produce electrophysiological potentiation would be a failure to enhance cAMP levels. It is likely that cAMP sensitive ion channels are involved in the early membrane effects of isoproterenol. Evaluating the dose dependency of cAMP increases in this system would test this hypothesis.

The site of interaction between β-adrenergic and serotonergic input remains to be identified at the cellular level. However, we suspect the interaction occurs in mitral cells because localized pCREB increases were observed in mitral cells in the olfactory bulbs

after conditioning (McLean et al., 1999). The results of the present study seem to suggest that electrophysiological alterations in functional circuitry will always accompany acquisition of odor preferences and other learning paradigm.

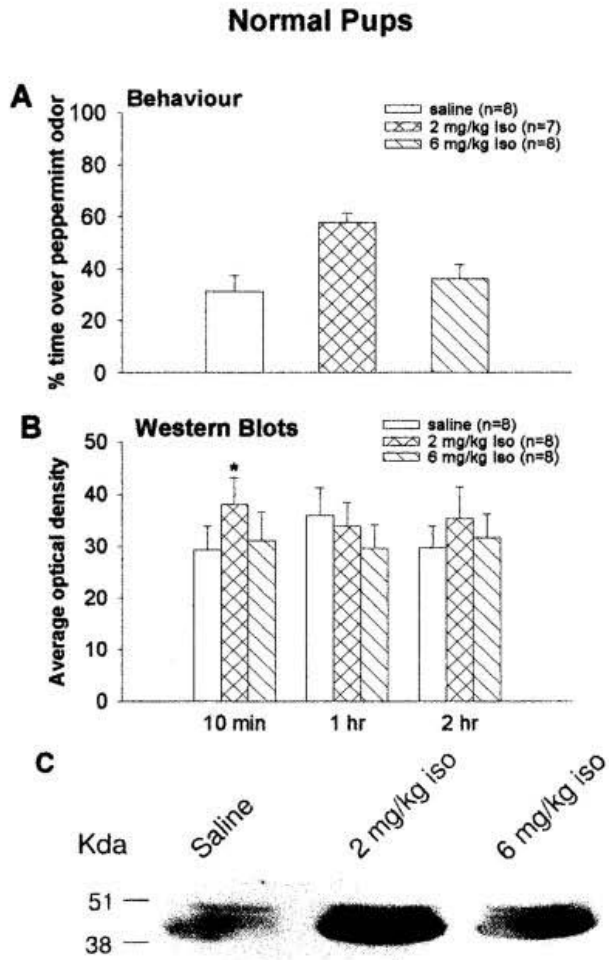

Figure 2.1 Odor preference test and Western blot results of pCREB in normal pups
A: Odor preference test in normal pups from the saline, 2 mg/kg and 6 mg/kg isoproterenol group after odor only training. **$p<0.01$ B: Western results showing the average optical density (mean ± S.E.M.) of pCREB in the olfactory bulbs of normal rat pups. *$p<0.05$ C: Representative Western blot showing pCREB levels in the normal olfactory bulbs from different treatment groups at 10 min after odor exposure. pCREB bands locate at 43 kD. (Iso) Isoproterenol.

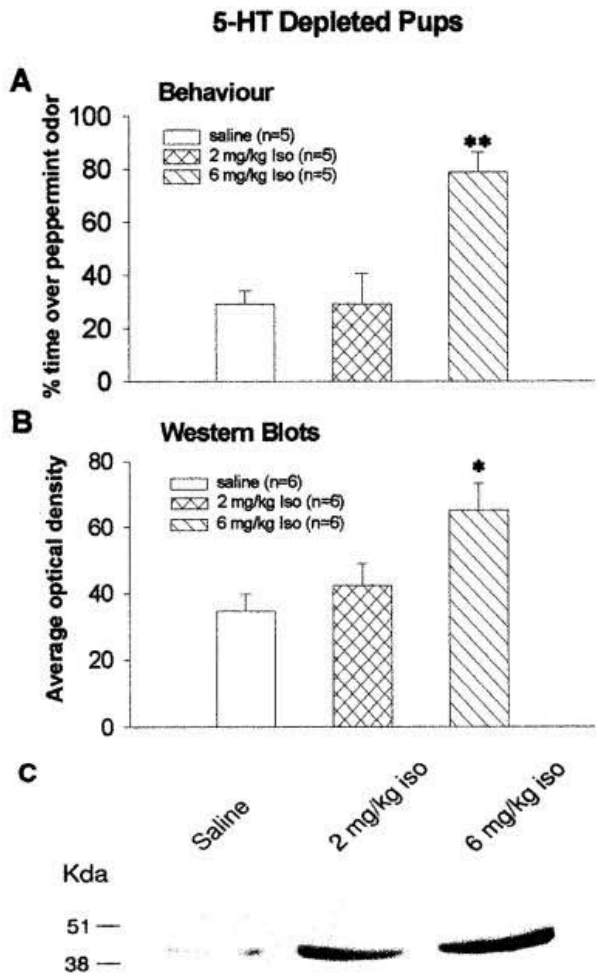

Figure 2.2 Odor preference test and Western blot results of pCREB in olfactory bulb 5-HT depleted pups

A: Odor preference test in bulbar 5-HT depleted pups after odor only training. **$p<0.01$ B: western results showing the average optical density (mean±S.E.M.) of pCREB in the olfactory bulbs of 5-HT depleted rat pups. *$p<0.05$ C: representative Western blot showing pCREB levels in the normal olfactory bulbs from different treatment groups at 10 min after odor exposure. (Iso) Isoproterenol

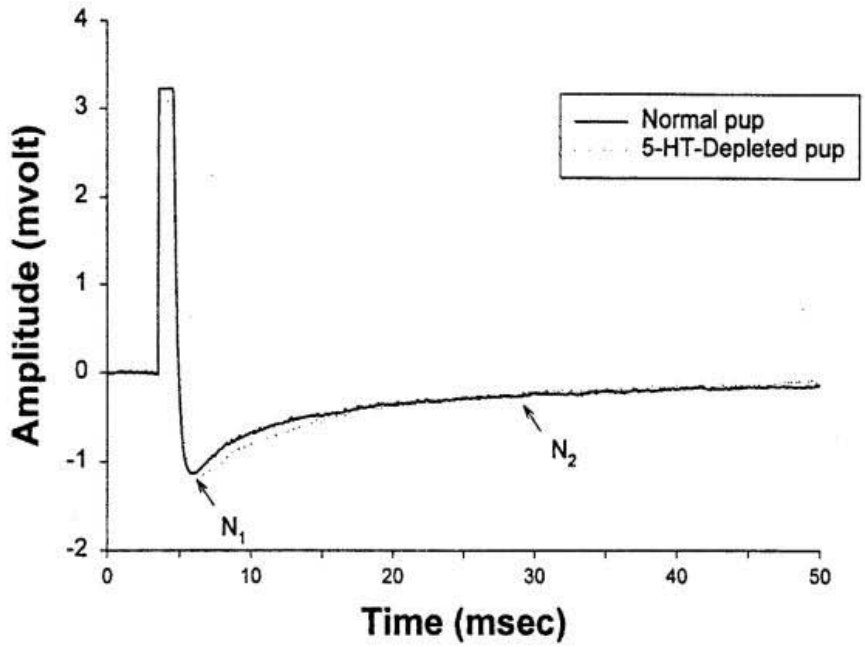

Figure 2.3 Characteristic waveforms of field potentials in the EPL of normal and 5-HT depleted olfactory bulbs by ON stimulation. N1 and N2 showing the kainate/AMPA receptor and NMDA receptor components described by Aroniadou-Anderjaska (Aroniadou-Anderjaska et al. 1997)

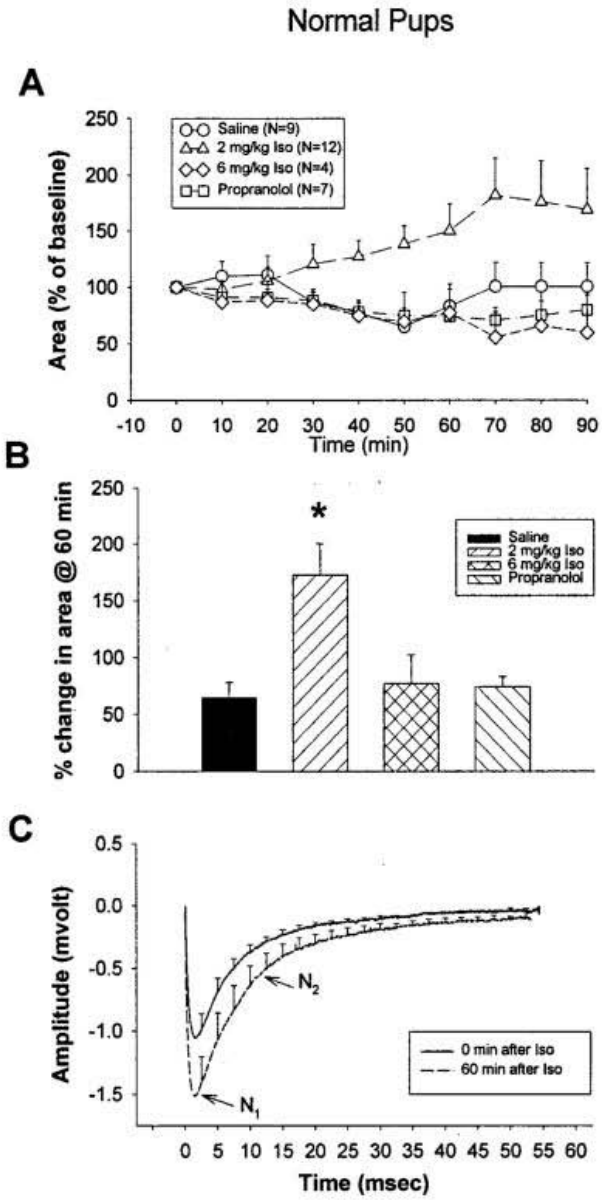

Figure 2.4 EFP recordings in normal rat pups
A: percentage change of EFP areas from baseline (0 min) among different treatment groups of normal pups at various time interval. (mean±S.E.M.) B: percentage change of EFP area at 60 min in normal pups. *$p<0.05$ C: average effect of 2 mg/kg isoproterenol on ON-evoked potentials at 0 min and 60 min. (Iso) Isoproterenol

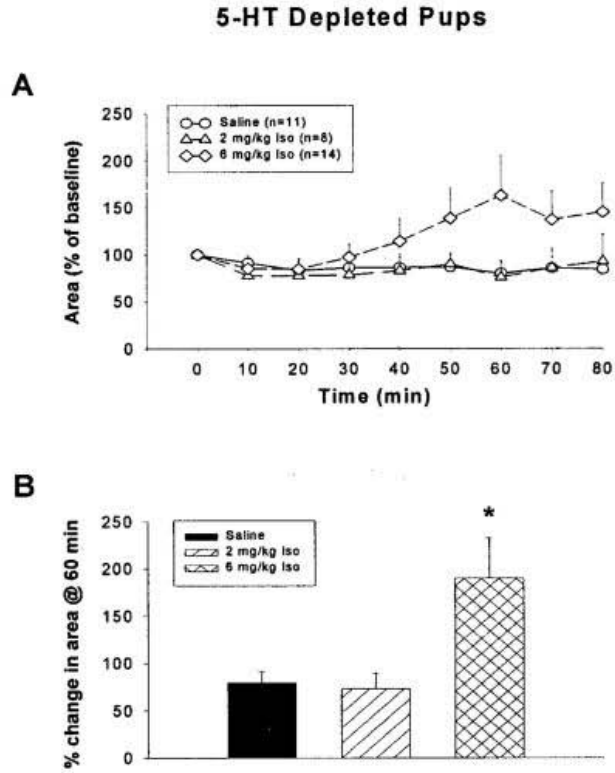

Figure 2.5 EFP recordings in olfactory bulb 5-HT depleted rat pups
A: percentage change of EFP areas from baseline (0 min) of bulbar 5-HT depleted pups at various time interval. (mean±S.E.M.) B: percentage change of EFP areas at 60 min in bulbar 5-HT depleted pups. *$p<0.05$ (Iso) Isoproterenol

Chapter 3 Optical Imaging of Odor Preference Memory in the Rat Olfactory Bulb

(Published in J.Neurophysiology. 87: 3156-3159, 2002)

3.1 Introduction

Neonate rats rapidly (one-trial learning) form a preference to an odor that is paired with a reinforcing tactile stimulus (Sullivan and Leon, 1987) that activates the locus coeruelus (Nakamura et al., 1987) or that is paired with the beta adrenergic agonist, isoproterenol (Sullivan et al., 1989a&b). Several lines of evidence suggest that the olfactory bulb itself is sufficient to mediate this early odor preference learning: activation of beta receptors locally in the bulb, concomitant with peppermint odor presentation, is both necessary (Sullivan et al., 1989a&b) and sufficient (Sullivan et al., 2000b) for odor preference learning to occur. Previous work has shown that odors induce focal uptake of [^{14}C]2- deoxyglucose (2-DG) within the glomerular layer of the olfactory bulb (OB) and that focal 2-DG uptake in the glomerular layer increases after early odor preference learning (Sullivan et al., 1991; Johnson and Leon, 1996). Effective odor preference training protocols increase cAMP response element binding protein (CREB) phosphorylation (McLean et al., 1999; Yuan et al., 2000b) in the bulb and selectively increase the field EPSP to olfactory nerve stimulation (Yuan et al., 2000b). Both the

NMDA and AMPA components of the olfactory nerve field EPSP are enhanced. Furthermore, odor conditioning enhances single-unit responses in mitral-tufted cells in areas that exhibit enhanced 2-DG labeling following exposure to a previously conditioned odor (Wilson and Leon, 1988).

Recent advances in optical imaging have facilitated our understanding of the spatial representation of odors in the olfactory bulb (Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001; Rubin and Katz, 2001). Responses to odors can be measured directly by optical recording of intrinsic signals from the dorsal surface of the OB (Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001; Rubin and Katz, 2001). Representations of odorants within the OB can be visualized at the level of glomeruli. The patterns of odor-induced optical signals are similar among different animals (Belluscio and Katz, 2001).

Intrinsic optical signals are due to activity-dependent hemodynamic changes and light scattering (Malonek et al., 1997; Meister and Bonhoeffer, 2001). Intrinsic signal imaging enables in vivo recording and multiple manipulations on anesthetized animals. Therefore, it may serve as a useful tool to explore training-dependent changes in stimulus-induced patterns of neuronal activity. In this study, we investigated the feasibility of using intrinsic signal imaging to detect training-dependent changes within the OB 24 hr after conditioned odor preference training. We performed intrinsic optical

imaging on the OBs of both trained and control one-week-old rat pups. An enhanced optical signal was observed in trained animals to the trained odor. The result demonstrated that intrinsic signal imaging could monitor training induced changes in neuronal activity.

3.2 Methods

3.2.1 Odor preference training

Eighteen Sprague-Dawley rat pups from five litters were used in this study. The procedure for conditioning has been previously described in detail (McLean et al., 1993; McLean et al., 1999). Briefly, on postnatal day 6 (PND6, the day of birth was considered PND 0), rat pups were removed from the dam and put on fresh bedding 10 min before odor exposure. In one group, pups were placed on peppermint-scented bedding (0.3 ml of peppermint/500 ml bedding) and stroked vigorously on the hind region using a sable brush every other 30 s for 30 s over a 10 min period (odor + stroking). In another group, the pups were only exposed to the peppermint bedding without being stroked (odor only). Immediately after training or odor exposure, the pups were returned to the dams. Previous studies (Sullivan and Leon, 1987; Sullivan et al., 1989a&b; Sullivan et al., 1991; McLean et al., 1993) have shown that rat pups subjected to the above conditioning procedure develop a predictable odor preference for the odor used.

3.2.2 Optical imaging

Rat pups were subjected to optical imaging the day after training. Rats were anesthetized with a 2.25 g/kg intraperitoneal injection of 20% urethane. Anesthetized rat pups were placed in a stereotaxic frame and the bone overlying the dorsal surface of the olfactory bulbs was carefully thinned until the blood vessels underneath the bone were visible (Uchida et al., 2000; Rubin and Katz, 2001).

The stereotaxic frame with the anesthetized rat pups was mounted below optics consisting of a 1x objective and a 1.6x projection lens. Odorants were diluted in glycerol and delivered by computer controlled pressure pulses into a stream of fresh air blowing over the rat's nose (Fig.3.1A). The bulbs were illuminated with red light (630 nm) via two light guides positioned lateral to the objective (Uchida et al., 2000; Rubin and Katz, 2001). The light was focused just below the blood vessels at the level of the glomeruli. Images (640 x 480 pixel) were acquired by a cooled CCD system (Sensicam, PCO Computer Optics GmbH, Germany) under control of Axon Imaging Workbench software (Axon Instruments, Inc., Foster city, CA) at a frame rate of 2 Hz. Different odors and no-odor recordings were interleaved and repeated 5-10 times. Odors were presented for 4 s with a 60 s intertrial interval. Time series of images were averaged (n= 5 to 10) and responses were expressed as odor-induced fractional change in reflected light intensity ($\Delta R/R$, see Fig.3.1B). Thresholding (Uchida et al., 2000; Rubin and Katz, 2001) or spatial

filtering techniques (Meister and Bonhoeffer, 2001) were not applied in order to avoid any interference between these data transformations and data quantification. Data processing and analysis were performed using Origin software (Origin Lab Corporation, MA) and custom-made software written in Interactive Data Language (IDL5.4, Research Systems, CO). The experimental protocol was approved by the Experimental Animal Committee of the RIKEN Institute (Wako Shi, Japan).

3.3 Results

Figure 3.1A shows a schematic illustration of the experimental design for imaging of OB responses to amyl acetate and peppermint. The dorsal surface of the OB was imaged and reflected light was sampled from the medio-rostral, latero-rostral, medio-caudal and latero-caudal quadrants. As shown in Figure 3.1B application of peppermint (10%) for 4 seconds induced a transient change in light reflectance after a delay of about 3 s. Peak amplitudes of these responses amounted to 0.2 % up to 1 % of the baseline light intensity. Signal sizes of the four quadrants did not differ significantly and, therefore, signals from the four quadrants were averaged in subsequent analysis.

The preceding experimental design was then applied using odor trained and control littermate pups (Fig.3.2A). Control animals exhibited amyl acetate and peppermint-induced intrinsic optical signals of comparable peak amplitudes (0.414% ±

6.61x10-4, and 0.354% ± 1.08x10-3, respectively; mean ± SE). Trained animals, however, exhibited larger signals to the trained odor (peppermint, 0.991% ± 2.26x10-3) as compared to the control odor (amyl acetate, 0.521% ± 1.5x10-3) applied to the same animals (Fig.3.2B). Trained animals also responded with significantly larger intrinsic signals to the trained odor than did control littermates to the same odor (Fig.3.2B). Furthermore, odor preference training significantly enhanced the ratio between the responses induced by peppermint and amyl acetate (Fig3.2C).

3.4 Discussion

In the present study, we investigated whether odor preference memory can be accessed by imaging of intrinsic signals at the level of the glomeruli and found that this was the case. This outcome is consistent with the earlier reports of enhanced 2-DG uptake at the glomerular level in the OB following peppermint preference training. It has been established that odor-induced intrinsic signals imaged from the OB involve "global", i.e. spatially less confined components as well as components that can resolve single glomeruli (Meister and Bonhoeffer, 2001). The present odor-induced responses were seen over the dorsal surface of the OB, i.e. at the "global" level, and only occasionally more localized response patterns emerged (not shown). There are several reasons we might expect primarily "global" signals in these experiments. The first is the age of the subjects. Intrinsic optical signals in the somatosensory barrel fields of rats less than 7

weeks of age are more diffuse than those in adults (Yazawa et al., 2001). This is attributed to horizontal interactions. Similarly only diffuse intrinsic optical signals are seen initially in the visual cortex of young ferrets when orientation maps are studied and there is considerable individual variation in the development of the more specific patterns (Chapman et al., 1999). Thus, the olfactory maps in week old rat pups may be more diffuse than in older rats even though glomerular organization has already developed at this age (Bailey et al., 1999). On the other hand, the same concentration of peppermint used here produces discrete 2-DG spots in week-old pups (Sullivan and Leon, 1987). 2-DG peppermint representations are, however, less sensitive to odorant concentration than optical signals appear to be (Carmi and Leon, 1991). Signals for amyl acetate, for example, have been measured at similar concentrations with both methods (Stewart et al., 1979; Rubin and Katz, 2001) and focal patterns are more discrete for higher concentrations with 2-DG (Stewart et al., 1979). In addition increased 2-DG uptake over the entire glomerular layer, as well as enhanced focal uptake, occurs following peppermint preference learning even in older pups (Johnson and Leon, 1996). It is unlikely the global increases seen here are due to respiratory changes to the learned odor since previous studies have found no change in respiration with peppermint preference learning (Sullivan et al., 1988). Finally, in 19 day old pups, 2-DG and c-Fos foci following extended odor preference training are primarily in the midlateral bulb (Woo et al., 1987; Johnson et al., 1995) that was not sampled here. In week old pups 2-DG (Sullivan and Leon, 1987; Yuan et al. 2000a) and pCREB (McLean et al., 1999) images

show dorsolateral foci as well. Thus a portion of the focal peppermint representation was included in the present study, although visualization of the midlateral bulb might have increased the probability of capturing a focal response.

These data are consistent with the evidence from earlier experiments showing an increase in the field EPSP to olfactory nerve input in pups of the same age that receive learning effective training conditions (Yuan et al., 2000b). The intrinsic signal change at the level of the glomeruli 24 hr later in the present study may indicate that the synaptic modification seen during acquisition conditions is sustained.

Creation of an olfactory preference in the rat pup may therefore be intimately related to an increase in synaptic strength at the level of the glomeruli. Such a hypothesis is consistent with the recent report of a Drosophila mutation that concomitantly produces an increase in glomerular synapses and the appearance of a behavioral preference for a normally neutral odor (Acebes and Ferrus, 2001). Transduction of the odor is not altered. Other evidence supporting a special role for the glomerular layer in odor preference learning is the report of increased glomerular size (Woo et al., 1987) (as in the Drosophila model) and of increased numbers of juxtaglomerular cells (Woo and Leon, 1991) following peppermint preference training.

Future studies might examine glomerular intrinsic signal changes at a longer

interval after training to assess focal alterations and to ask if the generalized response seen here is enduring as reported for 2-DG. Within-pup analysis of optical signals in an acquisition paradigm might permit an assessment of training-induced changes in discrete foci when they occur. This was precluded in the present between-group study due to the variability in the occurrence of discrete signals.

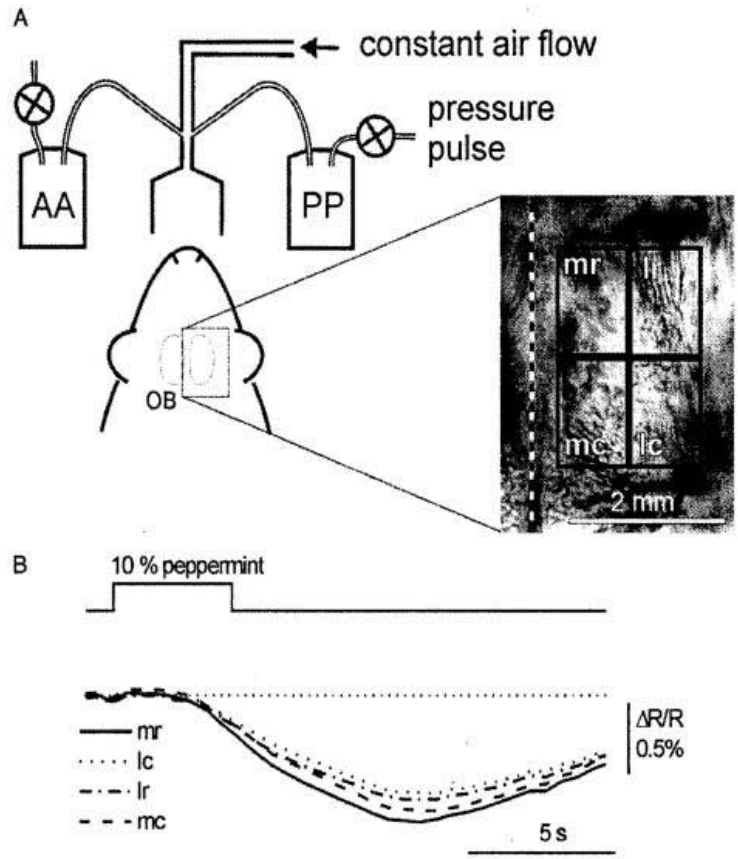

Figure 3.1 Intrinsic imaging setup and peppermint response recordings from the OB
A: Schematic illustration showing the experimental design for imaging of olfactory bulb (OB) responses to two different odors (amyl acetate, AA, and peppermint, PP). The dorsal surface of the OB was imaged and reflected light was sampled from the medio-rostral, latero-rostral, medio-caudal and latero-caudal quadrants. B: Responses obtained with application of peppermint (10%) for 4 seconds. Individual traces were obtained from the 4 quadrants indicated in A.

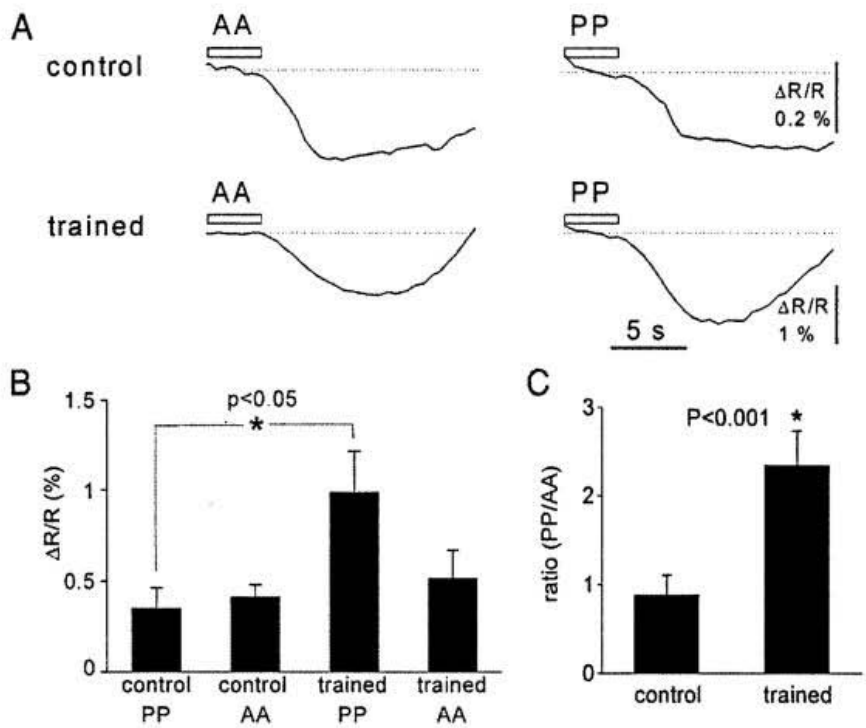

Figure 3.2 Optical imaging of OB responses to AA and PP in control and trained pups. A: Intrinsic OB responses to amyl acetate (AA) and peppermint (PP) in control and trained pups. Note larger response to PP in trained pups as compared to control animals and control odor (AA). Time courses of responses did not differ between the populations of control and trained pups. B: Statistical analysis of data (mean ±s.e.m.). C: Ratio of responses to PP and AA in control and trained rat pups. Asterisks indicate significant differences (One-Way ANOVA).

Chapter 4 Mitral Cell β_1 and 5-HT_{2A} Receptor co-Localization and cAMP co-Regulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb

(Accepted by Learning & Memory, 2002)

4.1 Introduction

The olfactory bulb is an excellent preparation for demonstrating classical conditioning. Odor preferences can be produced in rat pups as young as one week when an odor (conditioned stimulus or CS) is paired with any of several unconditioned stimuli (UCS) including milk, stroking or even mild foot shock (Sullivan and Wilson, 1994; Sullivan et al., 2000a). The learning is localized to the olfactory bulb (Sullivan et al., 2000b) and modifications of the electrical (Wilson et al., 1987; Wilson and Leon, 1988) and metabolic (Sullivan and Leon, 1987; Sullivan et al., 1991) activity of the olfactory bulb are observable after conditioning. Olfactory bulb norepinephrine (NE), acting through β-adrenoceptors, is both necessary and sufficient as neural substrate for the UCS (Sullivan et al., 2000b).

Based on these data and others, Sullivan and Wilson (1994) suggested that learning results from the disinhibition of mitral cells, which permits activation of NMDA

receptors and could promote long-term potentiation-like changes in the granule cell-mitral cell connection (Wilson and Sullivan, 1994). In this scenario NE input from the locus coeruleus to the olfactory bulb acts as the UCS by inhibiting granule cell interneurons in the bulb through β-adrenoceptors to produce disinhibition.

In contrast to the disinhibition model, other data suggest the action of the UCS occurs directly on mitral cells rather than through the intermediary granule cells (McLean et al., 1999). Granule cells show only weak responses to the β-adrenoceptor agonist, isoproterenol, but show much larger responses to α-adrenoceptor agonists (Trombley, 1992; Trombley and Shepherd, 1992; Trombley, 1994; Mouly et al., 1995; Czesnik et al., 2002) and disinhibition of mitral cells is also driven by α-adrenoceptor agonists (Trombley, 1992; Trombley and Shepherd, 1992; Trombley, 1994; Czesnik et al., 2002; but see Wilson and Leon, 1988; Okutani et al., 1998).

Stroking (tactile stimulation that increase NE levels, Rangel and Leon, 1995) or isoproterenol paired with an odor produces learning (Langdon et al., 1997), and the same parameters induce phosphorylation of cAMP response element binding protein (CREB) in the mitral cells (McLean et al., 1999; Yuan et al., 2000b). CREB phosphorylation is significantly increased in the olfactory quadrant that received the odor input (McLean et al., 1999). Likewise the conditioning procedure produces potentiation of the glutamatergic olfactory input to the mitral cells (Yuan et al., 2000b). An interesting

feature of the isoproterenol induced odor preference learning is that it benefits from co-activation of the serotonergic system. 5-HT depletion of the olfactory bulb prevents learning with typical doses of isoproterenol, but higher doses of isoproterenol, 4 mg/kg (Langdon et al., 1997) or 6 mg/kg (Yuan et al., 2000b), can overcome the deficit. Pharmacological studies using ritanserin and DOI suggest that the $5HT_{2A/2C}$ receptor is the critical receptor mediating the serotonin effect (Price et al., 1998), but $5HT_{2A/2C}$ receptor activation by itself does not produce learning (McLean et al., 1996). 5-HT acting through $5HT_{2A/2C}$ receptors, as assessed with ritanserin, ketanserin and DOI, has been observed to potentiate β-adrenoceptor activation in rat neocortex, resulting in enhanced cAMP production (Morin et al., 1992; Rovescalli et al., 1993).

We hypothesize that the critical UCS event in olfactory learning is the production of cAMP in mitral cells. CREB phosphorylation results from the convergence of the UCS cAMP signal and the CS arising from the odor stimulus and travelling via the olfactory nerve. This model parallels that proposed for sensory learning in Aplysia (Kandel et al. 2000).

In the present study we pursue three lines of evidence in support of a direct action of NE on mitral cells as the neural substrate for early olfactory learning. First, selective antibodies for the β_1-adrenoceptors and the 5-HT_{2A} receptor are used to examine the localization and co-localization of these two receptors in the olfactory bulb. Second, the

expression of cAMP following odor preference conditioning is examined with a cAMP assay. The dependence of β-adrenoceptors activation of cAMP signaling on 5-HT input was also tested using 5-HT depletion. Third, the localization of cAMP increases associated with odor preference conditioning is examined immunocytochemically in the olfactory bulb.

We predicted we would find mitral cell co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors in Experiment 1. We had three predictions with respect to variations in cAMP levels in Experiment 2. First, an effective UCS (e.g., stroking or 2 mg/kg isoproterenol in normal rat pups) would produce an increase in cAMP. Second, in rat pups with 5-HT depletion in the olfactory bulbs, these UCSs would no longer increase cAMP. Third, an ineffective UCS in normal pups (e.g, saline or 4 mg/kg isoproterenol) would be associated with lower levels of cAMP.

While β-adrenergic and serotonergic receptor localization on mitral cells, together with cAMP synergism, are key requirements of the present hypothesis, a more convincing demonstration of the hypothesis would include localization of the cAMP increase itself to the mitral cells. This is undertaken in Experiment 3 with cAMP immunocytochemistry following odor plus the learning-effective 2 mg/kg dose of isoproterenol. In the same pups one bulb was depleted of 5-HT. cAMP increases would not be predicted in mitral cells of the 5-HT depleted bulb.

4.2 Experiment 1 5-HT_{2A} receptor and β_1- adrenoceptor localization

4.2.1 Materials and methods

All experimental procedures were approved by the Memorial University Institutional Animal Care Committee and conform to the standards set by the Canadian Council on Animal Care.

4.2.1.1 Animals and sacrifice

Both young (postnatal day 6-12, PND 6-12) and older (PND 30-50) Sprague-Dawley rats totaling 26 rats from 22 litters were used in this study. The rats were anesthetized with an overdose of sodium pentobarbital and perfused as described previously (McLean et al., 1999).

4.2.1.2 Immunocytochemistry/Immunofluorescence

Frozen sections were cut coronally through the olfactory bulbs at 30 μm using a cryostat. The sections were either melted directly onto subbed slides or collected floating in cold PBS. The sections were processed for the β_1-adrenoceptor and/or the 5-HT_{2A} receptor using immunocytochemistry or immunofluorescence.

For the β_1-adrenoceptor immunocytochemistry, briefly, cryostat-mounted sections were air-dried at room temperature for 5-10 min, then incubated in primary antibody (β_1-adrenoreceptor Ab, 1:1000, Oncogene, Cambridge, MA) in 0.2% Triton X-100, 2% normal goat serum (NGS) in PBS at 4°C overnight. Secondary antibody processing and visualization using diaminobenzidine dihydrochloride (DAB) was as described previously (McLean et al., 1999).

For fluorescence double-labeling, the sections were collected free floating in 0.1 M PBS, followed by incubation overnight at 4°C in primary antibodies. Both the 5-HT_{2A} receptor antibody (1:500, Pharmingen, Mississauga, ON) and the β_1-adrenoceptor antibody (1:1000) were dissolved in 0.2% Triton X-100 and 2% normal goat serum (NGS) in PBS. After 3×10 min rinses in PBS, the sections were incubated in goat anti-mouse IgG conjugated to CY3 (1:400, Jackson ImmunoResearch, Mississauga, ON) and goat anti-rabbit IgG conjugated to FITC (1:50, Sigma, Mississauga, ON) or Alexa 488 (1:1000, Molecular Probes, Hornby, ON) dissolved in 2% NGS and 0.2% Triton X-100 in PBS for 1 hr. The sections were rinsed 3×10 min in PBS and mounted on subbed microscope slides.

To improve the results of immunocytochemistry and immunofluorescence, a heat-induced antigen retrieval protocol was employed. Heat assists in unmasking the epitopes or antigens that are hidden as a result of protein cross-linking induced by formaldehyde

fixation (Shi et al., 1991; Cattoretti et al., 1993; Jiao et al., 1999). In this study, microwave irradiation was used before the commencement of immunostaining. Briefly, both slide-mounted sections and free-floating sections were placed in a microwave in containers containing 0.1M PBS solution (pH 7.4). Irradiation at the maximum setting for 1-2 min raised the temperature of the PBS solution to 90-95°C after which the power setting was adjusted to keep the solution at a constant temperature of 90-95°C for 10 min. The sections were kept in the PBS for another 20 min to cool down. Standard immunocytochemical staining as described above was performed after the microwave irradiation. To exclude the possible non-specific staining resulting from microwave irradiation, sections with no primary antibody incubation were also included in the experiment.

4.2.1.3 Image processing

For DAB-stained sections, the olfactory bulbs were examined using bright-field microscopy. For fluorescence, two-channels of a confocal microscope (Olympus Fluoview) or an epifluorescence (mercury lamp) microscope were used. The confocal processing provided scans of 0.25 μm thickness, which enabled unequivocal cellular localization of the label. Images were captured digitally with either the Fluoview confocal software or with a Spot® digital camera.

4.2.2 Results

4.2.2.1 Microwave irradiation and β_1-adrenoceptor labeling

We observed substantially improved immunocytochemical labeling of β_1-adrenoceptors in olfactory bulb sections by using the microwave procedure described in the Methods (Fig.4.1A vs.4.1B). Strong immunocytochemical staining was observed in the mitral cells and tufted cells. Label was mainly confined to the cytoplasm of the somata. Fainter label was observed in periglomerular cells and small subsets of granule cells. Without microwave irradiation, only faint, punctuate labeling of cells was observed (Fig.4.1A). Thus, microwave treatment produced enhanced visualization of cells immunoreactive for β_1-adrenoceptors within the bulb. To control for possible non-specific staining resulting from microwave irradiation, some sections were incubated without the presence of the primary antibody. This procedure served as a negative control for β_1-adrenoceptor immunocytochemistry and produced no cellular label in the olfactory bulb, although non-specific label of fiber bundles within the deep granule cell layer was present (data not shown).

4.2.2.2 Immunofluorescence double label

To investigate the targets of NE and 5-HT action, immunofluorescence double

labeling of β_1-adrenoceptor and 5-HT_{2A} receptors was performed. Consistent with previous studies (Pompeiano et al., 1994; McLean et al., 1995; Hamada et al., 1998; Cornea-Hébert et al., 1999), the mitral cell and external plexiform layers were intensely labeled by the 5-HT_{2A} receptor antibody (Fig.4.2D). Labeled tufted cells were also found in the main olfactory bulb. Figure 4.3 shows CY3 immunofluorescence label of mitral cells in a PND 35 rat. In a few cells, both cell bodies and their dendrites were clearly labeled for the 5-HT_{2A} receptor.

By using two-channel confocal imaging, we observed substantial β_1-adrenoceptor and 5-HT_{2A} receptor double labeling of mitral and tufted cells in both young (eg. PND10, Fig.4.2A,B) and older animals. The label of both receptors was mainly cytoplasmic as shown by punctuate label within the cytoplasm of mitral cells as illustrated in Figures 4.2C & D. This observation is consistent with the observation that G-protein coupled receptors are normally internalized (Tang et al., 1999; Chakraborti et al., 2000).

4.3 Experiments 2A and 2B cAMP expression following odor preference training

4.3.1 Materials and methods

In Experiment 2A, 63 Sprague-Dawley rat pups of both sexes from 9 litters were

used. Seven training groups were included in this experiment. In Experiment 2B, ten rat pups from 5 litters were subjected to unilateral 5-HT depletions of the olfactory bulbs on PND 1 and given either 2 mg/kg or 6 mg/kg isoproterenol (β-adrenoceptor agonist, Sigma) injections before training. All litters were culled to 12 pups/litter. No more than one pup of either sex from each litter was assigned to each training group.

4.3.1.1 Odor conditioning and drug injection

The procedure for conditioning has been described in detail before (Langdon et al., 1997; Price et al., 1998; McLean et al., 1999; Yuan et al., 2000b). Briefly, on PND 6, saline or isoproterenol (1 mg/kg, 2 mg/kg, and 4 mg/kg for Experiment 2A; 2 mg/kg and 6 mg/kg for experiment 2B) was injected subcutaneously into normal pups (Experiment 2A) or pups with unilateral 5-HT depletion of olfactory bulbs (Experiment 2B) 40 min before their exposure to odor conditioning. The odor conditioning was performed by placing the pups on peppermint-scented bedding for a period of 10 min (0.3ml peppermint extract in 500 ml of fresh wood-chip bedding). Also, in this study, serotonergic fiber depletion was performed unilaterally in the olfactory bulbs. Either the low (2 mg/kg) or the high (6 mg/kg) doses of isoproterenol was injected systemically into the pups to investigate the synergistic effect of β-adrenergic and serotonergic receptor interaction in inducing cAMP cascade activation (Experiment 2B).

In Experiment 2A, some pups from the same litters were taken from their dams 10 min before they were subjected to one of the following three training conditions: odor+stroking (the pup was stroked by a sable brush every other 30 seconds for a period of 10 min while the pup was placed on peppermint-bedding), stroking only (the pup was subjected to stroking while it was placed on fresh bedding), and naive (the pup was placed on fresh bedding for 10 min). The purpose of this grouping was to investigate the cAMP levels when using a more natural learning paradigm than the isoproterenol-induced learning.

Immediately after training, the pups were sacrificed by decapitation, both olfactory bulbs were removed from the skull and frozen on dry ice. In Experiment 2A, each pair of olfactory bulbs from a pup was placed in 1.5 ml centrifuge tubes, whereas in Experiment 2B, olfactory bulbs from each pup were put individually into a microcentrifuge tube because in each pup one bulb was subjected to 5-HT depletion while the other was not. All samples were subsequently stored at -70°C until they were assayed for cAMP content.

4.3.1.2 5-HT depletion

Unilateral 5-HT depletions of olfactory bulbs were performed in order to provide intra-animal controls for the effect of 5-HT on isoproterenol induced cAMP expression.

The procedure of 5-HT depletion has been previously described in detail (McLean et al., 1993; McLean and Darby-King, 1994). Briefly, PND1 pups were removed from the dams, pretreated with10 mg/kg desipramine by intraperitoneal injection, and placed on fresh bedding. Forty-five min later, after being anesthetized by hypothermia on ice, the pups were placed in a modified stereotaxic instrument, and 150 nl of 5,7-dihydroxytryptamine (5,7-dHT) in Ringer's solution plus 0.02% ascorbic acid was injected unilaterally into the anterior olfactory nucleus. Immunocytochemistry performed on the olfactory bulbs of some 5-HT depleted pups confirmed 5-HT fiber depletion.

4.3.1.3 cAMP assay

Olfactory bulb samples were homogenized in 300 µl distilled water containing 4 mM EDTA. The homogenate was heated for 5 min in a boiling water bath to coagulate the protein, then centrifuged at 10,000 rpm for 5 min at 4°C. After centrifugation, the supernatant was removed and placed in a microcentrifuge tube. The pellet was kept for protein assay. cAMP in the supernatant was assayed using a radiolabeled cyclic AMP (^{3}H) assay kit (Amersham, Baie d'Urfé, PQ). The protein pellet was reconstituted by 500 µl of dH_2O. The protein content of the samples was determined by a BCA protein assay kit (Pierce, Rockford, IN). cAMP content is presented as pmole/mg protein.

4.3.2 Results

cAMP expression in the olfactory bulb is increased by effective odor preference training protocols (Fig.4.4A). It is also increased by protocols that do not produce odor preference learning. The groups receiving 2 mg/kg isoproterenol and 4 mg/kg isoproterenol paired with odor had significantly more cAMP than the odor only control group (Repeated measures ANOVA F_3=3.20, $p<.05$; least significant difference tests, $p<.05$). The 1 mg/kg isoproterenol group was intermediate. From the histogram (Fig.4.4A) it also appears that isoproterenol increases cAMP in a dose-related manner.

Pups receiving stroking paired with odor also had significantly more cAMP than the naive control group ($p<.01$, paired $t_{1\text{-tailed}}$-test). Pups with stroking alone had the same mean cAMP levels as those with odor pairing and were also different from the naive control group ($p<.05$, paired $t_{1\text{-tailed}}$-test). The mean cAMP levels of the odor only pups also did not differ from that of naive pups. This indicates that stroking, acting as the unconditioned stimulus, is sufficient to activate the cAMP cascade, while the conditioning stimulus (peppermint odor) appears to have no further influence on the level of cAMP expression during odor preference learning.

In 5-HT depleted olfactory bulbs, the level of cAMP was significantly ($p<.05$, $t_{2\text{-tailed}}$-test) reduced compared to non-depleted sides in both 2 mg/kg and 6 mg/kg

isoproterenol groups (Fig.4.4B). However, the dose-dependent profile of cAMP increase following isoproterenol injection was maintained. This indicates that 5-HT and norepinephrine act synergistically to activate cAMP during odor preference learning.

4.4 Experiment 3A and 3B cAMP immunocytochemistry following unilateral 5-HT depletion and isoproterenol injection

4.4.1 Materials and methods

4.4.1.1 Animal preparation

Twelve Sprague-Dawley rat pups of both sexes from 4 litters were used in this experiment. All pups were given 5-HT depletions of left olfactory bulbs on PND1 as described in Experiment 2. On PND6, in Experiment 3A, seven pups were subjected to 2 mg/kg isoproterenol injections (s.c.) 40 min before being placed on peppermint-scented bedding for 10 min. The pairing of 2 mg/kg isoproterenol and odor on PND6 normally induces odor preference in pups (Sullivan et al., 1989b; Langdon et al., 1997). Immediately after odor exposure, pups were sacrificed by decapitation. The brains were removed from the skulls, fixed in ice-cold 4% paraformaldehyde in 0.1M phosphate buffer for one hour, then kept overnight in 20% sucrose and 1.5% paraformaldehyde in 0.1M phosphate butter. The next day, the brains were transferred to a 20% sucrose

solution for one hour and then cut frozen at 30 μm using a cryostat. In Experiment 3B, 5 pups were sacrificed directly on PND6, to test whether unilateral 5-HT depletion itself affects the basal level of cAMP in the olfactory bulb. Immediately after sacrifice, the brains were removed from the skull, and processed as described in Experiment 3A.

4.4.1.2 Immunocytochemistry

Olfactory bulb sections were thawed onto subbed slides and processed using immunocytochemistry for cAMP and 5-HT (to confirm 5-HT depletions of the left olfactory bulbs). The cryostat-mounted sections were air-dried at room temperature for 5-10 min followed by 1 hr incubation in 2% NGS, 0.2% TritonX-100 in PBS at room temperature to block non-specific binding. Sections were incubated in the primary antibody (cAMP, Chemicon, Mississauga, ON, diluted 1:500; 1:1000; 1:3000; 1:5000 ; 5-HT, INCStar, Stillwater, MN, diluted 1:3000) in 0.2% Tx-100, 2% NGS in PBS at 4°C overnight followed by standard immunocytochemical methods.

4.4.1.3 Image processing and analysis

The 5-HT depletions were confirmed under bright-field microscopy. Consistent with previous results (McLean and Darby-King, 1994), it produced more than 80% depletion of the 5-HT fibers of the left olfactory bulb as shown by immunocytochemistry.

For cAMP immunocytochemistry, the relative amount of cAMP expression was quantified systematically by comparing the optical density (darkness) of label in the mitral cell layer from both bulbs. In each bulb, five sections at even intervals through the entire olfactory bulb were examined.

Image analysis was performed by tracing the medial region of the mitral cell layer and an adjacent background region in the internal plexiform layer. Relative optical density was achieved by determining the difference of optical density between the region of interest and the background region divided by the optical density of the background region. All the slides from Experiment 3A and 3B were coded so that the person analysing sections was blind to the treatment. The regional optical densities from both olfactory bulbs were compared statistically using the paired Student t-test.

4.4.2 Results

Consistent with β_1-adrenoceptor and 5-HT_{2A} immunocytochemical localization, strong cAMP immunocytochemical staining was observed mainly in the mitral cells (Fig.4.5) and tufted cells. Only a few periglomerular cells and granule cells were stained. This suggests that NE action through the β_1-adrenoceptor observed here increases cAMP mainly in the output cells of the olfactory bulb. To support our hypothesis that NE and 5-HT act synergistically to enhance cAMP signaling in the CREB phosphorylation pathway,

quantitative analysis of the relative optical density of the medial regions of mitral cell layers was performed on both the 5-HT depleted bulbs and the control sides. Figure 4.6A shows that after the pairing of isoproterenol and odor exposure, in 5-HT depleted olfactory bulbs there was significantly ($p<.01$, paired $t_{2\text{-tailed}}$-test, $n=7$) less cAMP staining in the mitral cell layer (reflected by relative optical density in medial regions) compared to that in the control side of the same animals. To determine if unilateral 5-HT depletion itself reduces the basal level of cAMP expression in mitral cells, cAMP immunocytochemistry was also performed on the olfactory bulbs of non-isoproterenol injected pups. Fig.4.6B shows that there is no significant difference in the relative optical density of cAMP immunocytochemical staining in mitral cells in the 5-HT depleted sides and the control sides. Comparison were only made within animals to avoid variability due to differences in the overall immunocytochemical reaction.

Therefore, in the present data set 5-HT depletion does not by itself reduce the basal level of cAMP expression, but it impaires the ability of isoproterenol to enhance cAMP signaling. This is consistent with our hypothesis that β-adrenoceptors and 5-HT_2 receptors are critical in early odor preference learning and interact via a synergistic promotion of cAMP in mitral cells of the olfactory bulb.

4.5 Discussion

The major findings of this study are that β_1-adrenoceptors and 5-HT_{2A} receptors are localized on mitral cells in the olfactory bulb and that interaction of these receptors affects cAMP processing in mitral cells. cAMP expression is not directly affected by the loss of serotonin but its up-regulation by either tactile stimulation or by stimulation of β_1-adrenoceptors in the rat pup is impaired. Below we discuss the potential relevance of such interactions for early olfactory preference learning.

4.5.1 Cellular localization of the β_1-adrenoceptor and the 5-HT_{2A} receptor

Localization of 5-HT_{2A} receptor protein (Hamada et al., 1998; Cornea-Hébert et al., 1999) and mRNA (McLean et al., 1995) has been previously shown in mitral and tufted cells. Our result using immunofluorescence to label the 5-HT_{2A} receptor is consistent with these previous studies.

With the increased sensitivity of the present heat-induced antigen retrieval method for immunocytochemistry, we are the first to report substantial neuronal localization of the β_1-adrenoceptors in the olfactory bulb output cells. The finding that the receptor is localized primarily in the output cells of the bulb (mitral and tufted) and co-localized with 5-HT_{2A} receptors is consistent with the demonstrated functional interaction of these

receptors in the olfactory bulb of the neonate rat and supports the present model of critical neural substrates.

Our finding that the two receptor subtypes remain co-localized in older animals implies possible functional 5-HT/NE interactions in adult rat olfactory bulb that merit further investigation.

β_1-adrenoceptor localization in the olfactory bulb of rat has been briefly described in two survey papers (Wanaka et al., 1989; Nicholas et al., 1993). Both papers suggested weak localization of β_1-adrenoceptors (in situ hybridization, Nicholas et al., 1993) or β-adrenoceptors (immunoctyochemistry, Wanaka et al., 1989) in the granule cell layer of the bulb as also seen in a small subset of cells in the present study. A developmental binding study targeted to the olfactory bulb also suggested most β_1-adrenoceptors binding occurred in layers other than the mitral cell layer and increased developmentally (Woo and Leon, 1995). In a later study the same authors reported locus coeruleus lesions increased β-adrenoceptors density in the glomerular layer (Woo et al., 1996), suggesting this region might be most responsive to locus coeruleus input. Such receptors could be on the dendrites of mitral cells projecting to the glomerular layer.

The reasons for the failure of the binding studies to identify mitral cells as important sites of β_1-adrenoceptors are unclear. As noted by Woo and Leon (1995),

iodopindolol is biased toward the detection of β_2-adrenoceptors, thus even examining radiolabeling in the presence of a β_2-adrenoceptor antagonist may not be sufficient to eliminate some binding of β_2-adrenoceptors, which are numerous in the olfactory bulb. The profiles of the two receptors seen in the binding studies were identical except for additional external plexiform labeling for β_2-adrenoceptors. Mitral cells also have prominent internalized β_1-adrenoceptor labeling in the present study. Internalized receptors have low binding affinity (Flugge et al., 1997) and their demonstration may depend critically on ligand concentration. Finally, Leon's laboratory, in a brief report (Ivins et al., 1993) using *in situ* hybridization methodology, identified β_1-adrenoceptor mRNA in mitral cells, consistent with the present observations.

Isoproterenol interacts with β_2, as well as β_1-adrenoceptors, in the olfactory bulb. The present study does not rule out a role for the β_2-adrenoceptors in olfactory preference learning. *In situ* hybridization study demonstrated that β_2-adrenoceptors are more widely expressed than β_1-adrenoceptors in the olfactory bulb, including the mitral cell layer (Nicholas et al., 1993), and can up-regulate cAMP. Their specific contribution to early olfactory learning remains to be identified.

4.5.2 Functional significance of cAMP activation via β_1-adrenoceptors and 5-HT_{2A} receptors in output cells of the olfactory bulb

cAMP increases accompanied both stroking and 2 mg/kg isoproterenol, the two effective UCSs, as predicted by the present hypothesis. However, odor+stroking did not produce increases greater than those of stroking alone, although the odor+stroking group was less variable. In contrast to the present failure to detect an increase in cAMP with odor pairing, the studies of pCREB using Western blots showed higher levels in the odor+stroking than the stroking only condition (McLean et al., 1999). With 10% peppermint as the odorant, cells involved in the odor representation appear widespread and were sufficient to demonstrate the pCREB effect. The pCREB result may argue that the failure to see pairing associated cAMP change is real. If cAMP is not increased by odor pairing, it suggests that, unlike the Aplysia model in which sensory input and a monoaminergic input converge on adenylyl cyclase activation, in the rat pup olfactory bulb the sensory input influences learning by convergence on the pCREB pathway (see Fig.4.7).

The hypothesis of receptor synergism in cAMP recruitment was tested in Experiment 2. As mentioned, 5-HT depletion prevents early olfactory preference learning produced by pairing odor with the normally optimal 2 mg/kg dose of the β-adrenoceptor agonist, isoproterenol. A higher, 4 mg/kg or 6 mg/kg dose of the β-adrenoceptors, can,

however, overcome the depletion effect and produce learning (Langdon et al., 1997; Yuan et al., 2000b). The higher 4 mg/kg dose of isoproterenol is normally ineffective as a UCS in normal pups (Langdon et al., 1997). A dose of 6 mg/kg has also been shown to be ineffective in learning and in producing CREB phosphorylation in normal pups (Yuan et al., 2000b). Thus, isoproterenol-induced learning and CREB phosphorylation show parallel inverted U curve profiles with increasing doses of isoproterenol. A similar inverted U curve profile has been described for stroking-induced learning (Sullivan et al., 1991), suggesting it is a basic property of the learning system.

The dose-dependent increase in cAMP with increasing isoproterenol did not support the initial hypothesis that biphasic agonist control of cAMP explains the inverted U curve seen behaviorally, electrophysiologically and biochemically. Although biphasic cAMP control of behavior has been reported in other models (Ozacmak et al., 2002), the present data suggest instead that there is an optimal level of cAMP activation which can be exceeded. In the present model three possibilities suggest themselves: (a) higher levels of cAMP recruit increased calcium entry which might favor calcineurin-induced dephosphorylation; (b) higher levels of cAMP promote greater phosphodiesterase 4 (PDE4) activation through PKA (Ang and Antoni, 2002) and this may critically shorten the duration of the cAMP signal; and (c) elevated cAMP promotes faster cAMP extrusion (Wiemer et al., 1982) which again would shorten the signal duration. CREB phosphorylation has been shown to be enhanced by longer durations of cAMP signaling

(Barad et al., 1998). Manipulation of calcineurin and measurements of the time course of cAMP elevation with varying doses of isoproterenol would test these hypotheses.

cAMP-regulated signaling pathways and the associated phosphorylation of CREB have been postulated as important mechanisms underlying learning and memory (Davis et al., 1995; Abel et al., 1997; Impey et al., 1998b; Mons et al., 1999; Wong et al., 1999). Aversive olfactory learning in the Drosophila depends critically on this cascade (Zhong et al., 1992; Davis et al., 1995; Mons et al., 1999) and has several parallels with the rat pup model of olfactory learning. Both cAMP increases and CREB phosphorylation are thought to mediate the acquisition of odor aversion in Drosophila. Although cAMP levels in Drosophila have not been measured directly with training as in the present study, systematic manipulations of the components of the cAMP cascade produce predictable deficits in olfactory learning and memory (Zhong et al., 1992; Davis et al., 1995). In addition, in the dunce mutants with reduced phosphodiesterase, cAMP levels are increased above normal and olfactory learning and memory are deficient (Byers et al., 1981). As in the present study an impairment of the normal temporal dynamic of cAMP has been suggested to underlie the impairment in olfactory learning in Drosophila mutants with higher levels of cAMP.

4.5.3 A new model of noradrenergic-mediated early olfactory preference learning in the rat pup

In the present study, we demonstrate that mitral cells, the main output cells in the olfactory bulb, are the postsynaptic cellular substrate for olfactory preference learning. Immunocytochemistry demonstrated the co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors in mitral cells. Manipulation of β-adrenoceptor and 5-HT receptor activation affected cAMP levels in mitral cells in a predictable pattern. This profile of results supports the hypothesis that the β_1-adrenoceptors and $5HT_{2A/2C}$ receptors, interact in early odor preference learning via a synergistic promotion of cAMP in mitral cells in the olfactory bulb, as they do in neocortex (Morin et al., 1992), and that the critical learning change occurs in the mitral cell processing of olfactory nerve input.

This new model of olfactory preference learning mediated by β_1-adrenoceptor activation is illustrated at the circuit and intracellular level in Figure 4.7. This model accounts for several aspects of what we have seen.

Isoproterenol paired at a learning effective dose with olfactory nerve input will potentiate the olfactory nerve EPSP including both NMDA and non-NMDA components (Yuan et al., 2000b). Such potentiation may be mediated by cAMP-initiated phosphorylation of NMDA and AMPA channels. Phosphorylation of L-Ca^{2+} channels

could also contribute to membrane depolarization and potentiation of NMDA currents. Closing of K^+ channels following isoproterenol which has been reported in other systems (Karle et al., 2002) is another mechanism by which depolarization might occur. The failure of higher doses of isoproterenol to produce the electrophysiological potentiation would again be related to an excess of dephosphorylation activity or a shortening of the elevation of cAMP.

Either odor-stroking pairings or odor-isoproterenol pairings also produce CREB phosphorylation (McLean et al., 1999; Yuan et al., 2000b). For this component an interaction of the cAMP cascade and Ca^{2+}/ calmodulin cascade is suggested to occur. In particular Ca^{2+}/calmodulin activates calmodulin kinases while PKA prevents phosphatase activation resulting in a net phosphorylation of CREB (Impey et al., 1998a; Wong et al., 1999). PKA may also enhance activity of the MAPK pathway (Impey et al., 1998a; Poser and Storm, 2001), but we have not yet characterized the role of this pathway in early olfactory learning. Again excessive activation of the cAMP pathway that fails to activate CREB phosphorylation may truncate the duration of the cAMP signal or have phosphatase promoting consequences.

The approach behavior of rat pups 24 hr following odor-UCS pairing is thought to be linked to these initiating events. Optical imaging experiments in trained pups suggest increased activation in the olfactory bulb to the conditioned odor, but not to a control

odor, 24 hr after training (Yuan et al., 2002). It is clear that pairing odor with local infusion of isoproterenol in the rat pup is sufficient to produce the approach response (Sullivan et al., 2000b). It appears that a change in the representation of the odor in the olfactory bulb determines the response. However, we do not even know why some odors are inherently attractive. A study of the bulbar representation of such odors might be helpful. Our data suggests the odor representation potentiates, both NMDA and AMPA components of olfactory nerve input are strengthened during acquisition procedures (Yuan et al., 2000b), while activation probed by optical imaging is stronger 24 hr later (Yuan et al., 2000). How this increased activation is coupled to approach behavior is unknown.

The present model with its emphasis on the role of the β_1-adrenoceptor on mitral cells does not rule out a role for disinhibition in normal learning. β-adrenoceptor mediated disinhibition (Wilson and Leon, 1988; Okutani et al., 1998) has been reported in pups and adults in the olfactory bulb and might occur via the β_2-adrenoceptor on granule cells (Nicholas et al., 1993). Other α-adrenoceptor-mediated disinhibitory effects have been documented (see introduction). Such disinhibition would further support the β_1-adrenoceptor mechanisms identified here.

Finally, the present model is closely related to the learning model well-described for Aplysia. Indeed, Kandel et al. originally suggested that norepinephrine in mammals

might play the role of serotonin in the Aplysia (Brunelli et al., 1976). The present model follows that suggestion. A key difference between the two models would appear to be in the co-incidence detection mechanism. In Aplysia adenylyl cyclase itself is the co-incidence detector for the CS and UCS and higher levels of cAMP determine the occurrence of learning. In the present model high levels of cAMP alone do not produce learning and the CS pathway appears to interact with the UCS pathway at a later stage.

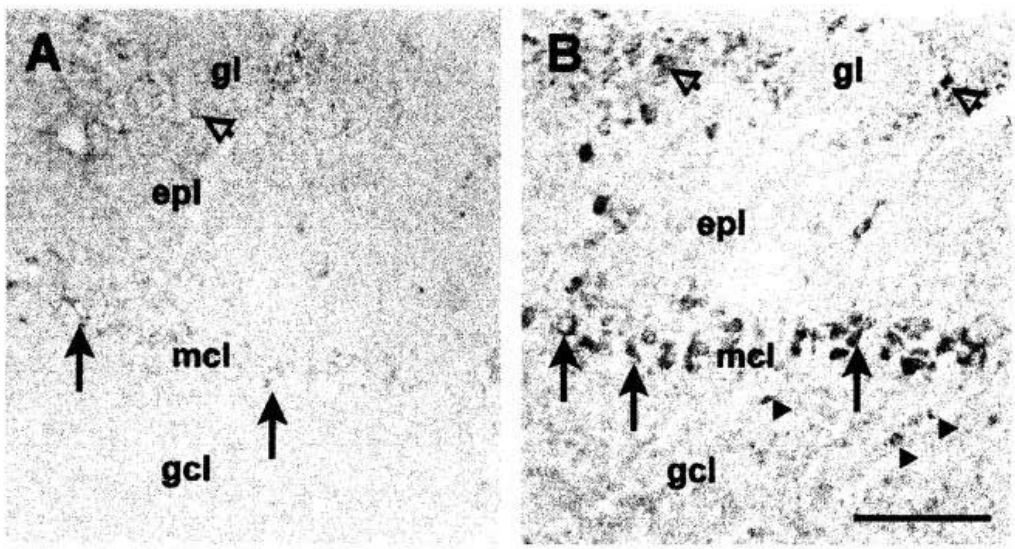

Figure 4.1 Localization of the β_1-adrenoceptor in the olfactory bulb by immunocytochemistry.
A: Visualization of the receptor without the use of microwave heating.
B: Visualization of the receptor after the use of microwave heating.
Note the faint label of mitral cells in A and the clear labeling of mitral and tufted cells and a small number of granule and periglomerular cells in B.
Abbreviations, epl, external plexiform layer; gcl, granule cell layer; gl, glomerular layer; mcl, mitral cell layer; . Bars, 100 μm.

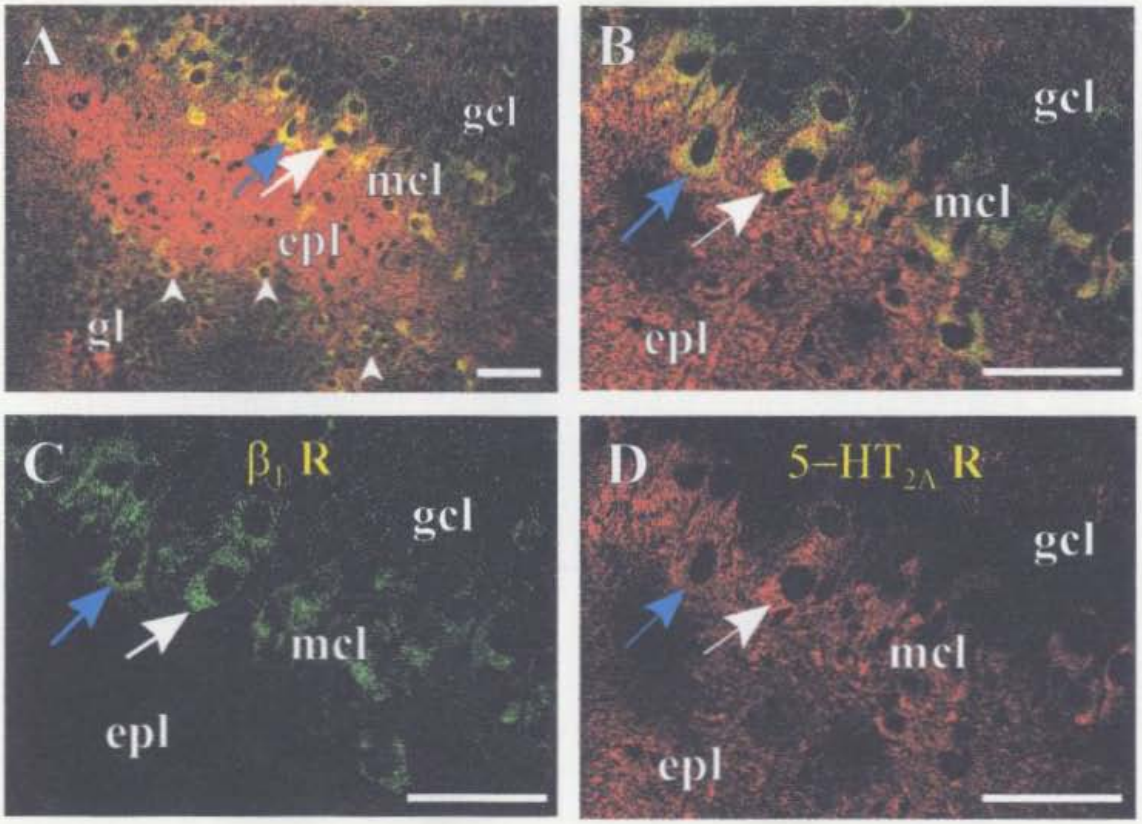

Figure 4.2 Confocal images of the olfactory bulb from a PND 10 pup.
Note the double labeled mitral cells (eg. at white and blue arrows) at low (A) and higher magnification (B-D). A&B show the combined label of β_1 & 5-HT_{2A} receptors. Double label was also observed in tufted cells (arrowheads) near the glomerular layer (A). Bars, 50 μm.

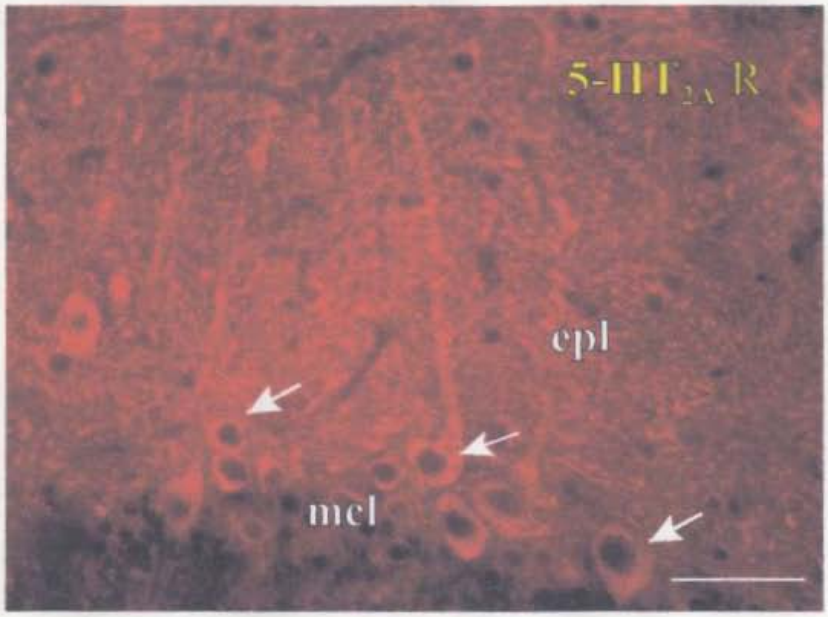

Figure 4.3 Immunofluoresence label of mitral cells in a PND35 rat using an antibody to the $5\text{-}HT_{2A}$ receptor. Note the mitral cell body (arrows) and dendritic label. Bar, 50 μm.

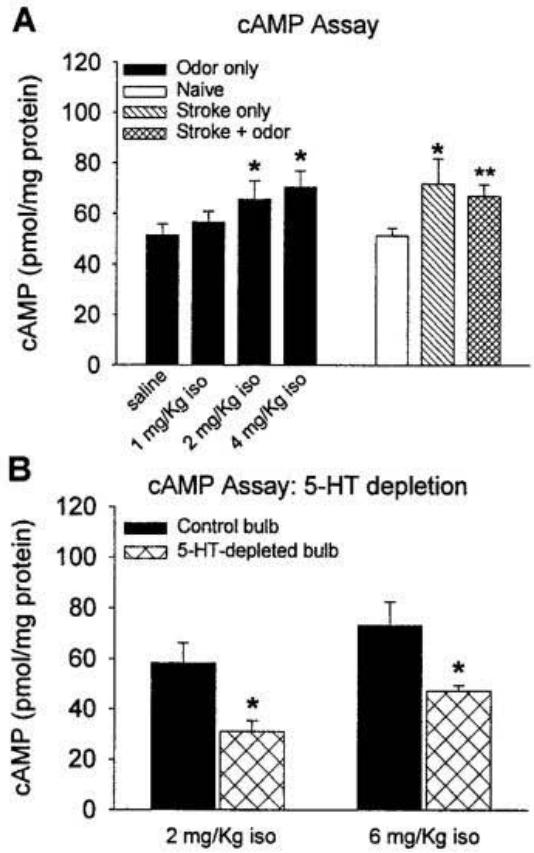

Figure 4.4 cAMP expression in the olfactory bulb of PND 6 pups immediately after various training sessions.

(A). An increase in cAMP expression is observed with increasing β-adrenoceptor activation (isoproterenol) compared to saline-injected controls exposed to odor only at the time of training (N=9 *, $p<0.05$ compared to the saline group). The act of stroking the pup appears to activate the cAMP to levels equivalent to pups given stroking plus odor (N=9 *, $p<0.05$; **, $p<0.01$). (B). 5-HT depletion reduces the isoproterenol induced cAMP expression which can be partially overcome by inducing more activation of β-adrenoceptors (via isoproterenol). *, $p<0.05$; 5-HT depleted bulb compared to normal control bulb. N=4 for 6 mg/kg iso control group, N= 5 for other three groups. Abbreviation: iso, isoproterenol.

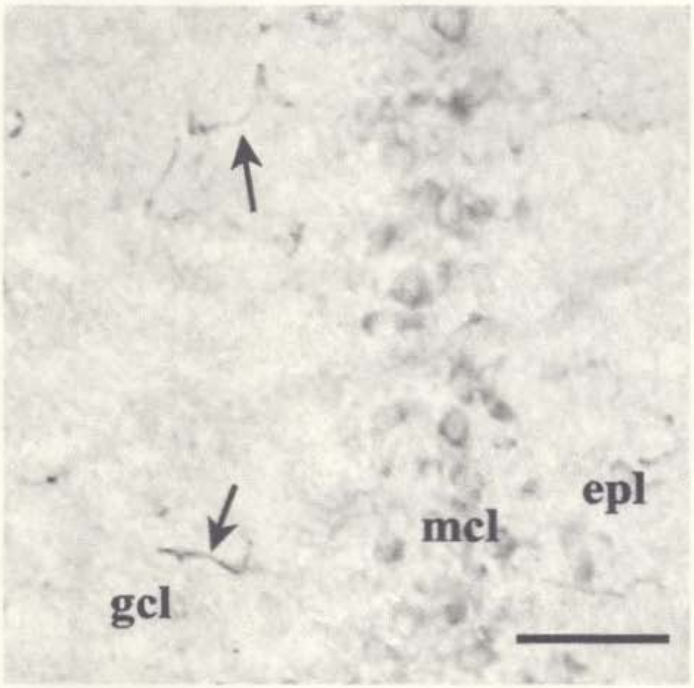

Figure 4.5 cAMP immunocytochemistry shows the cellular location of cAMP in the olfactory bulb. The immunocytochemical methods employed here showed selective cAMP expression in mitral cells of the mcl. Arrows indicate artifact labeling of blood vessels. Abbreviations. epl, external plexiform layer; gcl, granule cell layer; mcl, mitral cell layer. Bar, 50 μm

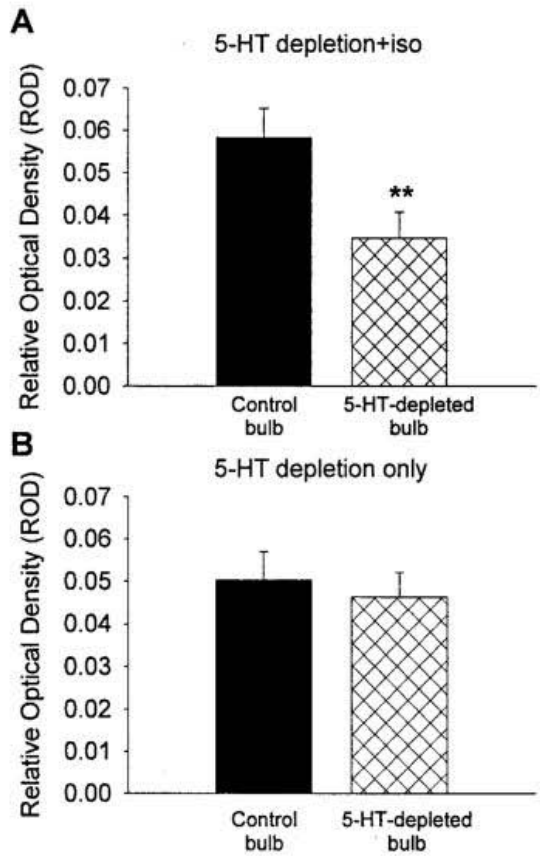

Figure 4.6 Relative optical density (ROD) measures of cAMP showing the influence of isoproterenol (iso) and/or unilateral bulbar 5-HT depletion on cAMP expression in the mitral cell layer.

(A). ROD of cAMP immunocytochemical staining in the 5-HT depleted olfactory bulbs and the control bulbs of the same animals after pairing of 2 mg/kg isoproterenol injections with odor exposure. Significantly less cAMP level is seen in 5-HT depleted olfactory bulbs compared to the control sides ($N=7$, $p<0.01$).

(B). ROD of cAMP immunocytochemical staining in the 5-HT depleted olfactory bulbs and the control bulbs of the same animals without isoproterenol injections or odor exposure. Note that there is no difference between these two groups ($N=5$, $p>0.05$) which suggests that 5-HT depletion by itself does not affect cAMP levels.

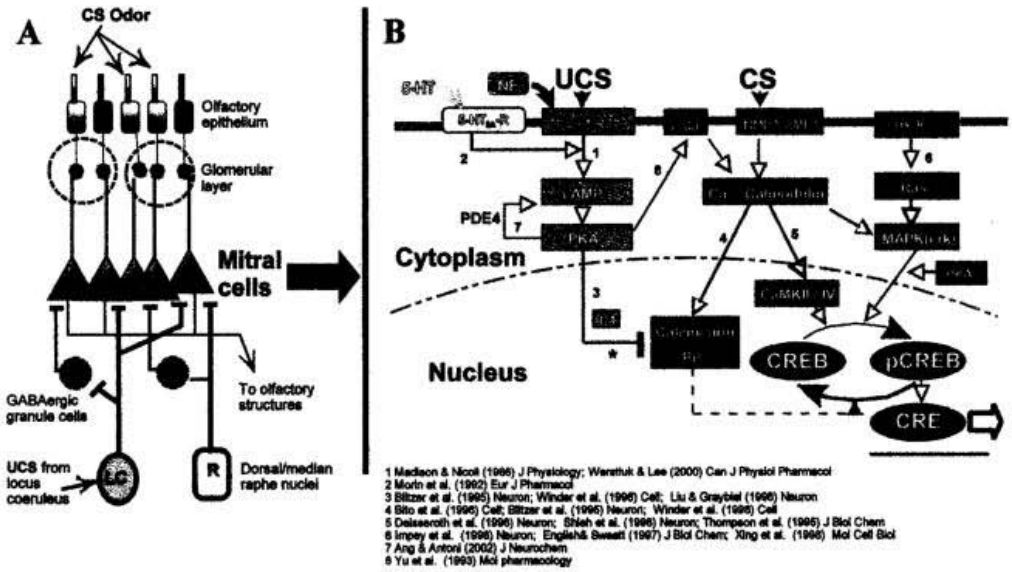

Figure 4.7 Proposed intercellular and intracellular pathways in the olfactory bulb activated by β_1 and 5-HT_{2A} receptors.

(A). Schematic diagram indicating the major circuitry in the olfactory bulb especially as it relates to the present odor learning model and identifying the convergence of odor input (via olfactory receptor cells), and noradrenergic and serotonergic input onto mitral cells.

(B). Intracellular circuitry of the mitral cell. β_1-adrenoceptors mediate the unconditioned stimulus (UCS) via either tactile stimulation or a β-adrenoceptor agonist. The conditioned stimulus (CS) is provided by odors which stimulate glutamate receptors on mitral cells. When proper stimulation occurs the pathways induce phosphorylation of CREB and learning. ★cAMP/PKA "gating" of the calcineurin dephosphorylation pathway of CREB (see discussion for further details of the pathways).

Chapter 5 Early Odor Preference Learning in the Rat: Bidirectional Effects of CREB and Mutant CREB Support a Causal Role for pCREB

(submitted to J.Neuroscience., 2002)

5.1 Introduction

Early odor preference learning offers a unique paradigm for the study of natural mammalian learning. In the neonate rat, neural circuitry changes that are critical for odor preference memory occur in the olfactory bulb (Wilson and Sullivan, 1994; Sullivan et al., 2000b). A change in the first synapse, the olfactory input to mitral cell connection, appears to underlie both the acquisition and expression of odor preference (Wilson et al., 1987; Woo et al., 1987; Wilson and Leon, 1988; Johnson et al., 1995; Woo et al., 1996; Yuan et al., 2000b; Yuan et al., 2000). Phosphorylation of cAMP response element binding protein (CREB), proposed as a universal "memory molecule" (Silva et al., 1998), is seen in mitral cells following olfactory preference training, but not in control conditions (McLean et al., 1999). The present study asks whether phosphorylation of CREB in the olfactory bulb can be shown to be causal in early olfactory preference learning.

In neonate rats, a single 10 min session of tactile stimulation such as stroking

(unconditioned stimulus, UCS) paired with an odor (conditioned stimulus, CS), typically peppermint, produces odor preference learning, seen as an approach to the odor 24 hr later (Sullivan and Leon, 1987; Wilson and Sullivan, 1994). Norepinephrine (NE) released from the locus coeruleus with tactile stimulation is known to act in the olfactory bulb via β-adrenoceptors (Wilson and Sullivan, 1994; Langdon et al., 1997; Yuan et al., 2000b) coupled to cAMP. The β-adrenoceptor agonist, isoproterenol, can substitute for stroking when given systemically, or directly into the olfactory bulb, to induce learning (Petralia et al., 1996; Langdon et al., 1997; Yuan et al., 2000b). NE and serotonin (5-HT) in the olfactory bulb have been shown to interact in early odor preference learning to promote increases in the cAMP-mediated CREB phosphorylation (Yuan et al., 2000b). pCREB increases are transient and occur selectively in the peppermint-encoding area of the mitral cell layer in the olfactory bulb following peppermint conditioning (McLean et al., 1999). Both learning and increases in pCREB also occur when odor is paired with a moderate dose of isoproterenol (2 mg/kg), but not with lower (1 mg/kg) or higher (4 or 6 mg/kg) doses of isoproterenol (Sullivan et al., 1989b; Sullivan et al., 1991; Langdon et al., 1997; Yuan et al., 2000b). The parallel inverted-U curve profiles in both isoproterenol-induced learning and CREB phosphorylation are shifted to the right following depletion of 5-HT in the olfactory bulb (Yuan et al., 2000b), such that a higher dose (6 mg/kg) of isoproterenol is now required for the induction of learning and the increase in pCREB.

Based on these studies, we proposed a causal role for CREB in neonate rat odor preference learning; but the evidence was correlational (McLean et al., 1999; Yuan et al., 2000b). Here we evaluate causality using a herpes simplex virus, HSV, to express additional CREB or dominant negative mutant CREB (single point mutation at the phosphorylation site Ser133) in neurons of the rat pup olfactory bulb. HSV-LacZ expressing E.coli β-galatosidase, was used to verify the expression of HSV-encoded proteins in the olfactory bulb, and as a control to determine whether virus injection itself would affect odor preference learning.

In the present study we ask whether additional CREB or mutant CREB in the olfactory bulb will alter normal odor preference learning and/or promote the occurrence of odor preference learning when sub- or supra-optimal doses of isoproterenol are given. We also measure the levels of pCREB to assess the link between the substrate and the transcription factor.

5.2 Material and methods

5.2.1 Animals

Sprague-Dawley rat pups of both sexes were used in this study. Litters were culled to 12 pups/litter on postnatal day 1 (PND1, the day of birth is considered PND0). The

dams were maintained under a 12hr light-dark cycle, with *ad libitum* access to food and water. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

5.2.2 Virus vector

HSV-LacZ, HSV-CREB (overexpression of CREB), and HSV-mCREB (overexpression of a dominant negative mutant CREB) were used in this study. The average titer of the recombinant virus stocks was $4.0x10^7$ infectious unit/ml (for viral vector preparation see Cattoretti et al., 1993; Carlezon, Jr. et al., 1998; Neve and Geller, 1999).

5.2.3 Virus injection

On PND4, rat pups were anaesthetized under hypothermia on ice and placed in a stereotaxic frame. The skull over the central region of each olfactory bulb was carefully removed by a dental drill. A total of 1µl virus stock/bulb was injected at four levels into each bulb over a 5 min period with a 27 gauge Hamilton syringe. Pups were then warmed up and returned to the dam after recovery.

5.2.4 Odor conditioning

The procedure for odor conditioning for natural learning has been described before (Sullivan et al., 1989b; Sullivan et al., 1991; McLean et al., 1993). Briefly, on PND6, rat pups were removed from the dam and put on fresh bedding 10 min before odor exposure. In the odor+stroking (O/S) group, pups were placed on peppermint scented bedding (0.3 ml peppermint/500 ml normal bedding) and stroked vigorously on the hind region using a sable brush every other 30 sec for 30 sec over a 10 min period. In the odor only (O/O) group, the pups were only exposed to the peppermint bedding without being stroked. The naive pups were placed on fresh bedding for a 10 min period. Immediately after these conditions, the pups were returned to the dams.

The procedure for odor conditioning using isoproterenol (1 mg/kg, 2 mg/kg or 4 mg/kg) has been described before (Langdon et al., 1997; Yuan et al., 2000b). Briefly, on PND6, saline or isoproterenol was injected subcutaneously into pups 40 min before exposed to the peppermint odor. The pup was removed from the dam 30 min after injection and placed on fresh bedding. Ten min later, The pup was placed on peppermint-scented bedding for 10 min. After odor exposure, The pup was returned to the dam.

5.2.5 Odor preference test

On PND7, pups were subjected to odor preference testing. A stainless steel test box (30x20x18cm) was placed on two boxes which were separated by a 2 cm neutral zone. One box contained fresh bedding; the other contained peppermint scented bedding. Each pup was removed from the dam and placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint scented bedding or normal bedding was recorded for five 1-min trials. The percentage of time the pup spent on peppermint scented bedding over the 5 min period was calculated. One-way ANOVAs were used for analysis.

5.2.6 X-gal histochemistry

LacZ expression in the olfactory bulb was revealed by visualizing its substrate β-galactosidase activity using X-gal histochemistry. Pups were given an overdose of sodium pentobarbital (80 mg/kg) and perfused transcardially with an ice-cold saline solution followed by a fixative solution (0.5% paraformaldehyde + 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4). Brains were removed from the skull, postfixed in the same solution for 1hr and transferred to a 30% sucrose solution overnight.

Coronal sections (40μm) were cut in a cryostat the next day. Sections were

mounted onto slides and air dried at room temperature. Alternate sections were collected for X-gal and Nissl staining. Slides containing olfactory bulb sections for X-gal staining were then incubated overnight with a solution containing 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 0.15 M NaCl, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, and 0.2 mg/ml X-gal (dissolved in N,N'-dimethyl formamide) in 10 mM phosphate buffer (pH7.4). An insoluble blue color indicated β-galactosidase activity. After a brief rinse in PBS, all slides were dehydrated and coverslipped with Permount (Sigma). Possible cytoarchitectural damage due to virus injections was investigated in Nissl stained sections.

5.2.7 Nuclear cell extract and CREB/pCREB assay

Pups used for the CREB/pCREB assay were anaesthetized with CO_2 and sacrificed by decapitation. Both olfactory bulbs were collected immediately on dry ice and stored in microcentrifuge tubes at -70°C. Olfactory bulb tissue was homogenized using 100 µL/sample of Buffer A containing 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithoithreitol (DTT), 1 mM PMSF, and 0.1 % NP-40. The samples were incubated on ice for 15 min, then centrifuged at 1,000x g at 4°C for 10 min. The supernatant was discarded. The pellet was resuspended in 500 µL of Buffer A without NP-40. Again, the samples were centrifuged at 1000x g for 10 min and the supernatant was discarded. The pellet was resuspended in 100 µL of TransAm lysis buffer (Active

Motif) containing DTT and a protease inhibitor cocktail. The samples were rocked at 4°C for 30 min, then centrifuged for 10 min at 14,000x g at 4°C in a microcentrifuge. The supernatant (nuclear extract) was collected. Protein determination was performed by a bicinchoninic acid (BCA) protein assay kit (Pierce).

CREB/pCREB protein content was determined using CREB/pCREB assays (Active Motif) according to manufacturer's instructions. A total of 10 μg protein was loaded into each well. CREB/pCREB was visualized and quantified by a colorimetric reaction and read by a spectrophotometer at 450nm. 2.5 μg forskolin-stimulated WI-38 cell extract was used as a positive control. The optical densities of the CREB/pCREB per mg protein were compared by a paired Student t-test between the two groups in each experimental condition.

5.2.8 Experimental procedures

5.2.8.1 Expression of HSV-LacZ in the olfactory bulb and its effect on odor preference learning

To determine whether transgenes are expressed in olfactory bulb neurons, and whether virus injection itself affects olfactory preference learning, forty-three rat pups of both sexes from eight litters were divided into 6 groups: 2 injection conditions (HSV-

LacZ & saline) X 3 training conditions (Odor+stroke, O/S; Odor only, O/O; Naive). X-gal histochemistry and Nissl staining were performed on the olfactory bulbs of HSV-LacZ injected pups after they were tested for odor preference. One-way ANOVAs were used to compare different training groups in the two injection conditions after the odor preference testing.

5.2.8.2 The causality of CREB in natural odor preference learning

Eighty-seven pups from 10 litters were divided into nine groups: 3 injection conditions (HSV-CREB, HSV-mCREB, HSV-lacZ) X 3 training conditions (O/S, O/O, naive). In each litter, no more than one pup was assigned to each group. Odor preference learning and testing were performed as described above. One-way ANOVAs were used to compare the performance of the three training groups in different injection conditions.

To test for the expected increase of CREB expression in the olfactory bulb at the time of learning following the viral injection, eighteen pups from three litters were injected bilaterally into olfactory bulbs with either HSV-CREB or HSV-LacZ on PND 4. On PND 6, the pups were sacrificed by decapitation. Both olfactory bulbs were collected in dry ice and stored in microcentrifuge tubes at -70°C until a CREB assay was performed.

To test if increased CREB substrate results in an enhanced pCREB expression following conditioning, further delineating the effects of CREB/pCREB levels on odor preference learning, a pCREB assay was performed on the olfactory bulbs of the rats from the O/S groups injected with either HSV-LacZ or HSV-CREB. Eighteen pups from three litters were used in this experiment. Previous work showed that pCREB increases maximally at 10 min after odor conditioning (McLean et al., 1999). Therefore, 10 min after being taken away from the peppermint bedding, the pups were sacrificed by decapitation. Both olfactory bulbs were collected for a pCREB assay.

5.2.8.3 The effects of CREB levels on isoproterenol-induced odor preference learning

Experiments were carried out to determine if additional wild type CREB (by HSV-CREB injection) or dominant negative CREB that could not be phosphorylated at the serine 133 site (by HSV-mCREB injection) changes the sensitivity of the system to the unconditioned stimulus, therefore shifting the isoproterenol effective inverted U-curve.

Ninety pups from eight litters were used. Twelve groups were created in this experiment: 3 virus injection conditions (HSV-lacZ, HSV-CREB, HSV-mCREB) X 4 drug/saline injection conditions (saline, 1 mg/kg isoproterenol, 2 mg/kg isoproterenol, and

4 mg/kg isoproterenol). No more than one pup from the same litter was assigned to the same group.

One way ANOVAs were used to compare the learning results from the training groups in different virus injection conditions. Subsequently, the learning effective groups (HSV-CREB+1 mg/kg isoproterenol and HSV-mCREB+4 mg/kg isoproterenol groups) were compared to their non-learning control groups (HSV-LacZ+1 mg/kg isoproterenol and HSV-LacZ+4 mg/kg isoproterenol groups) by a student t-test.

CREB phosphorylation has been proposed as a critical step in the acquisition of long-term memory (McLean et al., 1999). pCREB assays were performed on the olfactory bulbs of the rats from the groups that exhibited learning in the first set of the experiment: the 1 mg/kg HSV-CREB group, the 4 mg/kg HSV-mCREB group and their corresponding non-learning control groups : the 1 mg/kg HSV-LacZ and the 4 mg/kg HSV-LacZ groups. 36 pups from nine litters were used in this experiment. The optical densities of the pCREB/mg protein were compared by a paired Student t-test between the learning groups and their corresponding control groups.

5.3 Results

5.3.1 Expression of HSV-LacZ in the olfactory bulb and its effect on odor preference learning

To assess viral-mediated expression of LacZ in the olfactory bulb, β-galactosidase histochemical staining by X-gal was used to visualize the HSV-LacZ infected cells. Expression of the virus has been reported to be maximal at 2-4 days postinjection (Carlezon, Jr. et al., 1998). Dark blue cells were seen in all layers of the olfactory bulb using X-gal staining three days after HSV-LacZ injection (Fig.5.1). The area around the injection site was most heavily stained. HSV-LacZ spread well along the rostro-caudal axis. Control pups with saline injections did not show any X-gal staining. Microinjection of HSV-LacZ caused minimal damage to tissue structures as evaluated by Nissl staining (data not shown).

HSV-LacZ did not affect the animals' odor preference learning. As shown in Figure 5.2, HSV-LacZ injected pups demonstrated behavioural results comparable to those of the saline injected ones. HSV-LacZ injected pups in the O/S group demonstrated significant preference learning compared to the O/O or the naive groups ($p<0.01$). The same pattern of learning results applied to saline-injected pups.

5.3.2 The Causality of CREB in Natural Odor Preference Learning

Injection of HSV-mCREB prevented learning, as shown in Figure 5.3. HSV-mCREB injected animals in the O/S group failed to show a preference for peppermint after training, whereas the control littermate HSV-LacZ injected pups in the O/S group showed odor preference learning ($p<0.05$) compared to the O/O or the naive group. This suggests a causal role for CREB in odor preference learning. Mutant CREB binds to the DNA but does not promote transcription because it is not phosphorylated. Interestingly, HSV-CREB injection did not improve preference learning in the O/S group, rather the opposite occurred. Additional CREB impaired the ability of animals to acquire the odor preference, suggesting there is a window for CREB and implying an optimal window for pCREB functioning given that pCREB is the critical mediator for CREB pathway activation.

To confirm further the increase of CREB expression at the time of learning, a CREB assay was performed on the olfactory bulbs of the HSV-CREB injected pups that were sacrificed on the day of learning (2 days after HSV-CREB injection). The HSV-CREB injection group showed a 22.9% increase ($p<0.01$, Figure 5.4) in the optical density per mg of protein of CREB in olfactory bulb tissue relative to that of the HSV-LacZ control group.

Expression of pCREB was determined 10 min following O/S conditioning test if

increasing CREB substrate increased pCREB following learning. A positive result would suggest that too much pCREB interferes with learning, since HSV-CREB injection impaired odor preference learning in the O/S condition. Figure 5.5 shows that there was a significant increase in the optical density of pCREB (13.8%, $p<0.05$) in the HSV-CREB injected group compared to the HSV-LacZ group. This suggests that increasing CREB substrate by HSV-CREB injection enhances pCREB levels correspondingly, and that increasing CREB/pCREB beyond an optimal level interferes with learning.

5.3.3 The effects of CREB levels on isoproterenol-induced odor preference learning

Figure 5.6 demonstrates that increasing CREB expression by HSV-CREB injection enhanced the sensitivity of the system to the UCS, so that, an originally ineffective dose, 1 mg/kg isoproterenol, now induced learning when paired with odor ($p<0.05$, compared within the HSV-CREB injected groups). The normally optimal dose, 2 mg/kg, and the higher dose, 4 mg/kg, of isoproterenol, failed to induce learning in the HSV-CREB injected groups. Thus, CREB shifted the isoproterenol dose-response relationship to the left. This is consistent with the results in the previous experiment showing that a critical CREB/pCREB window exists, and that too much, as well as too little, CREB/pCREB can prevent learning. Surprisingly, the HSV-mCREB injected pups developed odor preferences when the higher dose of isoproterenol, 4 mg/kg, was used

($p<0.01$, compared within the HSV-mCREB injected groups). Thus, it appears that mCREB shifts the inverted U curve dose response relationship to the right. Both HSV-CREB + 1 mg/kg isoproterenol and HSV-mCREB + 4 mg/kg isoproterenol groups demonstrated significantly higher percentages of time spent over the peppermint side than their corresponding non-learning control groups: HSV-LacZ + 1 mg/kg isoproterenol and HSV-LacZ + 4 mg/kg isoproterenol groups ($P<0.01$, Student t-test).

More important than the CREB increase itself, is the phosphorylation of CREB, since pCREB is the initial step for CREB activation and the CRE-induced gene expression that underlies long-term synaptic plasticity and memory formation (McLean et al., 1999). Again we compared the pCREB levels in both learning groups with their non-learning controls. We were particularly interested to know whether 4 mg/kg isoproterenol increased pCREB in HSV-mCREB injected pups which would be consistent with our behavioural results. As seen in Figure 5.7, we found that, the learning group: HSV-CREB + 1 mg/kg isoproterenol had an 11.1% increase in the optical density of pCREB over that of the non-learning control group: HSV-LacZ + 1 mg/kg isoproterenol. Similarly, pCREB in the HSV-mCREB + 4 mg/kg isoproterenol learning group showed a significantly higher pCREB (11.7% increase in the optical density of pCREB, $p<0.01$) than that observed in the non-learning control group (HSV-LacZ + 4 mg/kg isoproterenol).

5.4 Discussion

Natural, stroking-induced odor learning or 2 mg/kg isoproterenol-induced odor learning was prevented by infusion of a HSV-mCREB into the olfactory bulb. This result critically implicates CREB as a mediator of early odor preference learning. Since the serine 133 site is the only one *not* available for phosphorylation (Josselyn et al., 2002), it also suggests phosphorylation of serine 133 may be critical in early odor preference learning.

Unexpectedly, a bi-directional effect of mCREB was observed in these experiments. The pairing of odor and a 4 mg/kg dose of isoproterenol, that normally does not produce learning, was a successful learning paradigm if HSV-mCREB was previously infused into the olfactory bulb. pCREB assay of this novel effective learning condition revealed higher levels of pCREB, as seen previously with learning, than those seen in rat pups receiving LacZ infusions. We have previously hypothesized that the failure of high doses of isoproterenol to produce learning and/or increased pCREB, might be related to enhanced protein kinase A activation of phosphatases (Yuan et al., 2000b). Since mCREB would provide a 'false' target for both kinase and phosphatase activity, it could alter the balance of enzyme activities in infected neurons to decrease, or increase, the likelihood of pCREB expression depending on the enzyme levels induced by training. Thus, with optimal enzyme levels (odor+ stroking or odor+2 mg/kg isoproterenol) mCREB is

deleterious, but with an excess of phosphatase activity (odor+4mg/kg isoproterenol), for example, it could be beneficial.

The ability of HSV-mCREB to alter downstream transcription and behavioural outcomes has been previously demonstrated in nucleus accumbens (Carlezon, Jr. et al., 1998; 2000). No effect of HSV-mCREB, however, was observed in an earlier study of long-term memory when it was infused into the amygdala (Guzowski and McGaugh, 1997).

HSV-CREB infusions lowered the threshold for isoproterenol-induced odor learning. Infusion of HSV-CREB in the olfactory bulb produced learning in rats pups given odor+ 1 mg/kg isoproterenol, normally an ineffective pairing for learning. The ability of CREB to lower the threshold for isoproterenol-induced odor learning further supports our hypothesis that CREB has a causal role in early odor preference learning. pCREB was also significantly increased in this novel learning condition as compared to rats pups receiving HSV-LacZ infusions. The ability of HSV-CREB to alter downstream genomic expression and behavior has been previously demonstrated in the nucleus accumbens (Carlezon, Jr. et al., 1998; 2000). HSV-CREB has also been shown to convert short term memory to long term memory in the amygdala, although it did not render a weak unconditioned stimulus more effective (Guzowski and McGaugh, 1997) as observed in the present experiment.

A bi-directional effect of CREB appeared when HSV-CREB was infused prior to normally effective learning conditions. Thus, rat pups given odor+stroking or odor+2 mg/kg isoproterenol did not learn if HSV-CREB was infused prior to training.

Over expression of CREB has been shown to interfere with learning in other paradigms (Guzowski and McGaugh, 1997; Josselyn et al., 2002). The present pattern of results is consistent with that literature. However, here, pCREB levels were also assessed in the odor+stroking group given CREB infusion. This group had higher pCREB levels than the LacZ controls that successfully learned the odor preference. This outcome supports the hypothesis of an optimal window for pCREB level in initiating the development of odor memory. It has been shown that the duration of pCREB activation critically influences downstream gene expression (Bito et al., 1996), and it has been proposed that overactivation of CREB might lead to increased repressor activity (Silva et al., 1998), but this is the first demonstration of a negative effect of elevated pCREB levels on learning and memory.

CREB is a target of the PKA/cAMP intracellular pathway. We have shown that cAMP is increased in mitral cells by stroking and by isoproterenol (Yuan et al., in press). We have suggested a model of early odor preference learning in which the locus coeruleus input activates beta receptors on mitral cells to trigger cAMP increases (Yuan et al, in press). This increase is hypothesized to interact with calcium currents activated by the

odor input to mitral cells to enhance pCREB in those same cells. Other evidence however demonstrates CREB phosphorylation at the Ser 133 site through a variety of protein kinases including those activated by calcium (Silva et al., 1998). An alternative model of odor preference learning suggests NE-induced disinhibition of mitral cells by granule cells, which could enhance NMDA currents from odor input onto mitral cells (Wilson and Sullivan, 1994). This model would also predict pCREB increases in mitral cells. Both mechanisms are likely to contribute to early odor preference learning.

Our data from optical imaging (Yuan et al., 2000) and from electrophysiological measurements of olfactory nerve evoked potentials (Yuan et al., 2000b) suggest a critical change during both acquisition and retrieval is the potentiation of the mitral cell responses to the odor input. An input potentiation model is also supported by earlier evidence of enhanced 2-DG (Woo et al., 1987; Johnson and Leon, 1996) and c-Fos (Johnson et al., 1995) during memory retrieval. Potentiation of the mitral cell responses to the odor input following appetitive olfactory conditioning has also been reported for the honeybee (Faber et al., 1999) and the sheep (Kendrick et al., 1992).

CREB and pCREB were first shown to have causal roles in the encoding of sensory memory in Aplysia. Using genetic tools, CREB has been shown to be causal in olfactory learning in Drosophila. This is the first report that CREB and pCREB have causal roles in mammalian olfactory learning.

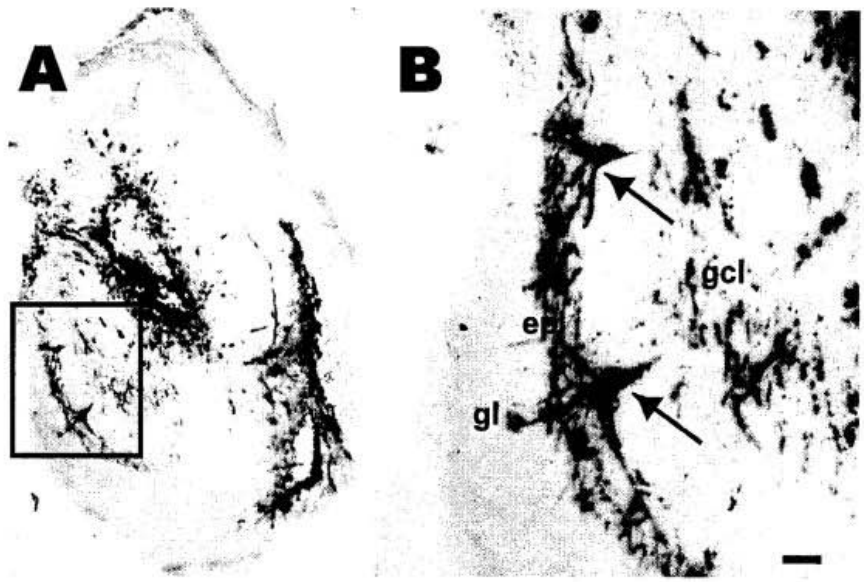

Figure 5.1 Visulization of β-galactosidase by X-gal staining showing expression of LacZ in many cells throughout the olfactory bulb. Labelled cells in A are shown at higher magnification in B. Mitral cells are indicated by arrows. Bar in B, 50μm
Abbreviation: gl, glomerular layer; epl, external plexform layer; gcl, granule cell layer

.

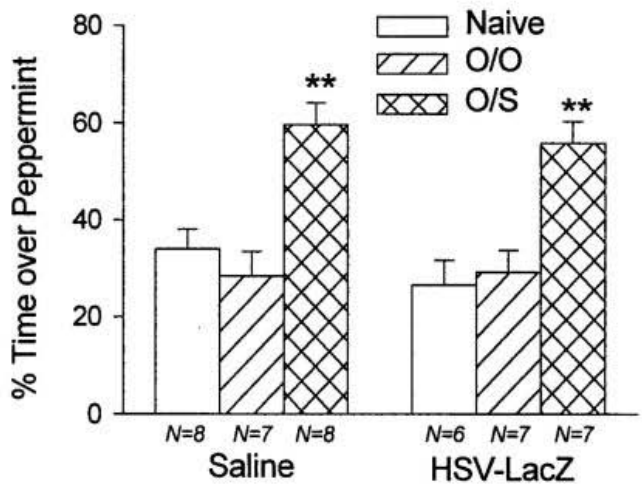

Figure 5.2 Odor preference test showing HSV-LacZ injection itself does not affect odor preference learning.

HSV-LacZ injected pups in the O/S group demonstrate significant preference learning when compared with those in either the O/O or the naïve group. The same pattern applies to the saline injected pups (**p<.01). Abbreviations: O/S, odor+stroke; O/O, odor only.

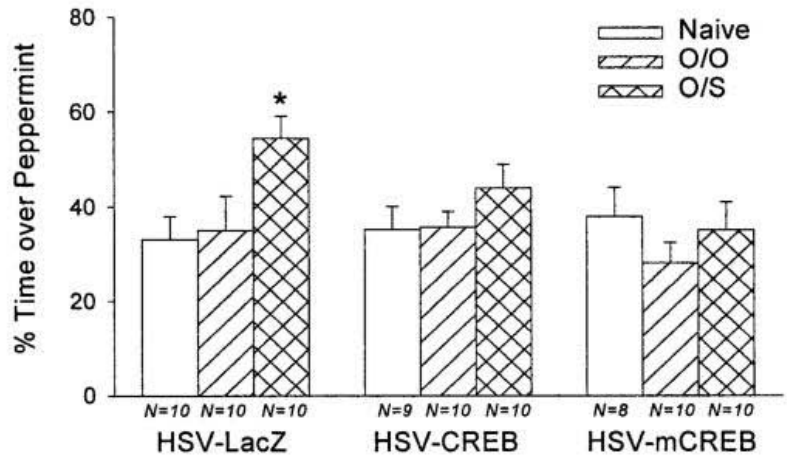

Figure 5.3 Odor preference test showing CREB and mCREB injections block odor preference learning in a naturally learning paradigm. Both HSV-CREB and HSV-mCREB injected pups show deficient odor preference learning compared to their HSV-LacZ control pups (*$p<0.05$)

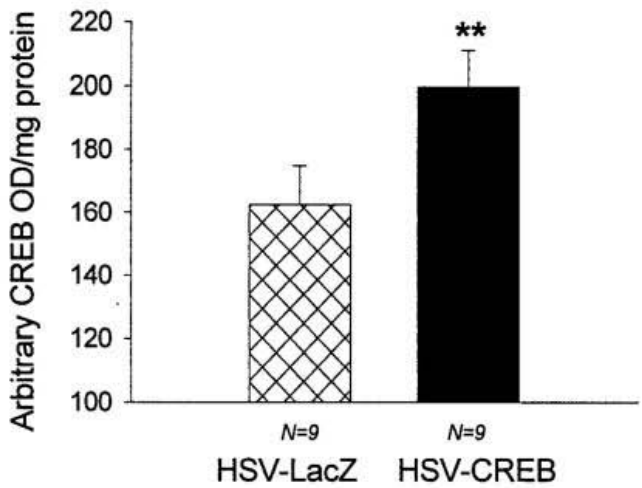

Figure 5.4 CREB assay showing CREB in the olfactory bulb is increased two days after HSV-CREB injection. CREB content is presented as arbitrary optical density/mg protein. (*p<.05).

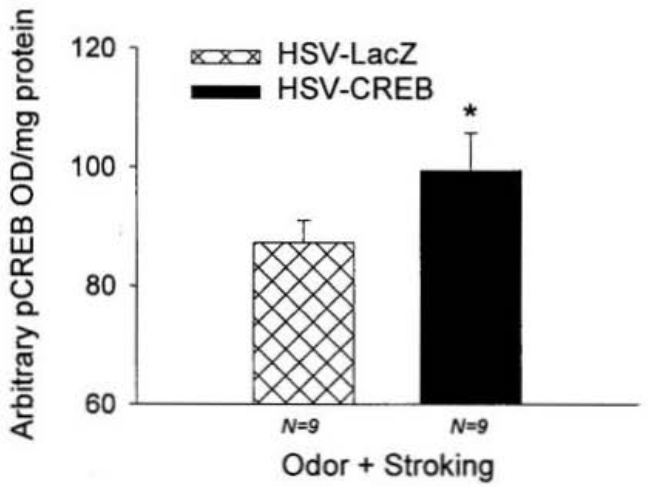

Figure 5.5 pCREB assay showing pCREB is significantly increased in the olfactory bulbs of the HSV-CREB injected group compared to those of the HSV-LacZ group 10 min after they are subjected to O/S. pCREB content is presented as arbitrary optical density/mg protein. (*p<.05)

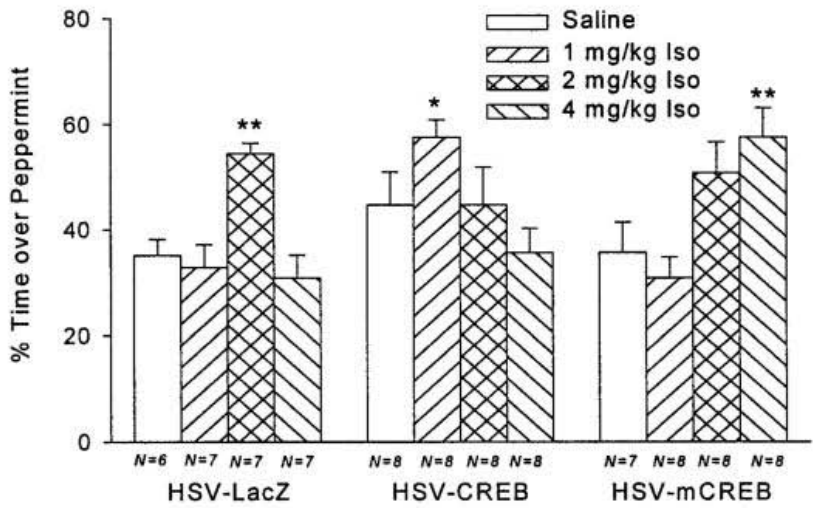

Figure 5.6 Odor preference test showing CREB and mCREB injections shift the isoproterenol inverted U-curve to the left and right respectively.
For the HSV-LacZ injected group, 2 mg/kg isoproterenol induces odor preference learning when paired with peppermint odor. In contrast, the HSV-CREB injected group shows that, a lower dose of isoproterenol (1 mg/kg) produces learning; for the HSV-mCREB injected group, a higher dose of isoproterenol (4 mg/kg) produces learning. (**p<.01, *p<.05)

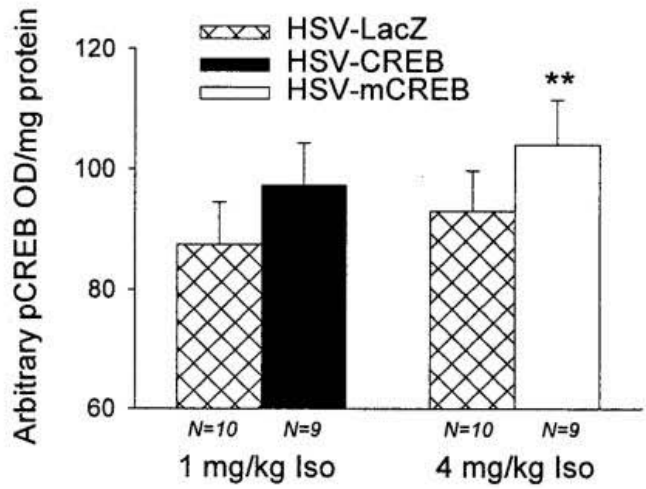

Figure 5.7 pCREB assay showing pCREB is increased in the olfactory bulbs of the learning groups.

CREB/1 mg/kg isoproterenol and mCREB/4 mg/kg isoproterenol learning groups demonstrate increased pCREB expression following training than their lacZ control groups.
pCREB content is presented by the pCREB arbitrary optical density/mg protein. (**p<.05)

Chapter 6 Summary

6.1 Research outcomes

6.1.1 Mitral cells are the postsynaptic substrate for learning

A fundamental question of my thesis work is where early odor preference learning occurs. It has been suggested that the granule cell was the primary site for NE-mediated learning plasticity in the olfactory bulb (Jahr and Nicoll, 1982; Trombley and Shepherd, 1992; Wilson and Sullivan, 1994). Granule cells provide feedback inhibition by releasing GABA onto the mitral cell. NE acts on granule cells to reduce this inhibitory feedback. Reduced inhibition in turn would permit an increased NMDA current in the mitral-granule cell synapses.

However, there are discrepancies regarding the receptors mediating the disinhibitory effect of NE. Whereas Sullivan et al (1994) demonstrated a β-adrenoceptor mediated odor preference learning model, α-, but not β-adrenoceptors mediate the disinhibition from granule to mitral cells. Moreover, the cellular localization of β-adrenoceptors was not clear. Binding studies are inherently inconclusive regarding the precise postsynaptic targets. A more defined immunocytochemical study of the cellular

location of β-adrenoceptors in the olfactory bulb could better elucidate this issue. Also, given that 5-HT_2 receptors and β-adrenoceptors interact in early odor preference learning, the precise postsynaptic target common to these receptors in the bulb was yet unknown.

We examined the cellular distribution of β_1-adrenoceptors and their co-localization with 5-HT_{2A} receptors in the olfactory bulb. Results from these experiments, together with others, e.g. the pCREB and cAMP localization experiments, lead us to suggest that mitral cells, the output cells of the olfactory bulb, serve as the neuronal substrate for a β_1-adrenoceptor-mediated odor preference learning.

The specific evidence is as follows: (a) β_1-adrenoceptors are primarily located on mitral/tufted cells of the olfactory bulb, with few located on granule cells. Fluorescence immunocytochemistry and confocal imaging demonstrate a clear co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors on mitral cells. (b) β-adrenoceptors are G-protein coupled and when activated, trigger a cAMP second messenger cascade. The interaction of NE (via β-adrenoceptors) and 5-HT in elevating cAMP levels occur in mitral cells as observed by cAMP immunocytochemistry. (c) The interaction of NE (β-adrenoceptors) and 5-HT leads to the phosphorylation of CREB. pCREB changes are also observed in the mitral cells of the olfactory bulb. These outcomes strongly suggest mitral cells are the postsynaptic targets for a β-adrenoceptor mediated, 5-HT receptor facilitated, intracellular cAMP signalling cascade.

6.1.2 From inverted-U curves to functional windows

As described before, an important and useful aspect of odor preference learning is the inverted-U curve property of the odor conditioning. Low doses of isoproterenol are ineffective, medium doses are effective, and high doses are again ineffective (Sullivan et al., 1991). This parallels evidence obtained with stroking. A subthreshold stroking input can summate with a subthreshold isoproterenol dose to produce learning, while an effective stroking stimulus becomes ineffective in inducing learning when a low dose of isoproterenol is also given (Sullivan et al., 1989b). The inverted-U curve of the isoproterenol UCS can be shifted by 5-HT manipulations (Langdon et al., 1997; Yuan et al., 2000b).

To understand the inverted-U curve function of UCS in odor learning and its mechanism was another goal of my thesis. The exploration of this issue has led to the discovery of functional windows in the cAMP-CREB signalling pathway.

First, when pCREB is measured in the olfactory bulb after odor conditioning, whereas an effective dose of isoproterenol, when paired with odor, enhances the pCREB expression in the olfactory bulb, either a lower dose, or a higher dose of isoproterenol, fails to enhance pCREB levels in the olfactory bulb. This suggests CREB activation exhibits a parallel inverted-U curve to that seen in behaviour. A critical window for

calcium and PKA co-activation of phosphorylation events may have been exceeded by pairing odor and a high dose (6 mg/kg) of isoproterenol in the normal rat pups. Interestingly, potentiation of the glutamatergic olfactory nerve input by systemic isoproterenol injection also demonstrates an inverted-U curve window. As discussed in Chapter 4, there are several possibilities for understanding these parallel effects of the isoproterenol UCS on both pCREB expression and ON input potentiation. (a) The failure to phosphorylate CREB may be a simple consequence of the failure of potentiation. (b) It is likely that phosphorylation-sensitive ion channels are involved in the early membrane effects of isoproterenol. The failure of appropriate phosphorylation activation could lead to the failure of ON input potentiation as well as the failure to increase pCREB. (c) Less probable are paralled mechanisms – one accounts for CREB phosphorylation, one for ON input potentiation.

Second, a functional window for cAMP occurs in NE-mediated odor preference learning. Because the known effects of isoproterenol are mediated via G-protein activation and recruitment of adenylate cyclase, we suspected the failure to induce learning, increase pCREB, and potentiate the ON evoked potential resulted from a failure to enhance cAMP by a high dose of isoproterenol. However, experiments using a cAMP assay showed that cAMP is dose-dependently increased by isoproterenol immediately after odor conditioning. Two conclusions are suggested by this outcome: (a) cAMP plays

a modulatory role rather than directly transmitting the learning signals, and (b) a more complicated spatial and temporal activation of cAMP is required for signal transduction during learning. For example, we hypothesize, the duration of cAMP action, rather than the absolute amount of cAMP, may be more important for learning to occur.

Third, there appears to be a window for effective pCREB levels. Our experiments using viral vector injections of either HSV-CREB or HSV-mCREB suggest too much, as well as too little CREB impairs odor preference learning. As discussed in 1.3.1, there are two regulation mechanisms for CREB activation. Firstly, CREB activation is determined by the balance between a Ca^{2+}/calmodulin phosphorylation pathway and a Ca^{2+}/calcineurin dephosphorylation pathway at the upstream stage. As described above and in Chapter 4, pCREB is optimal when a moderate, behaviourally effective dose of isoproterenol is used. A stronger stimulus, such as a higher dose of isoproterenol, may favour a dephosphorylation activation that eventually overrides the phosphorylation cascade. Secondly, the balance between CREB activators and repressors may be a second mechanism at the transcriptional stage. Activation of CREB may result in the increased expression of CREM isoforms, the accumulation of which may eventually lead to the repression of CREB-dependent transcription (Silva et al., 1998). Furthermore, our work is the first to exhibit in a *in vivo* mammal learning model, that too much pCREB is not helpful.

6.1.3 CREB is critical in odor preference learning

CREB has been hypothesized as a universal "memory molecule" (Silva et al., 1998). Its role in learning and memory has been extensively investigated in various species and a diversity of memory models in the last two decades. Establishing the causal role of CREB in odor preference learning was an important goal of my thesis. It is, also, a critical component of our hypothesized odor learning model, and furthermore, a basis for the future study of the downstream genomic, synaptic, and structural changes following odor learning.

CREB phosphorylation correlates with learning conditions. Odor + stroking conditioning increases pCREB transiently in a restricted odor-coding area in the olfactory bulb (McLean et al., 1999). An effective dose of isoproterenol, when paired with the odor, can substitute for stroking to increase pCREB.

More importantly here, we evaluated the causal role of CREB in odor preference learning using a Herpes viral vector, to insert additional CREB copies, or mutant CREB copies, into the neurons of the rat pup olfactory bulb. We had two important findings that suggest the causal role of CREB in odor preference learning. First, mutant CREB insertion, which could competitively reduce the phosphorylation of natural CREB in the olfactory bulb, blocks learning. Second, additional CREB insertion increases the

sensitivity of the system to UCS stimulation, shifting the isoproterenol inverted-U function curve to the left. A low, normally ineffective dose of isoproterenol, induces successful odor learning in HSV-CREB injected rat pups.

The use of a viral vector as a CREB delivery vehicle to manipulate the CREB level in the olfactory bulb of rats bypasses the potential problems of using transgenic, or gene knockout animals, to study learning and memory in other systems. It avoids the potential systemic, and/or developmental deficits caused by transgenic, or gene knock-out, manipulations. Recent studies using HSV vectors in other brain regions suggest it is a safe and effective means of incorporating genes into the host (Cattoretti et al., 1993; Carlezon, Jr. et al., 1998; Neve and Geller, 1999; Schutzer et al., 2000; Mower et al., 2002). We are the first to use this means of gene manipulation in the rat olfactory system to study natural learning in normal animals.

6.1.4 Biochemical and physiological changes induced by odor learning are long-lasting

Learning leaves traces which can be retrieved at the synaptic, and cellular levels during the memory phase. Early odor preference learning changes the single-unit responses of mitral cells during odor re-exposure (Wilson et al., 1987). Focal 2-DG uptake and c-fos expression in the glomerular layer increase after early odor preference

learning (Woo et al., 1987; Johnson and Leon, 1996). Increased focal 2-DG uptake may be due to increased glomerular size (Woo et al., 1987), increased numbers of juxtaglomerular cells (Woo and Leon, 1991) following peppermint learning, or simply due to increased activation by odor input.

Consistent with these changes, by using a novel technique, intrinsic optical imaging, we demonstrated that 24 hr after peppermint preference learning, there is an increase in intrinsic optical signals at the glomerular level. This result is consistent with the evidence showing an increase in the field evoked potential to the ON input in pups of the same age that receive learning effective training conditions. Creation of an olfactory preference in the rat pup may therefore be intimately related to an increase in synaptic strength at the level of glomeruli. The intrinsic signal change at the level of the glomeruli 24 hr later may indicate that the synaptic modification seen during acquisition conditions is sustained and reflected as an enhance synaptic excitation (Waldvogel et al., 2000). An increase in mitral cell excitation as a representative memory change is supported by evidence from lamb odor recognition studies in which β-adrenoceptor activation paired with lamb odor lead to an increase in mitral cell excitation to the lamb odor as well as an increase in inhibitory transmitter measurement in the sheep olfactory bulb (Kendrick et al., 1992).

Although rapid progress has been made in the past few years in applying optical

imaging techniques to explore odor coding in the olfactory bulbs of both mice and rats, we are the first to report a memory-associated change in the neonate rat olfactory bulb using this technique. We showed that optical imaging is an excellent technique to explore training-related odor presentation and changes in the olfactory bulb. Optical changes can be recorded from olfactory bulbs during both memory acquisition and retrieval phases in living animals.

6.2 A new model for odor preference learning

Based on my thesis work and previous work done in our laboratory, we proposed a new model for odor preference learning, in which we suggested that the β-adrenoceptors and $5HT_{2A/2C}$ receptors, critical in early odor preference learning, interact via a synergistic promotion of a cAMP cascade in mitral cells in the olfactory bulb to mediate CREB pathway activation, which critically underpins memory formation. The critical learning change occurs in the mitral cell processing of olfactory nerve input, and the olfactory circuitry and/or structural changes induced are long-lasting.

6.2.1 Comparison with a disinhibition model

Sullivan and Wilson (1994) proposed that learning results from the disinhibition of mitral cells, which permits activation of NMDA receptors on granule cells leading to

increased long-term inhibition of mitral cells and accompanying structural changes during the memory phase. In that model, NE input from the locus coeruleus to the olfactory bulb acts as the UCS by inhibiting granule cell interneurons in the bulb through β-adrenoceptors.

However, the discrepancy regarding the receptor subtypes in mediating mitral cell disinhibition and the lack of data regarding the cellular distribution of β-adrenoceptors in the olfactory bulb made it necessary to further explore the location and function of β-adrenoceptors in odor preference learning.

6.2.1.1 Evidence consistent with the mitral cell cAMP/PKA/pCREB model

We demonstrated in this thesis, first, either natural learning using odor+stroking (McLean et al., 1999), or learning using odor+effective doses of isoproterenol (see Chapter 2), enhanced pCREB expression in mitral cells of the olfactory bulb. Since β-adrenoceptor activation promotes a cAMP 2nd messenger cascade via G-proteins, we hypothesized that β-adrenoceptor activation could activate intracellular signal cascades to further promote Ca^{2+} entry through the olfactory nerve mediated NMDA or L-type Ca^{2+} channels, which, on one hand, could enhance the phosphorylation of ion channels and the depolarization of postsynaptic mitral cells; and on the other hand, could promote CREB phosphorylation and CRE-mediated downstream genomic changes in mitral cells. The

potentiation of the olfactory nerve evoked field potentials of mitral cells and the activation of intracellular signalling machinery during the learning phase result in long-lasting changes that can be recruited during the memory phase (see Chapter 3).

Second, the observation that the β_1-adrenoceptors and 5-HT_{2A} receptors co-localize in mitral/tufted cells and NE and 5-HT manipulations change the levels of cAMP in mitral cells further confirmed our hypothesis, suggesting NE via β_1-adrenoceptors activates a cAMP cascade in mitral cells of the olfactory bulb. 5-HT appears to promote the NE-induced cAMP signalling (see Chapter 4). Since isoproterenol is a non-specific β-adrenoceptor agonist, a role for the β_2-adrenoceptors in odor preference learning can not be ruled out. An *in situ* hybridization study demonstrates the β_2-adrenoceptors are more widely expressed in the olfactory bulb (Nicholas et al., 1993). Their specific contribution to early odor preference learning remains to be determined.

Third, we demonstrated a causal role of CREB activation in early odor preference learning. Learning itself causes the activation of CREB, while manipulations of CREB level change the ability of the UCS system to induce learning. Elevating CREB levels by viral vector injection of HSV-CREB shifts the isoproterenol inverted-U curve to the left so that a low, normally ineffective dose of isoproterenol causes learning. Importantly, reducing the levels of CREB by HSV-mCREB (single point mutation at phosphorylation site serine 133) injection blocks the O/S natural learning, which shifts the isoproterenol

inverted-U curve to the right in the isoproterenol mediated learning. These outcomes suggest a critical role of CREB phosphorylation in inducing learning (see Chapter 5).

Therefore, unlike the disinhibitory model proposed by Sullivan et al, here we demonstrate a model in which a cAMP cascade activated by a NE UCS via β_1-adrenoceptors promotes the excitation of the mitral cell itself and results in the intracellular signalling pathway activations which underpin long-term memory formation. Mitral cells, rather than granule cells, are the neuronal focus of β_1-adrenoceptor mediated odor learning in this model.

6.2.2 Comparison with cAMP-mediated learning models in other species

6.2.2.1 Aplysia and Drosophila

Identifying key molecular elements underpinning learning and memory in invertebrates has provided evidence for an important role of the cAMP signalling cascade in memory formation.

In Aplysia, a weak CS (a touch to the siphon) when repeatedly paired with a strong UCS (an electric shock to the tail), results in a greatly potentiated gill-withdrawal reflex elicited by a touch to the siphon (Carew and Sahley, 1986; Mons et al., 1999).

Studies of the mechanism of this system demonstrate that the CS is induced by Ca^{2+} influx through voltage-gated ion channels activated by the action potential in the sensory neuron. The UCS is induced by exciting facilitatory interneurons, which leads to the release of the modulatory neurotransmitter serotonin on to sensory neurons and the subsequent activation of a cAMP cascade via adenylyl cyclase (AC) in these neurons. In this system, long-term facilitation and synaptic changes are mediated by the synergistic interaction between Ca^{2+}/CaM and 5HT-αs GTP pathways that coactivate AC to produce the strong or prolonged cAMP signals that appear required for transcriptional activation. As discussed in 1.3.2.1, an increase in cAMP by repeated pulses of 5-HT gives rise to the translocation of the catalytic subunit of PKA to the nucleus, where it phosphorylates CREB and immediate early genes to regulate expression of late response genes, which encode new proteins that are critical for the persistent changes underlying the development of more stable and durable forms of memory (Frank and Greenberg, 1994).

In Drosophila system, the cAMP signalling cascade plays a critical role in long-term memory of olfactory avoidance learning in which the fruit fly Drosophila learns to distinguish a conditioned odor that has been paired with electric shock from a neutral odor. Deficits in olfactory associative learning and memory are observed in mutant flies that have changes in the cAMP-signalling cascade or CREB levels. In the Drosophila model, mushroom body neurons integrate sensory inputs from both olfactory cues (the CS, producing an increase in intracellular Ca^{2+}) and footshock (the UCS, activating a Gs-

coupled receptor which in turn activates the cAMP cascade) (Goodwin et al., 1997; Mons et al., 1999). As in Aplysia, elevated cAMP may cause the translocation of PKA to the nuclei where it phosphorylates CREB and initiates a cascade of gene expression responsible for long-term structural and functional changes at synaptic sites. As discussed in 1.3.2.2, mutations of cAMP pathway components cause behavioural deficits. Either elevating the cAMP level (*Dunce*, which lacks phosphodiesterase) or reducing the cAMP level (*Rutabaga*, which is defective in adenylyl cyclase) impairs odor avoidance learning in Drosophila. However, double mutants of *Dunce*- and *Rutabaga*-, which exhibit approximately normal levels of cAMP, still show deficit in learning. This suggests that complex spatial and temporal regulations of cAMP as opposed to absolute levels of cAMP may underpin memory formation in Drosophila (Mons et al., 1999), as is also suggested in the present thesis (see Chapter 4).

In both of these models, the coincident activation of two input pathways (CS and UCS) converge to produce a synergistic activation of Ca^{2+}/CaM stimulable ACs which in turn enhances cAMP levels and engages a crucial intracellular cascade for the establishment of the memory traces (Mons et al., 1999). The activation of the CREB transcriptional pathway may serve as a second convergent site for the CS and UCS inputs, with adenylyl cyclases being the first site (Dash et al., 1991).

The present odor preference learning model in neonate rats shares several

common components with invertebrate learning models described in both Aplysia and Drosophila. For example, cAMP increases and CREB pathway activation have been implicated both in the invertebrate learning models and the present rat odor preference learning model in rats. In addition, as in the Drosophila system, an optimal window appears to occur for cAMP functioning in early odor preference learning, with too high or too low levels of cAMP interfering with learning. Also, the present model suggests temporal and spatial patterns of cAMP activation may be important in the odor preference learning process. However, a key difference between the invertebrate models and the early odor preference learning model in rats appears to be the co-incidence detection mechanism. In both Aplysia and Drosophila, adenylyl cyclase is the co-incidence detector for the CS and UCS. The coincident activation of two inputs converge to produce a synergistic activation of Ca^{2+}/CaM stimulable adenylyl cyclases, which in turn enhances the cAMP level proposed as the primary mediator of downstream events that engage synaptic plasticity in learning and memory. In Aplysia, Ocorr et al (1985) showed that depolarization of the sensory neurons prior to exposure to 5-HT pulses increases levels of cAMP over those seen when CS and UCS are unpaired. In odor preference learning in neonate rats, high levels of cAMP alone do not produce learning; thus the CS pathway appears to interact with the UCS pathway at a later stage. The CS-UCS pairing does not provide an additional increase of cAMP compared to UCS stimulation alone (see Chapter 4). Thus, the role and mechanism of cAMP in learning are different between the model for rat odor learning and the invertebrate learning model. While in Aplysia and

Drosophila, cAMP activates PKA to translocate into the nucleus and phosphorylate CREB, therefore serving as a direct mediator, in our model for rat odor preference learning, the cAMP signalling appears to act as a modulatory "gating" system to regulate the Ca^{2+} signal induced phosphorylation pathways which will be discussed in 6.3.1.2 (see also Chapter 4).

6.2.2.2 LTP model in mammalian hippocampus

The mechanisms that generate LTP in the three major pathways in the hippocampus, namely perforant, mossy fiber and Schaffer collateral pathways have been extensively studied. LTP in the mossy fiber pathway is nonassociative and NMDA-independent. It requires Ca^{2+} influx into the presynaptic cell after the tetanus to activate Ca^{2+}/CaM stimulable adenylyl cyclase, and increase the cAMP/PKA activity in the presynaptic cell. Mossy fiber LTP can be enhanced by a noradrenergic input that engages β-adrenoceptors (Kandel et al. 2000, pp1260). In contrast, the associative, NMDA-dependent LTP present in the Schaffer collateral and the perforant pathways is mainly initiated by an elevation of postsynaptic Ca^{2+} influx, via NMDA receptors. However, ample evidence suggests that the cAMP-signalling pathway is also critically implicated as an intracellular mechanism that underlies both early and late phases of hippocampal LTP. During the early stage of Schaffer collateral LTP in the CA1 regions, the cAMP/PKA pathway has been proposed to play a modulatory role: instead of transmitting signals for

the generation of LTP, it gates the transcriptional phosphorylation pathways by reducing calcineurin dephosphorylation activity (Blitzer et al., 1995; Liu and Graybiel, 1996; Blitzer et al., 1998; Winder et al., 1998). cAMP activation by itself does not induce LTP, rather activated CaMKII is necessary and sufficient to generate early LTP (Blitzer et al., 1995). The late, or long-lasting LTP, which requires both transcriptional activation and new protein synthesis, however, is entirely dependent on an elevation of cAMP levels to trigger PKA-induced, MAPK co-phosphorylation of CREB. Long-lasting LTP can be blocked by PKA inhibitors (Impey et al., 1996) and PKA activation is sufficient to induce late LTP in the absence of electrical stimuli (Frey et al., 1993).

Consistent with this hippocampal LTP model, our model for neonate rat odor preference learning demonstrated a crucial role for the cAMP-CREB signalling pathway activation in long-term memory formation. Our data also suggest cAMP plays more of a modulatory role in odor learning. Higher, ineffective doses of the β-adrenoceptor agonist isoproterenol do not enhance pCREB, but appear to increase intracellular cAMP level in a dose-dependent manner. Enhanced Ca^{2+} signalling by the CS (odor) input does not induce an additional increase of cAMP, suggesting the cAMP cascade is activated independently by the UCS. Neither a cAMP increase (by stroking the body of the pups), or a hypothesized elevated Ca^{2+} signal (by odor input), appears sufficient to induce an odor preference. These outcomes suggest that in early odor preference learning, the association of the CS-UCS pathways is strictly regulated. Learning results from a

temporally synergistic activation of both CS and UCS intracellular signalling (see also Chapter 4).

Also consistent with the hippocampal LTP and long-term memory models, odor preference learning in neonate rats requires CREB phosphorylation as an initial step for downstream genomic and synaptic changes. CREB pathway activation is critically involved in long-term memory formation. CREB levels are delicately regulated in both systems (see also Chapter 2 and Chapter 5). Once memory is acquired, the memory substrates such as synaptic circuitry, or metabolic, changes in the olfactory bulb are long-lasting (see also Chapter 3).

6.3 Future directions

6.3.1 Mechanisms of cAMP functioning in odor preference learning

Although our proposed model and evidence argue for the role of a cAMP cascade in NE mediated odor preference learning, the mechanism of cAMP action in activating the CREB signalling pathway following learning is yet unclear and merits further exploration.

6.3.1.1 Duration of cAMP activation

Isoproterenol dose-dependently increases cAMP expression in the olfactory bulb, while pCREB activation exhibits an inverted-U curve parallel to that of the isoproterenol effects in learning. This suggests that there is an optimal level of cAMP activation which can be exceeded. More likely, we suggest that the duration and temporal pattern of cAMP activation are more critical than the absolute amount of cAMP as also suggested by the Drosophila mutant learning models. Higher levels of cAMP by a stronger UCS may shorten the duration of its own activation. For example, higher levels of cAMP promote greater PDE4 activation through PKA (Ang and Antoni, 2002) which may critically shorten the duration of cAMP signal. Elevated cAMP can also promote faster cAMP extrusion (Wiemer et al., 1982), which again, shortens the signal duration (see also Chapter 4).

Therefore, one of the future directions from my thesis is to measure the dynamic cAMP changes at different time points following odor preference training. Furthermore, the essential role of cAMP in mediating a pCREB signal and in inducing learning can be tested and further supported by manipulations of cAMP levels and the duration of its activation by rolipram, a PDE4 inhibitor. Rolipram can either increase the cAMP amount and/or prolong its activation by reducing PDE4 action. Rolipram application, in theory, should be able to shift the isoproterenol inverted-U curve to the left, but the overall effect

might be complicated given that the cAMP effect itself may be complex. The outcome is difficult to predict, but the experiment itself would be very interesting to explore.

6.3.1.2 cAMP "gating" phosphorylation by reducing calcineurin activity

Another means by which learning may be abolished when an optimal level of cAMP activation is exceeded is that higher levels of cAMP recruit increased calcium entry which may preferentially favor a calcineurin-induced dephosphorylation. The duration of CREB phosphorylation and related gene expression is dependent on phosphatase activity (Bito et al., 1996). If the cAMP cascade promotes CREB phosphorylation by reducing phosphatase activity, application of a calcineurin inhibitor such as FK506 should test the possibility. Specifically, first, FK506 should be able to at least partially substitute for cAMP in promoting CREB phosphorylation; second, FK506 should be able to synergise with cAMP activation; application of FK506 should in theory shift the isoproterenol inverted-U curve to the left. This series of experiments would provide us with clearer insight into how cAMP is functioning in promoting the CREB phosphorylation pathway as well as in promoting learning.

6.3.1.3 Phosphorylation of NMDA receptors following cAMP activation.

Another consequence of cAMP activation is hypothesized to be the

phosphorylation of phosphorylation-sensitive ion channels such as NMDA receptors to promote Ca^{2+} influx into the postsynaptic cells. Measurements of the phosphorylated NMDA receptor NR1 at different time following different training conditions (O/O, O/S, and naive etc.) would test this hypothesis.

6.3.1.4 Visualizing Ca^{2+} entry by optical imaging

Further experiments could be performed to explore the intracellular calcium signals predicted to be critical. The relationship between the UCS/cAMP cascade and postulated CS/Ca^{2+} signalling pathway needs to be examined. How the levels of cAMP influence the levels of intracellular Ca^{2+} could be tested by Ca^{2+} voltage-sensitive dye imaging. Multi-photon imaging could directly assess Ca^{2+} entry at the receptor/glomerular level where odor input is transmitted to the mitral cell dendrites both in the acquisition and memory retrieval phases. Understanding the Ca^{2+}/calmodulin pathway is essential for elucidating the actual intracellular changes and interactions underpinning odor learning.

6.3.2 Other types of adrenergic receptors involved in odor preference learning

We have proposed a new model in which NE and 5-HT synergistically activate a cAMP cascade to co-activate a CREB pathway when occurring with the glutamatergic olfactory nerve evoked Ca^{2+} signal in the mitral cells of the olfactory bulb. This does not

rule out a role for disinhibition or a role for other adrenoceptors or other modulators in odor preference learning. Whether, for example, β_2-adrenoceptors also promote a cAMP second messenger signalling, or provide disinhibition to mitral cells; and whether α-adrenoceptors play roles in mediating odor preference learning and whether they act via a disinhibitory effect could be explored behaviourally and electrophysiologically.

6.3.3 Downstream genes following CREB activation

We have demonstrated that the regulation of genomic expression by CREB is causal in producing preference learning. Future experiments could be designed to pursue the downstream changes regulated by CREB. A major direction would be in examining the downstream events involved in memory retrieval. Microarrays of genes that are highly expressed following associative learning would be helpful to screen the downstream candidates. The inverted U-curve properties of early odor preference learning offer particularly useful control conditions for comparison with effective learning conditions. However, known candidates such as the neurotrophin BDNF, adhesion molecules, and cytoskeletal proteins which are components of long-term plasticity implicated in other memory systems, might be first choice candidates for experimentation in this early odor preference learning model.

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MAKING MEMORY: NORADRENERGIC AND SEROTONERGIC INTERACTION LEADING TO cAMP RESPONSE ELEMENT BINDING PROTEIN ACTIVATION VIA cAMP MEDIATED 2nd MESSENGER SIGNALLING IN NEONATE RAT ODOR PREFERENCE LEARNING

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Making Memory: Noradrenergic and Serotonergic Interaction Leading to cAMP Response Element Binding Protein Activation via cAMP Mediated 2nd Messenger Signalling in Neonate Rat Odor Preference Learning

by

© Qi Yuan

A thesis submitted to the
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Ph.D.

Faculty of Medicine
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October, 2002

St. John's Newfoundland Canada

Abstract

Early odor preference learning offers a unique paradigm for the study of natural mammalian learning. Week-old rat pups form an approach response to an odor that is paired with a tactile stimulus such as stroking. In this associative conditioning paradigm, norepinephrine (NE) input to the olfactory bulb from the locus coeruleus serves as the unconditioned stimulus (UCS) while olfactory nerve stimulation by odor input serves as the conditioned stimulus (CS). β-adrenoceptors are critically implicated in NE-mediated UCS effects. The β-adrenoceptor agonist, isoproterenol, can substitute for stroking to induce early odor preference learning. Activation of β-adrenoceptors in the olfactory bulb is both necessary and sufficient to induce early odor preference learning. The effects of isoproterenol exhibit an inverted-U curve; while a moderate dose of isoproterenol is effective in inducing odor preference learning when paired with an odor, both higher, or lower doses of isoproterenol fail to induce learning. Serotonin (5-HT) depletion shifts the isoproterenol UCS curve to the right. In an earlier model for odor preference learning, Sullivan and Wilson proposed that early odor preference learning results from the NE disinhibition of mitral cells from granule cells via β-adrenoceptors. This strengthens the mitral to granule cell synapses and increases mitral cell inhibition during memory retrieval.

In the present thesis, I propose a new model for early odor preference learning. I

suggest that a cAMP cascade activated by the NE UCS, likely via β_1-adrenoceptors, directly modulates the olfactory nerve to mitral cell connections and results in CREB transcriptional activation in the mitral cell which underpins long-term memory formation. The new model is based on the following evidence: effective CS and UCS pairing enhances phosphorylated CREB (pCREB) expression in mitral cells of the olfactory bulb and potentiates the olfactory nerve evoked field potentials of mitral cells (Chapter 2). Odor preference learning produces long-lasting increases in blood flow at the level of the olfactory nerve to mitral cell synapses which are observed during memory retrieval, and which support the hypothesis of a stronger odor input signal during memory. This is demonstrated by intrinsic optical imaging showing an enhanced response at the level of glomeruli 24 hrs after odor conditioning (Chapter 3). β_1-adrenoceptors and 5-HT_{2A} receptors co-localize in mitral/tufted cells. 5-HT depletion decreases NE-induced elevations of cAMP in mitral cells. 5-HT appears to promote NE-induced learning through convergence on the cAMP cascade in mitral cells (Chapter 4). CREB plays a causal role in early odor preference learning. Manipulations of CREB levels by a viral vector injection directly into the olfactory bulb change the likelihood of learning and this is reflected in alterations in pCREB (Chapter 5).

Acknowledgement

I wish to thank my supervisor Dr. John McLean, who has provided me the opportunity to participate in the Neuroscience program at Faculty of Medicine, Memorial University of Newfoundland, and to complete my Ph.D thesis work in his lab; who has opened a door for me toward a scientific world; who has guided me patiently and persistently; who has pulled me through frustrations and celebrated with me every small success I have achieved. I came to Canada with limited English language skill and dreams for a scientific career; without him, I could not have achieved what I have dreamed.

I wish to thank my co-supervisor Dr. Carolyn Harley, whose spirit and scientific creativity have always inspired me; who has shared her intelligence and ideas with me; who has encouraged me to face the challenges; who has guided me, little by little, to pursue the exciting projects for my thesis and to explore the real meaning of doing Science.

I wish to thank Andrea Darby-King, who has not only provided me her expertise and assistance throughout my program but shared my feelings like a sister; who has taught me every technique in the beginning of my research and helped me throughout my projects; who has shared laughs and tears with me and held my hands whenever I needed.

I wish to thank Dr. Thomas Knopfel, who has provided me an opportunity and resources to learn optical imaging and work on an exciting project; whose love for Science and dedication to research have inspired me.

I wish to thank my parents Shen Yuan and Suming Zhan, who have not only nurtured me but taught me the very first lessons in my life; who have given me my spirit and encouraged me to pursue my dreams; whose endless love has always accompanied me.

I wish to thank Daniel Palomo, who has given me his love and faith; who has supported me through my thesis writing; who has shared with me the excitement of doing Science and, the meaning of life.

I wish to thank CIHR for having provided me the financial support throughout my program, and all the faculty members and students in the Neuroscience program who have given me help and support. Special thanks to Dr. Richard Neuman for the correction and valuable suggestions to my thesis.

Co-authorship Statement

I, Qi Yuan, hold a principle author status for all the manuscript chapters (Chapter 2-5) in my thesis. However, each manuscript is co-authored by my supervisors and co-workers, whose contributions have greatly facilitated the development of my hypotheses in the manuscripts, the practical aspects of my experiments and the manuscript writing as described below.

Chapter 2, titled "Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve-Evoked Potentials in Normal and 5-HT-Depleted Olfactory Bulbs in Rat Pups Only at Doses That Produce Odor Preference Learning", is co-authored by Carolyn W. Harley, Jamie C. Bruce, Andrea Darby-King, and John H. McLean. As the principle author, I participated in the experimental designs and accomplished all the experimental work in Experiment 1 and a portion of the work in Experiment 2 (olfactory nerve evoked field potential (ON-EFP) recordings from 10 pups in normal rat pup groups). I accomplished the first draft writing. Carolyn W. Harley and John H. McLean contributed greatly to the design of the research proposal in this paper, and the completion and improvement of the writing. Jamie C. Bruce performed most of the physiological recordings from olfactory bulb 5-HT depleted pups. Andrea Darby-King provided substantial technical supports in both experiments and participated in data analysis, especially in Experiment 2. Mark Power and Steve Milway also provided technical

assistance in this work.

Chapter 3, titled "Optical Imaging of Odor Preference Memory in the Rat Olfactory Bulb" is co-authored by Carolyn W. Harley, John H. McLean and Thomas Knopfel. As the principle author for this work, I accomplished all the actual experimental work and data analysis, participated in the initial design of the research proposal and actual experimental designs. I co-wrote the manuscript with Thomas Knopfel, whose dedication and assistance enabled the accomplishment of the project described in this paper. He provided substantial technical assistance and participated in the actual experiments. Carolyn W. Harley and John H. McLean participated in the initial design of this work and contributed substantially to the correction and improvement of the manuscript.

Chapter 4, titled "Mitral Cell β_1 and 5-HT_{2A} Receptor Co-localization and cAMP Co-regulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb" is co-authored by Carolyn W. Harley and John H. McLean. As the principle author, I participated in the development of model hypothesis and actual experiment designs, performed most of the experimental work and finished the first draft of the manuscript. Carolyn W. Harley contributed substantially to the improvement of the manuscript, and the initiation of the proposal for a new model for the neonate rat odor preference learning from this work and previous ones. John H. McLean contributed greatly to the

experimental designs and partial experimental work, and also assisted in writing and improvement of the manuscript. Andrea Darby-King assisted in final graph making. Mary Primmer and Dr. Reza Tabrizchi provided technical assistance in this work.

Chapter 5, titled "Early Odor Preference Learning in the Rat: Bidirectional Effects of CREB and Mutant CREB Support a Causal Role for CREB" is co-authored by Carolyn W. Harley, Andrea Darby-King, Rachael L. Neve, and John H. McLean. As the principal author, I participated in the experimental design and accomplished most of the experimental work. I finished the first draft of the manuscript. Andrea Darby-King provided technical support. Carolyn W. Harley and John H. McLean participated in the experimental design and writing. John H. McLean also assisted in the actual experimental work. Rachael Neve kindly provided the viral vectors used in this study and made valuable suggestions for the manuscript. Eric Nestler completed the HSV-CREB and HSV-mCREB amplicon constructions. Dr. Jun Chen provided technical assistance in this work.

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Chapter 2 Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve-Evoked Potential in Normal and 5-HT-Depleted Olfactory Bulbs in Rat Pups Only at Doses That Produce Odor Preference Learning

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Abbreviations

2-DG	2-deoxyglucose
5-HT	5-hydroxytryptamine;serotonin
5,7-dHT	5,7-dihydroxytryptamine
6-OHDA	6-hydroxydopamine
AA	amyl acetate
AC	adenylyl cyclase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid
ANOVA	analysis of variance
AOB	accessory olfactory bulb
AP-5	amino-5-phosphonopentanic acid
APV	D-2-amino-5-phosphonovaleric acid
ATF	activating transcription factor
BCA	bicinchoninic acid
bZip	leucine-zipper domain
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer binding protein
CF	centrifugal fiber
CNS	central nervous system
CREB	cAMP response element binding protein
CREM	cAMP response element modulater
CS	conditioned stimulus
DA	dopamine
DAB	diaminobenzidine dihydrochloride
DCG-IV	(2S, 2'R, 3'R)-2-(2'3'-dicarboxycycloproplyl) glycine
DGG	γ-D-glutamylglycine
DNQX	6,7-dinitroquinoxaline-2,3-dione
DOI	2,5-dimethoxy-4-iodoamphetamine hydrochloride

EFP	evoked field potential
EPL	external plexiform layer
fMRI	functional magnetic resonance imaging
GABA	gamma-amino butyric acid
$GABA_AR$	$GABA_A$ receptor
GC	granule cell
GCL	granule cell layer
GL	glomerular layer
Glu	glutamate
GluR	glutamate receptor
HSV	herpes simplex virus
ICER	inducible cAMP early repressor
IEG	immediate early gene
iGluR	ionotropic glutamate receptor
IPL	internal plexiform layer
LOT	lateral olfactory tract
Iso	isoproterenol
LTD	long-term depression
LTF	long-term facilitation
LTP	long-term potentiation
LTS	long-term sensitization
MCL	mitral cell layer
mCREB	mutant CREB
mGluR	metabotropic glutamate receptors
MC	mitral cell
MOB	main olfactory bulb
NE	norepinephrine
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
OB	olfactory bulb

ON	olfactory nerve
ORN	olfactory receptor neuron
O/O	odor only
O/S	odor+stroking
pCREB	phosphorylated CREB
PDE4	phosphodiesterase4
PKA	protein kinase A
PND	postnatal day
PP	peppermint
PP1	protein phosphatase 1
ROD	relative optical density
STF	short-term facilitation
UCS	unconditioned stimulus
VN	vomeronasal nerve

Chapter 1 Introduction

This introduction reviews, first, our understanding of the early odor preference learning literature up to the beginning of the experiments that comprise the thesis. The next sections review two additional topics of particular relevance for the present set of experiments: (1) the newer methodology of optical imaging and (2) the role of CREB as a putative "universal" memory molecule. The final section outlines the experiments undertaken here.

1.1 Odor preference learning in neonate rats

New born rat pups can not open their eyes until postnatal day (PND)10-12. During this early period of their life, they depend heavily on smell to locate and attach to the mother's nipples. In order to survive, a new born has to develop an attachment to its mother, regardless of the quality of care-giving (Sullivan et al., 2000a). The first postnatal week, therefore, has special meaning in the life of rats: they rely exclusively on olfactory and somatosensory stimuli to develop their initial relationship with their environment; their immature central nervous system (CNS) determines their potential for plasticity, both behaviourally and cellularly.

An approach response to an odor cue can be easily obtained by classical

conditioning in neonatal rats. This approach response in rat pups is quite stereotyped. Multiple stimuli, even aversive ones, such as foot shock and tail pinch, can produce a conditioned approach response to an odour (Camp and Rudy, 1988; Wilson and Sullivan, 1994; Sullivan et al., 2000a). The approach response can be tested 24 hr later by either Y-maze or two-odor testing protocols. This kind of learning is called odor preference learning. The classical conditioning paradigm of odor preference learning enables us to explore the neural substrates underlying learning associated changes. It provides information not only about the mechanisms of synaptic plasticity in the olfactory system but also about the mechanisms of learning and memory in general.

1.1.1 Behavioural paradigm

In 1986, Sullivan and Leon reported that early olfactory learning produced by pairing a novel odor with a reinforcing tactile stimulus from PND 1 to 18, led to an odor preference and enhanced 2-deoxyglucose (2-DG) uptake in specific areas of olfactory bulb glomeruli to subsequent presentations of that odor. In this paradigm, the tactile stimulus serves as an unconditioned stimulus (UCS); the odor itself serves as a conditioned stimulus (CS). The same changes were reported later by the same group after one-trial olfactory training (Sullivan and Leon, 1987). In this simplified paradigm, PND 6 rat pups are simultaneously subjected to a 10 min odor exposure and to a reinforcing tactile stimulation, stroking of their bodies using a brush. The control groups receive

either the odor only (CS only), or the stroking only (UCS only), or neither of these stimuli (naive). The next day, when the pups are assessed by a two odor choice test, the odor+stroking conditioned group demonstrates a preference for the conditioning odor, while the other groups fail to show approach responses.

Different strategies and stimuli have been explored since the initial work by Sullivan and her colleagues. A diversity of stimuli, including stroking, milk, tail pinch, the odor of maternal saliva, high humidity, mild foot shock, heat and intracranial brain stimulation have been used as UCSs (Wilson and Sullivan, 1994). An interesting characteristic of this early olfactory learning paradigm is that there is a sensitive period, during which various brain structures involved in emotion and learning are still immature and undergoing maturation. This sensitive period ends around postnatal day 10. During this period, rat pups have a high potential for acquiring approach behaviour, even when an aversive stimulus such as foot shock is used. After postnatal day 10, pups start to develop avoidance responses. Interestingly, young pups (<PND10) and older ones (>PND10) have similar responses to foot shock (vocalization, vigorous physical response etc.), although they show striking differences in the learned behaviour. Furthermore, the amygdala, a brain structure critically involved in the emotional aspect of learning and memory, does not demonstrate a change after shock-induced odor preference in PND8 pups as measured by 2-DG (Sullivan et al., 2000a), suggesting learning may occur primarily in the olfactory bulb itself during this sensitive period. This hypothesis is

reinforced by the observation of metabolic and electrophysiological changes in the olfactory bulb which will be discussed in 1.1.2.1.1.2.

1.1.2 Neural substrates for odor preference learning

1.1.2.1 Neuroanatomical structures and functions

1.1.2.1.1 Main olfactory bulb

In odor preference conditioning, as mentioned, learning associated changes have mainly been observed in the main olfactory bulb (MOB) (Wilson and Sullivan, 1994). The MOB is a simple cortical structure which receives direct sensory input from olfactory receptor neurons in the olfactory epithelium. The clear laminar structure and limited synaptic circuitry make it an excellent model system to study learning associated synaptic plasticity (Figure 1.1).

1.1.2.1.1.1 Basic structure

The sensory organ of the main olfactory system is the olfactory epithelium. The olfactory receptor neurons (ORNs) transmit the information of volatile compounds of odorants to the olfactory bulb via the olfactory nerve (ON). Located deep to the olfactory

nerve layer are distinctive spherical structures called glomeruli. Glomeruli are where the axons of ORNs synapse onto the apical dendrites of olfactory output neurons – mitral/tufted cells. The secondary dendrites of mitral cells form dendrodendritic synapses with the dendrites of inhibitory neurons -- granule cells in the external plexiform layer (EPL, Figure 1.1A). In rats, the ON forms glutamatergic synapses with the apical dendrites of mitral cells (Ennis et al., 1996; Aroniadou Anderjaska et al., 1997), the excitation of which is regulated by the GABAergic granule cells. The interaction between mitral cells and granule cells is through dendrodendritic synapses. The mitral cell releases glutamate onto granule cell dendrites upon activation; whereas granule cell dendrites, in turn, release gamma-amino butyric acid (GABA) back onto mitral cell dendrites to prevent their further excitation (Figure 1.1B). The chemosensory information is extensively processed and refined within the MOB before it is sent to the olfactory cortex.

The axons of mitral and deep tufted cells of the MOB project via the lateral olfactory tract (LOT) to the olfactory cortex (Schoenfeld et al., 1985; Scott, 1986). In rodents, the olfactory cortex includes the anterior olfactory nucleus; the piriform cortex; cortical and medial nuclei of the amygdala; the olfactory tubercle and the transitional entorhinal cortex. Olfactory information is also relayed to the thalamus and the orbitofrontal cortex via secondary and tertiary connections. The projection to the amygdala is importantly implicated in the mediation of the emotional and motivational aspects of smell and smell behaviour. Afferent projections to the orbitofrontal cortex via

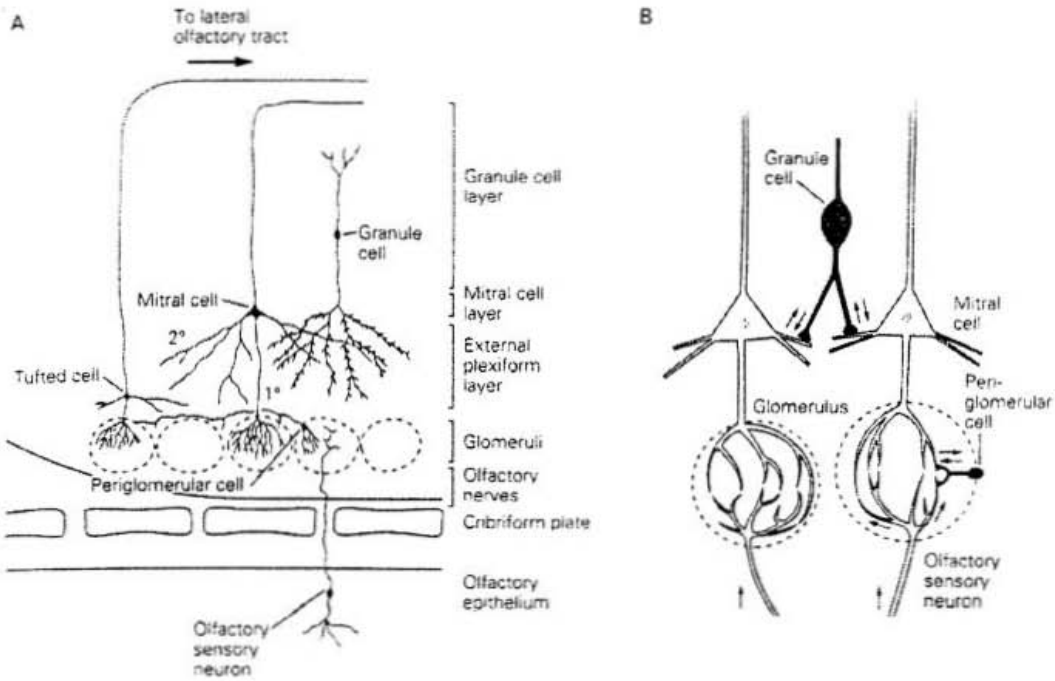

Figure 1.1 The basic structure of the main olfactory bulb (A) and the synaptic circuitry within the olfactory bulb (B). Adapted from Kandel et al. The Principles of Neuroscience. pp 631.

the thalamus are thought to be responsible for the perception and discrimination of the odors (Kandel et al. 2000, pp 633). At the present time no extrabulbar structures have been directly implicated in mediating early odor preference learning.

In addition to the editing of chemosensory information within the olfactory bulb through inhibitory interneurons, the multiple inputs to the olfactory bulb from the olfactory cortex as well as from the basal forebrain (horizontal limb of the diagonal band) and midbrain (locus coeruleus and raphe nuclei) also play modulating roles in olfactory bulb function. The same odor input can take on different behavioural significance depending on the physiological state of the animal (Kandel et al. 2000, pp 633). The activities of some of these systems play important roles in early olfactory conditioned learning which will be discussed in 1.1.2.2..

1.1.2.1.1.2 Changes at the bulbar level following odor preference learning

Neural changes occur at the level of the olfactory bulb following odor preference training. Rat pups that undergo odor conditioning show enhanced focal 2-DG uptake in the glomerular layer when subsequently exposed to the same odor (peppermint) compared to control pups (Coopersmith et al., 1986; Coopersmith and Leon, 1986; Sullivan and Leon, 1986). The increased 2-DG uptake is limited to the 2-DG foci in the midlateral region of the olfactory bulb, but not the ventrolateral 2-DG foci. The enhanced focal 2-

DG uptake may be related to training associated structural changes. For example, increased numbers of juxtaglomerular cells around the glomeruli and increased dendritic processes within glomeruli are also seen following odor conditioning (Woo et al., 1987; Woo and Leon, 1991). Furthermore, early odor preference training increases the density of Fos-immunopositive cells in midlateral glomerular regions underlying the high uptake 2-DG foci seen upon subsequent exposure to the learned odor (Johnson et al., 1995). These results suggest specific regions in the olfactory bulb that encode the conditioned odor are modified following the learning of that odor.

The olfactory responses to odors can be measured by single-unit recording of mitral/tufted cells. Wilson et al reported that granule cell suppression of mitral cell responses to a conditioned odor is increased by conditioning (Wilson et al., 1987; Wilson and Leon, 1988). The increase occurs in the region of the olfactory bulb that responds to the odor. The inhibition that is increased after conditioning may occur via lateral inhibition. The increased strength of lateral inhibition may refine the pattern of mitral cell activity induced by the learned odor (Brennan and Keverne, 1997).

The functional and structural changes in the olfactory bulb associated with odor preference learning occur only during the critical period; the changes cannot be induced after the second postnatal week although preferences can still be established (Woo et al., 1987). The olfactory bulb undergoes extensive growth after birth, the maturation of which

depends greatly on sensory input, which is analogous to the development of other systems, such as binocularity and orientation column formation in the visual system. In this sensitive developmental period, enriched odor environments enhance olfactory neuron survival and synapse formation, whereas unilateral naris occlusion leads to decreased surviving neurons in the deprived olfactory bulb (Meisami and Safari, 1981; Najbauer and Leon, 1995; McLean et al., 2001). Neonatal pups in this period thus have a high potential for neural, as well as behavioural, plasticity.

1.1.2.1.2 Other brain regions involved in odor preference learning

The MOB appears to be the major brain substrate for the acquisition of early conditioned odor preferences and for the processing of odor information during odor learning in general. However, a variety of other brain structures play roles in various aspects of odor learning in both neonate and adult rats (Brennan and Keverne, 1997). These will be briefly reviewed below.

The amgydala appears to be involved in neonate rat odor learning. Bilateral electrolytic lesions of the amygdala on PND5, mainly the cortical nucleus of the amygdala, disrupt the conditioned odor approach responses obtained on PND6, although rat pups still show conditioned behavioural activation (Sullivan and Wilson, 1993). However, the loss of the conditioned odor approach response can be compensated by

overtraining. With sufficient overtraining, pups can acquire both conditioned activation and an odor approach response, suggesting that the amygdala is not essential for this early odor preference learning, although it can facilitate the acquisition of an odor preference.

Interestingly, the amygdala is involved in a differential response to odor-shock conditioning as reported by Sullivan (2000). As mentioned in 1.1.1, there is a critical period for neonate odor preference learning. Odor-shock conditioning induces an approach response in rat pups before postnatal day 10, but results in odor aversion response after this critical period. Amygdala 2-DG uptake after odor-shock conditioning is enhanced in PND12 pups that develop an aversion response; but is not changed in PND8 pups that form an approach response following odor-shock. The olfactory projections to the amygdala are present near birth. Why the amygdala is not critically involved in the early odor-shock induced approach response is not clear, however, it seems to be necessary for the acquisition of an odor aversion in older rats when the same training protocol is used.

In addition to the amygdala, increases in 2-DG uptake have been found in both the anterior olfactory nucleus (Cierpial and Hall, 1988; Hamrick et al., 1993) and piriform cortex (Cierpial and Hall, 1988) following odor learning in rats. Long-term potentiation (LTP), a synaptic phenomena which has been suggested to be closely associated with long-term memory formation, can be induced in the piriform cortex in adult rats (Roman

et al., 1993).

Mediodorsal thalamus and hippocampal areas appear to play roles in adult odor learning. In an odor reversal learning model, water-deprived rats are trained on a go, no-go two-odor olfactory discrimination task to respond to one odor (S+) with water as a reward and to suppress responding to the other odor (S-). The rats are then tested for their ability to reverse the stimulus association. Lesions of mediodorsal thalamus disrupt odor reversal learning in rats (Slotnick and Risser, 1990), but odor discrimination is well preserved. The mediodorsal thalamus projects to the orbitofrontal cortex where olfactory information converges with gustatory and visual inputs and forms associations with rewards (Rolls and Baylis, 1994). In contrast, the parahippocampal areas are more involved with transient olfactory memory (Otto and Eichenbaum, 1992). Disruption of the olfactory input to the hippocampus does not affect odor reversal learning (Slotnick and Risser, 1990). The hippocampus is implicated in supporting the representations of relations among odor stimuli such as the formation of the spatial memory for odor cues (Eichenbaum, 1998). Interestingly, lesions to the above structures, as well as lesions to the amygdala, do not seem to disrupt a previously learned olfactory discrimination (Slotnick and Risser, 1990; Staubli et al., 1995; Brennan and Keverne, 1997).

1.1.2.2 Neurotransmitters implicated in early olfactory learning

As mentioned, the olfactory bulb receives a strong input from neuromodulatory systems: noradrenergic input from the locus coeruleus, serotonergic input from the raphe, and cholinergic input from the horizontal limb of the nucleus of the diagonal band. There are also many intrinsic dopaminergic neurons in the glomerular layer of the olfactory bulb. Different transmitters including NE, 5-HT, dopamine (DA), GABA and glutamate have been shown to play important roles in mediating odor preference learning which will be discussed in the following sections.

1.1.2.2.1 Norepinephrine

Noradrenergic neurons are located in the locus coeruleus. The locus coeruleus projects diffusely throughout the cortex, cerebellum and spinal cord (Kandel et al. 2000, pp 283). In mammals, ascending noradrenergic neurons project directly to the MOB and accessary olfactory bulb (AOB), which is present to a significant extent at birth (McLean and Shipley, 1991). NE terminals have the highest degree of laminar specificity in the MOB of any brain structure (McLean et al., 1989). Most NE fibers terminate in deep layers of the MOB, including the internal plexiform layer (IPL) and the granule cell layer (GCL) and to a lesser degree in the EPL, but it is sparse in the glomerular layer (GL). The ascending NE system is involved with attention and other complex cognitive functions

(Hasselmo, 1995). The role of NE in early olfactory learning has been extensively investigated.

1.1.2.2.1.1 Behavioural aspect

In rat pups, the pairing of a novel odor with tactile stimulation or stroking, which mimics maternal care, produces a conditioned odor preference for that odor. In this model, odor serves as the CS and stimulates the glutamatergic ON via olfactory receptor activation, while the tactile stimulus used as the UCS has been shown to activate the locus coeruleus (Nakamura et al., 1987) to release NE in the olfactory bulb (Rangel and Leon, 1995). The convergence of CS and UCS in the olfactory bulb is hypothesized to result in the structural, biological and physiological changes reported both at the level of glomeruli and the output neurons (as discussed in 1.1.2.1.1.2.).

Associative odor learning in neonate pups requires an intact NE input to the bulb . Bilateral lesions of the locus coeruleus by 6-hydroxydopamine (6-OHDA) significantly impair olfactory learning in rat pups (Sullivan et al., 1994). Systemic (Sullivan et al., 1994) or local bulbar (Sullivan et al., 1992) injection of the β-adrenoceptor antagonist propranolol, or timolol, prior to training blocks odor preference learning in a dose-dependent manner. This effect is not due to the loss of odor sensitivity or discrimination (Doty et al., 1988).

Stimulation of the NE input to the olfactory bulb is sufficient to induce an odor preference (Sullivan et al., 2000b). Putative stimulation of the locus coeruleus by idazoxan or acetylcholine produce an odor preference which could be blocked by a pretraining injection of propranolol. Association of odor with either systemic (Sullivan et al., 1989b; Sullivan et al., 1991) or intrabulbar (Sullivan et al., 2000b) injection of the β-adrenoceptor agonist isoproterenol can substitute for stroking to induce an odor preference in rat pups. Systemic injection of isoproterenol also results in the same enhanced 2-DG uptake within the olfactory bulb as that seen with the stroking+odor conditioning (Sullivan et al., 1991). β-adrenoceptor agonist and antagonist studies together suggest β-adrenoceptor activation is both necessary and sufficient for early odor preference learning. The evidence that intrabulbar β-adrenoceptor activation alone paired with odor is sufficient to induce an odor approach response also suggests that the critical CS-UCS seems to converge in the olfactory bulb during early odor preference learning (Sullivan et al., 2000b).

NE is not only involved in the acquisition, but also in the consolidation of odor preference memories in rat pups. Post-training injection of propranolol up to 1 hr after training blocks learned odor memory acquired through odor-milk association. NE does not appear to be necessary for expression of the learned response; propranolol injection immediately before testing (24 hr after training) does not affect memory (Sullivan and

Wilson, 1992).

While β-adrenoceptors have been extensively studied, other adrenoceptor subtypes have not been directly assessed in early odor preference learning.

1.1.2.2.1.2 β-adrenoceptor activation and the inverted U-curve for early odor preference learning

Interestingly, although the association of an odor with either stroking or isoproterenol results in an odor approach response, the effect is dose-dependent. The dose-response curve for this effect has an inverted U-shape function. Only an optimal activation of β-adrenoceptor produces learning (Sullivan et al., 1989b; Sullivan et al., 1991; Langdon et al., 1997). A moderate dose of isoproterenol, 2 mg/kg, when paired with an odor, can substitute for stroking to induce an approach response; whereas higher doses, 4 mg/kg and 6 mg/kg, or a low dose, 1 mg/kg, of isoproterenol fail to produce odor learning. These outcomes have suggested that the balance between inhibition and disinhibition of output cells of the olfactory bulb may be critical as will be discussed in 1.1.3.1.3 and Chapter 2. Additionally, a critical window for calcium and cyclic adenosine monophosphate (cAMP) coactivation of intracellular phosphorylation pathways may occur with a moderate level of β-adrenoceptors stimulation as will be discussed later in Chapter 2.

The effect of isoproterenol is additive to stroking in supporting early odor learning (Sullivan et al., 1991). An odor paired with a moderate level of either of these stimuli produces learning. However, a combination of 2 mg/kg isoproterenol stimulation with a normal magnitude of stroking does not produce learning. Combination of suboptimal levels of both of these stimuli results in learning. These outcomes suggests that β-adrenoceptor activation and the stroking UCS share a common substrate for inducing early odor learning.

1.1.2.2.1.3 Physiological effects of NE implicated in early odor preference learning

Early olfactory learning depends on MOB noradrenergic neurotransmission as discussed earlier. Studies of NE actions in the MOB are controversial (Jiang et al., 1996). In early work, NE was found to inhibit mitral cell discharge in rabbits and cats (Salmoiraghi et al., 1964; McLennan, 1971), although no pharmacological studies on receptor subtypes were done. More recently, NE was reported to disinhibit mitral cells in the adult turtle olfactory bulb slice preparation (Jahr and Nicoll, 1982). Trombley et al demonstrated the same disinhibition effect on cultured mitral cells from the rat MOB (Trombley, 1992; Trombley and Shepherd, 1992). This disinhibitory effect is mediated by an α-, but not a β-adrenoceptor, presynaptic mechanism (Trombley, 1994). NE application results in the disinhibition of mitral cells from granule cells through inhibition

of presynaptic calcium influx in the mitral cells. In vivo Jiang et al (1996) reported that locus coeruleus activation increased mitral cell responses to weak olfactory bulb stimulation, which is consistent with the disinhibition hypothesis. More importantly, in present concern, an *in vivo* study in rat pups reported activation of β-adrenoceptors in the olfactory bulb decreased granule cell inhibition (Wilson and Leon, 1988). By examining the effects of the β-adrenoceptor agonist, isoproterenol, and the antagonist, propranolol, on paired-pulse inhibition at the granule cell/mitral cell reciprocal synapse, Wilson and Leon (1988) found activation of β-adrenoceptors caused disinhibition of mitral cells, while its blockade resulted in increased inhibition. The conflicting results may be attributable to methodological differences (Jiang et al., 1996). Differences in drug concentration, duration and sites of action in different preparations may all influence the actual NE effect observed. More work is needed on the pharmacology and mechanism of NE modulation of inhibition in the rat pup. The in vitro work suggests there is NE mediated disinhibition via α-adrenoceptors, but the role of α-adrenoceptors in early olfactory learning is unknown. α-adrenoceptors disinhibition has been implicated in another form of olfactory learning (Brennan and Keverne, 1997).

Early odor preference learning in the MOB is blocked by β-adrenoceptor antagonists. Norepinephrine has been proposed to promote learning-dependent behavioural and neural changes through β-adrenoceptors (Wilson and Sullivan, 1994). The results of Wilson and Leon in the rat pup support a β-adrenoceptor-mediated

disinhibition as possibly playing a critical role in early odor preference learning. Based on the physiological results together with the behavioural results and bulbar changes following behaviour, Sullivan and Wilson (1994) proposed that during associative odor preference learning, the UCS activates NE input to the olfactory bulb, resulting in disinhibition of mitral cells via a β-adrenoceptor effect. This UCS disinhibition facilitates the excitation of mitral cells and could produce an enhancement of the CS excitatory effect on mitral cells. However, in the Sullivan and Wilson model, the critical learning related change is hypothesized to be a strengthening of the mitral cell glutamatergic input to the granule cell such that stronger inhibition of mitral cells is seen with retrieval of the odor memory. This parallels other odor learning models as will be discussed in 1.1.3.1.

The discrepancy between the α-, and β-adrenoceptor mediated disinhibition again, may result from different experimental preparations (Brennan and Keverne, 1997). However, the presence of multiple receptor types for NE in the olfactory bulb suggests their roles in odor preference learning might be diverse and merit further investigation. For example, besides a possible inhibitory role, another mechanism of NE action might be to directly enhance mitral cell excitability (Jiang et al., 1996; Hayar et al., 2001) by blocking spike accommodation (Jiang et al., 1996). In addition, β-adrenoceptor mediated activation of the cAMP cascade has also been hypothesized to be critically involved in early preference learning as will be discussed in 1.1.3.2.1 and Chapter 2, 4, and 5.

1.1.2.2.2 Serotonin

Another olfactory bulb modulating system is the 5-HT input from the dorsal and median raphe nuclei (McLean and Shipley, 1987). Serotonergic fibers are extensively distributed in the olfactory bulb, however, only 5-$HT_{2A/2C}$ receptor subtypes have been examined in early odor preference learning. 5-HT_{2A} receptor mRNA (McLean et al., 1995) and protein (Hamada et al., 1998) are localized in mitral and tufted cells, and to a lesser extent, in periglomerular and granule cells (Hamada et al., 1998).

Earlier work from McLean's lab suggested that 5-HT acting through 5-$HT_{2A/2C}$ receptors promotes noradrenergic-induced plasticity (McLean et al., 1999). Although NE input appears to act as the critical UCS for odor preference learning, the effect depends on an intact serotonergic input. 5-HT_2 activation itself does not induce learning, but depletion of olfactory bulb 5-HT fibers by 5,7-dihydroxytryptamine (5,7-dHT) injection into the anterior olfactory nucleus on PND2 prevents the acquisition of odor preference induced by a normally effective dose of isoproterenol (McLean et al., 1993). Sufficient β-adrenoceptor activation using a higher dose of isoproterenol can overcome the requirement for 5-HT input during acquisition (Langdon et al., 1997) and an increase of 5-HT_2 receptor activation using the receptor agonist 2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) can promote learning when a subthreshold isoproterenol is given (Price et al., 1998).

These results led us to the hypothesis that noradrenergic and serotonergic inputs may act synergistically on the same cells to promote a cAMP/protein kinase A (PKA) phosphorylation cascade which has been critically implicated in other memory system such as in Aplysia and Drosophila models (Silva et al., 1998). β_1-adrenoceptors stimulate adenyl cyclase induced cAMP production via a G-protein (Prisco et al., 1993; McLean et al., 1999). In contrast, 5-HT receptor activation by itself does not increase cAMP expression (Morin et al., 1992; Rovescalli et al., 1993), which is consistent with the behavioural failure to induce learning by 5-HT activation alone. However, 5-HT_2 activation can enhance isoproterenol-induced intracellular cAMP expression through other second messenger systems (Rovescalli et al., 1993). Therefore, 5-HT could play a permissive role in conditioned odor learning induced by norepinephrine; 5-HT appears to set the stage for an effective noradrenergic action.

1.1.2.2.3 Other transmitters

1.1.2.2.3.1 Dopamine

There is a large population of DA neurons in the olfactory bulb glomerular layer: the cells surround the glomeruli and are known as periglomerular cells (Wilson and Sullivan, 1994). The role of DA in the odor preference learning has been investigated in one study. Systemic injection of the D_1 antagonist SKF 83566 immediately after

odor+stroking blocks odor preference formation. However, the effects of pretraining injections of SKF 83566 are blocked by post-training injections of the DA agonist apomorphine (Weldon et al., 1991). Thus, the role of DA in the odor preference learning may be limited to the post-training consolidation phase. However, more experiments are needed to clarify the role and mechanism of dopamine in early odor preference learning.

1.1.2.2.3.2 GABA

The granule cell GABAergic interneurons may play an important role in the olfactory circuit by providing modulation to the output neurons, mitral cells, during odor preference learning as suggested in the previous section. Granule cells inhibit mitral cells by releasing GABA onto mitral cells through dendrodendritic synapses between these two cell types. Blockage of GABA activation in the olfactory bulb by a pretraining injection of picrotoxin, which blocks $GABA_A$ receptor, disrupts early odor preference learning (Wilson and Sullivan, 1994).

Work with GABAergic antagonists in older pups suggests a possibly complex relationship between level of inhibition and learning. A study of GABAergic control of olfactory learning in 14-day-old rats (Okutani et al., 1999) showed that a low dose of bicuculline, a $GABA_A$ receptor antagonist, when infused into the olfactory bulb, induces an odor preference; whereas a high dose induces an odor aversion. A moderate dose of

bicuculline, however, is ineffective in inducing odor learning of either type. This result, argues for a critical balance of inhibition vs. disinhibition in initiating odor preference learning.

Since NE has also been proposed to induce odor learning through the promotion of disinhibition of mitral cells from granule cell interneurons, NE and GABA regulating pathways may share common local mechanisms (Brennan and Keverne, 1997).

1.1.2.2.3.3 Glutamate

The role of glutamate and glutamatergic receptors have been extensively studied in long-term synaptic plasticity such as LTP formation in the hippocampus. There are two classes of receptors: ionotropic glutamate receptors (iGluR) that are linked directly to ion channels (N-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA) receptors and kainate receptors) and metabotropic glutamate receptors (mGluRs) that are linked to G-proteins (6533).

The glutamatergic olfactory nerve synaptic input has both an AMPA receptor mediated component and an NMDA component (Aroniadou Anderjaska et al., 1997). Mitral/tufted cells are also glutamatergic neurons, which release glutamate onto GluRs on granule cells. However, the role of glutamate and glutamatergic receptors in olfactory

learning has only been extensively studied in the AOB pheromonal learning system. The pheromonal learning paradigm in mice is known as the Bruce effect, in which exposure of newly mated female mice to males, different from those that they had been mated with, causes pregnancy failure (Kaba et al., 1989).

In parallel with the effect of bicuculline, AOB infusion of iGluR antagonists cause disinhibition of mitral cells and can influence pheromonal learning. However, only the non-selective antagonist γ-D-glutamylglycine (DGG) or a combination of the selective NMDA antagonist D-2-amino-5-phosponovaleric acid (APV) and the selective AMPA antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) disrupt pheromonal memory formation, while blockade of either receptor alone, only results in pregnancy failure without disrupting memory formation (Brennan and Keverne, 1997).

In early olfactory preference learning dependent on the MOB, systemic injection of amino-5-phosphonopentanoic acid (AP5) for the first 18 days blocks the acquisition of an odor preference. Pre-training APV injection suppresses both the behavioural preference and enhanced olfactory bulb response to the learned odor (Lincoln, et al., 1988). However, NMDA antagonists may disrupt normal bulb function (Wilson and Sullivan, 1994; Brennan and Keverne, 1997), therefore more work should be done to clarify the role of the NMDA/AMPA receptors in olfactory preference learning.

Among the many subtypes of mGluRs, the role of mGluR2 is most studied in the AOB. mGluR2 are located in the dendritic spines of granule cells in AOB (Hayashi et al., 1993). By using the specific mGluR2 agonist (2S,2'R,3'R)-2-(2'3'-dicarboxycyclopropyl) glycine (DCG-IV), it was reported that DCG-IV markedly reduced the GABA-mediated inhibitory current from granule cell activation in slices of rat AOB (Hayashi et al., 1993). Kaba et al (1994) investigated whether AOB infusions of DCG-IV could promote pheromonal memory formation by reducing mitral cell inhibition from granule cells. Their results showed that DCG-IV infusions into AOB result in memory formation for male odors without mating. Figure 1.2 summaries the neural circuitry of the mGluR2 in the AOB.

mGluR2 also occurs in the main olfactory bulb (Petralia et al., 1996). One recent study showed that DCG-IV infusion into the MOBs of 1-week-old rat pups, induced an odor preference for the conditioned odor, peppermint (Rumsey et al., 2001). In both cases, DCG-IV acts as a UCS. These results suggest mGluR mediated disinhibition of mitral cells is an additional mechanism for the induction of early olfactory preference learning.

1.1.3 Candidate mechanisms underlying odor preference learning

Research on mechanisms of the neurobiology of learning and memory has focused

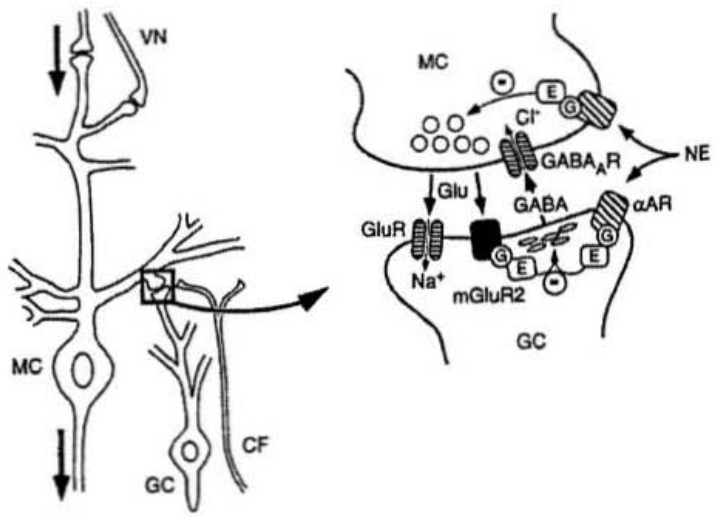

Figure 1.2 Neural circuitry and neurotransmitters between the mitral and granule cells in the AOB. Abbreviations: αAR, α-adrenergic receptor; CF, centrifugal fiber of norepinephrine projection from locus ceruleus; GABA, gamma-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; GC, granule cell; Glu, glutamate; GluR, glutamate receptor; mGluR, metabotropic glutamate receptor; MC, mitral cell; NE, norepinephrine; VN, vomeronasal nerve. Adapted from Kaba et al. Science. 265(5169):262-4 (1994)

on two levels: intercellular synaptic plasticity and intracellular signaling pathways. In terms of intercellular plasticity, in early odor preference learning, neuronal circuits between olfactory bulb output neurons and interneurons, especially the interaction between mitral cells and granule cells via dendrodendritic synapses has been studied extensively. A disinhibition model has been proposed in both pheromonal learning in the AOB and odor preference learning in the MOB.

In terms of intracellular signalling, long-term memory formation requires gene expression and new protein synthesis. cAMP response element binding protein (CREB) and CRE-mediated gene expression pathways have been critically implicated in long-term memory formation in animals as diverse as Aplysia, Drosophila, rats, and mice (Silva et al., 1998). A role for Ca^{2+}/cAMP-mediated phosphorylation signalling and protein phosphatase-mediated dephosphorylation has been suggested in a variety of olfactory learning paradigms (Brennan and Keverne, 1997).

1.1.3.1 Intercellular synaptic plasticity

This section briefly revisits the evidence for a role of disinhibition in olfactory learning. Reduction of inhibitory neurotransmission by manipulations of either NE, or GABA or $mGluR_2$ appears to be a common feature for the induction of different types of olfactory memory mechanisms, such as pheromonal memory formation in mice and odor

preference learning in rat pups, both of which have been characterized by an initial disinhibition of mitral cells from granule cell inhibition during the acquisition of the memory, followed by an enhanced inhibitory gain of the mitral – granule cell reciprocal synapses during the retrieval of the memory (Brennan and Keverne, 1997).

1.1.3.1.1 The AOB disinhibition model

In the AOB, the association of a mating male's pheromones with increased levels of NE after mating is thought to result in a long-lasting increase in the inhibitory gain of the mitral/granule cell synapses (Kaba et al., 1994; Brennan and Keverne, 1997). During subsequent pheromonal exposure, the mitral cells responding to the mating male's pheromones would be subject to greater self-inhibition and thus prevent the signal of the mating male's pheromones from being transmitted to activate central neuroendocrine mechanisms that cause pregnancy failure. The disinhibition effect of NE in AOB pheromonal learning system is mediated by α-adrenoceptor activation (Kaba et al., 1994).

1.1.3.1.2 The MOB disinhibition model

In the MOB, β-adrenoceptor activation induces a disinhibitory effect on granule cell inhibition during LOT (Wilson and Leon, 1988) stimulation. Norepinephrine has been proposed to promote learning and learning-dependent changes in the MOB by

disinhibiting mitral cells (Wilson and Sullivan, 1994). Odor learning induced changes include an increase in inhibitory neurotransmission upon odor re-exposure (Figure 1.3). Single-unit recording shows increased depression of mitral cell activation to the conditioned odor (Wilson et al., 1985; Wilson et al., 1987). The increased inhibition during odor re-exposure may be due to increased lateral inhibition, sharpening the mitral cell signal induced by the learned odor (Brennan and Keverne, 1997).

1.1.3.1.3 Disinhibition and the inverted U-curve

A simple disinhibiton model would not explain all the findings in early olfactory learning, such as the inverted U dose dependency curve observed with the β-adrenoceptor agonist isoproterenol, or the behavioural consequences resulting from different doses of bicuculline infused into the olfactory bulbs (Okutani et al., 1999). There may be a requirement for a critical balance between inhibition and disinhibition for learning to take place. Increased reciprocal inhibition induced by an increased intensity of mitral cell activation may override increased excitability resulting from granule cell disinhibition.

1.1.3.2 Intracellular signalling

Ca^{2+} and cAMP-dependent signalling cascades have been implicated in long-term memory formation in a variety of species in numerous studies which will be discussed in

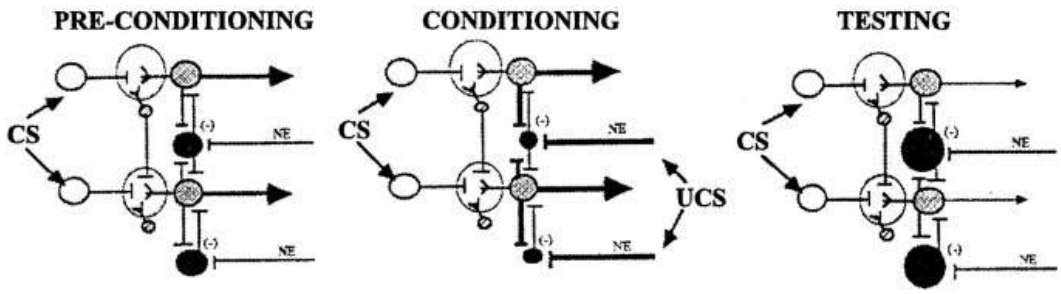

Figure 1.3 The disinhibition model for early odor preference learning proposed by Wilson and Sullivan. Adapted from Wilson and Sullivan. Behavioral and Neural Biology 61, 1-18 (1994)

1.3. Below is the hypothesis for the convergence of Ca^{2+} and cAMP-dependent signalling cascades in inducing the CREB pathway in early odor preference learning.

1.1.3.2.1 The cAMP/PKA/CREB hypothesis of early odor preference learning

McLean et al. (1999) proposed an intracellular model for odor preference learning in which a calcium signal initiated by the odor input via the olfactory nerve and a cAMP signal initiated by NE are required synergistically in mitral cells to elevate phosphorylated CREB (pCREB) and produce olfactory learning. The proposed model fits a Hebbian mechanism in that simultaneous activation in both the pre- and postsynaptic cells results in a long-lasting synaptic facilitation (Hebb,1949; Kandel et al. 2000, pp1260). While the disinhibition model of NE in odor preference learning predicts that granule cells are the critical substrates for learning, McLean et al (1999) hypothesized that mitral cells are the postsynaptic substrate for odor preference learning. The NE effect results from β-adrenoceptor activation of cAMP cascade in mitral cells. The association of Ca^{2+} entry through NMDA channel activation by odor input and cAMP signalling results in an enhanced olfactory nerve EPSP in the postsynaptic mitral cells and may promote a selectively enhanced mitral cell response to ON input during the memory phase.

1.2 A new strategy to study odor preference learning – optical imaging

The development of advanced new techniques has enriched our knowledge of olfactory coding and information processing within the olfactory bulb. The topography of the olfactory bulb has been explored with various techniques that permit visualization of the activity of olfactory neurons from metabolic mapping of 2-DG (Coopersmith and Leon, 1984; Johnson and Leon, 1996; Johnson et al., 1998) or immediate-early gene c-Fos expression (Matsuda et al., 1990; Guthrie et al., 1993; Guthrie and Gall, 1995), to recent utilization of optical imaging (Katz and Rubin, 1999; Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001), and functional magnetic resonance imaging (fMRI) (Yang et al., 1998; Xu et al., 2000). Among these techniques, optical imaging provides high spatial (intrinsic imaging) and temporal resolution (calcium imaging) (Galizia et al., 1999; Katz and Rubin, 1999). This method permits fast, noninvasive in vivo visualization of neuronal activity and repeated measurements from the same animal. Its utilization has been extensively explored in the visual system, the barrel cortex, and recently, in the olfactory system. It provides a new methodology to explore neuronal changes following odor learning.

1.2.1 Olfactory encoding

Olfactory encoding is particularly amenable to optical imaging because the initial

encoding of odor information takes place in the glomeruli, which is immediately below the surface of the bulb. An odorant is first detected by olfactory receptor neurons located in the olfactory epithelium. In the mammalian olfactory epithelium, each sensory neuron expresses only one olfactory receptor gene. In rats, a large multigene family codes for more than 1000 olfactory receptors. Neurons expressing an olfactory receptor are confined to one of the four olfactory receptor expression zones in the epithelium, where they are scattered throughout the zone with neurons expressing other olfactory receptors (Dudai, 1999; Kandel et al. 2000, pp630). The receptor neurons transmit odor information via unmyelinated axons to the glomeruli of the olfactory bulb (approximately 1000 glomeruli in mouse olfactory bulb, 2000 in rat bulb. Dudai, 1999). In the bulb, axons of receptor neurons expressing the same olfactory receptor converge onto a few glomeruli. Glomeruli are considered as the functional units of the olfactory bulb. Information about different odorants are coded in the glomeruli, in the sense that one odorant is coded by a combinational activation pattern in glomeruli: 1) each glomerulus recognizes one component (molecular feature) of the odorant; and 2) the pattern of the presentation of an odorant in a group of glomeruli determines odor coding. Therefore, the first step in central olfactory processing involves transformation of a chemical code (molecular features of an odorant deciphered by its binding to the olfactory receptors) into a distributed place code (Dudai, 1999).

1.2.2 Intrinsic signal imaging

Intrinsic signal imaging is a powerful method of analysing activity-dependent patterns of neuronal populations in the brain. Since Rubin and Katz (1999) first employed this technique to explore olfactory encoding in the rat, numerous studies have endeavoured to decipher odor coding in the olfactory bulb. Intrinsic signals are due to activity-dependent neuronal changes, which are reflected as optical changes of the imaged tissue. Activity-dependent changes involve hemodynamic changes such as a change in blood volume, oxygenation of hemoglobin, or light scattering changes caused by the local movement of ions and released transmitters (Dudai, 1999). Intrinsic imaging has an excellent spatial resolution (<50μm) which allows the measurement of neuronal changes at the level of glomeruli on the dorsal surface of the olfactory bulb.

Optical imaging of intrinsic signals in the rat olfactory bulb has revealed detailed spatial patterns of glomerular activation representing different odorants, the concentration dependence of glomerular activation, and the molecular receptive range of specific glomeruli (Dudai, 1999; Katz and Rubin, 1999; Uchida et al., 2000). The functional representation of odorant molecules revealed by intrinsic imaging conforms to a series of basic principles: 1) bilateral symmetry of odor representations; 2) local clustering of glomeruli activation; and, 3) local variability of odor presentations between animals (Katz and Rubin, 1999; Uchida et al., 2000; Belluscio and Katz, 2001).

Intrinsic optical imaging has its limits. One shortcoming is that only odor-induced activity changes on the surface of the olfactory bulb are revealed, not the cellular source of the activity (Dudai, 1999). The odor representation patterns reported from intrinsic imaging studies are postulated to occur in glomeruli based on the location, size, and shape of the active regions. However, a combination of optical imaging with voltage-sensitive dyes or electrophysiological measurement would help identify the cellular source of the neuronal activity. Another limit of intrinsic imaging is its relatively slow temporal resolution (seconds after odor application). In contrast, calcium imaging, has better temporal resolution.

1.2.3 Implications of optical techniques for the study of odor learning

Dudai, in his review paper (1999), predicted that the power of intrinsic optical imaging in the olfactory bulb is not only in illuminating the functional architecture of the bulb, but it is a promising technology to visualize the olfactory brain in action. As mentioned previously, one characteristic of intrinsic imaging is its non-invasiveness. Activity-dependent changes can be visualized through thinned bone or intact dura and repeated imaging can be applied to the same animal over a protracted period. Therefore, it would be especially beneficial for exploring experience-dependent modification of odor representation in the olfactory bulb, which may underlie odor learning and memory. Recording brain activity in vivo during learning is fundamental to understanding how

memories are formed (Faber et al., 1999).

Activity-dependent changes with odor experience have been reported in honeybee antennal lobes (an equivalent structure to the olfactory bulb in mammals). Faber et al.(1999) trained individual bees to discriminate a rewarded odor from an unrewarded odor. The rewarded odor (CS) is paired with sucrose solution (UCS), applied to the proboscis. Acquisition of the odor-reward association leads to an increase of the representation of the rewarded odor and different activity patterns representing rewarded and unrewarded stimuli. This is promising for similar research in the mammalian olfactory bulb. The present thesis takes advantage of this methodology to explore odor encoding following memory formation in early odor preference learning.

1.3 CREB, synaptic plasticity and memory

Learning and memory are strongly associated with synaptic plasticity in the CNS. Long-term memory formation requires new protein synthesis. CREB- dependent intracellular pathways are thought to be pivotal in mediating the transition from short-term memory, which lasts only 1-2 hours, to long-term memory, which lasts days, or even a lifetime. In a variety of species from Aplysia, Drosophila to mice and rats, CREB-dependent transcription appears to be a crucial component underpinning long-term memory formation (Silva et al., 1998). CREB levels appear to be delicately regulated in

memory systems; disrupting normal CREB functioning impairs long-term memory.

1.3.1 CREB and transcription

CREB is a member of a large family of transcriptional factors that bind to promoter cAMP responsive element (CRE) sites (Silva et al., 1998; Walton and Dragunow, 2000). The CREB transcriptional family of proteins consists of three functional domains: a leucine-zipper domain (bZIP) that mediates dimerization, a DNA-binding domain, and the transcriptional activation domain. Based on the differentiation of bZIP, members of the CREB family can be divided into three groups: activator CREB, repressor cAMP response element modulator (CREM), and activating transcription factor (ATF). CREB protein has three alternatively spliced isoforms, α, β and Δ. The repressor CREM gene consists of at least four different factors: CREM α, β, γ and inducible cAMP early repressor (ICER).

CREB has been implicated in the transcriptional control of numerous genes, such as immediate early genes c-Fos, c-jun, Egr-1, Bcl-2 (Walton and Dragunow, 2000). Many of these genes are expressed rapidly in response to an elevation of intracellular cAMP or Ca^{2+} levels. The transcriptional control of CREB on gene expression has been shown to be involved in a variety of biophysiological phenomena such as neuronal survival (Walton and Dragunow, 2000), learning and memory (Silva et al., 1998), drug addiction

(Lane-Ladd et al., 1997) and tumorogenesis.(Xie et al., 1997).

1.3.1.1 CREB phosphorylation and transcriptional activation

The transcriptional activation of CREB is crucially dependent on phosphorylation of Ser 133 by various protein kinases such as PKA, Ca^{2+}/CaM kinases, ribosomal S6 kinase 2, or mitogen-activated protein-kinase activated protein kinase 2 (MAPKK2) (Silva et al., 1998). The phosphorylation of CREB by different kinase pathways may be a mechanism for the convergence of these pathways to regulate downstream gene expression (Sheng et al., 1991; Silva et al., 1998).

CREB phosophorylation has been postulated as an initial step in the transcriptional control of synaptic plasticity underlying learning and memory (McLean et al., 1999). In invertebrates such as Aplysia, multiple pulses of 5-HT result in an increase in cAMP in the sensory neuron. This in turn activates the catalytic subunits of PKA to translocate into the nucleus, where they phosphorylate CREB and thus activate the transcription of IEGs. IEG activation may turn on the transcription of late response genes such as cytoskeletal proteins, adhesion molecules and neurotrophins/receptors which might encode proteins necessary for long-term synaptic plasticity (Frank and Greenberg, 1994).

CREB activation in the mammalian hippocampus appears to be more complicated than that observed in the invertebrate CNS. Studies from Bito's lab (Bito et al., 1996; Deisseroth et al., 1996) have focused on the cellular processes that regulate the phosphorylation state of CREB in hippocampal neurons. When pCREB was monitored at the single-cell level with an antibody specific for the Ser-133 phosphorylation site, they reported two important occurrences. First, NMDA- dependent synaptic stimulation, but not action potential firing, results in CREB phosphorylation, suggesting that CREB phosphorylation is a specific synaptic signalling marker engaged by both NMDA receptors and L-type calcium channels (Deisseroth et al., 1996; Mermelstein et al., 2000). Second, phosphorylation of CREB is necessary, but not sufficient, for the stimulation of CRE-mediated gene expression in the hippocampus; only sustained phosphorylation of CREB by a prolonged stimulus (5Hz for 180s instead of 18s) induced gene expression such as c-Fos and ss-14 (Bito et al., 1996). Furthermore, they provided strong evidence for the involvement of the Ca^{2+}/CaM pathway in inducing CREB phosphorylation in hippocampal neurons. CaM kinase IV (CaMKIV) is expressed in the nucleus at a time consistent with the appearance of pCREB (Bito et al., 1996) Both pCREB-CREB binding protein (CBP) formation and CaMKIV were blocked by the CaMK inhibitor KN-93, but a PKA inhibitor, KT5720, failed to block CREB phosphorylation (Bito et al., 1996; Deisseroth et al., 1996).

In contrast, Impey et al. (1996) argued for a critical role of PKA in activating

CREB-dependent transcription in hippocampal slices from transgenic mice, CRE-LacZ. PKA inhibitors blocked CREB-dependent transcription in hippocampal slices from the CRE-lacZ mice (Impey et al., 1996). PKA facilitates the MAPK (ERK) pathway phosphorylation of CREB (Impey et al., 1998a). The controversial outcomes may be explained by the different kinetics of protein kinases (Impey et al., 1998a). CaMKIV may mediate an early phase of CREB phosphorylation, while the sustained or late phase of CREB phosphorylation may require PKA/MAPK co-activation. Alternatively, different protein kinase pathway interactions may exist. For example, CaMKIV can also activate MAP kinases (Enslen et al., 1996). It is likely that CaMKIV and MAPK-dependent pathways co-operate to induce pCREB mediated gene activation (Silva et al., 1998).

1.3.1.2 Transcriptional repression

Two processes are responsible for regulating CREB phosphorylation and CRE-mediated gene expression.

First, dephosphorylation of pCREB at Ser133 is important for the inactivation of CREB. The protein phosphatases, calcineurin and protein phosphatase-1 (PP1), are thought to be involved in dephosphorylation of CREB (Blitzer et al., 1995; Liu and Graybiel, 1996; Blitzer et al., 1998; Winder et al., 1998). Treatment with FK506, a calcineurin inhibitor, enhances the duration of CREB phosphorylation and therefore

induces c-Fos and ss-14 expression by a previous ineffective brief stimulus (18s) in hippocampal cultures (Bito et al., 1996). One role of PKA is thought to be to "gate" the Ca^{2+}/CaMK phosphorylation pathway by suppressing phosphatase calcineurin via inhibitor-1 (Blitzer et al., 1998; Winder et al., 1998).

Second, CREB repressors such as CREM α, β, γ and ICER block the activation of CREB. CREM α, β and γ do not have the transcriptional domain, but compete with CREB to bind CRE sites (Foulkes et al., 1991). CREB repressors can be upregulated by CREB activation or CREB mutation (Silva et al., 1998). The ratio of CREB activator to repressor appears to be important in regulating memory formation (Silva et al., 1998).

1.3.2 The role of CREB in memory

Rapid progress has been made in understanding the molecular mechanisms of learning and memory by advanced genetic, pharmacological, and eletrophysiological techniques. For example, synaptic plasticity such as the phenomena of LTP was reported decades ago (Bliss et al., 1973). A brief high-frequency train of stimuli (a tetanus) to any of the three major synaptic pathways of the hippocampus increases the amplitude of the excitatory postsynaptic potentials in the target hippocampal neurons. Results from different studies all suggest that the intracellular cAMP-regulated CREB pathway plays a key role in these forms of LTP and in the long-term memory formation.

1.3.2.1 Long-term facilitation in Aplysia

The early studies on the invertebrate mollusk Aplysia have been invaluable for our understanding of the basic forms of associative learning. Aplysia withdraws its gill and siphon when a noxious stimulus is applied to its tail. A single stimulus produces a short-term sensitization (mins) to a subsequent stimulus, while repeated stimulation leads to long-term (hours to days) sensitization. The facilitation of the synapse between the sensory and the motor neurons is thought to be critical in mediating behavioural sensitization. The neurotransmitter serotonin, released from interneurons after stimulation of the Aplysia tail, leads to an enhanced synaptic transmission between the sensory and the motor neurons (Montarolo et al., 1986).

This Aplysia model for learning can be replicated by a reduced preparation in vitro. When a single Aplysia sensory neuron is co-cultured with a motor neuron, the two cells form a synapse. Multiple applications of 5-HT lead to both a long-term facilitation (LTF) of synaptic function and a long-term sensitization (LTS) behaviourally. Serotonin application results in the activation of adenylyl cyclase, which in turn, activates the cAMP second messenger system. cAMP activation of PKA leads to the subsequent translocation of the catalytic subunits of PKA to the nucleus to active CREB-dependent transcription of genes, which eventually leads to the growth of new synaptic connections (Frank and Greenberg, 1994; Silva et al., 1998). Direct injection of cAMP into the sensory neuron

triggers both short- and long-term facilitation (Schacher et al., 1988), which can be blocked by PKA inhibitors (Ghirardi et al., 1992) .

The first study to suggest that CREB is required for this LTS formation was reported by Dash et al (1990). Oligonucleotides with CRE sequences injected into cultured sensory neurons selectively blocked LTF, but not short-term facilitation (STF). A more recent study also showed that induction of LTF triggers CREB activation and CRE-mediated transcription by using a lacZ reporter gene transferred into individual Aplysia sensory neurons (Kaang et al., 1993)

The repeated pulses of 5-HT initiate a gene activation cascade that leads ultimately to the growth of new synaptic connections (Martin et al., 1997). Several genes have been identified in this process, including apCREB1, apCREB2, apCCAAT/enhancer binding protein (C/EBP), and the cell adhesion molecule apCAM. ApCREB2 represses ApCREB1-mediated transcription (Bartsch et al., 1995). Opposing forms of CREB (activator CREB1a vs. repressors CREB1b and CREB2) produce opposite effects on long-term facilitation (Bartsch et al., 1995; Bartsch et al., 1998). The injection of antibodies to ApCREB1 or antisense oligonucleotides to ApCREB1 into the sensory neurons selectively blocked long-term facilitation. On the other hand, injection of phosphorylated CREB-1 into the cell body (Barco et al., 2002) or injection of an antiserum against a CREB repressor (ApCREB2) (Bartsch et al., 1995) gives rise to long-

term memory by one pulse of serotonin, which normally only results in short-term facilitation in normal conditions. With five pulses of 5-HT, PKA recruits MAP kinase and both translocate to the nucleus, where they activate ApCREB1 and de-repress ApCREB2, leading to the induction of a set of immediate-early genes which are associated with late genes that are responsible for the growth of new synaptic connections (Martin et al., 1997).

1.3.2.2 cAMP and Drosophila

The importance of cAMP signalling and the CREB pathway for memory formation has been demonstrated in the fruitfly, Drosophila. Drosophila form robust and reliable olfactory discrimination memories. When Drosophila are exposed to two odors, if one is paired with an electric shock, they learn to avoid the paired odor in a T-maze test (Tully, 1991).

The involvement of the cAMP pathway has been shown in Drosophila olfactory learning by genetic manipulations. Four mutants that showed abnormal levels of cAMP also exhibited learning deficits: 1) *Dunce*, which lacks phosphodiesterase, an enzyme that degrades cAMP and therefore has a high level of cAMP; 2) *Rutabaga*, which is defective in adenylyl cyclase and therefore has a low level of cAMP; 3) *Amnesiac* which lacks a peptide transmitter acting on adenylyl cyclase, and, 4) PKA-R1 which is defective in

PKA (Kandel et al. 2000, pp1257). Furthermore, when Drain et al.(1991) generated a transgene that blocked the catalytic subunit of PKA under an heat-sensitive inducible promoter, they found that even transient blockade of PKA interfered with the fly's ability to learn and form short-term olfactory memories. These outcomes from Drosophila mutants suggest an important role for cAMP/PKA involvement in Drosophila olfactory learning.

Long-term associative memory in Drosophila requires CREB pathway activation and new protein synthesis. As in Aplysia, Drosophila has both a CREB activator and a CREB-2 repressor. Overexpression of the CREB activator enhances long-term memory, whereas over-expression of the repressor selectively blocks long-term memory without disrupting short-term memory (Yin et al., 1994; 1995).

1.3.2.3 CREB in transgenic mice

The role of CREB in synaptic plasticity and memory formation has been greatly advanced by neurogenetic manipulations in mice. The role of cAMP/PKA signalling in LTP and long-term memory was demonstrated by using a transgenic approach to reduce PKA activity in the hippocampus by using R(AB), a dominant negative form of the RI_{∂} regulatory subunit of PKA (Brandon et al., 1995). Recordings from slices of R(AB) mice showed impaired Late-LTP (L-LTP, lasts for at least 24 hours) but not Early-LTP (E-

LTP, lasts only for 1-2 hours) induced in the Schaffer collateral pathway. In parallel, when R(AB) mice were tested for their memory function, they exhibited normal short-term memory, but deficient long-term memory for contextual fear conditioning. These results suggest that L-LTP and long-term memory require cAMP/PKA second-messenger pathways in the hippocampus.

In $CREB^{\alpha\Delta-}$ mice, a neomycin resistance gene insertion causes the loss of the two main CREB isoforms α and Δ. However, CREB β and CREM isoforms showed enhanced expression levels in $CREB^{\alpha\Delta-}$ mice (Hummler et al., 1994). The issue of whether the $CREB^{\alpha\Delta-}$ mutation affected memory was tested in three behavioural tasks, each thought to be dependent upon hippocampal function (Silva et al., 1998): contextual fear conditioning, the Morris water maze, and the social transmission of food preferences. $CREB^{\alpha\Delta-}$ mice showed severe contextual memory deficits when tested 24 hr, but not 30 min, after training (Bourtchuladze et al., 1994). Also, the $CREB^{\alpha\Delta-}$ mice in the social transmission of food preference task showed impaired long-term, but not short term memory (Kogan et al., 1997). $CREB^{\alpha\Delta-}$ mice demonstrated profound spatial learning and memory deficits in the Morris water maze test, but not in a visible platform version of the water maze, which does not depend on hippocampal function (Silva et al., 1998). Importantly, these behavioural deficits did not appear to result from the more generalized impact of the mutation on CNS development (Bourtchuladze et al., 1994).

Unfortunately, different isoforms of CREB as well as CREM up-regulation and the potential developmental and general impact of such mutations have made the interpretation of behavioural results complicated. Restricted and regulated expression of a constitutively active form of CREB, has been constructed in hippocampal CA1 neurons of VP16-CREB mice (Barco et al., 2002). The induction of the VP16-CREB transgene lowers the threshold for eliciting Late-LTP in the Schaffer collateral pathway. Synaptic tagging and capture have been outlined as a novel property of hippocampal LTP (Frey and Morris, 1998; Barco et al., 2002). The induction of LTP is associated with the setting of a "synaptic tag" at activated synapses, whose role is to sequester plasticity-related proteins that then serve to stabilize temporary synaptic changes and so extend their persistence (Frey and Morris, 1998). Pharmacological and two-pathway experiments suggest a model in which VP16-CREB activates the transcription of CRE-driven genes and leads to a cell-wide distribution of proteins that prime the synapses for subsequent synapse-specific capture of Late-LTP by a weak stimulus. This result argues that activation of a CRE-driven pathway may be sufficient for consolidation of LTP (Barco et al., 2002).

1.3.2.4 CREB studies in rats

The design of CREB antisense oligonucleotides enables the acute modulation of CREB levels in specific brain areas in rats. Intrahippocampal infusion of these

oligonucleotides prior to training does not disrupt short-term spatial memory, but does affect long-term memory for the water maze tested two days after training (Guzowski and McGaugh, 1997). This is consistent with other studies showing CREB pathway involvement in long-term memory. Furthermore, the same study shows the critical period of CREB function is shortly after training, since infusion of the oligonucleotides one day after training does not affect memory two days after training. In another study (Lamprecht et al., 1997), the neural mechanisms of a conditioned taste aversion were investigated in amygdala. Local injection of CREB oligodeoxynucleotide antisense into the rat amygdala several hours before conditioned taste aversion training transiently reduces the level of CREB protein during training and impairs memory when tested 3-5 days later. CREB antisense in the amygdala has no effect on retrieval of a conditioned taste aversion memory once it has been acquired and does not affect short-term memory.

Long-term memory formation requires de novo protein synthesis and CREB may be one of the transcription factors which is required for the new protein synthesis. It therefore is proposed as a molecular switch for the formation of long-term memory. From invertebrates (e.g., Aplysia and Drosophila) to mammals, spaced training (repeated training trials presented with optimal rest intervals) is more effective than massed training (the same training protocal presented with no or shorter rest intervals) in producing long-term memory. Massed fear conditioning in rats produces no or weak long-term memory. However, increasing CREB levels, specifically in the basolateral amygdala via viral

vector- mediated gene transfer, significantly increases long-term memory after massed fear training (Josselyn et al., 2002). In contrast, overexpression of CREB does not alter short-term memory produced by massed training or long-term memory produced by spaced training.

As mentioned before, the levels of CREB are controlled at both the activation level (phosphorylation vs. dephosphorylation) and the transcription level (activator vs. repressor). At the first level, it has been reported that it takes 3-8 min for synaptic activation to trigger maximal CREB phosphorylation (Moore et al., 1996). Additionally, the longer intervals between training trials may result in optimal inactivation of phosphatases (e.g. calcineurin), which may produce longer CREB phosphorylation (Bito et al., 1996; Liu and Graybiel, 1996; Silva et al., 1998). The duration, but not necessarily the amount of CREB phosphorylation, is critical in producing CRE-mediated gene expression (Bito et al., 1996). At the second level, massed training may result in excessive activation of CREB repressors, reducing the ratio of activator/repressor activation. Application of additional CREB activator into the amygdala by virus vector injection therefore enhanced learning from massed training by enhancing activator/repressor ratio (Josselyn et al., 2002). Alternatively, activators can assume the role of repressors on occasion. An excess of activator may overwhelm the upstream kinases and result in excessive nonphosphorylated CREB which can act as a repressor of C/EBP- induced transcription (Vallejo et al., 1995; Silva et al., 1998).

Phosphorylation of CREB is thought to be important in processes underlying long-term memory. Overexpression of mutant CREB, with a single point mutation at Ser133, does not facilitate long-term memory (Josselyn et al., 2002). Moreover, CREB is phosphorylated in the CA1 pyramidal cells following electrical stimuli that induce LTP and after training in hippocampal-dependent tasks (Impey et al., 1998b). Increased pCREB is also present in the olfactory bulb shortly after conditioned odor preference training (McLean et al., 1999). CREB can be phosphorylated by various cascades, such as PKA, CaMKIV, MAPK, all of which have been implicated in late-LTP. Therefore, CREB is a strong candidate for the activation of CRE-driven gene expression observed during memory formation (Barco et al., 2002).

1.4 Rationale and hypotheses for the present thesis

McLean et al (1999) hypothesized an intracellular model for early odor preference learning. They proposed that NE and 5-HT interact in mitral cells of the olfactory bulb to elevate cAMP levels, which in turn, synergise with a calcium signal initiated by the odor input to activate the CREB phosphorylation pathway crucially implicated in memory formation. In addition to suggesting that mitral cells are the primary target of CS-UCS interaction, the model implies that the strengthening of the odor input to mitral cell connection and a subsequent increase in mitral cell response to the CS is a critical aspect of the memory representation. The work in this thesis evaluates and refines this model.

As previously reviewed, pairing odor with stroking or β-adrenoceptor activation induces early odor preference learning. McLean et al (1999) had demonstrated that stroking-induced preference learning was associated with increased levels of pCREB in mitral cells. In first series of experiments (Chapter 2), we hypothesize that NE and 5-HT act synergistically to increase CREB phosphorylation during odor conditioning and that their interaction potentiates olfactory nerve input to mitral cells. In this set of experiments, we ask if significant pCREB increases are also associated with isoproterenol-induced learning and whether pCREB follows the inverted U pattern seen with learning (Sullivan et al., 1991) or is simply dose-dependently related to β-adrenoceptor activation. Electrophysiological indices of odor nerve input before and after injection of isoproterenol in both normal pups and olfactory bulb 5-HT depleted pups are also evaluated.

The second series of experiments focuses on identifying changes in the response to odor input that occur during memory retrieval using intrinsic optical imaging. These experiments test the hypothesis that a strengthening of the CS-mitral cell input in the glomerular layer characterizes the memory representation. Previous work on the early odor preference model (Wilson and Sullivan, 1994) and in the accessory olfactory bulb (Brennan and Keverne, 1997) suggested instead that increased inhibition of mitral cells was the primary associate of the memory representation. The second series of experiments (Chapter 3) uses intrinsic optical imaging to examine odor-induced

activation at the glomerular level at the time of retrieval to further evaluate this issue.

In the third series of experiments (Chapter 4), the hypothesis that NE and 5-HT interact synergistically to produce an optimal cAMP level in the mitral cells of the olfactory bulb in early odor preference learning is examined. The changes in cAMP associated with stroking and isoproterenol-induced learning are examined with particular attention to the effects of 5-HT depletion. In earlier experiments it had been shown that prior depletion of 5-HT in the olfactory bulb prevents learning (McLean et al., 1993) unless a higher, normally ineffective dose of isoproterenol is used (Langdon et al., 1997). In these 5-HT depletion experiments the $5HT_{2A/2C}$ receptor was identified as playing a critical role, both by using DOI as an agonist to restore normal learning (Price et al., 1998) and ritanserin as an antagonist to prevent normal learning (McLean et al., 1996). Experiments in rat neocortical slices had demonstrated that cAMP activation by isoproterenol is enhanced by activation of the $5HT_{2A/2C}$ receptor and that in the presence of a $5HT_{2A/2C}$ antagonist there is reduced production of cAMP induced by isoproterenol (Morin et al., 1992). These results paralleled the 5-HT effects seen in the rat pup with the same drugs. Thus, we ask here, if cAMP levels relate to the behavioural effects of stroking and isoproterenol in normal learning and whether 5-HT depletion alters cAMP levels in the olfactory bulb and, specifically, in the mitral cells as predicted by our hypothesis.

The final set of experiments (Chapter 5) evaluates the causality of CREB's role in early odor preference learning by directly manipulating CREB using an HSV viral vector. Although previous work (McLean et al., 1999) demonstrated CREB phosphorylation following odor preference learning, the evidence for a role for pCREB was correlational rather than causal. We hypothesize in this study that increased CREB substrate would shift the isoproterenol dose-dependent learning curve to the left, and mutant CREB substrate would interefere with normal learning. The relation of the effects of CREB in early odor preference learning to pCREB levels is also assessed.

Chapter 2. Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve Evoked Potentials in Normal and Bulbar 5HT-Depleted Rat Pups only at Doses that Produce Odor Preference Learning

(Published in Learning & Memory 7:413-421, 2000)

2.1 Introduction

The neonate rat forms an odor preference to odors that are paired with either tactile stimulation (stroking) or 2 mg/kg of the β-adrenoceptor agonist isoproterenol (Sullivan et al., 1989b; Sullivan and Wilson, 1991; Langdon et al., 1997). In this early olfactory learning paradigm, stroking has been shown to activate the locus coeruleus (Nakamura et al., 1987), which releases norepinephrine in the main olfactory bulb, and engages β-adrenoceptors (Sullivan and Wilson, 1994; Woo et al., 1996). Intrabulbar infusion of the β-adrenoceptor antagonist, propranolol, prevents the development of the conditioned odor preference (Sullivan et al., 1989b). Co-activation of the glutamatergic olfactory nerve input (Berkowicz et al., 1994; Ennis et al., 1996) and β-adrenoceptors is hypothesized to be critical for triggering the long-term change in olfactory bulb processing which mediates conditioned odor preference learning (McLean et al., 1999). The pairing of odor with the β-adrenoceptor agonist exhibits an inverted U-curve with

both a lower dose (1 mg/kg) and a higher dose (4 mg/kg) of isoproterenol being ineffective relative to the moderate dose (2 mg/kg) that is optimal for learning (Sullivan et al., 1989b; Langdon et al., 1997). Selective serotonin (5-HT) fiber depletion in the olfactory bulb of rat pups shifts the isoproterenol inverted U-curve such that a higher dose (6 mg/kg) is now required for learning while the normal optimal moderate dose (2 mg/kg) is ineffective (Langdon et al., 1997).

Electrophysiological recordings carried out in the olfactory bulb of rat pups that have undergone conditioned odor preference training have revealed a significant decrease in the ratio of excitation to inhibition in single unit mitral cell activity recorded in the olfactory bulb (Wilson and Sullivan, 1991). However, it was not possible in the unit recording studies to know if the mitral cells encoding the learned odor were selectively sampled. The increased inhibition might reflect increased lateral inhibition concomitant with stronger signalling in the conditioned odor pathway. Disinhibition of mitral cells from granular cell GABAergic effects at dendrodendritic granule cell-mitral cell synapses has been suggested to play a critical role in conditioned olfactory learning (Jahr and Nicoll, 1982; Okutani et al., 1999). Such an effect might be expected to potentiate, rather than inhibit olfactory nerve (ON) throughput during acquisition. The occurrence of disinhibition is supported by evidence that norepinephrine (NE) applied to the external plexiform layer (EPL) decreases inhibitory postsynaptic potentials recorded in the granular cell layer by stimulating olfactory nerves (Jahr and Nicoll, 1982). Paired pulse

inhibition of the mitral cells is also suppressed by injections of NE or isoproterenol (Jahr and Nicoll, 1982; Wilson and Leon, 1988).

In another direction, extensive research has been carried out to identify the molecular components of synaptic plasticity underlying memory formation. Emerging from these studies, cAMP response element binding protein (CREB) has been identified as an important modulator of memory formation (Silva et al., 1998). Its activation is required to initiate the cellular events underlying long-term memory formation in a variety of species (Yin and Tully, 1996; Abel and Kandel, 1998; Bartsch et al., 1998; Silva et al., 1998). CREB phosphorylation at ser 133 by different protein kinases has been implicated as the initial step of CRE-related gene transcription (Walton and Dragunow, 2000). Down regulation of phosphorylated CREB (pCREB) or of related protein kinases impairs long-term memory formation (Silva et al., 1998). Electrophysiological evidence in hippocampal cultures (Bito et al., 1996; Deisseroth et al., 1998; Mermelstein et al., 2000) suggests CREB phosphorylation responds to specific synaptic signals engaging both NMDA receptors and L-type calcium channels. Increased pCREB activation has been shown to occur in the mitral cells of rat pup olfactory bulbs that are pre-treated with effective pairings of odor and stroking (McLean et al., 1999).

In the present study, we asked, first, whether the pairing of odor with a behaviourally effective dose of isoproterenol (2 mg/kg) could produce the same pCREB

increase seen previously with stroking. Further, we examined whether a higher dose of isoproterenol (6mg/kg), which cannot induce preference learning in normal rat pups, when paired with odor, could still increase pCREB. Then, the importance of pCREB in the mediation of serotonergic modulation of NE-induced odor preference in rat pups was examined by using selective serotonergic depletion of the olfactory bulb. If pCREB is specifically involved in learning, a higher dose of isoproterenol (6 mg/kg) should now be required to enhance pCREB expression. This would parallel the previous behavioural model in which selective 5-HT depletion of olfactory bulbs shifted the effective dose of isoproterenol, so that a higher dose (6 mg/kg) was required to induce odor preference learning.

Second, to illuminate possible changes in synaptic transmission in olfactory circuitry, which may trigger the subcellular signal transduction underlying associative learning, or functionally support such learning, we asked whether behaviourally optimal doses of isoproterenol altered ON-evoked potentials. Normal rat pups, and pups with bulbar 5-HT depletion, were again tested.

If pCREB is critical for odor preference learning, and odor preference learning alters the response to ON input, we expected that only behaviourally optimal doses of isoproterenol would selectively change both pCREB expression and the ON-evoked potential in rat pups with either normal or 5-HT depleted olfactory bulbs.

2.2 Experiment 1. Increased pCREB expressions following manipulation of NE and 5-HT inputs to the olfactory bulb correlate with odor preference learning in neonate rats.

To test the hypothesis that pairing odor with a behaviourally effective dose of the β-adrenoceptor agonist isoproterenol triggers phosphorylation of CREB (a postulated signalling substrate for learning), we subcutaneously injected isoproterenol into normal rat pups 40 min before conditioned pairing with odor (**Experiment 1a**). Isoproterenol can completely substitute for stroking as an unconditioned stimulus during odor conditioning (Sullivan et al., 1989b). This effect is consistent with the observed activation of locus coeruleus neurons, the source of NE in neonate rat olfactory bulbs, by somatosensory stimulation (Nakamura et al., 1987). Since a selective pCREB increase was observed in olfactory conditioned preference learning (increased pCREB in the bulbs of odor conditioned pups, but not in the bulbs of the pups that were trained by odor or stroke alone, McLean et al., 1999), we hypothesized that the intracellular cAMP second messenger system, activated by NE input to the β-receptor, works synergistically with the Ca^{2+} entry triggered by glutamatergic olfactory input, to influence CREB phosphorylation and the downstream gene transcription which are necessary for long-term memory formation. In the present study, direct activation of the β-receptor by isoproterenol should have the same effect as the tactile stimulation in odor preference learning. In addition, given the observation that 5-HT normally promotes the efficacy of β-adrenoceptor agonist

isoproterenol as an unconditioned stimulus during olfactory learning, we predicted that a higher dose of isoproterenol would be required in 5-HT depleted animals to induce a comparable increase in pCREB to that observed in normal animals (**Experiment 1b**).

2.2.1 Methods

In **Experiment 1a**, a total of 95 Sprague-Dawley rats of both sexes from 8 litters were used. In **Experiment 1b**, 33 rat pups from 6 litters were used. Three groups were included in each experiment: a saline group, a 2 mg/kg isoproterenol group and a 6 mg/kg isoproterenol group. Litters were culled to 12 pups/litter on PND1 (the day of birth is considered PND0). The dams were maintained under a 12hr light-dark cycle, with *ad libitum* access to food and water. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

2.2.1.1 Odor conditioning and drug injection

The procedure for conditioning has been described previously (Langdon et al., 1997). Briefly, on PND6, saline or isoproterenol 2 mg/kg or 6 mg/kg (Research Biochemicals) was injected subcutaneously into normal pups (**Experiment 1a**) or bulbar 5-HT depleted pups (**Experiment 1b**) 40 min prior to odor exposure. The treated pups were removed from the dam 30 min after injection and put on fresh wood bedding. Ten

minutes later, pups were placed on peppermint-scented bedding (0.3 ml peppermint/500 ml bedding) for a period of 10 min. Following training, one normal pup from each treatment condition was sacrificed at various intervals (10min, 1hr, 2hr, **experiment 1a**), whereas 5-HT depleted pups were sacrificed only at 10 min after odor conditioning (**experiment 1b**). After sacrifice, both olfactory bulbs were removed quickly from the skull, immediately frozen on dry ice and subsequently stored at -70°C in microcentrifuge tubes. Other treated littermates were used for odour preference testing the next day (PND7).

2.2.1.2 Preference testing

A stainless steel test box (30×20×18cm) with a polypropylene mesh screen inside was placed on two trays, which were separated by a 2 cm neutral zone. One tray contained fresh bedding; the other contained peppermint-scented bedding. Each pup was removed from the dam and placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint bedding or normal bedding was recorded for five 1 min trials. A timer was started when a pup moved its nose and one paw into one side of the test box. The percentage time the pup spent on peppermint bedding over the five minute period was calculated. One-way analysis of variances (ANOVA) were used to compare different treatment groups, and post hoc tests were performed using the Tukey-Kramer test.

2.2.1.3 5-HT depletion

The procedure for 5-HT depletion of the olfactory bulb has been described elsewhere (McLean et al., 1993). Briefly, PND1 pups were removed from the dams, pretreated with 10 mg/kg desipramine by intraperitoneal injection and placed on fresh bedding. Forty five min later, after being anaesthetized by hypothermia on ice, the pups were placed in a modified stereotaxic instrument, and 150 nl of 5,7-dihydroxytryptamine (5,7-dHT) in Ringer's solution plus 0.02% ascorbic acid were injected bilaterally into the anterior olfactory nucleus. The pups were returned to the dams after recovery. Immunocytochemistry was performed on the brains of some of the animals to confirm depletion of 5-HT. This procedure has been shown to produce greater than 80% 5-HT fiber depletion in the olfactory bulb and is specific for the serotonergic fibers (McLean et al., 1993).

2.2.1.4 Protein determination and Western blot analysis

pCREB protein expression was determined by Western blot using previously published methods (McLean et al., 1999). Briefly, each pair of olfactory bulbs were placed in microcentrifuge tubes and ground in 100 μl lysis buffer containing 0.1% SDS, 1% NP-40, 20 mM PMSF, 10% glycine, and 1.37 mM sodium chloride with 1 μl/ml leupeptin, 2 mM PMSF, 8.9 U/ml aprotinin, and 1 mM sodium orthovanadate. The

homogenate was placed on a rotator for 30 min and then centrifuged at 13,500 rpm for 15 min at 4°C. The clear lysate supernatant was stored in 50 μl aliquots at -70°C. A bicinchoninic acid (BCA) protein assay kit was used to determine the protein concentration from each pair of olfactory bulbs.

After protein determination, 20 μl of prepared samples were boiled, cooled on ice and loaded into each lane of a SDS-PAGE gel for each blot. In each sample, 4 μl of 5× sample buffer (0.25 M Tris-HCL, 10% SDS, 50% glycerol, 0.025% bromophenol blue, and 0.5 M dithiothreitol added prior to use) and sufficient water were added to volumes of lysate that contained equal amounts of protein. 10 μl of colour coded molecular standard (Bio-Rad) was loaded into a separate lane for each blot. Following sample loading, each gel apparatus was attached to a Bio-Rad power supply set to 100 mV for 10 min, then the voltage was reset to 150 mV until all the samples were loaded completely. The gel running buffer contained 25 mM Tris, 250 mM glycine, and 3.5 mM SDS (pH 8.3). Protein transfer to nitrocellulose paper (Hybond ECL, Amersham) was performed at 0.2 A for 1 hr in transfer solution (25 mM tris, 192 mM glycine, and 20% methanol). After transfer, the nitrocellulose blots were processed for detection of pCREB. Briefly, after 3×5 min rinses in PBS contains 0.05% Tween-20 (PBST), the blots were blocked for non-specific proteins using 5% milk in PBST for 1 hr. Following 3×5 min rinses in PBST, the blots were treated with a rabbit polyclonal pCREB antibody (1/1,500, Upstate Biotechnology) in PBST overnight at 4°C. The specificity and sensitivity of this antibody

has been shown before (McLean et al., 1999). After 3×5 min rinses in PBST and incubation in anti-rabbit IgG conjugated to horseradish peroxidase for 1 hr, the blots were rinsed and visualized by ECL chemiluminescence (Amersham). Then, the blots papers were immersed in Poncean S solution to determine if equal amounts of protein were loaded.

The analysis of Western blots was carried out using a ChemiImager (Alpha Innotech Corp.). The average optical density (AVG = integrated density value/area) was recorded in the defined region of the pCREB bands. The background integrated optical density was automatically subtracted from each defined area. One way repeated measure ANOVAs were used to compare different treatment groups at various intervals.

2.2.2 Results

Experiment 1a Figure 2.1 shows the change of pCREB in the olfactory bulbs produced by pairing two doses of the β-adrenoceptor agonist isoproterenol with odor, and the odor preference results in normal rat pups. Behavioural results showed that 2 mg/kg isoproterenol induced significant odor preference learning compared to either the saline group ($p<0.01$) or the 6 mg/kg isoproterenol group ($p<0.05$)(Fig.2.1A). Correspondingly, the olfactory bulbs of pups that were trained by pairing odor with 2 mg/kg isoproterenol injection showed increased pCREB expression 10 min after training compared to pups

from other treatment groups (Fig.2.1B & 2.1C). Statistical analysis revealed a significant treatment effect. ($F_{2,21}$=3.87, p=0.046). In the post hoc Tukey-Kramer tests, the 2 mg/kg isoproterenol group (AVG=58.0) showed significantly higher pCREB ($p<0.05$) than the saline group (AVG=31.2), whereas there was no significant difference between saline and 6 mg/kg isoproterenol (AVG=36.3). Analysis of Western blots from longer time durations (1 hr and 2 hr) after conditioning did not show any significant difference among the various treatment groups (Fig.2.1B).

Experiment 1b Odor preference tests showed that only the 6 mg/kg isoproterenol group exhibited a significant increase in odor preference when compared to either the saline or the 2mg/kg isoproterenol groups ($p<0.01$, 2.2A) in bulbar 5-HT depleted pups. The same pCREB measurements on bulbar 5-HT depleted animals were only examined at the10 min interval after training for Western blots because we had not shown any difference of pCREB expression at longer time intervals in **experiment 1a**. In the present experiment, we found the 6 mg/kg isoproterenol group, but not the 2 mg/kg group, showed significantly increased pCREB (Fig.2.2B & 2.2C). Following a one-way ANOVA analysis ($F_{2,15}$=5.61, p=0.015), Tukey-Kramer tests revealed a significant difference ($p<0.05$) between the 6 mg/kg isoproterenol group (AVG=65.1) and the saline group (AVG=34.6). No significant difference was found between the saline and the 2 mg/kg isoproterenol group (AVG=42.3) (Fig.2.2B).

2.2.3 Discussion

Enhanced pCREB expression has been shown in neonate rat olfactory bulbs following pairing of odour with stroking (McLean et al., 1999), a procedure demonstrated to induce reliable preference learning (Sullivan et al., 1989a&b; Sullivan et al., 1991; McLean et al. 1992). It is hypothesized that the observed increases in pCREB induced by stroking were the result of tactile stimulation enhancing NE release from the locus coeruleus and activating β-adrenoceptors in the olfactory bulb concomitant with glutamatergic receptor activation by odor input. Here we demonstrate that exogenous injection of 2 mg/kg isoproterenol, a dose that completely substitutes for stroking in odor preference learning, when paired with peppermint odor, increased pCREB expression in the olfactory bulbs of normal pups.

In contrast, a higher dose of isoproterenol, 6 mg/kg was required to increase pCREB, as well as to induce odor preference, in bulbar 5-HT depleted animals. These results corroborate our previous work suggesting that 5-HT acting through 5-HT_2 receptors normally promotes noradrenergic-induced plasticity in the olfactory bulb (McLean et al., 1999). In the mammalian model, 5-HT receptor activation does not by itself increase cAMP (Morin et al., 1992), but 5-HT_2 activation potentiates isoproterenol or adenylate cyclase induced cytoplasmic cAMP levels through the phosphatidyl inositol system (Rovescalli et al., 1993). The localized increases of pCREB in mitral cells in the

previous study (McLean et al., 1999) suggest that the 5-HT and NE interaction might occur in mitral cells.

Although a late phase of pCREB was observed in another hippocampal LTP plasticity paradigm (Schulz et al., 1999), we failed to show a 2nd peak of pCREB during the later time. It is possible that the high level of stimulation used in the previous hippocampal LTP paradigm may have led to seizure activity, thus recruiting the second peak of pCREB activation. Double peaks have not yet been reported in a natural learning paradigm.

Our previous study showed that an effective conditioning pairing, odor plus stroking, or in the present experiment, odor plus 2 mg/kg isoproterenol, but not odor alone or stroking alone, enhances pCREB expression. These data suggest both a glutamate-initiated calcium signal (Bozza and Kauer, 1998) triggered by odor input, and a cAMP/PKA signal initiated by β-receptor activation are required to significantly elevate pCREB. The striking result in the present experiment, however, is that odor plus 6 mg/kg of isoproterenol is ineffective in producing either odor preference learning or enhanced pCREB expression. This outcome argues that a critical window for calcium and PKA co-activation of phosphorylation events has been exceeded by pairing odor and the 6 mg/kg dose of isoproterenol in the normal rat pup. The argument that 6 mg/kg isoproterenol might be producing pharmacological effects that directly interfere with odor learning is

countered by the effectiveness of this dose in bulbar 5-HT depleted pups. The notion of critical windows for intracellular plasticity cascades is not novel. Long-term depression and long-term potentiation occur variously as a function of specific levels of intracellular calcium (Foehring and Lorenzon, 1999; Yang et al., 1999). Competition at the level of CREB factors leading to a failure of plasticity (Ptashne, 1988) has been described, but it has not been suggested previously that a narrow band window exists for the events triggering CREB phosphorylation itself. The present data demonstrate a strong correlation between effective conditioned stimulus plus unconditioned stimulus pairing and pCREB. Experiments are under way to probe a causal role for pCREB in this learning model.

2.3 Experiment 2. Increased ON-evoked synaptic potentials following manipulation of NE and 5-HT inputs to the olfactory bulb correlate with the requirements for conditioned odor preference learning in neonate rats.

We have established that a β-adrenoceptor agonist, isoproterenol, can completely substitute for tactile stimulation as the unconditioned stimulus in olfactory preference learning, while bulbar serotonin appears to facilitate this noradrenergic action (Langdon et al., 1997). In experiment 1, we hypothesized, and further showed, that the interaction of NE and 5-HT to produce odor preference learning might act through the cAMP 2nd message system to induce CREB phosphorylation, which in turn, would trigger transcriptional activation of downstream proteins. Here we examined the

electrophysiological changes induced by these modulating neurotransmitter interactions in the olfactory bulb. We hypothesize that the electrophysiological changes we observed reflect critical changes underlying odor preference learning.

2.3.1 Method

A total of 65 Sprague-Dawley rats of both sexes were used in this study. Thirty three rat pups had their left olfactory bulbs depleted of 5-HT on PND1 or 2 prior to electrophysiological recording on PND5-10 (see 5-HT depletion method in experiment 1). In **Experiment 2a**, normal pups were divided into four groups: a saline group, a 2 mg/kg isoproterenol group, a 6 mg/kg isoproterenol group and a 20 mg/kg propranolol group. In **Experiment 2b**, three groups as above (the propranolol group was excluded) were included using bulbar 5-HT depleted pups.

2.3.1.1 Surgery

On PND 5-10, pups were anaesthetized with a 2.25 g/kg intraperitoneal injection of 20% urethane. Each pup was placed in a modified stereotaxic apparatus using pressure exerted by the reverse side of normal ear bars to hold the head. The body was supported in a polymer mould through which water warmed to 37°C was continually pumped to maintain the body temperature of the pup. The nasal bone overlying the left olfactory bulb

was removed using a dental drill.

2.3.1.2 Electrophysiology

A bipolar twisted Teflon coated stainless electrode (MS303, Plastic One) was placed on the rostrolateral surface of the exposed olfactory bulb to stimulate the ON. The stimuli consisted of three square bipolar 40V pulses of 0.2 ms duration, 10 sec apart. Extracellular field potentials were recorded at varying depths with a saline filled glass electrode with a tip diameter around 50 μm. After a depth profile, the recording electrode was usually placed in the EPL, approximately 200-300 μm deep to the dorsal surface, to maximize the ON-evoked field potential (EFP). Either saline, or 2 mg/kg or 6 mg/kg isoproterenol, or 20 mg/kg propranolol in 50 μl volume was subcutaneously injected into the pup. Starting from time zero (the time of injection), three recordings with a 10 sec interval were taken every 10 min for a total time of 80-90 min using a Labmaster A-D board. Asyst software was used to deliver the stimulation and collect and store the EFPs. Kruskal-Wallis nonparametric ANOVA tests were performed to compare the EFP areas of each group at every time interval.

2.3.2 Results

A characteristic waveform of a field potential in the EPL of normal olfactory bulb

stimulated by olfactory nerve stimulation is shown in Fig.2.3. The field potentials recorded 200-300 μm below the surface were long lasting (>50 msec) and displayed N_1 (kainate/AMPA receptor mediated) and N_2 (NMDA receptor mediated) components comparable to those described *in vitro* by Aroniadou-Anderjaska et al (Aroniadou Anderjaska et al., 1997). Bulbar 5-HT depletion did not appear to alter the ON-evoked potential (Fig.2.3)

In **Experiment 2a**, 2 mg/kg isoproterenol produced long-lasting increases in olfactory nerve EFP area that were evident beginning 30 min after injection and most prominent at 60 min (Fig.2.4A & 2.4B). ON-EFPs showed little change following injections of saline, 6 mg/kg isoproterenol or 20 mg/kg propranolol. At 60 min, the 2 mg/kg isoproterenol group showed significantly increased percentage EFP area from 0 min ($F_{3,28}$=5.897, p=0.003) compared to either the saline group ($p<0.01$), or the 6 mg/kg group ($p<0.05$) or the propranolol group ($p<0.05$). Waveform changes suggest both N1 and N2 components (Aroniadou Anderjaska et al., 1997) contributed to the increase of EFP area. N1 and N2 change ratios were separately estimated in the 2 mg/kg isoproterenol group by selecting a time point and assessing increases at that time point relative to the same point during the initial measurement (0 minutes). The average effect of 2 mg/kg isoproterenol on ON-evoked potentials at 0 min and 60 min is illustrated in Fig.2.4C.

In **Experiment 2b**, 2 mg/kg isoproterenol was ineffective in 5-HT-depleted bulbs, as was saline, in producing increases in the ON-EFP area (Fig.2.5A). In contrast, 6 mg/kg isoproterenol produced a significant increase in EFP area at 60 min ($F_{2,30}$=4.317, p=0.025) when compared to the saline group ($p<0.05$, Fig.2.5B).

2.3.3 Discussion

Isoproterenol at a dose, 2 mg/kg, which normally produces an effective conditioned odor preference in the 5-10 day old rat pup, and which also increases pCREB expression in olfactory bulbs after conditioned odor preference training (as shown in **Experiment 1**), potentiates the ON-EFP in urethane-anesthetized pups of the same age. This effect was specific for the 2 mg/kg dose of isoproterenol. The 6 mg/kg dose of isoproterenol, which does not produce effective odor preference learning in the rat pup, did not potentiate the ON-EFP. However, in the olfactory bulb of rat pups depleted of bulbar 5-HT, 6 mg/kg of isoproterenol, but not 2 mg/kg isoproterenol, was required to potentiate the ON-EFP. This change in the effective potentiating dose of isoproterenol with 5-HT depletion parallels the result produced by bulbar 5-HT depletion on the acquisition of early conditioned odor preference learning and the activation of pCREB in the olfactory bulbs. These results also suggest that during early conditioned odor preference acquisition the glutamatergic ON input is potentiated.

Potentiation of the N_1 component of the ON glutamatergic input by its pairing with an effective β-adrenergic activation, for example, enhancement of ON depolarization (Kawai et al., 1999), enhancement of postsynaptic glutamate responses (Segal, 1982), possibly through increases in membrane resistance, or increased synaptic glutamate through decreased reuptake (Hansson and Ronnback, 1991). Potentiation of the N_2 component of ON synaptic input may be related to those factors and also to enhanced phosphorylation of NMDA channels related to cAMP elevation and to disinhibition of the mitral cells as the result of β-adrenergic suppression of granule cell feedback (Wilson and Leon, 1988).

An important result of Experiment 2 was the failure of the 6 mg/kg dose of isoproterenol to increase the ON-EFP in normal rat pups. This outcome suggests that the electrophysiological potentiation of ON input is in some way dose dependent. While dose-dependency has not been described for the direct actions of β-receptor activation, it has been suggested that NE alters mitral cell excitability primarily by indirect actions (Jahr and Nicoll, 1982). NE decreases GABA release from granular cells resulting in less inhibition from granule cells to mitral cells, whereas glutamate released from mitral cells enhances GABA release from granular cells and increases feedback mitral cell inhibition. An increase in the glutamate released onto granular cells that in turn enhances GABA release at the higher dose of isoproterenol may overcome a net disinhibition produced by a lower isoproterenol dose. Calcium influx through NMDA receptors also directly evokes

GABA release in olfactory bulb granular cells (Halabisky et al., 2000). The balancing of inhibition and disinhibition in olfactory bulb circuitry may explain the failure to induce a potentiated ON-EFP response using a higher dose (6 mg/kg) of isoproterenol in normal olfactory bulbs.

The effectiveness of 6 mg/kg isoproterenol in increasing the ON-EFP in bulbar 5-HT-depleted rat pups suggests that phosphorylation cascades are critical for the electrophysiological effects. If potentiated responses depend on phosphorylation of ion channels, then the failure to recruit intracellular phosphorylation would impair the production of electrophysiological potentiation as well as lead to the failure in CREB phosphorylation.

The present data suggest the ON input mediating a specific odor is strengthened by preference training during acquisition and, likely, more enduringly (Hebb 1949). Combined with the observed pCREB increase in Experiment 1 and earlier evidence that pairing of stroking with peppermint odor selectively induces pCREB increases, the electrophysiological data support the hypothesis that an enhancement of the odor representation is part of odor preference learning.

2.4 General discussion

We have investigated the 5-HT and NE interactions underlying olfactory preference learning from behavior to synaptic transmission to post synaptic biological signal transduction. Among numerous lines of evidence, we are one of the few research groups to look at changes in pCREB during natural learning. In the present study, we have also tried to illuminate the nature of the electrophysiological changes in the olfactory circuitry that accompany acquisition of odor preference in the neonate rat by pairing an electrical odor input (stimulation of olfactory nerve) with behaviorally effective doses of isoproterenol. This procedure mimics the biological components underlying natural learning. We found a remarkably robust correlation in which a behavioral effective dose (2 mg/kg), but not a higher ineffective dose (6 mg/kg), of isoproterenol potentiated the EPSP induced by glutamatergic olfactory nerve input, and selectively enhanced CREB phosphorylation in neonate rat olfactory bulbs. A bulbar depletion of 5-HT shifted the dose-dependent effect of NE so that a higher dose (6 mg/kg) of isoproterenol was required to overcome 5-HT deficiency in the olfactory bulbs for both the electrophysiological change and the increased CREB phosphorylation triggered by behaviorally effective pairing.

There are at least two ways in which the inverted U curve for isoproterenol might be understood. (1) The factors that determine the failure to produce electrophysiological

potentiation to olfactory nerve input are the critical factors in understanding the inverted U curve for CREB phosphorylation. The failure to phosphorylate CREB is a simple consequence of the failure of potentiation. (2) there are two parallel actions of ineffective pairings of odor input and β-receptor activation: one that influences the electrophysiology of the bulb; and one that influences the ability of intracellular cascades to promote CREB phosphorylation. The parallel mechanism hypothesis is less parsimonious and seems less likely.

In attempting to understand the failure of electrophysiological potentiation to occur, we have entertained two hypotheses. The failure to produce odor preference learning may relate to an imbalance in mitral cell inhibition/disinhibition accompanying ineffective doses of isoproterenol. Alternatively, since the known effects of isoproterenol are mediated via G-protein activation and recruitment of adenylate cyclase, another possible node for the failure to produce electrophysiological potentiation would be a failure to enhance cAMP levels. It is likely that cAMP sensitive ion channels are involved in the early membrane effects of isoproterenol. Evaluating the dose dependency of cAMP increases in this system would test this hypothesis.

The site of interaction between β-adrenergic and serotonergic input remains to be identified at the cellular level. However, we suspect the interaction occurs in mitral cells because localized pCREB increases were observed in mitral cells in the olfactory bulbs

after conditioning (McLean et al., 1999). The results of the present study seem to suggest that electrophysiological alterations in functional circuitry will always accompany acquisition of odor preferences and other learning paradigm.

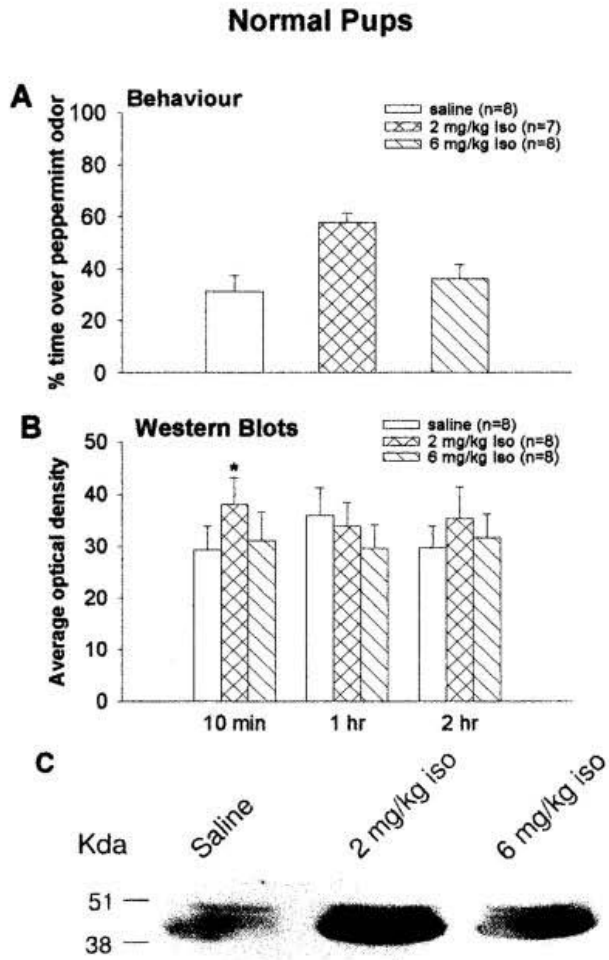

Figure 2.1 Odor preference test and Western blot results of pCREB in normal pups
A: Odor preference test in normal pups from the saline, 2 mg/kg and 6 mg/kg isoproterenol group after odor only training. **$p<0.01$ B: Western results showing the average optical density (mean ± S.E.M.) of pCREB in the olfactory bulbs of normal rat pups. *$p<0.05$ C: Representative Western blot showing pCREB levels in the normal olfactory bulbs from different treatment groups at 10 min after odor exposure. pCREB bands locate at 43 kD. (Iso) Isoproterenol.

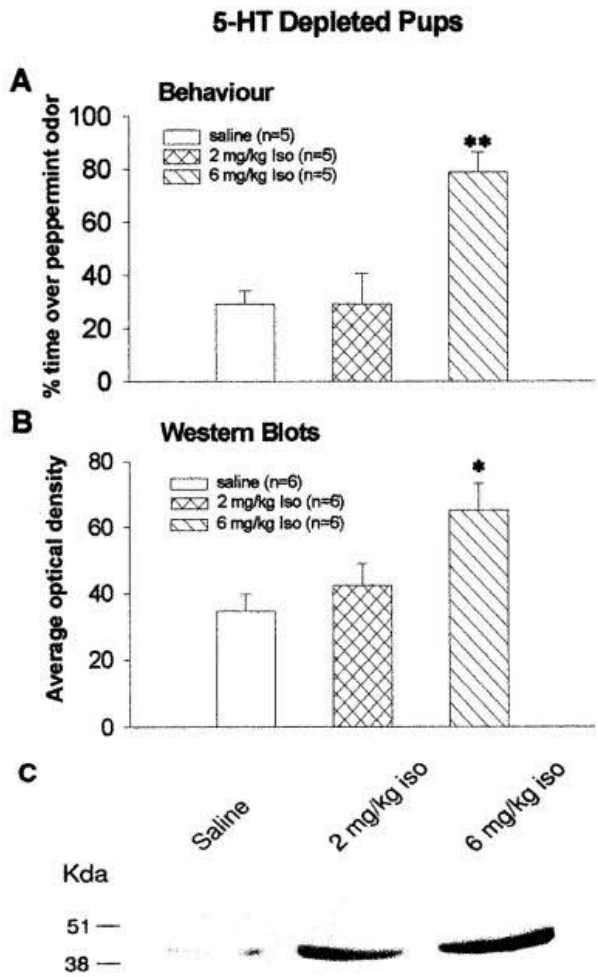

Figure 2.2 Odor preference test and Western blot results of pCREB in olfactory bulb 5-HT depleted pups

A: Odor preference test in bulbar 5-HT depleted pups after odor only training. **$p<0.01$ B: western results showing the average optical density (mean±S.E.M.) of pCREB in the olfactory bulbs of 5-HT depleted rat pups. *$p<0.05$ C: representative Western blot showing pCREB levels in the normal olfactory bulbs from different treatment groups at 10 min after odor exposure. (Iso) Isoproterenol

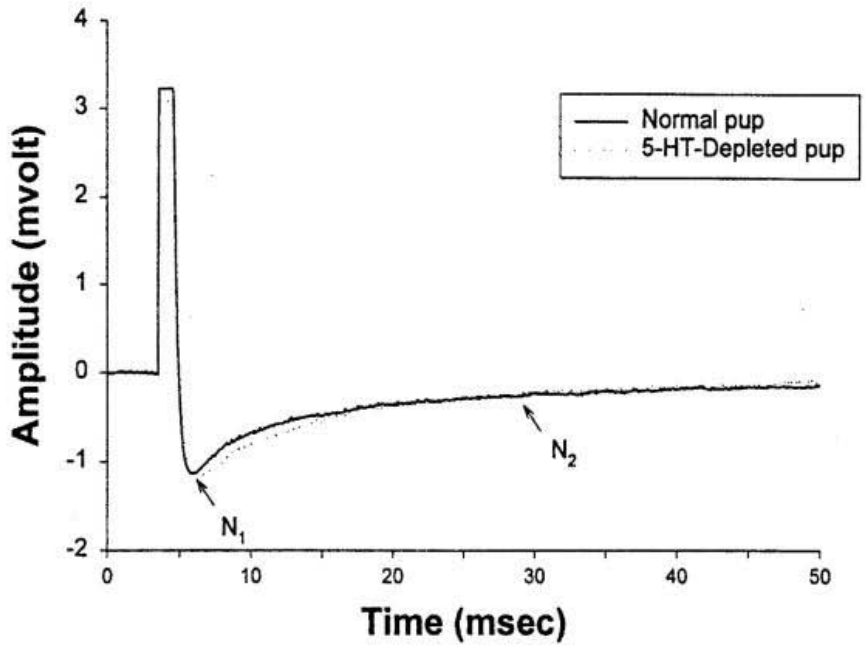

Figure 2.3 Characteristic waveforms of field potentials in the EPL of normal and 5-HT depleted olfactory bulbs by ON stimulation. N1 and N2 showing the kainate/AMPA receptor and NMDA receptor components described by Aroniadou-Anderjaska (Aroniadou-Anderjaska et al. 1997)

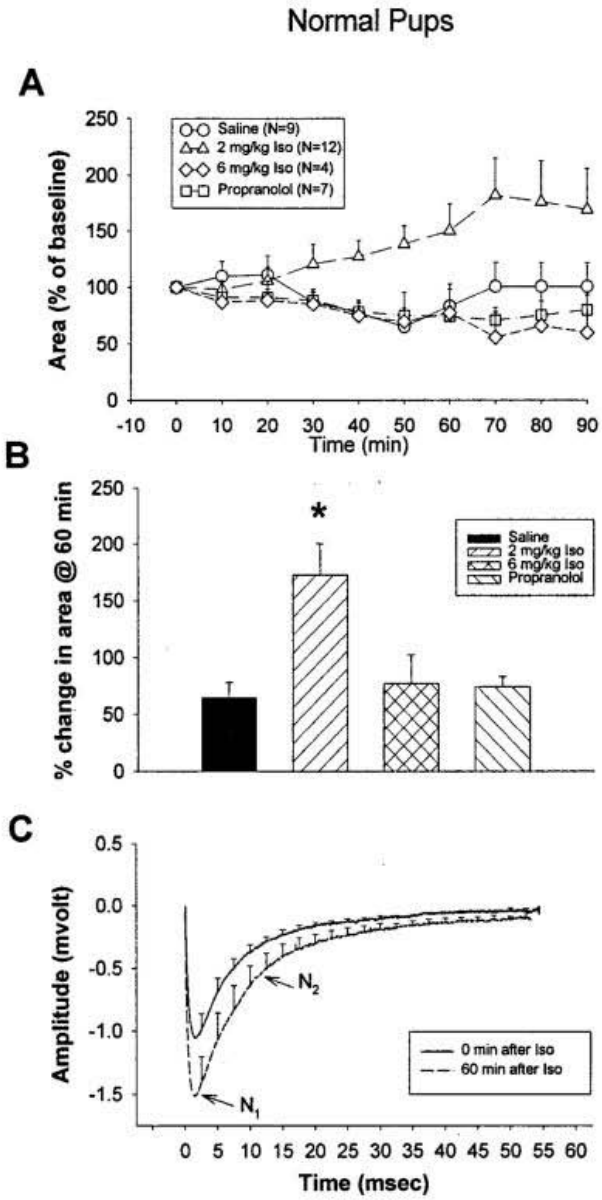

Figure 2.4 EFP recordings in normal rat pups
A: percentage change of EFP areas from baseline (0 min) among different treatment groups of normal pups at various time interval. (mean±S.E.M.) B: percentage change of EFP area at 60 min in normal pups. *$p<0.05$ C: average effect of 2 mg/kg isoproterenol on ON-evoked potentials at 0 min and 60 min. (Iso) Isoproterenol

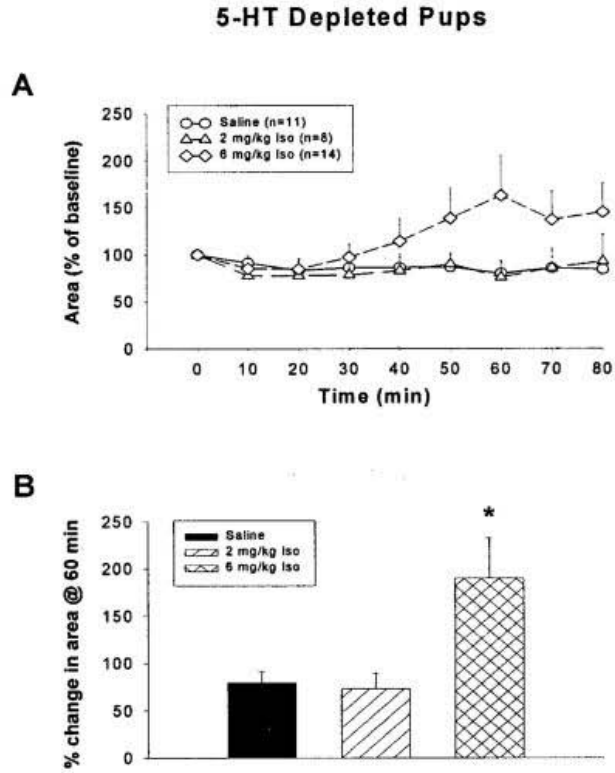

Figure 2.5 EFP recordings in olfactory bulb 5-HT depleted rat pups
A: percentage change of EFP areas from baseline (0 min) of bulbar 5-HT depleted pups at various time interval. (mean±S.E.M.) B: percentage change of EFP areas at 60 min in bulbar 5-HT depleted pups. *$p<0.05$ (Iso) Isoproterenol

Chapter 3 Optical Imaging of Odor Preference Memory in the Rat Olfactory Bulb

(Published in J.Neurophysiology. 87: 3156-3159, 2002)

3.1 Introduction

Neonate rats rapidly (one-trial learning) form a preference to an odor that is paired with a reinforcing tactile stimulus (Sullivan and Leon, 1987) that activates the locus coeruelus (Nakamura et al., 1987) or that is paired with the beta adrenergic agonist, isoproterenol (Sullivan et al., 1989a&b). Several lines of evidence suggest that the olfactory bulb itself is sufficient to mediate this early odor preference learning: activation of beta receptors locally in the bulb, concomitant with peppermint odor presentation, is both necessary (Sullivan et al., 1989a&b) and sufficient (Sullivan et al., 2000b) for odor preference learning to occur. Previous work has shown that odors induce focal uptake of [^{14}C]2- deoxyglucose (2-DG) within the glomerular layer of the olfactory bulb (OB) and that focal 2-DG uptake in the glomerular layer increases after early odor preference learning (Sullivan et al., 1991; Johnson and Leon, 1996). Effective odor preference training protocols increase cAMP response element binding protein (CREB) phosphorylation (McLean et al., 1999; Yuan et al., 2000b) in the bulb and selectively increase the field EPSP to olfactory nerve stimulation (Yuan et al., 2000b). Both the

NMDA and AMPA components of the olfactory nerve field EPSP are enhanced. Furthermore, odor conditioning enhances single-unit responses in mitral-tufted cells in areas that exhibit enhanced 2-DG labeling following exposure to a previously conditioned odor (Wilson and Leon, 1988).

Recent advances in optical imaging have facilitated our understanding of the spatial representation of odors in the olfactory bulb (Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001; Rubin and Katz, 2001). Responses to odors can be measured directly by optical recording of intrinsic signals from the dorsal surface of the OB (Uchida et al., 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001; Rubin and Katz, 2001). Representations of odorants within the OB can be visualized at the level of glomeruli. The patterns of odor-induced optical signals are similar among different animals (Belluscio and Katz, 2001).

Intrinsic optical signals are due to activity-dependent hemodynamic changes and light scattering (Malonek et al., 1997; Meister and Bonhoeffer, 2001). Intrinsic signal imaging enables in vivo recording and multiple manipulations on anesthetized animals. Therefore, it may serve as a useful tool to explore training-dependent changes in stimulus-induced patterns of neuronal activity. In this study, we investigated the feasibility of using intrinsic signal imaging to detect training-dependent changes within the OB 24 hr after conditioned odor preference training. We performed intrinsic optical

imaging on the OBs of both trained and control one-week-old rat pups. An enhanced optical signal was observed in trained animals to the trained odor. The result demonstrated that intrinsic signal imaging could monitor training induced changes in neuronal activity.

3.2 Methods

3.2.1 Odor preference training

Eighteen Sprague-Dawley rat pups from five litters were used in this study. The procedure for conditioning has been previously described in detail (McLean et al., 1993; McLean et al., 1999). Briefly, on postnatal day 6 (PND6, the day of birth was considered PND 0), rat pups were removed from the dam and put on fresh bedding 10 min before odor exposure. In one group, pups were placed on peppermint-scented bedding (0.3 ml of peppermint/500 ml bedding) and stroked vigorously on the hind region using a sable brush every other 30 s for 30 s over a 10 min period (odor + stroking). In another group, the pups were only exposed to the peppermint bedding without being stroked (odor only). Immediately after training or odor exposure, the pups were returned to the dams. Previous studies (Sullivan and Leon, 1987; Sullivan et al., 1989a&b; Sullivan et al., 1991; McLean et al., 1993) have shown that rat pups subjected to the above conditioning procedure develop a predictable odor preference for the odor used.

3.2.2 Optical imaging

Rat pups were subjected to optical imaging the day after training. Rats were anesthetized with a 2.25 g/kg intraperitoneal injection of 20% urethane. Anesthetized rat pups were placed in a stereotaxic frame and the bone overlying the dorsal surface of the olfactory bulbs was carefully thinned until the blood vessels underneath the bone were visible (Uchida et al., 2000; Rubin and Katz, 2001).

The stereotaxic frame with the anesthetized rat pups was mounted below optics consisting of a 1x objective and a 1.6x projection lens. Odorants were diluted in glycerol and delivered by computer controlled pressure pulses into a stream of fresh air blowing over the rat's nose (Fig.3.1A). The bulbs were illuminated with red light (630 nm) via two light guides positioned lateral to the objective (Uchida et al., 2000; Rubin and Katz, 2001). The light was focused just below the blood vessels at the level of the glomeruli. Images (640 x 480 pixel) were acquired by a cooled CCD system (Sensicam, PCO Computer Optics GmbH, Germany) under control of Axon Imaging Workbench software (Axon Instruments, Inc., Foster city, CA) at a frame rate of 2 Hz. Different odors and no-odor recordings were interleaved and repeated 5-10 times. Odors were presented for 4 s with a 60 s intertrial interval. Time series of images were averaged (n= 5 to 10) and responses were expressed as odor-induced fractional change in reflected light intensity ($\Delta R/R$, see Fig.3.1B). Thresholding (Uchida et al., 2000; Rubin and Katz, 2001) or spatial

filtering techniques (Meister and Bonhoeffer, 2001) were not applied in order to avoid any interference between these data transformations and data quantification. Data processing and analysis were performed using Origin software (Origin Lab Corporation, MA) and custom-made software written in Interactive Data Language (IDL5.4, Research Systems, CO). The experimental protocol was approved by the Experimental Animal Committee of the RIKEN Institute (Wako Shi, Japan).

3.3 Results

Figure 3.1A shows a schematic illustration of the experimental design for imaging of OB responses to amyl acetate and peppermint. The dorsal surface of the OB was imaged and reflected light was sampled from the medio-rostral, latero-rostral, medio-caudal and latero-caudal quadrants. As shown in Figure 3.1B application of peppermint (10%) for 4 seconds induced a transient change in light reflectance after a delay of about 3 s. Peak amplitudes of these responses amounted to 0.2 % up to 1 % of the baseline light intensity. Signal sizes of the four quadrants did not differ significantly and, therefore, signals from the four quadrants were averaged in subsequent analysis.

The preceding experimental design was then applied using odor trained and control littermate pups (Fig.3.2A). Control animals exhibited amyl acetate and peppermint-induced intrinsic optical signals of comparable peak amplitudes (0.414% ±

6.61x10-4, and 0.354% ± 1.08x10-3, respectively; mean ± SE). Trained animals, however, exhibited larger signals to the trained odor (peppermint, 0.991% ± 2.26x10-3) as compared to the control odor (amyl acetate, 0.521% ± 1.5x10-3) applied to the same animals (Fig.3.2B). Trained animals also responded with significantly larger intrinsic signals to the trained odor than did control littermates to the same odor (Fig.3.2B). Furthermore, odor preference training significantly enhanced the ratio between the responses induced by peppermint and amyl acetate (Fig3.2C).

3.4 Discussion

In the present study, we investigated whether odor preference memory can be accessed by imaging of intrinsic signals at the level of the glomeruli and found that this was the case. This outcome is consistent with the earlier reports of enhanced 2-DG uptake at the glomerular level in the OB following peppermint preference training. It has been established that odor-induced intrinsic signals imaged from the OB involve "global", i.e. spatially less confined components as well as components that can resolve single glomeruli (Meister and Bonhoeffer, 2001). The present odor-induced responses were seen over the dorsal surface of the OB, i.e. at the "global" level, and only occasionally more localized response patterns emerged (not shown). There are several reasons we might expect primarily "global" signals in these experiments. The first is the age of the subjects. Intrinsic optical signals in the somatosensory barrel fields of rats less than 7

weeks of age are more diffuse than those in adults (Yazawa et al., 2001). This is attributed to horizontal interactions. Similarly only diffuse intrinsic optical signals are seen initially in the visual cortex of young ferrets when orientation maps are studied and there is considerable individual variation in the development of the more specific patterns (Chapman et al., 1999). Thus, the olfactory maps in week old rat pups may be more diffuse than in older rats even though glomerular organization has already developed at this age (Bailey et al., 1999). On the other hand, the same concentration of peppermint used here produces discrete 2-DG spots in week-old pups (Sullivan and Leon, 1987). 2-DG peppermint representations are, however, less sensitive to odorant concentration than optical signals appear to be (Carmi and Leon, 1991). Signals for amyl acetate, for example, have been measured at similar concentrations with both methods (Stewart et al., 1979; Rubin and Katz, 2001) and focal patterns are more discrete for higher concentrations with 2-DG (Stewart et al., 1979). In addition increased 2-DG uptake over the entire glomerular layer, as well as enhanced focal uptake, occurs following peppermint preference learning even in older pups (Johnson and Leon, 1996). It is unlikely the global increases seen here are due to respiratory changes to the learned odor since previous studies have found no change in respiration with peppermint preference learning (Sullivan et al., 1988). Finally, in 19 day old pups, 2-DG and c-Fos foci following extended odor preference training are primarily in the midlateral bulb (Woo et al., 1987; Johnson et al., 1995) that was not sampled here. In week old pups 2-DG (Sullivan and Leon, 1987; Yuan et al. 2000a) and pCREB (McLean et al., 1999) images

show dorsolateral foci as well. Thus a portion of the focal peppermint representation was included in the present study, although visualization of the midlateral bulb might have increased the probability of capturing a focal response.

These data are consistent with the evidence from earlier experiments showing an increase in the field EPSP to olfactory nerve input in pups of the same age that receive learning effective training conditions (Yuan et al., 2000b). The intrinsic signal change at the level of the glomeruli 24 hr later in the present study may indicate that the synaptic modification seen during acquisition conditions is sustained.

Creation of an olfactory preference in the rat pup may therefore be intimately related to an increase in synaptic strength at the level of the glomeruli. Such a hypothesis is consistent with the recent report of a Drosophila mutation that concomitantly produces an increase in glomerular synapses and the appearance of a behavioral preference for a normally neutral odor (Acebes and Ferrus, 2001). Transduction of the odor is not altered. Other evidence supporting a special role for the glomerular layer in odor preference learning is the report of increased glomerular size (Woo et al., 1987) (as in the Drosophila model) and of increased numbers of juxtaglomerular cells (Woo and Leon, 1991) following peppermint preference training.

Future studies might examine glomerular intrinsic signal changes at a longer

interval after training to assess focal alterations and to ask if the generalized response seen here is enduring as reported for 2-DG. Within-pup analysis of optical signals in an acquisition paradigm might permit an assessment of training-induced changes in discrete foci when they occur. This was precluded in the present between-group study due to the variability in the occurrence of discrete signals.

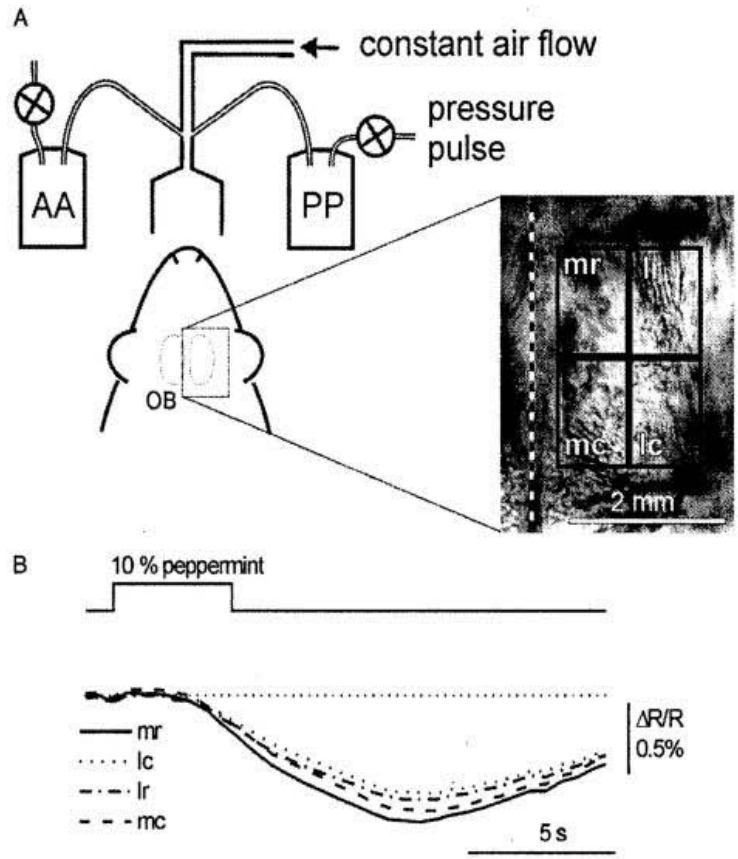

Figure 3.1 Intrinsic imaging setup and peppermint response recordings from the OB
A: Schematic illustration showing the experimental design for imaging of olfactory bulb (OB) responses to two different odors (amyl acetate, AA, and peppermint, PP). The dorsal surface of the OB was imaged and reflected light was sampled from the medio-rostral, latero-rostral, medio-caudal and latero-caudal quadrants. B: Responses obtained with application of peppermint (10%) for 4 seconds. Individual traces were obtained from the 4 quadrants indicated in A.

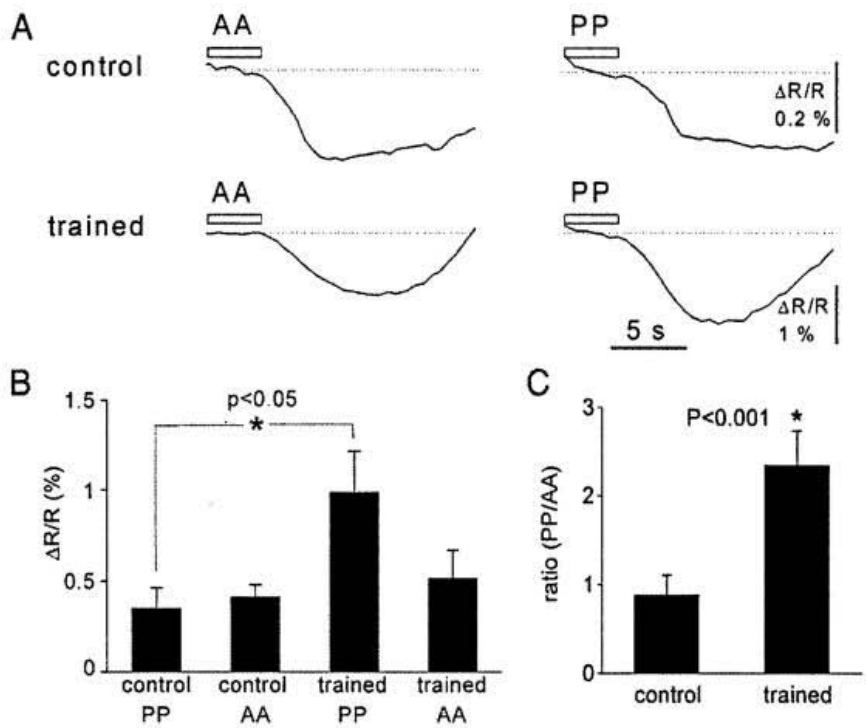

Figure 3.2 Optical imaging of OB responses to AA and PP in control and trained pups. A: Intrinsic OB responses to amyl acetate (AA) and peppermint (PP) in control and trained pups. Note larger response to PP in trained pups as compared to control animals and control odor (AA). Time courses of responses did not differ between the populations of control and trained pups. B: Statistical analysis of data (mean ±s.e.m.). C: Ratio of responses to PP and AA in control and trained rat pups. Asterisks indicate significant differences (One-Way ANOVA).

Chapter 4 Mitral Cell β_1 and 5-HT_{2A} Receptor co-Localization and cAMP co-Regulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb

(Accepted by Learning & Memory, 2002)

4.1 Introduction

The olfactory bulb is an excellent preparation for demonstrating classical conditioning. Odor preferences can be produced in rat pups as young as one week when an odor (conditioned stimulus or CS) is paired with any of several unconditioned stimuli (UCS) including milk, stroking or even mild foot shock (Sullivan and Wilson, 1994; Sullivan et al., 2000a). The learning is localized to the olfactory bulb (Sullivan et al., 2000b) and modifications of the electrical (Wilson et al., 1987; Wilson and Leon, 1988) and metabolic (Sullivan and Leon, 1987; Sullivan et al., 1991) activity of the olfactory bulb are observable after conditioning. Olfactory bulb norepinephrine (NE), acting through β-adrenoceptors, is both necessary and sufficient as neural substrate for the UCS (Sullivan et al., 2000b).

Based on these data and others, Sullivan and Wilson (1994) suggested that learning results from the disinhibition of mitral cells, which permits activation of NMDA

receptors and could promote long-term potentiation-like changes in the granule cell-mitral cell connection (Wilson and Sullivan, 1994). In this scenario NE input from the locus coeruleus to the olfactory bulb acts as the UCS by inhibiting granule cell interneurons in the bulb through β-adrenoceptors to produce disinhibition.

In contrast to the disinhibition model, other data suggest the action of the UCS occurs directly on mitral cells rather than through the intermediary granule cells (McLean et al., 1999). Granule cells show only weak responses to the β-adrenoceptor agonist, isoproterenol, but show much larger responses to α-adrenoceptor agonists (Trombley, 1992; Trombley and Shepherd, 1992; Trombley, 1994; Mouly et al., 1995; Czesnik et al., 2002) and disinhibition of mitral cells is also driven by α-adrenoceptor agonists (Trombley, 1992; Trombley and Shepherd, 1992; Trombley, 1994; Czesnik et al., 2002; but see Wilson and Leon, 1988; Okutani et al., 1998).

Stroking (tactile stimulation that increase NE levels, Rangel and Leon, 1995) or isoproterenol paired with an odor produces learning (Langdon et al., 1997), and the same parameters induce phosphorylation of cAMP response element binding protein (CREB) in the mitral cells (McLean et al., 1999; Yuan et al., 2000b). CREB phosphorylation is significantly increased in the olfactory quadrant that received the odor input (McLean et al., 1999). Likewise the conditioning procedure produces potentiation of the glutamatergic olfactory input to the mitral cells (Yuan et al., 2000b). An interesting

feature of the isoproterenol induced odor preference learning is that it benefits from co-activation of the serotonergic system. 5-HT depletion of the olfactory bulb prevents learning with typical doses of isoproterenol, but higher doses of isoproterenol, 4 mg/kg (Langdon et al., 1997) or 6 mg/kg (Yuan et al., 2000b), can overcome the deficit. Pharmacological studies using ritanserin and DOI suggest that the $5HT_{2A/2C}$ receptor is the critical receptor mediating the serotonin effect (Price et al., 1998), but $5HT_{2A/2C}$ receptor activation by itself does not produce learning (McLean et al., 1996). 5-HT acting through $5HT_{2A/2C}$ receptors, as assessed with ritanserin, ketanserin and DOI, has been observed to potentiate β-adrenoceptor activation in rat neocortex, resulting in enhanced cAMP production (Morin et al., 1992; Rovescalli et al., 1993).

We hypothesize that the critical UCS event in olfactory learning is the production of cAMP in mitral cells. CREB phosphorylation results from the convergence of the UCS cAMP signal and the CS arising from the odor stimulus and travelling via the olfactory nerve. This model parallels that proposed for sensory learning in Aplysia (Kandel et al. 2000).

In the present study we pursue three lines of evidence in support of a direct action of NE on mitral cells as the neural substrate for early olfactory learning. First, selective antibodies for the β_1-adrenoceptors and the 5-HT_{2A} receptor are used to examine the localization and co-localization of these two receptors in the olfactory bulb. Second, the

expression of cAMP following odor preference conditioning is examined with a cAMP assay. The dependence of β-adrenoceptors activation of cAMP signaling on 5-HT input was also tested using 5-HT depletion. Third, the localization of cAMP increases associated with odor preference conditioning is examined immunocytochemically in the olfactory bulb.

We predicted we would find mitral cell co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors in Experiment 1. We had three predictions with respect to variations in cAMP levels in Experiment 2. First, an effective UCS (e.g., stroking or 2 mg/kg isoproterenol in normal rat pups) would produce an increase in cAMP. Second, in rat pups with 5-HT depletion in the olfactory bulbs, these UCSs would no longer increase cAMP. Third, an ineffective UCS in normal pups (e.g, saline or 4 mg/kg isoproterenol) would be associated with lower levels of cAMP.

While β-adrenergic and serotonergic receptor localization on mitral cells, together with cAMP synergism, are key requirements of the present hypothesis, a more convincing demonstration of the hypothesis would include localization of the cAMP increase itself to the mitral cells. This is undertaken in Experiment 3 with cAMP immunocytochemistry following odor plus the learning-effective 2 mg/kg dose of isoproterenol. In the same pups one bulb was depleted of 5-HT. cAMP increases would not be predicted in mitral cells of the 5-HT depleted bulb.

4.2 Experiment 1 5-HT_{2A} receptor and β_1- adrenoceptor localization

4.2.1 Materials and methods

All experimental procedures were approved by the Memorial University Institutional Animal Care Committee and conform to the standards set by the Canadian Council on Animal Care.

4.2.1.1 Animals and sacrifice

Both young (postnatal day 6-12, PND 6-12) and older (PND 30-50) Sprague-Dawley rats totaling 26 rats from 22 litters were used in this study. The rats were anesthetized with an overdose of sodium pentobarbital and perfused as described previously (McLean et al., 1999).

4.2.1.2 Immunocytochemistry/Immunofluorescence

Frozen sections were cut coronally through the olfactory bulbs at 30 μm using a cryostat. The sections were either melted directly onto subbed slides or collected floating in cold PBS. The sections were processed for the β_1-adrenoceptor and/or the 5-HT_{2A} receptor using immunocytochemistry or immunofluorescence.

For the β_1-adrenoceptor immunocytochemistry, briefly, cryostat-mounted sections were air-dried at room temperature for 5-10 min, then incubated in primary antibody (β_1-adrenoreceptor Ab, 1:1000, Oncogene, Cambridge, MA) in 0.2% Triton X-100, 2% normal goat serum (NGS) in PBS at 4°C overnight. Secondary antibody processing and visualization using diaminobenzidine dihydrochloride (DAB) was as described previously (McLean et al., 1999).

For fluorescence double-labeling, the sections were collected free floating in 0.1 M PBS, followed by incubation overnight at 4°C in primary antibodies. Both the 5-HT_{2A} receptor antibody (1:500, Pharmingen, Mississauga, ON) and the β_1-adrenoceptor antibody (1:1000) were dissolved in 0.2% Triton X-100 and 2% normal goat serum (NGS) in PBS. After 3×10 min rinses in PBS, the sections were incubated in goat anti-mouse IgG conjugated to CY3 (1:400, Jackson ImmunoResearch, Mississauga, ON) and goat anti-rabbit IgG conjugated to FITC (1:50, Sigma, Mississauga, ON) or Alexa 488 (1:1000, Molecular Probes, Hornby, ON) dissolved in 2% NGS and 0.2% Triton X-100 in PBS for 1 hr. The sections were rinsed 3×10 min in PBS and mounted on subbed microscope slides.

To improve the results of immunocytochemistry and immunofluorescence, a heat-induced antigen retrieval protocol was employed. Heat assists in unmasking the epitopes or antigens that are hidden as a result of protein cross-linking induced by formaldehyde

fixation (Shi et al., 1991; Cattoretti et al., 1993; Jiao et al., 1999). In this study, microwave irradiation was used before the commencement of immunostaining. Briefly, both slide-mounted sections and free-floating sections were placed in a microwave in containers containing 0.1M PBS solution (pH 7.4). Irradiation at the maximum setting for 1-2 min raised the temperature of the PBS solution to 90-95°C after which the power setting was adjusted to keep the solution at a constant temperature of 90-95°C for 10 min. The sections were kept in the PBS for another 20 min to cool down. Standard immunocytochemical staining as described above was performed after the microwave irradiation. To exclude the possible non-specific staining resulting from microwave irradiation, sections with no primary antibody incubation were also included in the experiment.

4.2.1.3 Image processing

For DAB-stained sections, the olfactory bulbs were examined using bright-field microscopy. For fluorescence, two-channels of a confocal microscope (Olympus Fluoview) or an epifluorescence (mercury lamp) microscope were used. The confocal processing provided scans of 0.25 μm thickness, which enabled unequivocal cellular localization of the label. Images were captured digitally with either the Fluoview confocal software or with a Spot® digital camera.

4.2.2 Results

4.2.2.1 Microwave irradiation and β_1-adrenoceptor labeling

We observed substantially improved immunocytochemical labeling of β_1-adrenoceptors in olfactory bulb sections by using the microwave procedure described in the Methods (Fig.4.1A vs.4.1B). Strong immunocytochemical staining was observed in the mitral cells and tufted cells. Label was mainly confined to the cytoplasm of the somata. Fainter label was observed in periglomerular cells and small subsets of granule cells. Without microwave irradiation, only faint, punctuate labeling of cells was observed (Fig.4.1A). Thus, microwave treatment produced enhanced visualization of cells immunoreactive for β_1-adrenoceptors within the bulb. To control for possible non-specific staining resulting from microwave irradiation, some sections were incubated without the presence of the primary antibody. This procedure served as a negative control for β_1-adrenoceptor immunocytochemistry and produced no cellular label in the olfactory bulb, although non-specific label of fiber bundles within the deep granule cell layer was present (data not shown).

4.2.2.2 Immunofluorescence double label

To investigate the targets of NE and 5-HT action, immunofluorescence double

labeling of β_1-adrenoceptor and 5-HT_{2A} receptors was performed. Consistent with previous studies (Pompeiano et al., 1994; McLean et al., 1995; Hamada et al., 1998; Cornea-Hébert et al., 1999), the mitral cell and external plexiform layers were intensely labeled by the 5-HT_{2A} receptor antibody (Fig.4.2D). Labeled tufted cells were also found in the main olfactory bulb. Figure 4.3 shows CY3 immunofluorescence label of mitral cells in a PND 35 rat. In a few cells, both cell bodies and their dendrites were clearly labeled for the 5-HT_{2A} receptor.

By using two-channel confocal imaging, we observed substantial β_1-adrenoceptor and 5-HT_{2A} receptor double labeling of mitral and tufted cells in both young (eg. PND10, Fig.4.2A,B) and older animals. The label of both receptors was mainly cytoplasmic as shown by punctuate label within the cytoplasm of mitral cells as illustrated in Figures 4.2C & D. This observation is consistent with the observation that G-protein coupled receptors are normally internalized (Tang et al., 1999; Chakraborti et al., 2000).

4.3 Experiments 2A and 2B cAMP expression following odor preference training

4.3.1 Materials and methods

In Experiment 2A, 63 Sprague-Dawley rat pups of both sexes from 9 litters were

used. Seven training groups were included in this experiment. In Experiment 2B, ten rat pups from 5 litters were subjected to unilateral 5-HT depletions of the olfactory bulbs on PND 1 and given either 2 mg/kg or 6 mg/kg isoproterenol (β-adrenoceptor agonist, Sigma) injections before training. All litters were culled to 12 pups/litter. No more than one pup of either sex from each litter was assigned to each training group.

4.3.1.1 Odor conditioning and drug injection

The procedure for conditioning has been described in detail before (Langdon et al., 1997; Price et al., 1998; McLean et al., 1999; Yuan et al., 2000b). Briefly, on PND 6, saline or isoproterenol (1 mg/kg, 2 mg/kg, and 4 mg/kg for Experiment 2A; 2 mg/kg and 6 mg/kg for experiment 2B) was injected subcutaneously into normal pups (Experiment 2A) or pups with unilateral 5-HT depletion of olfactory bulbs (Experiment 2B) 40 min before their exposure to odor conditioning. The odor conditioning was performed by placing the pups on peppermint-scented bedding for a period of 10 min (0.3ml peppermint extract in 500 ml of fresh wood-chip bedding). Also, in this study, serotonergic fiber depletion was performed unilaterally in the olfactory bulbs. Either the low (2 mg/kg) or the high (6 mg/kg) doses of isoproterenol was injected systemically into the pups to investigate the synergistic effect of β-adrenergic and serotonergic receptor interaction in inducing cAMP cascade activation (Experiment 2B).

In Experiment 2A, some pups from the same litters were taken from their dams 10 min before they were subjected to one of the following three training conditions: odor+stroking (the pup was stroked by a sable brush every other 30 seconds for a period of 10 min while the pup was placed on peppermint-bedding), stroking only (the pup was subjected to stroking while it was placed on fresh bedding), and naive (the pup was placed on fresh bedding for 10 min). The purpose of this grouping was to investigate the cAMP levels when using a more natural learning paradigm than the isoproterenol-induced learning.

Immediately after training, the pups were sacrificed by decapitation, both olfactory bulbs were removed from the skull and frozen on dry ice. In Experiment 2A, each pair of olfactory bulbs from a pup was placed in 1.5 ml centrifuge tubes, whereas in Experiment 2B, olfactory bulbs from each pup were put individually into a microcentrifuge tube because in each pup one bulb was subjected to 5-HT depletion while the other was not. All samples were subsequently stored at -70°C until they were assayed for cAMP content.

4.3.1.2 5-HT depletion

Unilateral 5-HT depletions of olfactory bulbs were performed in order to provide intra-animal controls for the effect of 5-HT on isoproterenol induced cAMP expression.

The procedure of 5-HT depletion has been previously described in detail (McLean et al., 1993; McLean and Darby-King, 1994). Briefly, PND1 pups were removed from the dams, pretreated with10 mg/kg desipramine by intraperitoneal injection, and placed on fresh bedding. Forty-five min later, after being anesthetized by hypothermia on ice, the pups were placed in a modified stereotaxic instrument, and 150 nl of 5,7-dihydroxytryptamine (5,7-dHT) in Ringer's solution plus 0.02% ascorbic acid was injected unilaterally into the anterior olfactory nucleus. Immunocytochemistry performed on the olfactory bulbs of some 5-HT depleted pups confirmed 5-HT fiber depletion.

4.3.1.3 cAMP assay

Olfactory bulb samples were homogenized in 300 µl distilled water containing 4 mM EDTA. The homogenate was heated for 5 min in a boiling water bath to coagulate the protein, then centrifuged at 10,000 rpm for 5 min at 4°C. After centrifugation, the supernatant was removed and placed in a microcentrifuge tube. The pellet was kept for protein assay. cAMP in the supernatant was assayed using a radiolabeled cyclic AMP (^{3}H) assay kit (Amersham, Baie d'Urfé, PQ). The protein pellet was reconstituted by 500 µl of dH_2O. The protein content of the samples was determined by a BCA protein assay kit (Pierce, Rockford, IN). cAMP content is presented as pmole/mg protein.

4.3.2 Results

cAMP expression in the olfactory bulb is increased by effective odor preference training protocols (Fig.4.4A). It is also increased by protocols that do not produce odor preference learning. The groups receiving 2 mg/kg isoproterenol and 4 mg/kg isoproterenol paired with odor had significantly more cAMP than the odor only control group (Repeated measures ANOVA F_3=3.20, $p<.05$; least significant difference tests, $p<.05$). The 1 mg/kg isoproterenol group was intermediate. From the histogram (Fig.4.4A) it also appears that isoproterenol increases cAMP in a dose-related manner.

Pups receiving stroking paired with odor also had significantly more cAMP than the naive control group ($p<.01$, paired $t_{1\text{-tailed}}$-test). Pups with stroking alone had the same mean cAMP levels as those with odor pairing and were also different from the naive control group ($p<.05$, paired $t_{1\text{-tailed}}$-test). The mean cAMP levels of the odor only pups also did not differ from that of naive pups. This indicates that stroking, acting as the unconditioned stimulus, is sufficient to activate the cAMP cascade, while the conditioning stimulus (peppermint odor) appears to have no further influence on the level of cAMP expression during odor preference learning.

In 5-HT depleted olfactory bulbs, the level of cAMP was significantly ($p<.05$, $t_{2\text{-tailed}}$-test) reduced compared to non-depleted sides in both 2 mg/kg and 6 mg/kg

isoproterenol groups (Fig.4.4B). However, the dose-dependent profile of cAMP increase following isoproterenol injection was maintained. This indicates that 5-HT and norepinephrine act synergistically to activate cAMP during odor preference learning.

4.4 Experiment 3A and 3B cAMP immunocytochemistry following unilateral 5-HT depletion and isoproterenol injection

4.4.1 Materials and methods

4.4.1.1 Animal preparation

Twelve Sprague-Dawley rat pups of both sexes from 4 litters were used in this experiment. All pups were given 5-HT depletions of left olfactory bulbs on PND1 as described in Experiment 2. On PND6, in Experiment 3A, seven pups were subjected to 2 mg/kg isoproterenol injections (s.c.) 40 min before being placed on peppermint-scented bedding for 10 min. The pairing of 2 mg/kg isoproterenol and odor on PND6 normally induces odor preference in pups (Sullivan et al., 1989b; Langdon et al., 1997). Immediately after odor exposure, pups were sacrificed by decapitation. The brains were removed from the skulls, fixed in ice-cold 4% paraformaldehyde in 0.1M phosphate buffer for one hour, then kept overnight in 20% sucrose and 1.5% paraformaldehyde in 0.1M phosphate butter. The next day, the brains were transferred to a 20% sucrose

solution for one hour and then cut frozen at 30 μm using a cryostat. In Experiment 3B, 5 pups were sacrificed directly on PND6, to test whether unilateral 5-HT depletion itself affects the basal level of cAMP in the olfactory bulb. Immediately after sacrifice, the brains were removed from the skull, and processed as described in Experiment 3A.

4.4.1.2 Immunocytochemistry

Olfactory bulb sections were thawed onto subbed slides and processed using immunocytochemistry for cAMP and 5-HT (to confirm 5-HT depletions of the left olfactory bulbs). The cryostat-mounted sections were air-dried at room temperature for 5-10 min followed by 1 hr incubation in 2% NGS, 0.2% TritonX-100 in PBS at room temperature to block non-specific binding. Sections were incubated in the primary antibody (cAMP, Chemicon, Mississauga, ON, diluted 1:500; 1:1000; 1:3000; 1:5000 ; 5-HT, INCStar, Stillwater, MN, diluted 1:3000) in 0.2% Tx-100, 2% NGS in PBS at 4°C overnight followed by standard immunocytochemical methods.

4.4.1.3 Image processing and analysis

The 5-HT depletions were confirmed under bright-field microscopy. Consistent with previous results (McLean and Darby-King, 1994), it produced more than 80% depletion of the 5-HT fibers of the left olfactory bulb as shown by immunocytochemistry.

For cAMP immunocytochemistry, the relative amount of cAMP expression was quantified systematically by comparing the optical density (darkness) of label in the mitral cell layer from both bulbs. In each bulb, five sections at even intervals through the entire olfactory bulb were examined.

Image analysis was performed by tracing the medial region of the mitral cell layer and an adjacent background region in the internal plexiform layer. Relative optical density was achieved by determining the difference of optical density between the region of interest and the background region divided by the optical density of the background region. All the slides from Experiment 3A and 3B were coded so that the person analysing sections was blind to the treatment. The regional optical densities from both olfactory bulbs were compared statistically using the paired Student t-test.

4.4.2 Results

Consistent with β_1-adrenoceptor and 5-HT_{2A} immunocytochemical localization, strong cAMP immunocytochemical staining was observed mainly in the mitral cells (Fig.4.5) and tufted cells. Only a few periglomerular cells and granule cells were stained. This suggests that NE action through the β_1-adrenoceptor observed here increases cAMP mainly in the output cells of the olfactory bulb. To support our hypothesis that NE and 5-HT act synergistically to enhance cAMP signaling in the CREB phosphorylation pathway,

quantitative analysis of the relative optical density of the medial regions of mitral cell layers was performed on both the 5-HT depleted bulbs and the control sides. Figure 4.6A shows that after the pairing of isoproterenol and odor exposure, in 5-HT depleted olfactory bulbs there was significantly ($p<.01$, paired $t_{2\text{-tailed}}$-test, $n=7$) less cAMP staining in the mitral cell layer (reflected by relative optical density in medial regions) compared to that in the control side of the same animals. To determine if unilateral 5-HT depletion itself reduces the basal level of cAMP expression in mitral cells, cAMP immunocytochemistry was also performed on the olfactory bulbs of non-isoproterenol injected pups. Fig.4.6B shows that there is no significant difference in the relative optical density of cAMP immunocytochemical staining in mitral cells in the 5-HT depleted sides and the control sides. Comparison were only made within animals to avoid variability due to differences in the overall immunocytochemical reaction.

Therefore, in the present data set 5-HT depletion does not by itself reduce the basal level of cAMP expression, but it impaires the ability of isoproterenol to enhance cAMP signaling. This is consistent with our hypothesis that β-adrenoceptors and 5-HT_2 receptors are critical in early odor preference learning and interact via a synergistic promotion of cAMP in mitral cells of the olfactory bulb.

4.5 Discussion

The major findings of this study are that β_1-adrenoceptors and 5-HT_{2A} receptors are localized on mitral cells in the olfactory bulb and that interaction of these receptors affects cAMP processing in mitral cells. cAMP expression is not directly affected by the loss of serotonin but its up-regulation by either tactile stimulation or by stimulation of β_1-adrenoceptors in the rat pup is impaired. Below we discuss the potential relevance of such interactions for early olfactory preference learning.

4.5.1 Cellular localization of the β_1-adrenoceptor and the 5-HT_{2A} receptor

Localization of 5-HT_{2A} receptor protein (Hamada et al., 1998; Cornea-Hébert et al., 1999) and mRNA (McLean et al., 1995) has been previously shown in mitral and tufted cells. Our result using immunofluorescence to label the 5-HT_{2A} receptor is consistent with these previous studies.

With the increased sensitivity of the present heat-induced antigen retrieval method for immunocytochemistry, we are the first to report substantial neuronal localization of the β_1-adrenoceptors in the olfactory bulb output cells. The finding that the receptor is localized primarily in the output cells of the bulb (mitral and tufted) and co-localized with 5-HT_{2A} receptors is consistent with the demonstrated functional interaction of these

receptors in the olfactory bulb of the neonate rat and supports the present model of critical neural substrates.

Our finding that the two receptor subtypes remain co-localized in older animals implies possible functional 5-HT/NE interactions in adult rat olfactory bulb that merit further investigation.

β_1-adrenoceptor localization in the olfactory bulb of rat has been briefly described in two survey papers (Wanaka et al., 1989; Nicholas et al., 1993). Both papers suggested weak localization of β_1-adrenoceptors (in situ hybridization, Nicholas et al., 1993) or β-adrenoceptors (immunoctyochemistry, Wanaka et al., 1989) in the granule cell layer of the bulb as also seen in a small subset of cells in the present study. A developmental binding study targeted to the olfactory bulb also suggested most β_1-adrenoceptors binding occurred in layers other than the mitral cell layer and increased developmentally (Woo and Leon, 1995). In a later study the same authors reported locus coeruleus lesions increased β-adrenoceptors density in the glomerular layer (Woo et al., 1996), suggesting this region might be most responsive to locus coeruleus input. Such receptors could be on the dendrites of mitral cells projecting to the glomerular layer.

The reasons for the failure of the binding studies to identify mitral cells as important sites of β_1-adrenoceptors are unclear. As noted by Woo and Leon (1995),

iodopindolol is biased toward the detection of β_2-adrenoceptors, thus even examining radiolabeling in the presence of a β_2-adrenoceptor antagonist may not be sufficient to eliminate some binding of β_2-adrenoceptors, which are numerous in the olfactory bulb. The profiles of the two receptors seen in the binding studies were identical except for additional external plexiform labeling for β_2-adrenoceptors. Mitral cells also have prominent internalized β_1-adrenoceptor labeling in the present study. Internalized receptors have low binding affinity (Flugge et al., 1997) and their demonstration may depend critically on ligand concentration. Finally, Leon's laboratory, in a brief report (Ivins et al., 1993) using *in situ* hybridization methodology, identified β_1-adrenoceptor mRNA in mitral cells, consistent with the present observations.

Isoproterenol interacts with β_2, as well as β_1-adrenoceptors, in the olfactory bulb. The present study does not rule out a role for the β_2-adrenoceptors in olfactory preference learning. *In situ* hybridization study demonstrated that β_2-adrenoceptors are more widely expressed than β_1-adrenoceptors in the olfactory bulb, including the mitral cell layer (Nicholas et al., 1993), and can up-regulate cAMP. Their specific contribution to early olfactory learning remains to be identified.

4.5.2 Functional significance of cAMP activation via β_1-adrenoceptors and 5-HT_{2A} receptors in output cells of the olfactory bulb

cAMP increases accompanied both stroking and 2 mg/kg isoproterenol, the two effective UCSs, as predicted by the present hypothesis. However, odor+stroking did not produce increases greater than those of stroking alone, although the odor+stroking group was less variable. In contrast to the present failure to detect an increase in cAMP with odor pairing, the studies of pCREB using Western blots showed higher levels in the odor+stroking than the stroking only condition (McLean et al., 1999). With 10% peppermint as the odorant, cells involved in the odor representation appear widespread and were sufficient to demonstrate the pCREB effect. The pCREB result may argue that the failure to see pairing associated cAMP change is real. If cAMP is not increased by odor pairing, it suggests that, unlike the Aplysia model in which sensory input and a monoaminergic input converge on adenylyl cyclase activation, in the rat pup olfactory bulb the sensory input influences learning by convergence on the pCREB pathway (see Fig.4.7).

The hypothesis of receptor synergism in cAMP recruitment was tested in Experiment 2. As mentioned, 5-HT depletion prevents early olfactory preference learning produced by pairing odor with the normally optimal 2 mg/kg dose of the β-adrenoceptor agonist, isoproterenol. A higher, 4 mg/kg or 6 mg/kg dose of the β-adrenoceptors, can,

however, overcome the depletion effect and produce learning (Langdon et al., 1997; Yuan et al., 2000b). The higher 4 mg/kg dose of isoproterenol is normally ineffective as a UCS in normal pups (Langdon et al., 1997). A dose of 6 mg/kg has also been shown to be ineffective in learning and in producing CREB phosphorylation in normal pups (Yuan et al., 2000b). Thus, isoproterenol-induced learning and CREB phosphorylation show parallel inverted U curve profiles with increasing doses of isoproterenol. A similar inverted U curve profile has been described for stroking-induced learning (Sullivan et al., 1991), suggesting it is a basic property of the learning system.

The dose-dependent increase in cAMP with increasing isoproterenol did not support the initial hypothesis that biphasic agonist control of cAMP explains the inverted U curve seen behaviorally, electrophysiologically and biochemically. Although biphasic cAMP control of behavior has been reported in other models (Ozacmak et al., 2002), the present data suggest instead that there is an optimal level of cAMP activation which can be exceeded. In the present model three possibilities suggest themselves: (a) higher levels of cAMP recruit increased calcium entry which might favor calcineurin-induced dephosphorylation; (b) higher levels of cAMP promote greater phosphodiesterase 4 (PDE4) activation through PKA (Ang and Antoni, 2002) and this may critically shorten the duration of the cAMP signal; and (c) elevated cAMP promotes faster cAMP extrusion (Wiemer et al., 1982) which again would shorten the signal duration. CREB phosphorylation has been shown to be enhanced by longer durations of cAMP signaling

(Barad et al., 1998). Manipulation of calcineurin and measurements of the time course of cAMP elevation with varying doses of isoproterenol would test these hypotheses.

cAMP-regulated signaling pathways and the associated phosphorylation of CREB have been postulated as important mechanisms underlying learning and memory (Davis et al., 1995; Abel et al., 1997; Impey et al., 1998b; Mons et al., 1999; Wong et al., 1999). Aversive olfactory learning in the Drosophila depends critically on this cascade (Zhong et al., 1992; Davis et al., 1995; Mons et al., 1999) and has several parallels with the rat pup model of olfactory learning. Both cAMP increases and CREB phosphorylation are thought to mediate the acquisition of odor aversion in Drosophila. Although cAMP levels in Drosophila have not been measured directly with training as in the present study, systematic manipulations of the components of the cAMP cascade produce predictable deficits in olfactory learning and memory (Zhong et al., 1992; Davis et al., 1995). In addition, in the dunce mutants with reduced phosphodiesterase, cAMP levels are increased above normal and olfactory learning and memory are deficient (Byers et al., 1981). As in the present study an impairment of the normal temporal dynamic of cAMP has been suggested to underlie the impairment in olfactory learning in Drosophila mutants with higher levels of cAMP.

4.5.3 A new model of noradrenergic-mediated early olfactory preference learning in the rat pup

In the present study, we demonstrate that mitral cells, the main output cells in the olfactory bulb, are the postsynaptic cellular substrate for olfactory preference learning. Immunocytochemistry demonstrated the co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors in mitral cells. Manipulation of β-adrenoceptor and 5-HT receptor activation affected cAMP levels in mitral cells in a predictable pattern. This profile of results supports the hypothesis that the β_1-adrenoceptors and $5HT_{2A/2C}$ receptors, interact in early odor preference learning via a synergistic promotion of cAMP in mitral cells in the olfactory bulb, as they do in neocortex (Morin et al., 1992), and that the critical learning change occurs in the mitral cell processing of olfactory nerve input.

This new model of olfactory preference learning mediated by β_1-adrenoceptor activation is illustrated at the circuit and intracellular level in Figure 4.7. This model accounts for several aspects of what we have seen.

Isoproterenol paired at a learning effective dose with olfactory nerve input will potentiate the olfactory nerve EPSP including both NMDA and non-NMDA components (Yuan et al., 2000b). Such potentiation may be mediated by cAMP-initiated phosphorylation of NMDA and AMPA channels. Phosphorylation of L-Ca^{2+} channels

could also contribute to membrane depolarization and potentiation of NMDA currents. Closing of K^+ channels following isoproterenol which has been reported in other systems (Karle et al., 2002) is another mechanism by which depolarization might occur. The failure of higher doses of isoproterenol to produce the electrophysiological potentiation would again be related to an excess of dephosphorylation activity or a shortening of the elevation of cAMP.

Either odor-stroking pairings or odor-isoproterenol pairings also produce CREB phosphorylation (McLean et al., 1999; Yuan et al., 2000b). For this component an interaction of the cAMP cascade and Ca^{2+}/ calmodulin cascade is suggested to occur. In particular Ca^{2+}/calmodulin activates calmodulin kinases while PKA prevents phosphatase activation resulting in a net phosphorylation of CREB (Impey et al., 1998a; Wong et al., 1999). PKA may also enhance activity of the MAPK pathway (Impey et al., 1998a; Poser and Storm, 2001), but we have not yet characterized the role of this pathway in early olfactory learning. Again excessive activation of the cAMP pathway that fails to activate CREB phosphorylation may truncate the duration of the cAMP signal or have phosphatase promoting consequences.

The approach behavior of rat pups 24 hr following odor-UCS pairing is thought to be linked to these initiating events. Optical imaging experiments in trained pups suggest increased activation in the olfactory bulb to the conditioned odor, but not to a control

odor, 24 hr after training (Yuan et al., 2002). It is clear that pairing odor with local infusion of isoproterenol in the rat pup is sufficient to produce the approach response (Sullivan et al., 2000b). It appears that a change in the representation of the odor in the olfactory bulb determines the response. However, we do not even know why some odors are inherently attractive. A study of the bulbar representation of such odors might be helpful. Our data suggests the odor representation potentiates, both NMDA and AMPA components of olfactory nerve input are strengthened during acquisition procedures (Yuan et al., 2000b), while activation probed by optical imaging is stronger 24 hr later (Yuan et al., 2000). How this increased activation is coupled to approach behavior is unknown.

The present model with its emphasis on the role of the β_1-adrenoceptor on mitral cells does not rule out a role for disinhibition in normal learning. β-adrenoceptor mediated disinhibition (Wilson and Leon, 1988; Okutani et al., 1998) has been reported in pups and adults in the olfactory bulb and might occur via the β_2-adrenoceptor on granule cells (Nicholas et al., 1993). Other α-adrenoceptor-mediated disinhibitory effects have been documented (see introduction). Such disinhibition would further support the β_1-adrenoceptor mechanisms identified here.

Finally, the present model is closely related to the learning model well-described for Aplysia. Indeed, Kandel et al. originally suggested that norepinephrine in mammals

might play the role of serotonin in the Aplysia (Brunelli et al., 1976). The present model follows that suggestion. A key difference between the two models would appear to be in the co-incidence detection mechanism. In Aplysia adenylyl cyclase itself is the co-incidence detector for the CS and UCS and higher levels of cAMP determine the occurrence of learning. In the present model high levels of cAMP alone do not produce learning and the CS pathway appears to interact with the UCS pathway at a later stage.

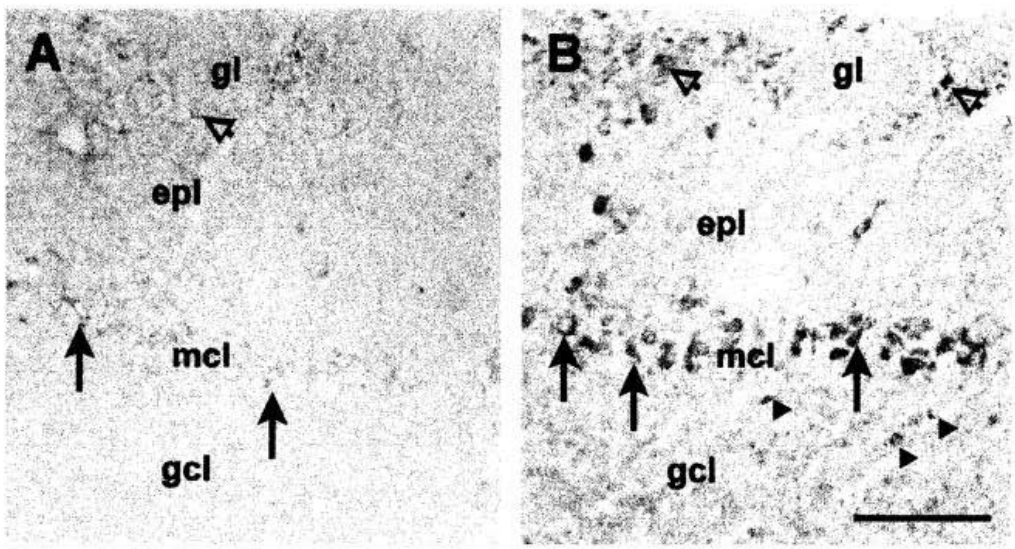

Figure 4.1 Localization of the β_1-adrenoceptor in the olfactory bulb by immunocytochemistry.
A: Visualization of the receptor without the use of microwave heating.
B: Visualization of the receptor after the use of microwave heating.
Note the faint label of mitral cells in A and the clear labeling of mitral and tufted cells and a small number of granule and periglomerular cells in B.
Abbreviations, epl, external plexiform layer; gcl, granule cell layer; gl, glomerular layer; mcl, mitral cell layer; . Bars, 100 μm.

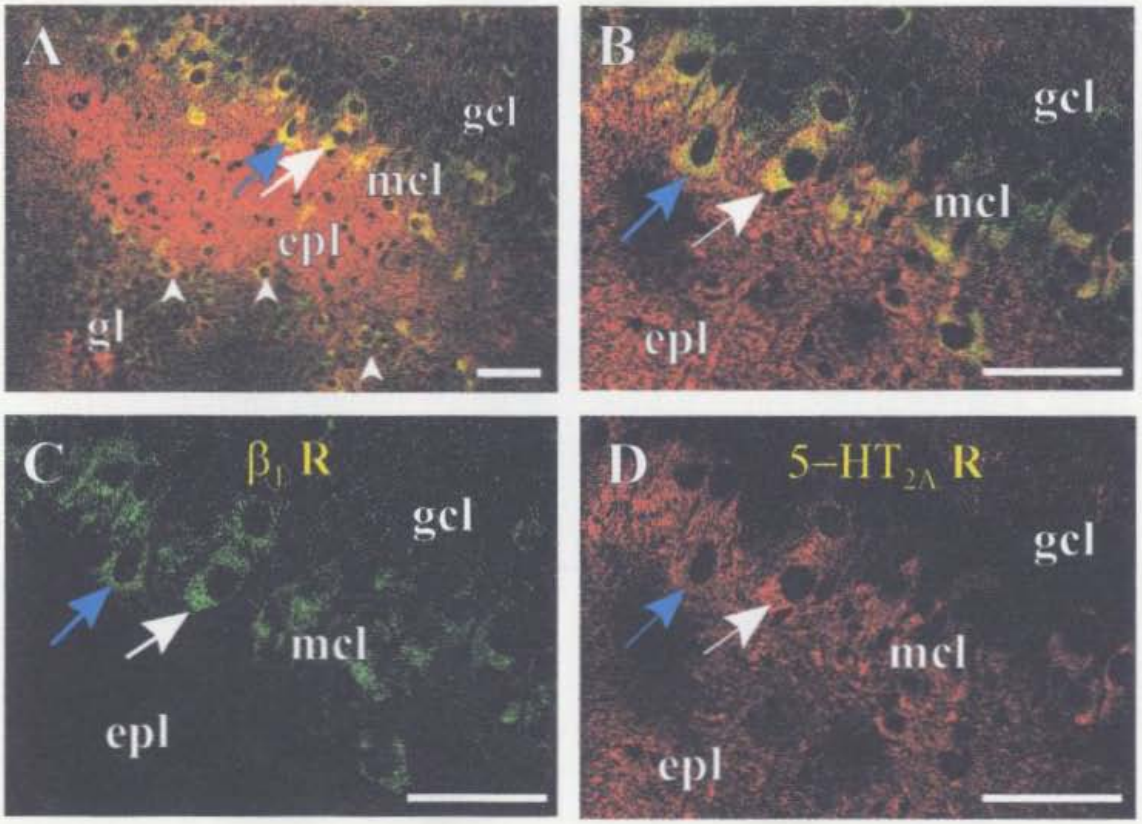

Figure 4.2 Confocal images of the olfactory bulb from a PND 10 pup.
Note the double labeled mitral cells (eg. at white and blue arrows) at low (A) and higher magnification (B-D). A&B show the combined label of β_1 & 5-HT_{2A} receptors. Double label was also observed in tufted cells (arrowheads) near the glomerular layer (A). Bars, 50 μm.

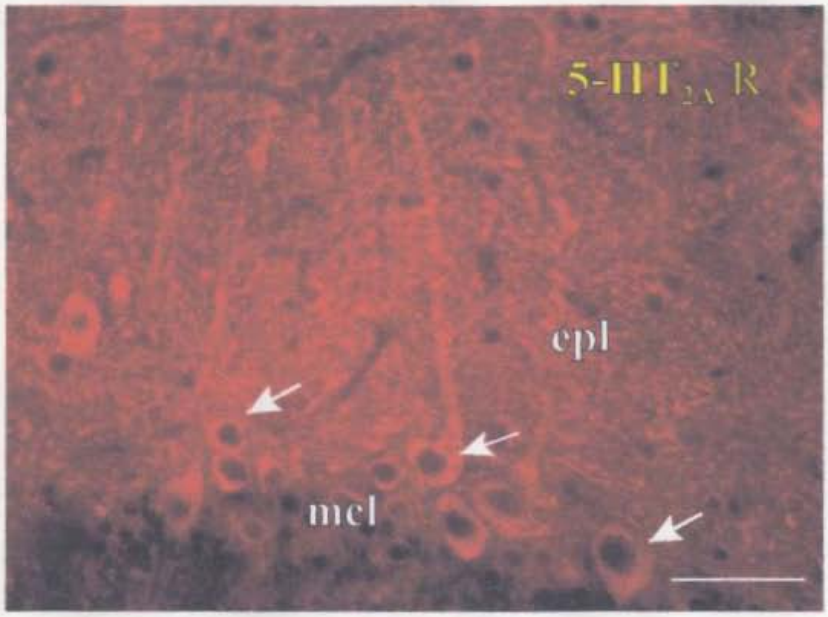

Figure 4.3 Immunofluoresence label of mitral cells in a PND35 rat using an antibody to the $5\text{-}HT_{2A}$ receptor. Note the mitral cell body (arrows) and dendritic label. Bar, 50 μm.

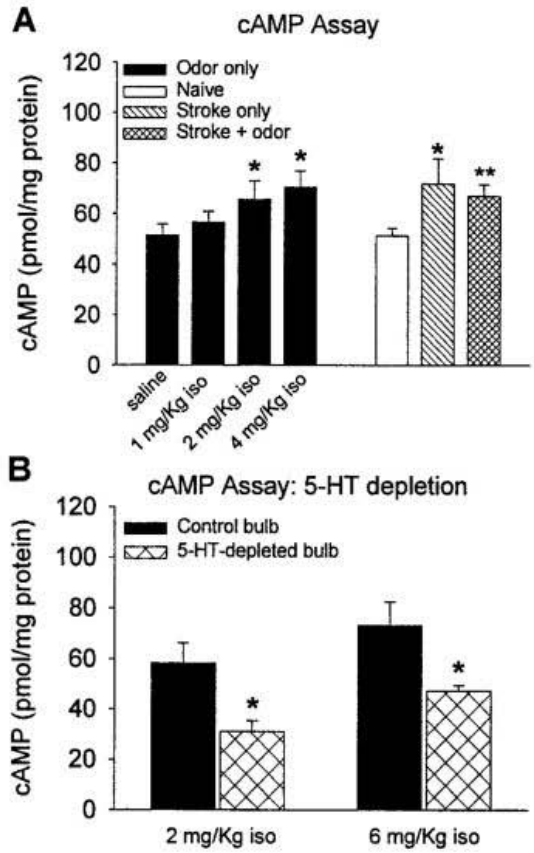

Figure 4.4 cAMP expression in the olfactory bulb of PND 6 pups immediately after various training sessions.

(A). An increase in cAMP expression is observed with increasing β-adrenoceptor activation (isoproterenol) compared to saline-injected controls exposed to odor only at the time of training (N=9 *, $p<0.05$ compared to the saline group). The act of stroking the pup appears to activate the cAMP to levels equivalent to pups given stroking plus odor (N=9 *, $p<0.05$; **, $p<0.01$). (B). 5-HT depletion reduces the isoproterenol induced cAMP expression which can be partially overcome by inducing more activation of β-adrenoceptors (via isoproterenol). *, $p<0.05$; 5-HT depleted bulb compared to normal control bulb. N=4 for 6 mg/kg iso control group, N= 5 for other three groups. Abbreviation: iso, isoproterenol.

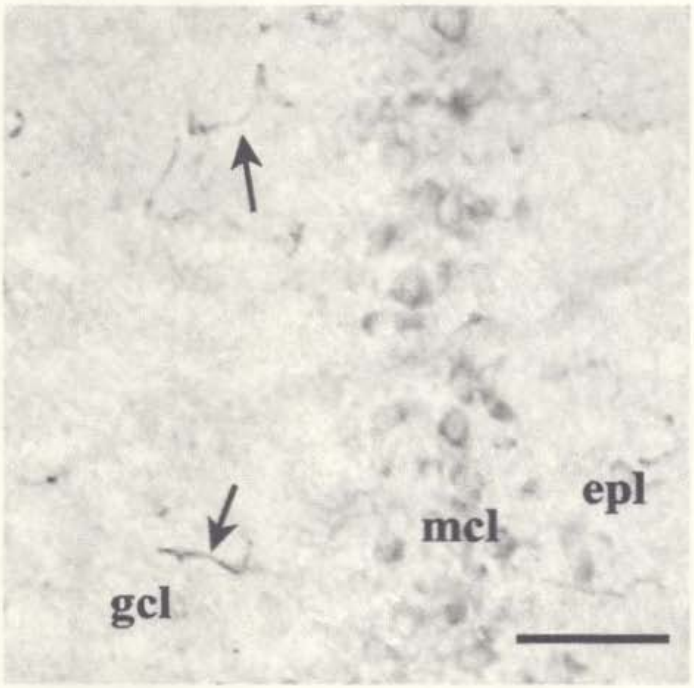

Figure 4.5 cAMP immunocytochemistry shows the cellular location of cAMP in the olfactory bulb. The immunocytochemical methods employed here showed selective cAMP expression in mitral cells of the mcl. Arrows indicate artifact labeling of blood vessels. Abbreviations. epl, external plexiform layer; gcl, granule cell layer; mcl, mitral cell layer. Bar, 50 μm

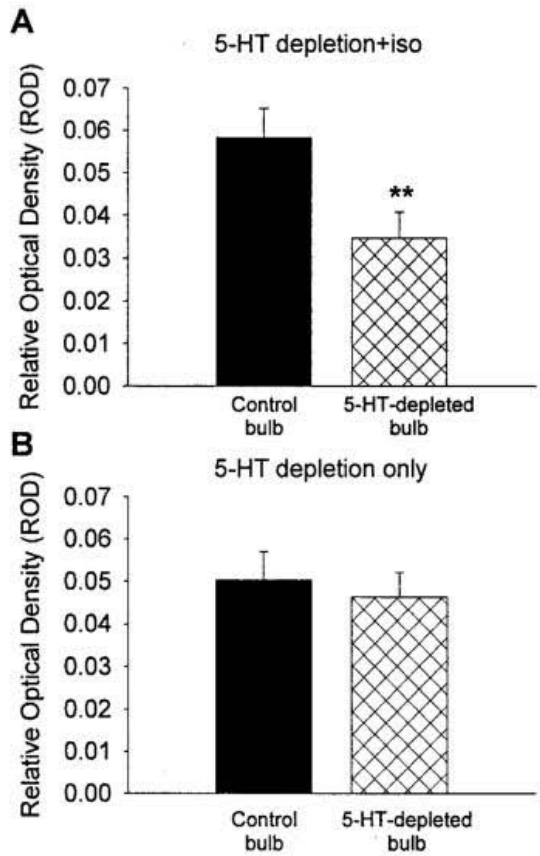

Figure 4.6 Relative optical density (ROD) measures of cAMP showing the influence of isoproterenol (iso) and/or unilateral bulbar 5-HT depletion on cAMP expression in the mitral cell layer.

(A). ROD of cAMP immunocytochemical staining in the 5-HT depleted olfactory bulbs and the control bulbs of the same animals after pairing of 2 mg/kg isoproterenol injections with odor exposure. Significantly less cAMP level is seen in 5-HT depleted olfactory bulbs compared to the control sides ($N=7$, $p<0.01$).

(B). ROD of cAMP immunocytochemical staining in the 5-HT depleted olfactory bulbs and the control bulbs of the same animals without isoproterenol injections or odor exposure. Note that there is no difference between these two groups ($N=5$, $p>0.05$) which suggests that 5-HT depletion by itself does not affect cAMP levels.

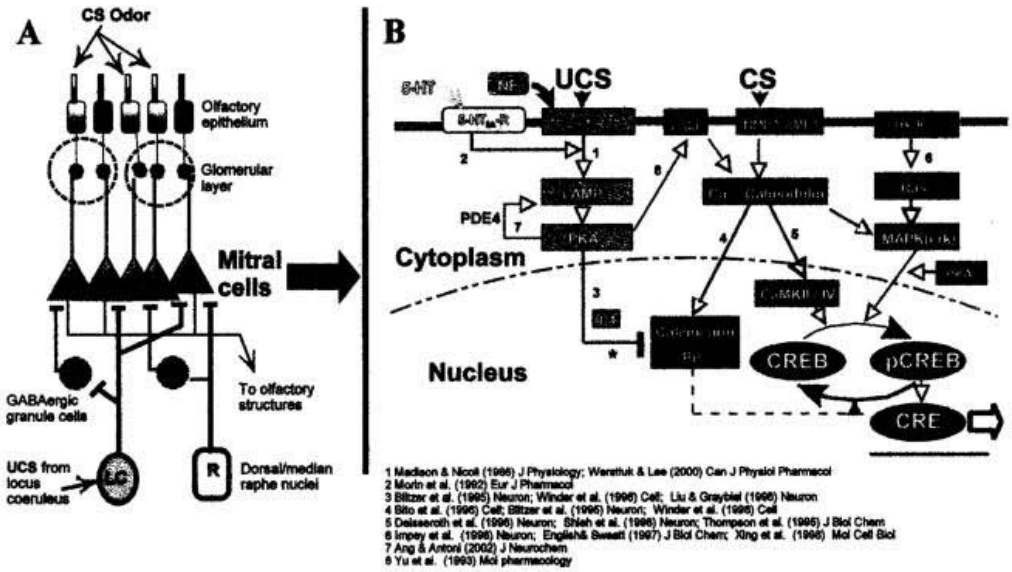

Figure 4.7 Proposed intercellular and intracellular pathways in the olfactory bulb activated by β_1 and 5-HT_{2A} receptors.

(A). Schematic diagram indicating the major circuitry in the olfactory bulb especially as it relates to the present odor learning model and identifying the convergence of odor input (via olfactory receptor cells), and noradrenergic and serotonergic input onto mitral cells.

(B). Intracellular circuitry of the mitral cell. β_1-adrenoceptors mediate the unconditioned stimulus (UCS) via either tactile stimulation or a β-adrenoceptor agonist. The conditioned stimulus (CS) is provided by odors which stimulate glutamate receptors on mitral cells. When proper stimulation occurs the pathways induce phosphorylation of CREB and learning. ★cAMP/PKA "gating" of the calcineurin dephosphorylation pathway of CREB (see discussion for further details of the pathways).

Chapter 5 Early Odor Preference Learning in the Rat: Bidirectional Effects of CREB and Mutant CREB Support a Causal Role for pCREB

(submitted to J.Neuroscience., 2002)

5.1 Introduction

Early odor preference learning offers a unique paradigm for the study of natural mammalian learning. In the neonate rat, neural circuitry changes that are critical for odor preference memory occur in the olfactory bulb (Wilson and Sullivan, 1994; Sullivan et al., 2000b). A change in the first synapse, the olfactory input to mitral cell connection, appears to underlie both the acquisition and expression of odor preference (Wilson et al., 1987; Woo et al., 1987; Wilson and Leon, 1988; Johnson et al., 1995; Woo et al., 1996; Yuan et al., 2000b; Yuan et al., 2000). Phosphorylation of cAMP response element binding protein (CREB), proposed as a universal "memory molecule" (Silva et al., 1998), is seen in mitral cells following olfactory preference training, but not in control conditions (McLean et al., 1999). The present study asks whether phosphorylation of CREB in the olfactory bulb can be shown to be causal in early olfactory preference learning.

In neonate rats, a single 10 min session of tactile stimulation such as stroking

(unconditioned stimulus, UCS) paired with an odor (conditioned stimulus, CS), typically peppermint, produces odor preference learning, seen as an approach to the odor 24 hr later (Sullivan and Leon, 1987; Wilson and Sullivan, 1994). Norepinephrine (NE) released from the locus coeruleus with tactile stimulation is known to act in the olfactory bulb via β-adrenoceptors (Wilson and Sullivan, 1994; Langdon et al., 1997; Yuan et al., 2000b) coupled to cAMP. The β-adrenoceptor agonist, isoproterenol, can substitute for stroking when given systemically, or directly into the olfactory bulb, to induce learning (Petralia et al., 1996; Langdon et al., 1997; Yuan et al., 2000b). NE and serotonin (5-HT) in the olfactory bulb have been shown to interact in early odor preference learning to promote increases in the cAMP-mediated CREB phosphorylation (Yuan et al., 2000b). pCREB increases are transient and occur selectively in the peppermint-encoding area of the mitral cell layer in the olfactory bulb following peppermint conditioning (McLean et al., 1999). Both learning and increases in pCREB also occur when odor is paired with a moderate dose of isoproterenol (2 mg/kg), but not with lower (1 mg/kg) or higher (4 or 6 mg/kg) doses of isoproterenol (Sullivan et al., 1989b; Sullivan et al., 1991; Langdon et al., 1997; Yuan et al., 2000b). The parallel inverted-U curve profiles in both isoproterenol-induced learning and CREB phosphorylation are shifted to the right following depletion of 5-HT in the olfactory bulb (Yuan et al., 2000b), such that a higher dose (6 mg/kg) of isoproterenol is now required for the induction of learning and the increase in pCREB.

Based on these studies, we proposed a causal role for CREB in neonate rat odor preference learning; but the evidence was correlational (McLean et al., 1999; Yuan et al., 2000b). Here we evaluate causality using a herpes simplex virus, HSV, to express additional CREB or dominant negative mutant CREB (single point mutation at the phosphorylation site Ser133) in neurons of the rat pup olfactory bulb. HSV-LacZ expressing E.coli β-galatosidase, was used to verify the expression of HSV-encoded proteins in the olfactory bulb, and as a control to determine whether virus injection itself would affect odor preference learning.

In the present study we ask whether additional CREB or mutant CREB in the olfactory bulb will alter normal odor preference learning and/or promote the occurrence of odor preference learning when sub- or supra-optimal doses of isoproterenol are given. We also measure the levels of pCREB to assess the link between the substrate and the transcription factor.

5.2 Material and methods

5.2.1 Animals

Sprague-Dawley rat pups of both sexes were used in this study. Litters were culled to 12 pups/litter on postnatal day 1 (PND1, the day of birth is considered PND0). The

dams were maintained under a 12hr light-dark cycle, with *ad libitum* access to food and water. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

5.2.2 Virus vector

HSV-LacZ, HSV-CREB (overexpression of CREB), and HSV-mCREB (overexpression of a dominant negative mutant CREB) were used in this study. The average titer of the recombinant virus stocks was $4.0x10^7$ infectious unit/ml (for viral vector preparation see Cattoretti et al., 1993; Carlezon, Jr. et al., 1998; Neve and Geller, 1999).

5.2.3 Virus injection

On PND4, rat pups were anaesthetized under hypothermia on ice and placed in a stereotaxic frame. The skull over the central region of each olfactory bulb was carefully removed by a dental drill. A total of 1µl virus stock/bulb was injected at four levels into each bulb over a 5 min period with a 27 gauge Hamilton syringe. Pups were then warmed up and returned to the dam after recovery.

5.2.4 Odor conditioning

The procedure for odor conditioning for natural learning has been described before (Sullivan et al., 1989b; Sullivan et al., 1991; McLean et al., 1993). Briefly, on PND6, rat pups were removed from the dam and put on fresh bedding 10 min before odor exposure. In the odor+stroking (O/S) group, pups were placed on peppermint scented bedding (0.3 ml peppermint/500 ml normal bedding) and stroked vigorously on the hind region using a sable brush every other 30 sec for 30 sec over a 10 min period. In the odor only (O/O) group, the pups were only exposed to the peppermint bedding without being stroked. The naive pups were placed on fresh bedding for a 10 min period. Immediately after these conditions, the pups were returned to the dams.

The procedure for odor conditioning using isoproterenol (1 mg/kg, 2 mg/kg or 4 mg/kg) has been described before (Langdon et al., 1997; Yuan et al., 2000b). Briefly, on PND6, saline or isoproterenol was injected subcutaneously into pups 40 min before exposed to the peppermint odor. The pup was removed from the dam 30 min after injection and placed on fresh bedding. Ten min later, The pup was placed on peppermint-scented bedding for 10 min. After odor exposure, The pup was returned to the dam.

5.2.5 Odor preference test

On PND7, pups were subjected to odor preference testing. A stainless steel test box (30x20x18cm) was placed on two boxes which were separated by a 2 cm neutral zone. One box contained fresh bedding; the other contained peppermint scented bedding. Each pup was removed from the dam and placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint scented bedding or normal bedding was recorded for five 1-min trials. The percentage of time the pup spent on peppermint scented bedding over the 5 min period was calculated. One-way ANOVAs were used for analysis.

5.2.6 X-gal histochemistry

LacZ expression in the olfactory bulb was revealed by visualizing its substrate β-galactosidase activity using X-gal histochemistry. Pups were given an overdose of sodium pentobarbital (80 mg/kg) and perfused transcardially with an ice-cold saline solution followed by a fixative solution (0.5% paraformaldehyde + 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4). Brains were removed from the skull, postfixed in the same solution for 1hr and transferred to a 30% sucrose solution overnight.

Coronal sections (40μm) were cut in a cryostat the next day. Sections were

mounted onto slides and air dried at room temperature. Alternate sections were collected for X-gal and Nissl staining. Slides containing olfactory bulb sections for X-gal staining were then incubated overnight with a solution containing 3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 0.15 M NaCl, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, and 0.2 mg/ml X-gal (dissolved in N,N'-dimethyl formamide) in 10 mM phosphate buffer (pH7.4). An insoluble blue color indicated β-galactosidase activity. After a brief rinse in PBS, all slides were dehydrated and coverslipped with Permount (Sigma). Possible cytoarchitectural damage due to virus injections was investigated in Nissl stained sections.

5.2.7 Nuclear cell extract and CREB/pCREB assay

Pups used for the CREB/pCREB assay were anaesthetized with CO_2 and sacrificed by decapitation. Both olfactory bulbs were collected immediately on dry ice and stored in microcentrifuge tubes at -70°C. Olfactory bulb tissue was homogenized using 100 µL/sample of Buffer A containing 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithoithreitol (DTT), 1 mM PMSF, and 0.1 % NP-40. The samples were incubated on ice for 15 min, then centrifuged at 1,000x g at 4°C for 10 min. The supernatant was discarded. The pellet was resuspended in 500 µL of Buffer A without NP-40. Again, the samples were centrifuged at 1000x g for 10 min and the supernatant was discarded. The pellet was resuspended in 100 µL of TransAm lysis buffer (Active

Motif) containing DTT and a protease inhibitor cocktail. The samples were rocked at 4°C for 30 min, then centrifuged for 10 min at 14,000x g at 4°C in a microcentrifuge. The supernatant (nuclear extract) was collected. Protein determination was performed by a bicinchoninic acid (BCA) protein assay kit (Pierce).

CREB/pCREB protein content was determined using CREB/pCREB assays (Active Motif) according to manufacturer's instructions. A total of 10 μg protein was loaded into each well. CREB/pCREB was visualized and quantified by a colorimetric reaction and read by a spectrophotometer at 450nm. 2.5 μg forskolin-stimulated WI-38 cell extract was used as a positive control. The optical densities of the CREB/pCREB per mg protein were compared by a paired Student t-test between the two groups in each experimental condition.

5.2.8 Experimental procedures

5.2.8.1 Expression of HSV-LacZ in the olfactory bulb and its effect on odor preference learning

To determine whether transgenes are expressed in olfactory bulb neurons, and whether virus injection itself affects olfactory preference learning, forty-three rat pups of both sexes from eight litters were divided into 6 groups: 2 injection conditions (HSV-

LacZ & saline) X 3 training conditions (Odor+stroke, O/S; Odor only, O/O; Naive). X-gal histochemistry and Nissl staining were performed on the olfactory bulbs of HSV-LacZ injected pups after they were tested for odor preference. One-way ANOVAs were used to compare different training groups in the two injection conditions after the odor preference testing.

5.2.8.2 The causality of CREB in natural odor preference learning

Eighty-seven pups from 10 litters were divided into nine groups: 3 injection conditions (HSV-CREB, HSV-mCREB, HSV-lacZ) X 3 training conditions (O/S, O/O, naive). In each litter, no more than one pup was assigned to each group. Odor preference learning and testing were performed as described above. One-way ANOVAs were used to compare the performance of the three training groups in different injection conditions.

To test for the expected increase of CREB expression in the olfactory bulb at the time of learning following the viral injection, eighteen pups from three litters were injected bilaterally into olfactory bulbs with either HSV-CREB or HSV-LacZ on PND 4. On PND 6, the pups were sacrificed by decapitation. Both olfactory bulbs were collected in dry ice and stored in microcentrifuge tubes at -70°C until a CREB assay was performed.

To test if increased CREB substrate results in an enhanced pCREB expression following conditioning, further delineating the effects of CREB/pCREB levels on odor preference learning, a pCREB assay was performed on the olfactory bulbs of the rats from the O/S groups injected with either HSV-LacZ or HSV-CREB. Eighteen pups from three litters were used in this experiment. Previous work showed that pCREB increases maximally at 10 min after odor conditioning (McLean et al., 1999). Therefore, 10 min after being taken away from the peppermint bedding, the pups were sacrificed by decapitation. Both olfactory bulbs were collected for a pCREB assay.

5.2.8.3 The effects of CREB levels on isoproterenol-induced odor preference learning

Experiments were carried out to determine if additional wild type CREB (by HSV-CREB injection) or dominant negative CREB that could not be phosphorylated at the serine 133 site (by HSV-mCREB injection) changes the sensitivity of the system to the unconditioned stimulus, therefore shifting the isoproterenol effective inverted U-curve.

Ninety pups from eight litters were used. Twelve groups were created in this experiment: 3 virus injection conditions (HSV-lacZ, HSV-CREB, HSV-mCREB) X 4 drug/saline injection conditions (saline, 1 mg/kg isoproterenol, 2 mg/kg isoproterenol, and

4 mg/kg isoproterenol). No more than one pup from the same litter was assigned to the same group.

One way ANOVAs were used to compare the learning results from the training groups in different virus injection conditions. Subsequently, the learning effective groups (HSV-CREB+1 mg/kg isoproterenol and HSV-mCREB+4 mg/kg isoproterenol groups) were compared to their non-learning control groups (HSV-LacZ+1 mg/kg isoproterenol and HSV-LacZ+4 mg/kg isoproterenol groups) by a student t-test.

CREB phosphorylation has been proposed as a critical step in the acquisition of long-term memory (McLean et al., 1999). pCREB assays were performed on the olfactory bulbs of the rats from the groups that exhibited learning in the first set of the experiment: the 1 mg/kg HSV-CREB group, the 4 mg/kg HSV-mCREB group and their corresponding non-learning control groups : the 1 mg/kg HSV-LacZ and the 4 mg/kg HSV-LacZ groups. 36 pups from nine litters were used in this experiment. The optical densities of the pCREB/mg protein were compared by a paired Student t-test between the learning groups and their corresponding control groups.

5.3 Results

5.3.1 Expression of HSV-LacZ in the olfactory bulb and its effect on odor preference learning

To assess viral-mediated expression of LacZ in the olfactory bulb, β-galactosidase histochemical staining by X-gal was used to visualize the HSV-LacZ infected cells. Expression of the virus has been reported to be maximal at 2-4 days postinjection (Carlezon, Jr. et al., 1998). Dark blue cells were seen in all layers of the olfactory bulb using X-gal staining three days after HSV-LacZ injection (Fig.5.1). The area around the injection site was most heavily stained. HSV-LacZ spread well along the rostro-caudal axis. Control pups with saline injections did not show any X-gal staining. Microinjection of HSV-LacZ caused minimal damage to tissue structures as evaluated by Nissl staining (data not shown).

HSV-LacZ did not affect the animals' odor preference learning. As shown in Figure 5.2, HSV-LacZ injected pups demonstrated behavioural results comparable to those of the saline injected ones. HSV-LacZ injected pups in the O/S group demonstrated significant preference learning compared to the O/O or the naive groups ($p<0.01$). The same pattern of learning results applied to saline-injected pups.

5.3.2 The Causality of CREB in Natural Odor Preference Learning

Injection of HSV-mCREB prevented learning, as shown in Figure 5.3. HSV-mCREB injected animals in the O/S group failed to show a preference for peppermint after training, whereas the control littermate HSV-LacZ injected pups in the O/S group showed odor preference learning ($p<0.05$) compared to the O/O or the naive group. This suggests a causal role for CREB in odor preference learning. Mutant CREB binds to the DNA but does not promote transcription because it is not phosphorylated. Interestingly, HSV-CREB injection did not improve preference learning in the O/S group, rather the opposite occurred. Additional CREB impaired the ability of animals to acquire the odor preference, suggesting there is a window for CREB and implying an optimal window for pCREB functioning given that pCREB is the critical mediator for CREB pathway activation.

To confirm further the increase of CREB expression at the time of learning, a CREB assay was performed on the olfactory bulbs of the HSV-CREB injected pups that were sacrificed on the day of learning (2 days after HSV-CREB injection). The HSV-CREB injection group showed a 22.9% increase ($p<0.01$, Figure 5.4) in the optical density per mg of protein of CREB in olfactory bulb tissue relative to that of the HSV-LacZ control group.

Expression of pCREB was determined 10 min following O/S conditioning test if

increasing CREB substrate increased pCREB following learning. A positive result would suggest that too much pCREB interferes with learning, since HSV-CREB injection impaired odor preference learning in the O/S condition. Figure 5.5 shows that there was a significant increase in the optical density of pCREB (13.8%, $p<0.05$) in the HSV-CREB injected group compared to the HSV-LacZ group. This suggests that increasing CREB substrate by HSV-CREB injection enhances pCREB levels correspondingly, and that increasing CREB/pCREB beyond an optimal level interferes with learning.

5.3.3 The effects of CREB levels on isoproterenol-induced odor preference learning

Figure 5.6 demonstrates that increasing CREB expression by HSV-CREB injection enhanced the sensitivity of the system to the UCS, so that, an originally ineffective dose, 1 mg/kg isoproterenol, now induced learning when paired with odor ($p<0.05$, compared within the HSV-CREB injected groups). The normally optimal dose, 2 mg/kg, and the higher dose, 4 mg/kg, of isoproterenol, failed to induce learning in the HSV-CREB injected groups. Thus, CREB shifted the isoproterenol dose-response relationship to the left. This is consistent with the results in the previous experiment showing that a critical CREB/pCREB window exists, and that too much, as well as too little, CREB/pCREB can prevent learning. Surprisingly, the HSV-mCREB injected pups developed odor preferences when the higher dose of isoproterenol, 4 mg/kg, was used

($p<0.01$, compared within the HSV-mCREB injected groups). Thus, it appears that mCREB shifts the inverted U curve dose response relationship to the right. Both HSV-CREB + 1 mg/kg isoproterenol and HSV-mCREB + 4 mg/kg isoproterenol groups demonstrated significantly higher percentages of time spent over the peppermint side than their corresponding non-learning control groups: HSV-LacZ + 1 mg/kg isoproterenol and HSV-LacZ + 4 mg/kg isoproterenol groups ($P<0.01$, Student t-test).

More important than the CREB increase itself, is the phosphorylation of CREB, since pCREB is the initial step for CREB activation and the CRE-induced gene expression that underlies long-term synaptic plasticity and memory formation (McLean et al., 1999). Again we compared the pCREB levels in both learning groups with their non-learning controls. We were particularly interested to know whether 4 mg/kg isoproterenol increased pCREB in HSV-mCREB injected pups which would be consistent with our behavioural results. As seen in Figure 5.7, we found that, the learning group: HSV-CREB + 1 mg/kg isoproterenol had an 11.1% increase in the optical density of pCREB over that of the non-learning control group: HSV-LacZ + 1 mg/kg isoproterenol. Similarly, pCREB in the HSV-mCREB + 4 mg/kg isoproterenol learning group showed a significantly higher pCREB (11.7% increase in the optical density of pCREB, $p<0.01$) than that observed in the non-learning control group (HSV-LacZ + 4 mg/kg isoproterenol).

5.4 Discussion

Natural, stroking-induced odor learning or 2 mg/kg isoproterenol-induced odor learning was prevented by infusion of a HSV-mCREB into the olfactory bulb. This result critically implicates CREB as a mediator of early odor preference learning. Since the serine 133 site is the only one *not* available for phosphorylation (Josselyn et al., 2002), it also suggests phosphorylation of serine 133 may be critical in early odor preference learning.

Unexpectedly, a bi-directional effect of mCREB was observed in these experiments. The pairing of odor and a 4 mg/kg dose of isoproterenol, that normally does not produce learning, was a successful learning paradigm if HSV-mCREB was previously infused into the olfactory bulb. pCREB assay of this novel effective learning condition revealed higher levels of pCREB, as seen previously with learning, than those seen in rat pups receiving LacZ infusions. We have previously hypothesized that the failure of high doses of isoproterenol to produce learning and/or increased pCREB, might be related to enhanced protein kinase A activation of phosphatases (Yuan et al., 2000b). Since mCREB would provide a 'false' target for both kinase and phosphatase activity, it could alter the balance of enzyme activities in infected neurons to decrease, or increase, the likelihood of pCREB expression depending on the enzyme levels induced by training. Thus, with optimal enzyme levels (odor+ stroking or odor+2 mg/kg isoproterenol) mCREB is

deleterious, but with an excess of phosphatase activity (odor+4mg/kg isoproterenol), for example, it could be beneficial.

The ability of HSV-mCREB to alter downstream transcription and behavioural outcomes has been previously demonstrated in nucleus accumbens (Carlezon, Jr. et al., 1998; 2000). No effect of HSV-mCREB, however, was observed in an earlier study of long-term memory when it was infused into the amygdala (Guzowski and McGaugh, 1997).

HSV-CREB infusions lowered the threshold for isoproterenol-induced odor learning. Infusion of HSV-CREB in the olfactory bulb produced learning in rats pups given odor+ 1 mg/kg isoproterenol, normally an ineffective pairing for learning. The ability of CREB to lower the threshold for isoproterenol-induced odor learning further supports our hypothesis that CREB has a causal role in early odor preference learning. pCREB was also significantly increased in this novel learning condition as compared to rats pups receiving HSV-LacZ infusions. The ability of HSV-CREB to alter downstream genomic expression and behavior has been previously demonstrated in the nucleus accumbens (Carlezon, Jr. et al., 1998; 2000). HSV-CREB has also been shown to convert short term memory to long term memory in the amygdala, although it did not render a weak unconditioned stimulus more effective (Guzowski and McGaugh, 1997) as observed in the present experiment.

A bi-directional effect of CREB appeared when HSV-CREB was infused prior to normally effective learning conditions. Thus, rat pups given odor+stroking or odor+2 mg/kg isoproterenol did not learn if HSV-CREB was infused prior to training.

Over expression of CREB has been shown to interfere with learning in other paradigms (Guzowski and McGaugh, 1997; Josselyn et al., 2002). The present pattern of results is consistent with that literature. However, here, pCREB levels were also assessed in the odor+stroking group given CREB infusion. This group had higher pCREB levels than the LacZ controls that successfully learned the odor preference. This outcome supports the hypothesis of an optimal window for pCREB level in initiating the development of odor memory. It has been shown that the duration of pCREB activation critically influences downstream gene expression (Bito et al., 1996), and it has been proposed that overactivation of CREB might lead to increased repressor activity (Silva et al., 1998), but this is the first demonstration of a negative effect of elevated pCREB levels on learning and memory.

CREB is a target of the PKA/cAMP intracellular pathway. We have shown that cAMP is increased in mitral cells by stroking and by isoproterenol (Yuan et al., in press). We have suggested a model of early odor preference learning in which the locus coeruleus input activates beta receptors on mitral cells to trigger cAMP increases (Yuan et al, in press). This increase is hypothesized to interact with calcium currents activated by the

odor input to mitral cells to enhance pCREB in those same cells. Other evidence however demonstrates CREB phosphorylation at the Ser 133 site through a variety of protein kinases including those activated by calcium (Silva et al., 1998). An alternative model of odor preference learning suggests NE-induced disinhibition of mitral cells by granule cells, which could enhance NMDA currents from odor input onto mitral cells (Wilson and Sullivan, 1994). This model would also predict pCREB increases in mitral cells. Both mechanisms are likely to contribute to early odor preference learning.

Our data from optical imaging (Yuan et al., 2000) and from electrophysiological measurements of olfactory nerve evoked potentials (Yuan et al., 2000b) suggest a critical change during both acquisition and retrieval is the potentiation of the mitral cell responses to the odor input. An input potentiation model is also supported by earlier evidence of enhanced 2-DG (Woo et al., 1987; Johnson and Leon, 1996) and c-Fos (Johnson et al., 1995) during memory retrieval. Potentiation of the mitral cell responses to the odor input following appetitive olfactory conditioning has also been reported for the honeybee (Faber et al., 1999) and the sheep (Kendrick et al., 1992).

CREB and pCREB were first shown to have causal roles in the encoding of sensory memory in Aplysia. Using genetic tools, CREB has been shown to be causal in olfactory learning in Drosophila. This is the first report that CREB and pCREB have causal roles in mammalian olfactory learning.

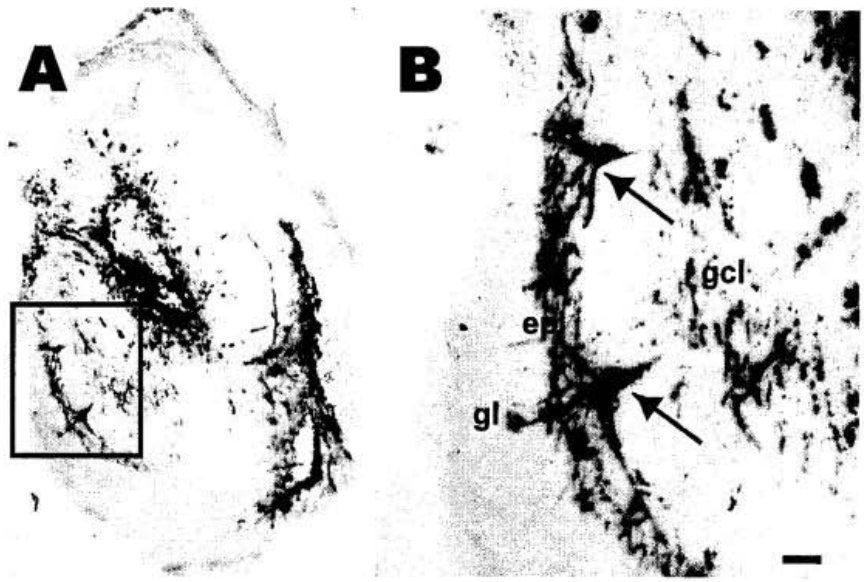

Figure 5.1 Visulization of β-galactosidase by X-gal staining showing expression of LacZ in many cells throughout the olfactory bulb. Labelled cells in A are shown at higher magnification in B. Mitral cells are indicated by arrows. Bar in B, 50μm
Abbreviation: gl, glomerular layer; epl, external plexform layer; gcl, granule cell layer

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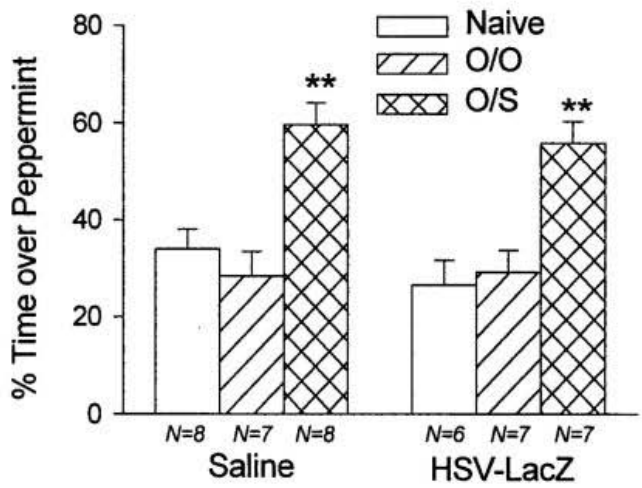

Figure 5.2 Odor preference test showing HSV-LacZ injection itself does not affect odor preference learning.

HSV-LacZ injected pups in the O/S group demonstrate significant preference learning when compared with those in either the O/O or the naïve group. The same pattern applies to the saline injected pups (**p<.01). Abbreviations: O/S, odor+stroke; O/O, odor only.

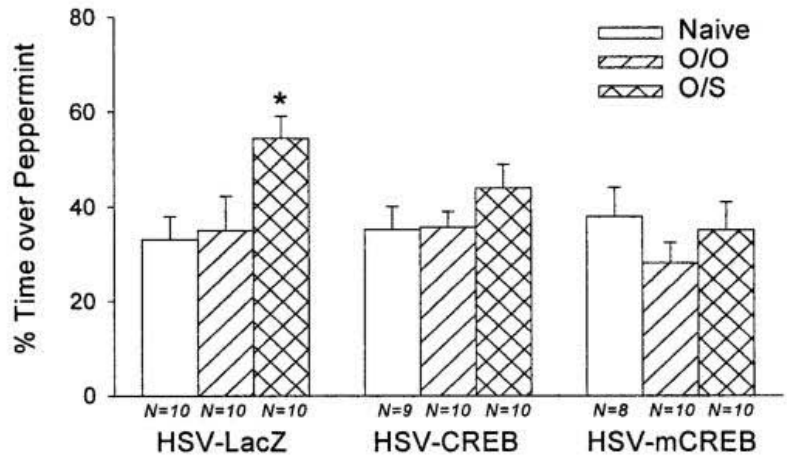

Figure 5.3 Odor preference test showing CREB and mCREB injections block odor preference learning in a naturally learning paradigm. Both HSV-CREB and HSV-mCREB injected pups show deficient odor preference learning compared to their HSV-LacZ control pups (*$p<0.05$)

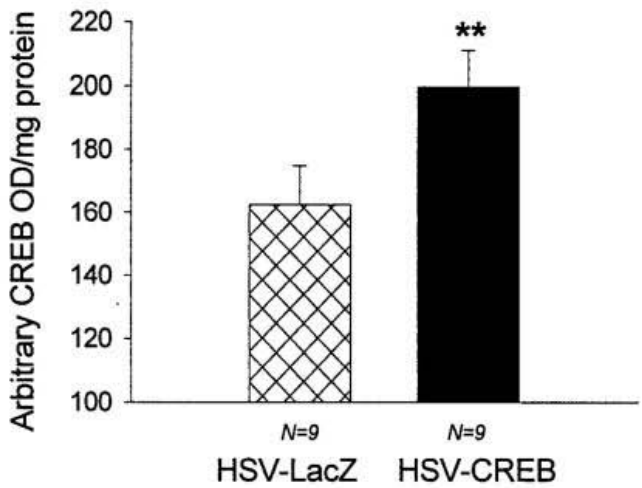

Figure 5.4 CREB assay showing CREB in the olfactory bulb is increased two days after HSV-CREB injection. CREB content is presented as arbitrary optical density/mg protein. (*p<.05).

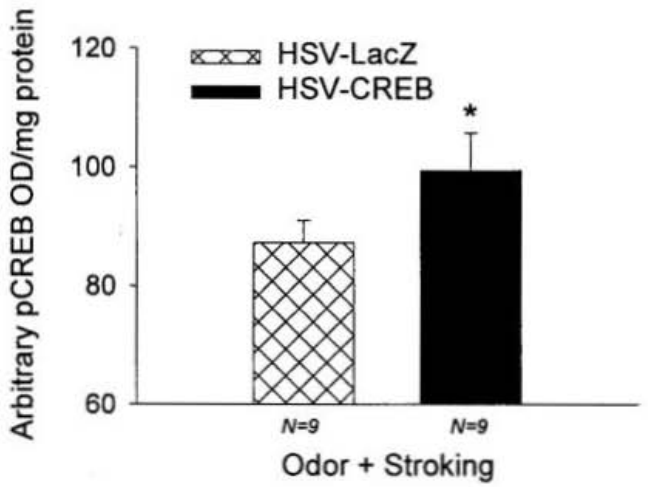

Figure 5.5 pCREB assay showing pCREB is significantly increased in the olfactory bulbs of the HSV-CREB injected group compared to those of the HSV-LacZ group 10 min after they are subjected to O/S. pCREB content is presented as arbitrary optical density/mg protein. (*p<.05)

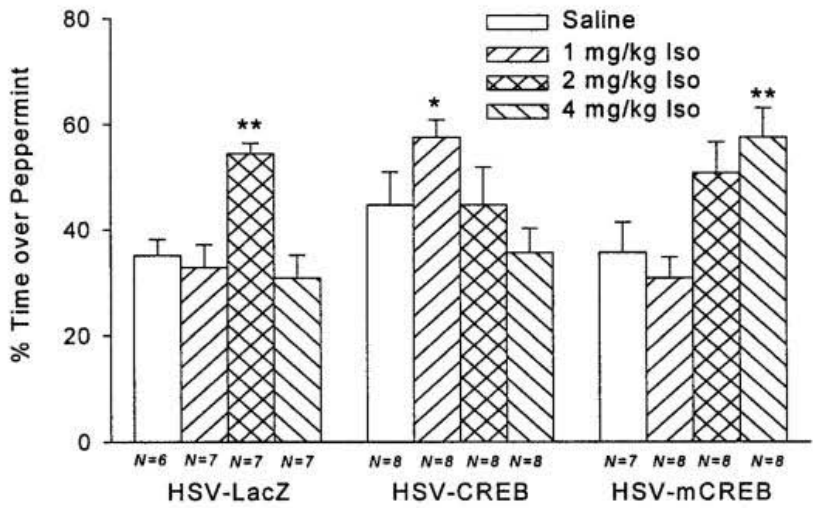

Figure 5.6 Odor preference test showing CREB and mCREB injections shift the isoproterenol inverted U-curve to the left and right respectively.
For the HSV-LacZ injected group, 2 mg/kg isoproterenol induces odor preference learning when paired with peppermint odor. In contrast, the HSV-CREB injected group shows that, a lower dose of isoproterenol (1 mg/kg) produces learning; for the HSV-mCREB injected group, a higher dose of isoproterenol (4 mg/kg) produces learning. (**p<.01, *p<.05)

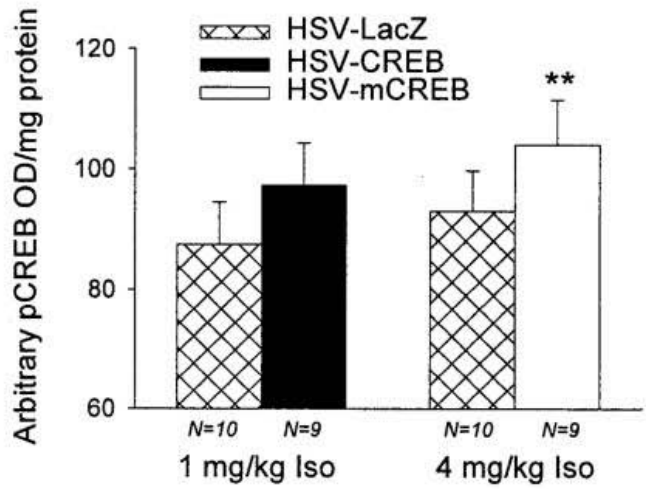

Figure 5.7 pCREB assay showing pCREB is increased in the olfactory bulbs of the learning groups.

CREB/1 mg/kg isoproterenol and mCREB/4 mg/kg isoproterenol learning groups demonstrate increased pCREB expression following training than their lacZ control groups.
pCREB content is presented by the pCREB arbitrary optical density/mg protein. (**p<.05)

Chapter 6 Summary

6.1 Research outcomes

6.1.1 Mitral cells are the postsynaptic substrate for learning

A fundamental question of my thesis work is where early odor preference learning occurs. It has been suggested that the granule cell was the primary site for NE-mediated learning plasticity in the olfactory bulb (Jahr and Nicoll, 1982; Trombley and Shepherd, 1992; Wilson and Sullivan, 1994). Granule cells provide feedback inhibition by releasing GABA onto the mitral cell. NE acts on granule cells to reduce this inhibitory feedback. Reduced inhibition in turn would permit an increased NMDA current in the mitral-granule cell synapses.

However, there are discrepancies regarding the receptors mediating the disinhibitory effect of NE. Whereas Sullivan et al (1994) demonstrated a β-adrenoceptor mediated odor preference learning model, α-, but not β-adrenoceptors mediate the disinhibition from granule to mitral cells. Moreover, the cellular localization of β-adrenoceptors was not clear. Binding studies are inherently inconclusive regarding the precise postsynaptic targets. A more defined immunocytochemical study of the cellular

location of β-adrenoceptors in the olfactory bulb could better elucidate this issue. Also, given that 5-HT_2 receptors and β-adrenoceptors interact in early odor preference learning, the precise postsynaptic target common to these receptors in the bulb was yet unknown.

We examined the cellular distribution of β_1-adrenoceptors and their co-localization with 5-HT_{2A} receptors in the olfactory bulb. Results from these experiments, together with others, e.g. the pCREB and cAMP localization experiments, lead us to suggest that mitral cells, the output cells of the olfactory bulb, serve as the neuronal substrate for a β_1-adrenoceptor-mediated odor preference learning.

The specific evidence is as follows: (a) β_1-adrenoceptors are primarily located on mitral/tufted cells of the olfactory bulb, with few located on granule cells. Fluorescence immunocytochemistry and confocal imaging demonstrate a clear co-localization of β_1-adrenoceptors and 5-HT_{2A} receptors on mitral cells. (b) β-adrenoceptors are G-protein coupled and when activated, trigger a cAMP second messenger cascade. The interaction of NE (via β-adrenoceptors) and 5-HT in elevating cAMP levels occur in mitral cells as observed by cAMP immunocytochemistry. (c) The interaction of NE (β-adrenoceptors) and 5-HT leads to the phosphorylation of CREB. pCREB changes are also observed in the mitral cells of the olfactory bulb. These outcomes strongly suggest mitral cells are the postsynaptic targets for a β-adrenoceptor mediated, 5-HT receptor facilitated, intracellular cAMP signalling cascade.

6.1.2 From inverted-U curves to functional windows

As described before, an important and useful aspect of odor preference learning is the inverted-U curve property of the odor conditioning. Low doses of isoproterenol are ineffective, medium doses are effective, and high doses are again ineffective (Sullivan et al., 1991). This parallels evidence obtained with stroking. A subthreshold stroking input can summate with a subthreshold isoproterenol dose to produce learning, while an effective stroking stimulus becomes ineffective in inducing learning when a low dose of isoproterenol is also given (Sullivan et al., 1989b). The inverted-U curve of the isoproterenol UCS can be shifted by 5-HT manipulations (Langdon et al., 1997; Yuan et al., 2000b).

To understand the inverted-U curve function of UCS in odor learning and its mechanism was another goal of my thesis. The exploration of this issue has led to the discovery of functional windows in the cAMP-CREB signalling pathway.

First, when pCREB is measured in the olfactory bulb after odor conditioning, whereas an effective dose of isoproterenol, when paired with odor, enhances the pCREB expression in the olfactory bulb, either a lower dose, or a higher dose of isoproterenol, fails to enhance pCREB levels in the olfactory bulb. This suggests CREB activation exhibits a parallel inverted-U curve to that seen in behaviour. A critical window for

calcium and PKA co-activation of phosphorylation events may have been exceeded by pairing odor and a high dose (6 mg/kg) of isoproterenol in the normal rat pups. Interestingly, potentiation of the glutamatergic olfactory nerve input by systemic isoproterenol injection also demonstrates an inverted-U curve window. As discussed in Chapter 4, there are several possibilities for understanding these parallel effects of the isoproterenol UCS on both pCREB expression and ON input potentiation. (a) The failure to phosphorylate CREB may be a simple consequence of the failure of potentiation. (b) It is likely that phosphorylation-sensitive ion channels are involved in the early membrane effects of isoproterenol. The failure of appropriate phosphorylation activation could lead to the failure of ON input potentiation as well as the failure to increase pCREB. (c) Less probable are paralled mechanisms – one accounts for CREB phosphorylation, one for ON input potentiation.

Second, a functional window for cAMP occurs in NE-mediated odor preference learning. Because the known effects of isoproterenol are mediated via G-protein activation and recruitment of adenylate cyclase, we suspected the failure to induce learning, increase pCREB, and potentiate the ON evoked potential resulted from a failure to enhance cAMP by a high dose of isoproterenol. However, experiments using a cAMP assay showed that cAMP is dose-dependently increased by isoproterenol immediately after odor conditioning. Two conclusions are suggested by this outcome: (a) cAMP plays

a modulatory role rather than directly transmitting the learning signals, and (b) a more complicated spatial and temporal activation of cAMP is required for signal transduction during learning. For example, we hypothesize, the duration of cAMP action, rather than the absolute amount of cAMP, may be more important for learning to occur.

Third, there appears to be a window for effective pCREB levels. Our experiments using viral vector injections of either HSV-CREB or HSV-mCREB suggest too much, as well as too little CREB impairs odor preference learning. As discussed in 1.3.1, there are two regulation mechanisms for CREB activation. Firstly, CREB activation is determined by the balance between a Ca^{2+}/calmodulin phosphorylation pathway and a Ca^{2+}/calcineurin dephosphorylation pathway at the upstream stage. As described above and in Chapter 4, pCREB is optimal when a moderate, behaviourally effective dose of isoproterenol is used. A stronger stimulus, such as a higher dose of isoproterenol, may favour a dephosphorylation activation that eventually overrides the phosphorylation cascade. Secondly, the balance between CREB activators and repressors may be a second mechanism at the transcriptional stage. Activation of CREB may result in the increased expression of CREM isoforms, the accumulation of which may eventually lead to the repression of CREB-dependent transcription (Silva et al., 1998). Furthermore, our work is the first to exhibit in a *in vivo* mammal learning model, that too much pCREB is not helpful.

6.1.3 CREB is critical in odor preference learning

CREB has been hypothesized as a universal "memory molecule" (Silva et al., 1998). Its role in learning and memory has been extensively investigated in various species and a diversity of memory models in the last two decades. Establishing the causal role of CREB in odor preference learning was an important goal of my thesis. It is, also, a critical component of our hypothesized odor learning model, and furthermore, a basis for the future study of the downstream genomic, synaptic, and structural changes following odor learning.

CREB phosphorylation correlates with learning conditions. Odor + stroking conditioning increases pCREB transiently in a restricted odor-coding area in the olfactory bulb (McLean et al., 1999). An effective dose of isoproterenol, when paired with the odor, can substitute for stroking to increase pCREB.

More importantly here, we evaluated the causal role of CREB in odor preference learning using a Herpes viral vector, to insert additional CREB copies, or mutant CREB copies, into the neurons of the rat pup olfactory bulb. We had two important findings that suggest the causal role of CREB in odor preference learning. First, mutant CREB insertion, which could competitively reduce the phosphorylation of natural CREB in the olfactory bulb, blocks learning. Second, additional CREB insertion increases the

sensitivity of the system to UCS stimulation, shifting the isoproterenol inverted-U function curve to the left. A low, normally ineffective dose of isoproterenol, induces successful odor learning in HSV-CREB injected rat pups.

The use of a viral vector as a CREB delivery vehicle to manipulate the CREB level in the olfactory bulb of rats bypasses the potential problems of using transgenic, or gene knockout animals, to study learning and memory in other systems. It avoids the potential systemic, and/or developmental deficits caused by transgenic, or gene knock-out, manipulations. Recent studies using HSV vectors in other brain regions suggest it is a safe and effective means of incorporating genes into the host (Cattoretti et al., 1993; Carlezon, Jr. et al., 1998; Neve and Geller, 1999; Schutzer et al., 2000; Mower et al., 2002). We are the first to use this means of gene manipulation in the rat olfactory system to study natural learning in normal animals.

6.1.4 Biochemical and physiological changes induced by odor learning are long-lasting

Learning leaves traces which can be retrieved at the synaptic, and cellular levels during the memory phase. Early odor preference learning changes the single-unit responses of mitral cells during odor re-exposure (Wilson et al., 1987). Focal 2-DG uptake and c-fos expression in the glomerular layer increase after early odor preference

learning (Woo et al., 1987; Johnson and Leon, 1996). Increased focal 2-DG uptake may be due to increased glomerular size (Woo et al., 1987), increased numbers of juxtaglomerular cells (Woo and Leon, 1991) following peppermint learning, or simply due to increased activation by odor input.

Consistent with these changes, by using a novel technique, intrinsic optical imaging, we demonstrated that 24 hr after peppermint preference learning, there is an increase in intrinsic optical signals at the glomerular level. This result is consistent with the evidence showing an increase in the field evoked potential to the ON input in pups of the same age that receive learning effective training conditions. Creation of an olfactory preference in the rat pup may therefore be intimately related to an increase in synaptic strength at the level of glomeruli. The intrinsic signal change at the level of the glomeruli 24 hr later may indicate that the synaptic modification seen during acquisition conditions is sustained and reflected as an enhance synaptic excitation (Waldvogel et al., 2000). An increase in mitral cell excitation as a representative memory change is supported by evidence from lamb odor recognition studies in which β-adrenoceptor activation paired with lamb odor lead to an increase in mitral cell excitation to the lamb odor as well as an increase in inhibitory transmitter measurement in the sheep olfactory bulb (Kendrick et al., 1992).

Although rapid progress has been made in the past few years in applying optical

imaging techniques to explore odor coding in the olfactory bulbs of both mice and rats, we are the first to report a memory-associated change in the neonate rat olfactory bulb using this technique. We showed that optical imaging is an excellent technique to explore training-related odor presentation and changes in the olfactory bulb. Optical changes can be recorded from olfactory bulbs during both memory acquisition and retrieval phases in living animals.

6.2 A new model for odor preference learning

Based on my thesis work and previous work done in our laboratory, we proposed a new model for odor preference learning, in which we suggested that the β-adrenoceptors and $5HT_{2A/2C}$ receptors, critical in early odor preference learning, interact via a synergistic promotion of a cAMP cascade in mitral cells in the olfactory bulb to mediate CREB pathway activation, which critically underpins memory formation. The critical learning change occurs in the mitral cell processing of olfactory nerve input, and the olfactory circuitry and/or structural changes induced are long-lasting.

6.2.1 Comparison with a disinhibition model

Sullivan and Wilson (1994) proposed that learning results from the disinhibition of mitral cells, which permits activation of NMDA receptors on granule cells leading to

increased long-term inhibition of mitral cells and accompanying structural changes during the memory phase. In that model, NE input from the locus coeruleus to the olfactory bulb acts as the UCS by inhibiting granule cell interneurons in the bulb through β-adrenoceptors.

However, the discrepancy regarding the receptor subtypes in mediating mitral cell disinhibition and the lack of data regarding the cellular distribution of β-adrenoceptors in the olfactory bulb made it necessary to further explore the location and function of β-adrenoceptors in odor preference learning.

6.2.1.1 Evidence consistent with the mitral cell cAMP/PKA/pCREB model

We demonstrated in this thesis, first, either natural learning using odor+stroking (McLean et al., 1999), or learning using odor+effective doses of isoproterenol (see Chapter 2), enhanced pCREB expression in mitral cells of the olfactory bulb. Since β-adrenoceptor activation promotes a cAMP 2nd messenger cascade via G-proteins, we hypothesized that β-adrenoceptor activation could activate intracellular signal cascades to further promote Ca^{2+} entry through the olfactory nerve mediated NMDA or L-type Ca^{2+} channels, which, on one hand, could enhance the phosphorylation of ion channels and the depolarization of postsynaptic mitral cells; and on the other hand, could promote CREB phosphorylation and CRE-mediated downstream genomic changes in mitral cells. The

potentiation of the olfactory nerve evoked field potentials of mitral cells and the activation of intracellular signalling machinery during the learning phase result in long-lasting changes that can be recruited during the memory phase (see Chapter 3).

Second, the observation that the β_1-adrenoceptors and 5-HT_{2A} receptors co-localize in mitral/tufted cells and NE and 5-HT manipulations change the levels of cAMP in mitral cells further confirmed our hypothesis, suggesting NE via β_1-adrenoceptors activates a cAMP cascade in mitral cells of the olfactory bulb. 5-HT appears to promote the NE-induced cAMP signalling (see Chapter 4). Since isoproterenol is a non-specific β-adrenoceptor agonist, a role for the β_2-adrenoceptors in odor preference learning can not be ruled out. An *in situ* hybridization study demonstrates the β_2-adrenoceptors are more widely expressed in the olfactory bulb (Nicholas et al., 1993). Their specific contribution to early odor preference learning remains to be determined.

Third, we demonstrated a causal role of CREB activation in early odor preference learning. Learning itself causes the activation of CREB, while manipulations of CREB level change the ability of the UCS system to induce learning. Elevating CREB levels by viral vector injection of HSV-CREB shifts the isoproterenol inverted-U curve to the left so that a low, normally ineffective dose of isoproterenol causes learning. Importantly, reducing the levels of CREB by HSV-mCREB (single point mutation at phosphorylation site serine 133) injection blocks the O/S natural learning, which shifts the isoproterenol

inverted-U curve to the right in the isoproterenol mediated learning. These outcomes suggest a critical role of CREB phosphorylation in inducing learning (see Chapter 5).

Therefore, unlike the disinhibitory model proposed by Sullivan et al, here we demonstrate a model in which a cAMP cascade activated by a NE UCS via β_1-adrenoceptors promotes the excitation of the mitral cell itself and results in the intracellular signalling pathway activations which underpin long-term memory formation. Mitral cells, rather than granule cells, are the neuronal focus of β_1-adrenoceptor mediated odor learning in this model.

6.2.2 Comparison with cAMP-mediated learning models in other species

6.2.2.1 Aplysia and Drosophila

Identifying key molecular elements underpinning learning and memory in invertebrates has provided evidence for an important role of the cAMP signalling cascade in memory formation.

In Aplysia, a weak CS (a touch to the siphon) when repeatedly paired with a strong UCS (an electric shock to the tail), results in a greatly potentiated gill-withdrawal reflex elicited by a touch to the siphon (Carew and Sahley, 1986; Mons et al., 1999).

Studies of the mechanism of this system demonstrate that the CS is induced by Ca^{2+} influx through voltage-gated ion channels activated by the action potential in the sensory neuron. The UCS is induced by exciting facilitatory interneurons, which leads to the release of the modulatory neurotransmitter serotonin on to sensory neurons and the subsequent activation of a cAMP cascade via adenylyl cyclase (AC) in these neurons. In this system, long-term facilitation and synaptic changes are mediated by the synergistic interaction between Ca^{2+}/CaM and 5HT-αs GTP pathways that coactivate AC to produce the strong or prolonged cAMP signals that appear required for transcriptional activation. As discussed in 1.3.2.1, an increase in cAMP by repeated pulses of 5-HT gives rise to the translocation of the catalytic subunit of PKA to the nucleus, where it phosphorylates CREB and immediate early genes to regulate expression of late response genes, which encode new proteins that are critical for the persistent changes underlying the development of more stable and durable forms of memory (Frank and Greenberg, 1994).

In Drosophila system, the cAMP signalling cascade plays a critical role in long-term memory of olfactory avoidance learning in which the fruit fly Drosophila learns to distinguish a conditioned odor that has been paired with electric shock from a neutral odor. Deficits in olfactory associative learning and memory are observed in mutant flies that have changes in the cAMP-signalling cascade or CREB levels. In the Drosophila model, mushroom body neurons integrate sensory inputs from both olfactory cues (the CS, producing an increase in intracellular Ca^{2+}) and footshock (the UCS, activating a Gs-

coupled receptor which in turn activates the cAMP cascade) (Goodwin et al., 1997; Mons et al., 1999). As in Aplysia, elevated cAMP may cause the translocation of PKA to the nuclei where it phosphorylates CREB and initiates a cascade of gene expression responsible for long-term structural and functional changes at synaptic sites. As discussed in 1.3.2.2, mutations of cAMP pathway components cause behavioural deficits. Either elevating the cAMP level (*Dunce*, which lacks phosphodiesterase) or reducing the cAMP level (*Rutabaga*, which is defective in adenylyl cyclase) impairs odor avoidance learning in Drosophila. However, double mutants of *Dunce*- and *Rutabaga*-, which exhibit approximately normal levels of cAMP, still show deficit in learning. This suggests that complex spatial and temporal regulations of cAMP as opposed to absolute levels of cAMP may underpin memory formation in Drosophila (Mons et al., 1999), as is also suggested in the present thesis (see Chapter 4).

In both of these models, the coincident activation of two input pathways (CS and UCS) converge to produce a synergistic activation of Ca^{2+}/CaM stimulable ACs which in turn enhances cAMP levels and engages a crucial intracellular cascade for the establishment of the memory traces (Mons et al., 1999). The activation of the CREB transcriptional pathway may serve as a second convergent site for the CS and UCS inputs, with adenylyl cyclases being the first site (Dash et al., 1991).

The present odor preference learning model in neonate rats shares several

common components with invertebrate learning models described in both Aplysia and Drosophila. For example, cAMP increases and CREB pathway activation have been implicated both in the invertebrate learning models and the present rat odor preference learning model in rats. In addition, as in the Drosophila system, an optimal window appears to occur for cAMP functioning in early odor preference learning, with too high or too low levels of cAMP interfering with learning. Also, the present model suggests temporal and spatial patterns of cAMP activation may be important in the odor preference learning process. However, a key difference between the invertebrate models and the early odor preference learning model in rats appears to be the co-incidence detection mechanism. In both Aplysia and Drosophila, adenylyl cyclase is the co-incidence detector for the CS and UCS. The coincident activation of two inputs converge to produce a synergistic activation of Ca^{2+}/CaM stimulable adenylyl cyclases, which in turn enhances the cAMP level proposed as the primary mediator of downstream events that engage synaptic plasticity in learning and memory. In Aplysia, Ocorr et al (1985) showed that depolarization of the sensory neurons prior to exposure to 5-HT pulses increases levels of cAMP over those seen when CS and UCS are unpaired. In odor preference learning in neonate rats, high levels of cAMP alone do not produce learning; thus the CS pathway appears to interact with the UCS pathway at a later stage. The CS-UCS pairing does not provide an additional increase of cAMP compared to UCS stimulation alone (see Chapter 4). Thus, the role and mechanism of cAMP in learning are different between the model for rat odor learning and the invertebrate learning model. While in Aplysia and

Drosophila, cAMP activates PKA to translocate into the nucleus and phosphorylate CREB, therefore serving as a direct mediator, in our model for rat odor preference learning, the cAMP signalling appears to act as a modulatory "gating" system to regulate the Ca^{2+} signal induced phosphorylation pathways which will be discussed in 6.3.1.2 (see also Chapter 4).

6.2.2.2 LTP model in mammalian hippocampus

The mechanisms that generate LTP in the three major pathways in the hippocampus, namely perforant, mossy fiber and Schaffer collateral pathways have been extensively studied. LTP in the mossy fiber pathway is nonassociative and NMDA-independent. It requires Ca^{2+} influx into the presynaptic cell after the tetanus to activate Ca^{2+}/CaM stimulable adenylyl cyclase, and increase the cAMP/PKA activity in the presynaptic cell. Mossy fiber LTP can be enhanced by a noradrenergic input that engages β-adrenoceptors (Kandel et al. 2000, pp1260). In contrast, the associative, NMDA-dependent LTP present in the Schaffer collateral and the perforant pathways is mainly initiated by an elevation of postsynaptic Ca^{2+} influx, via NMDA receptors. However, ample evidence suggests that the cAMP-signalling pathway is also critically implicated as an intracellular mechanism that underlies both early and late phases of hippocampal LTP. During the early stage of Schaffer collateral LTP in the CA1 regions, the cAMP/PKA pathway has been proposed to play a modulatory role: instead of transmitting signals for

the generation of LTP, it gates the transcriptional phosphorylation pathways by reducing calcineurin dephosphorylation activity (Blitzer et al., 1995; Liu and Graybiel, 1996; Blitzer et al., 1998; Winder et al., 1998). cAMP activation by itself does not induce LTP, rather activated CaMKII is necessary and sufficient to generate early LTP (Blitzer et al., 1995). The late, or long-lasting LTP, which requires both transcriptional activation and new protein synthesis, however, is entirely dependent on an elevation of cAMP levels to trigger PKA-induced, MAPK co-phosphorylation of CREB. Long-lasting LTP can be blocked by PKA inhibitors (Impey et al., 1996) and PKA activation is sufficient to induce late LTP in the absence of electrical stimuli (Frey et al., 1993).

Consistent with this hippocampal LTP model, our model for neonate rat odor preference learning demonstrated a crucial role for the cAMP-CREB signalling pathway activation in long-term memory formation. Our data also suggest cAMP plays more of a modulatory role in odor learning. Higher, ineffective doses of the β-adrenoceptor agonist isoproterenol do not enhance pCREB, but appear to increase intracellular cAMP level in a dose-dependent manner. Enhanced Ca^{2+} signalling by the CS (odor) input does not induce an additional increase of cAMP, suggesting the cAMP cascade is activated independently by the UCS. Neither a cAMP increase (by stroking the body of the pups), or a hypothesized elevated Ca^{2+} signal (by odor input), appears sufficient to induce an odor preference. These outcomes suggest that in early odor preference learning, the association of the CS-UCS pathways is strictly regulated. Learning results from a

temporally synergistic activation of both CS and UCS intracellular signalling (see also Chapter 4).

Also consistent with the hippocampal LTP and long-term memory models, odor preference learning in neonate rats requires CREB phosphorylation as an initial step for downstream genomic and synaptic changes. CREB pathway activation is critically involved in long-term memory formation. CREB levels are delicately regulated in both systems (see also Chapter 2 and Chapter 5). Once memory is acquired, the memory substrates such as synaptic circuitry, or metabolic, changes in the olfactory bulb are long-lasting (see also Chapter 3).

6.3 Future directions

6.3.1 Mechanisms of cAMP functioning in odor preference learning

Although our proposed model and evidence argue for the role of a cAMP cascade in NE mediated odor preference learning, the mechanism of cAMP action in activating the CREB signalling pathway following learning is yet unclear and merits further exploration.

6.3.1.1 Duration of cAMP activation

Isoproterenol dose-dependently increases cAMP expression in the olfactory bulb, while pCREB activation exhibits an inverted-U curve parallel to that of the isoproterenol effects in learning. This suggests that there is an optimal level of cAMP activation which can be exceeded. More likely, we suggest that the duration and temporal pattern of cAMP activation are more critical than the absolute amount of cAMP as also suggested by the Drosophila mutant learning models. Higher levels of cAMP by a stronger UCS may shorten the duration of its own activation. For example, higher levels of cAMP promote greater PDE4 activation through PKA (Ang and Antoni, 2002) which may critically shorten the duration of cAMP signal. Elevated cAMP can also promote faster cAMP extrusion (Wiemer et al., 1982), which again, shortens the signal duration (see also Chapter 4).

Therefore, one of the future directions from my thesis is to measure the dynamic cAMP changes at different time points following odor preference training. Furthermore, the essential role of cAMP in mediating a pCREB signal and in inducing learning can be tested and further supported by manipulations of cAMP levels and the duration of its activation by rolipram, a PDE4 inhibitor. Rolipram can either increase the cAMP amount and/or prolong its activation by reducing PDE4 action. Rolipram application, in theory, should be able to shift the isoproterenol inverted-U curve to the left, but the overall effect

might be complicated given that the cAMP effect itself may be complex. The outcome is difficult to predict, but the experiment itself would be very interesting to explore.

6.3.1.2 cAMP "gating" phosphorylation by reducing calcineurin activity

Another means by which learning may be abolished when an optimal level of cAMP activation is exceeded is that higher levels of cAMP recruit increased calcium entry which may preferentially favor a calcineurin-induced dephosphorylation. The duration of CREB phosphorylation and related gene expression is dependent on phosphatase activity (Bito et al., 1996). If the cAMP cascade promotes CREB phosphorylation by reducing phosphatase activity, application of a calcineurin inhibitor such as FK506 should test the possibility. Specifically, first, FK506 should be able to at least partially substitute for cAMP in promoting CREB phosphorylation; second, FK506 should be able to synergise with cAMP activation; application of FK506 should in theory shift the isoproterenol inverted-U curve to the left. This series of experiments would provide us with clearer insight into how cAMP is functioning in promoting the CREB phosphorylation pathway as well as in promoting learning.

6.3.1.3 Phosphorylation of NMDA receptors following cAMP activation.

Another consequence of cAMP activation is hypothesized to be the

phosphorylation of phosphorylation-sensitive ion channels such as NMDA receptors to promote Ca^{2+} influx into the postsynaptic cells. Measurements of the phosphorylated NMDA receptor NR1 at different time following different training conditions (O/O, O/S, and naive etc.) would test this hypothesis.

6.3.1.4 Visualizing Ca^{2+} entry by optical imaging

Further experiments could be performed to explore the intracellular calcium signals predicted to be critical. The relationship between the UCS/cAMP cascade and postulated CS/Ca^{2+} signalling pathway needs to be examined. How the levels of cAMP influence the levels of intracellular Ca^{2+} could be tested by Ca^{2+} voltage-sensitive dye imaging. Multi-photon imaging could directly assess Ca^{2+} entry at the receptor/glomerular level where odor input is transmitted to the mitral cell dendrites both in the acquisition and memory retrieval phases. Understanding the Ca^{2+}/calmodulin pathway is essential for elucidating the actual intracellular changes and interactions underpinning odor learning.

6.3.2 Other types of adrenergic receptors involved in odor preference learning

We have proposed a new model in which NE and 5-HT synergistically activate a cAMP cascade to co-activate a CREB pathway when occurring with the glutamatergic olfactory nerve evoked Ca^{2+} signal in the mitral cells of the olfactory bulb. This does not

rule out a role for disinhibition or a role for other adrenoceptors or other modulators in odor preference learning. Whether, for example, β_2-adrenoceptors also promote a cAMP second messenger signalling, or provide disinhibition to mitral cells; and whether α-adrenoceptors play roles in mediating odor preference learning and whether they act via a disinhibitory effect could be explored behaviourally and electrophysiologically.

6.3.3 Downstream genes following CREB activation

We have demonstrated that the regulation of genomic expression by CREB is causal in producing preference learning. Future experiments could be designed to pursue the downstream changes regulated by CREB. A major direction would be in examining the downstream events involved in memory retrieval. Microarrays of genes that are highly expressed following associative learning would be helpful to screen the downstream candidates. The inverted U-curve properties of early odor preference learning offer particularly useful control conditions for comparison with effective learning conditions. However, known candidates such as the neurotrophin BDNF, adhesion molecules, and cytoskeletal proteins which are components of long-term plasticity implicated in other memory systems, might be first choice candidates for experimentation in this early odor preference learning model.

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