cAMP SHOWS AN OSCILLATORY PATTERN WITH ODOR PREFERENCE CONDITIONING IN NEONATAL RATS

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by

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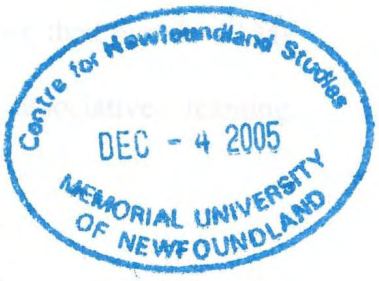

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ABSTRACT

Phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (pCREB) is critical in early odor preference learning. However, it is not known how cAMP levels relate to learning. Here, I test cAMP expression during and after the pairing of a conditioned stimulus (CS) odor with an unconditioned stimulus (US), isoproterenol. I find when odor is paired with a dose of isoproterenol previously shown to be optimal for learning, peaks and troughs of cAMP occur at 5 minute intervals. Pairing the odor with a higher isoproterenol dose, that does not produce learning, produces only a linear increase in cAMP. The oscillations only occur when the CS and the optimal US are paired. These data demonstrate a role for cAMP signaling in the acquisition of odor preference learning, and suggest cAMP oscillatory patterns, rather than simple cAMP concentrations, may be required to initiate mammalian associative learning.

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ABBREVIATION

2-DG	2-deoxyglucose
4-AP	4-aminopyridine
5-HT	serotonin
AC	adenylyl cyclase
ACh	acetylcholine
AChE	acetylchotinesterase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid
AON	anterior olfactory nucleus
ATP	adenosine triphosphate
BCA	bicinchoninic acid
C/EBP	CCAAT/enhancer binding protein
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CNG	cyclic nucleotide-gated
CNS	central nervous system
CREB	cyclic amp response element-binding protein
CS	conditioned stimulus
DA	dopamine
DAG	diacylglycerol
DCG-IV	(2S, 2'R, 3'R)-2-(2'3-dicarboxycycloproplyl) glycine
dn-CREB	dominant-negative mutant CREB
DOI	2,5-dimethoxy-4-iodoamphetamine hydrochloride
EIA	enzyme immunoassay
GABA	gamma-amino butyric acid
GnRH	gonadotropin-releasing hormone
HEK-293	Human Embryonic Kidney-293
HSV	herpes simplex virus
IP_3	inositol 1,4,5-trisphosphate
L-LTP	long-lasting LTP
LOT	lateral olfactory tract
LSD	least significant differences
LTP	long-term potentiation
MB	mushroom body
NE	norepinephrine
NMDA	N-methyl-D-aspartate
ON-EFPs	nerve-evoked field potentials
OSN	olfactory sensory neuron
pCREB	phosphorylated CREB
PDE	phosphodiesterase

PG	periglomerular
PIP_2	phosphatidylinositol 1,4,5-bisphosphate
PKA	cyclic amp-dependent protein kinase A
PKC	protein kinas C
PLC	phospholipase C
PND	postnatal day
TCA	trichloroacetic acid
TTX	Tetrodotoxin
US	unconditioned stimulus
wt-CREB	wild type CREB

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CHAPTER I INTRODUCTION

1 Background

Neonates need the food, protection, and comfort from their mother for growing and developing. Therefore, they must learn to form a bond with their mothers, to approach their mothers' nipples, and to attach to their only source of food (Leon, 1992). During the early period of their life, the first 10-12 days after birth, rat pups have not opened their eyes. They depend heavily on their olfactory sense to locate and attach to their mother's nipples, regardless of the quality of maternal care (Sullivan et al., 2000). Olfactory and somatosensory stimuli are particularly important in pups developing their initial relationship with the environment. During the first 12 days, the pup's immature central nervous system (CNS) has a large potential for plasticity, both behaviorally and cellularly.

Mothers can produce different odors, and these olfactory cues are partially dependent on the mother's diet (Leon, 1975). Thus, pups must have an adaptation mechanism to identify their mothers as odor cues change with the mother's diet.

Rat pups can acquire recognition of maternal odors through a classical conditioning mechanism. They associate tactile stimulation, milk and warmth from the mother with

maternal odors (Johanson and Hall, 1982; Johanson and Teicher, 1980; Pedersen et al., 1982; Sullivan et al., 1986). During the first postnatal week, pups appear to demonstrate a heightened probability of forming this association (Woo and Leon, 1987).

2 Behavioral paradigm

2.1 Classical conditioning

Classical conditioning involves learning a relationship between two stimuli: the unconditioned stimulus (US) and the conditioned stimulus (CS). Classical conditioning was best described by Pavlov, (1960). In his experiment, the famous Pavlov dog was trained by pairing an auditory cue with the presentation of meat. After many of these pairings, the meat was withheld and the animal salivated when it heard the sound alone. Thus, the dog learned an association between the sound and the meat. The US, such as the meat, usually produces a response without learning, such as salivation. The path of this inborn response has already been formed at birth. The CS, such as the sound, produces a response usually unrelated to what will be learned. The path of the conditioned response has to be completed in the higher nervous centers through training. Training is the way to establish new nervous connections during postnatal experience (Pavlov, 1960).

Pairing the CS with the US is critical in training. Only when the CS is followed by the US in a proper period, can the CS evoke the learned response. If the US is rewarding, such as food, the learned response will be approach to the cue. If the US is noxious, such as an electrical shock, the learned response will usually be avoidance of the cue. Repeated pairing of the CS and the US causes the CS to become a signal predicting the US. Thus, by classical conditioning, animals learn to anticipate events surrounding a stimulus. If the CS is repeatedly presented without the US, the learned response will decrease. This process is known as extinction (Pavlov, 1960). Extinction is important for animals to survive because it does not help animals to keep responding to signals that are no longer meaningful. Extinction is also a learning process. Animals learn that the US will not follow the CS any more. This classical conditioning makes it possible for animals to identify events that always take place together, rather than those associated only by chance, creating a mechanism that helps animals adapt and survive.

2.2 The olfactory preference learning paradigm in neonatal rats

Olfactory preference learning occurs when rat pups are presented with an odor together with either intraoral milk infusion (Brake, 1981; Johanson et al., 1984; Johanson and Hall, 1979; Johanson and Hall, 1982; Johanson and Teicher, 1980; Sullivan and Hall, 1988), tail pressure (Sullivan et al., 1986), high humidity (Do et al., 1988), warmth (Alberts and May, 1984), suckling (Amsel et al., 1976; Kenny and Blass, 1977), the odor

of maternal saliva (Sullivan et al., 1986), mild footshock (Camp and Rudy, 1988), heat (Pedersen et al., 1982), or intracranial brain stimulation (Wilson and Sullivan, 1990).

Acquisition of a preference for an odor signal in pups can be described as a classical conditioning paradigm. It was reported that pairing a novel odor with a reinforcing tactile stimulus for rat pups from postnatal day (PND) 1 to 18 could produce olfactory preference learning (Sullivan and Leon, 1986). This pairing not only led to an odor preference, but also enhanced glucose uptake [visualized by using radioactive 2-deoxyglucose (2-DG)] in specific areas of the glomerular layer in the olfactory bulb when that odor was presented again. In this paradigm, the tactile stimulus serves as the US and the odor serves as the CS. In 1987, they reported the same changes after one-trial olfactory training (Sullivan and Leon, 1987). The procedure of this simplified olfactory conditioning paradigm includes removing PND 6 rat pups from the nest and placing them individually in a holding nest for 10 minutes. The pups are then trained by CS-US pairings for 10 minutes: concurrently exposing the pups to an odor and stroking their bodies by a brush. The control groups include odor only (CS only), stroking only (US only), neither of these stimuli (naive), random CS-US pairing, and backward US-CS pairing. Odor preference memory is tested 24 hours after preference training, using either a Y-maze or a two-odor choice test. In the Y-maze test, the CS odor is supplied down one arm and the familiar odor of clean pine chips is supplied down the other. Pups are put in a start box and allowed to enter the arm of their choice. The two-odor choice test consists of a small arena in which the floor is divided in two by a narrow neutral zone. On either

side of the neutral zone, there is the trained odor (CS) or the familiar odor from clean pine chips. In every trial, pups are put on the neutral zone and allowed to explore in the area for 60 seconds. The amount of time spent over CS odor and over familiar odor is recorded. In both the Y-maze and two-odor choice test, pups receive three to five trials, depending on the experiment. Pups trained in the odor + stroking conditioned group choose the CS scented arm significantly more than control pups in the Y-maze test and spend significantly more time over the CS odor in two-odor choice test (Sullivan and Leon, 1987). Other control groups fail to show preference for the CS odor. So, newborn rats are notably capable of olfactory associative learning behavior.

Sullivan et al., (1989) found an optimal level of isoproterenol (β-adrenoceptor agonist) could replace tactile stimulation to serve as the US. They systemically injected different doses of isoproterenol: 1 mg/kg, 2 mg/kg and 4 mg/kg. Only the pairing of a 2 mg/kg dose of isoproterenol and odor produced a learned behavioral odor preference, enhanced uptake of C^{14}-2-DG in the odor-specific foci within the bulb and modified single-cell recording of mitral/tufted cells. Both the lower dose (1 mg/kg) and the higher dose (4 mg/kg) were ineffective in facilitating learning (Sullivan et al., 1989). The intracellular mechanisms by which different isoproterenol doses act in this early learning paradigm are not known. This thesis will address some of the potential mechanisms.

2.3 Advantages of the olfactory preference paradigm in neonatal rats

The early olfactory learning paradigm provides an easily assessable classical conditioning paradigm in neonate rats, in which an odorant CS is temporally paired with one of a myriad of potential US, e.g. milk, tactile stimulation etc., to produce conditioned responses to the odorant. Learned behaviors include behavioral activity and approach responses to the CS odor. During training, acquisition of the conditioned behavior can be quantified with a 5-point behavioral activity rating scale (Hall, 1979) to describe the amount of responsiveness pups display to the training stimuli. The learned approach responses can be assayed in Y-maze or two-odor choice tests. Early olfactory learning is robust; it can be readily shown in pups and is critical for their survival. Controls can be easily established to identify the role of odor exposure only, random CS-US pairings, or backward US-CS pairing. In summary, early olfactory associative learning has proven useful for identifying circuit, cellular, and molecular mechanisms of learning.

3 Neural connections

3.1 Olfactory sensory pathways in the olfactory system

3.1.1 Within the olfactory bulb circuitry

In the rat, the olfactory bulb has a simple ring-like structure. From outside in, there is the glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, granule cell layer, and ependymal zone. The olfactory sensory signals input to the glomerular layer of the olfactory bulb is from the olfactory epithelium via the olfactory sensory nerve. The glomerular layer is composed of clusters of spherical round structures called glomeruli, formed by the terminals of the olfactory sensory axons and the dendritic branches of mitral, tufted and periglomerular (PG) cells. In the glomerulus, axons of olfactory sensory neurons form synapses with the dendrites of the PG cell, and with the primary dendrites of mitral and tufted cells (Buck, 1996; Scott et al., 1993; Shipley et al., 1995; Trombley and Shepherd, 1993). Individual olfactory sensory neurons express only a single type of odorant receptor (Buck, 2000; Chess et al., 1994; Malnic et al., 1999; Mombaerts, 1999). Axons of olfactory sensory neurons expressing the same type of odorant receptor converge in a few fixed glomeruli. That is, each glomerulus receives inputs from olfactory neurons with a single type (or at most a small number) of odorant receptor(s) (Mombaerts et al., 1996; Mori and Yoshihara, 1995; Ressler et al., 1994;

Vassar et al., 1994). Each mitral/tufted cell projects a single primary dendrite to a single glomerulus, and thus receives inputs from axons of olfactory sensory neurons expressing same odorant receptor (Shepherd, 1990). Glomerular activation shows odor-specific spatial patterns across the bulb surface (Nagao et al., 2000; Ressler et al., 1994; Vassar et al., 1994).

Two types of inhibitory interneurons are involved in control mechanisms inside the olfactory bulb: dopaminergic and GABAergic PG cells, and GABAergic granule cells. Dopaminergic PG cells release dopamine (DA) onto olfactory sensory axons, thus inhibiting excitatory neurotransmission between olfactory sensory neurons and olfactory bulb mitral/tufted cells and PG cells (Davila et al., 2003). PG cells also release gamma-amino butyric acid (GABA) to directly inhibit mitral/tufted cells through their dendrodendritic synapses (Getchell and Shepherd, 1975; Mugnaini et al., 1984; Shepherd, 1971; Shepherd, 1972; White, 1972). GABAergic granule cells form reciprocal synapses with secondary dendrites of mitral/tufted cells. Excited mitral/tufted cells release glutamate to granule cells and then granule cells release GABA to inhibit the excitation of mitral/tufted cells forming a negative feedback loop (Jahr and Nicoll, 1982; Nicoll, 1971; Trombley and Westbrook, 1990; Yokoi et al., 1995). Each granule cell contacts numerous mitral/tufted cells. This inhibitory mechanism means that excitation of mitral/tufted cells receiving inputs from one glomerulus may inhibit mitral/tufted cells receiving inputs from neighboring glomeruli (Buck, 1996; Meredith, 1986; Mori, 1987; Scott et al., 1993;

Wilson and Leon, 1987). Thus, olfactory signals are modified in the olfactory bulb before being sent to higher olfactory cortices (Figure 1.1).

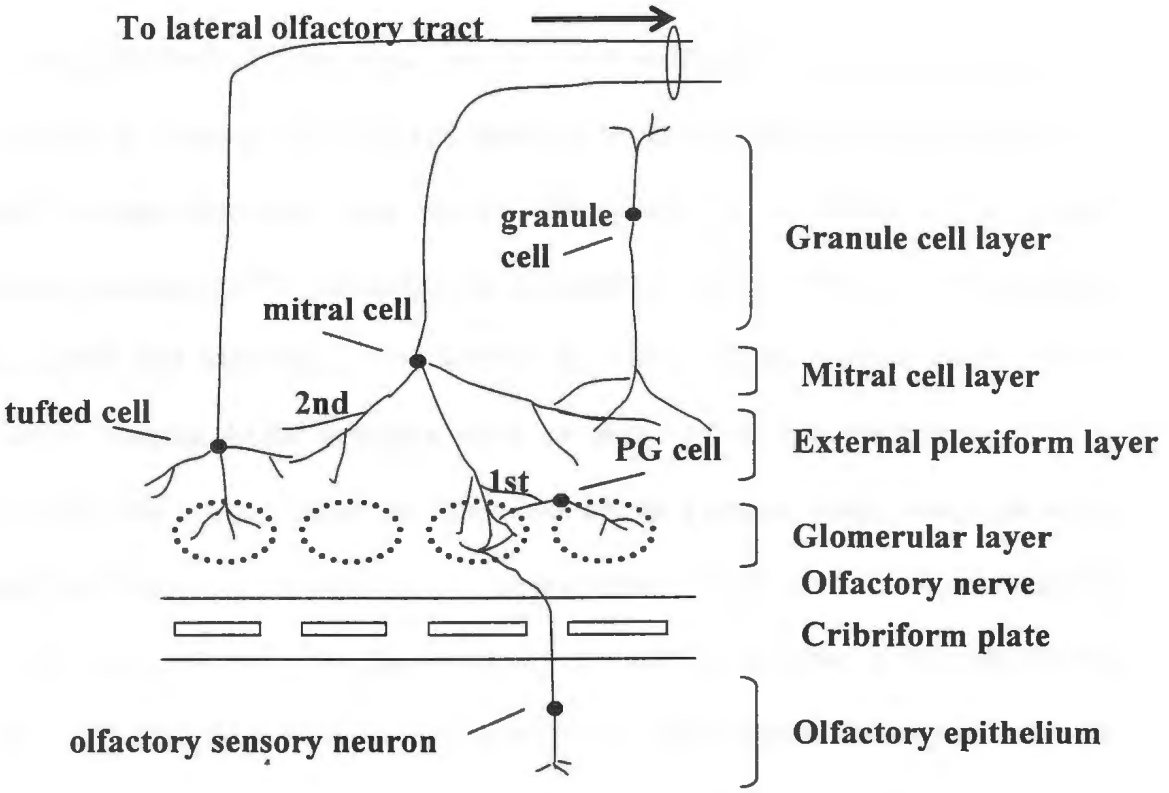

Figure 1.1 The basic structure and synaptic circuitry in the olfactory bulb

3.1.2 Central targets of the olfactory bulb

From the olfactory bulb, the output sensory signals project to the central olfactory cortex via the lateral olfactory tract (LOT) composed of axons from mitral/tufted cells. Olfactory cortex includes five main areas (Buck, 1996; Kandel et al., 2000): 1) the anterior olfactory nucleus (AON), connecting the symmetrical regions of the two olfactory bulbs (Schoenfeld and Macrides, 1984; Scott et al., 1985); 2) the piriform cortex; 3) the olfactory tubercle; 4) the amygdala which projects into the hypothalamus; and 5) the entorhinal area which projects into the hippocampus. From all areas, except the AON, axons will project to the orbitofrontal cortex either directly, or indirectly through the thalamus (Buck, 1996). The input pathways through the thalamus to the orbitofrontal cortex are thought to account for the perception and discrimination of odors, whereas the pathways through amygdala and hypothalamus are thought to mediate the emotional and motivational aspects of smell (Kandel et al., 2000). In addition to receiving inputs from each of the olfactory cortices, the olfactory bulb receives information from the locus coeruleus, raphe nucleus, and horizontal limb of the diagonal band (Shipley et al., 1995; Shipley, 1985). Thus, the olfactory bulb has widespread influence on the brain through its output and is influenced itself through many inputs.

3.2 Neural consequences of olfactory learning within the olfactory bulb

3.2.1 2-DG uptake marks neuronal activity

Learned behavioral reactions in olfactory learning correspond with specific neural changes in olfactory bulbs. The olfactory bulb adjusts its activation to learned odors. This neural change can be measured with C^{14}-2-DG autoradiography. Coopersmith et al., (1986) trained pups with odor-tactile paired stimulation from PND1-18. On PND 19, they found the uptake of 2-DG was increased in specific regions in the glomerular layer of the olfactory bulb, when pups responded to the learned odor. The neural response was specific to the odor used as a CS (Coopersmith et al., 1986), i.e., training with one odor can not increase the 2-DG uptake for another odor. It is even specific to the trained odor concentration (Carmi and Leon, 1991), i.e., training with a high odor concentration results in increased 2-DG uptake to the high odor concentration, compared with low odor concentration or clean air. The neural change (2-DG uptake increase) can also occur on the day after one-trial pairing of odor and tactile stimulation, during the first postnatal week (Sullivan and Leon, 1987). Only an odor-tactile stimulation paired training session induces the increase of 2-DG uptake. Either odor alone or backward presentation of tactile stimulation and odor was ineffective (Sullivan and Leon, 1986). There was no difference in respiration between pups that showed the increased uptake of 2-DG and those that did not. Only pups that had learned to prefer the odor showed an increased

uptake of 2-DG (Sullivan et al., 1988). Therefore, the increased uptake of 2-DG is not caused by increased stimulation of the olfactory system itself (e.g. through increased breathing), but is specific to the CS used.

In addition to the metabolic changes mentioned above, there are olfactory bulb structural changes caused by olfactory associated learning. Both trained and control pups have the same number of glomeruli within the 2-DG foci. However, trained pups have wider glomerular layers and a greater size of individual glomeruli with no differences in the nonfocal regions of the glomerular layer itself (Woo et al., 1987). The number of glomerular layer cells within the 2-DG foci of trained pups was increased notably compared with the control pups. These glomerular layer cells could consist of glial, short-axon, small external tufted and/or periglomerular cells. An increase in the number of any or all of these cell types could contribute to the enhanced 2-DG uptake noted by adding to the number of metabolically active cells (Woo and Leon, 1991). No difference was observed outside the 2-DG foci in the glomerular layer (Johnson et al., 1995; Woo and Leon, 1991). Thus, structural changes only happen at the area where olfactory inputs occur.

3.2.2 Optical imaging shows increased blood flow with odor activity

Another means of showing brain activity is by the use of optical imaging to detect changes in blood flow, thus changes in brain activity. Yuan et al., (2002) investigated

whether optical imaging could be used to detect changes in intrinsic signals at the glomerular level of the olfactory bulb in the early olfactory learning paradigm. They trained PND 6 rat pups by either pairing odor (peppermint) and tactile stimulation (stroke), or peppermint only (control littermates). Both conditioned pups and control littermates underwent imaging of intrinsic optical signals one day after the training trial. During the imaging, all anesthetized pups were presented with odors. Conditioned pups showed significantly greater intrinsic signals to the peppermint odor compared to control littermates exposed to peppermint odor but not paired with stroking. However, the intrinsic signal of conditioned pups responding to a control odor (amyl acetate) was not significantly different from that of control littermates. These findings illustrate that odor preference memory can be detected by optical imaging techniques (Yuan et al., 2002). The results were consistent with the earlier observations from 2-DG uptake indicating that learning increases metabolic activity in the olfactory bulb.

3.2.3 Other changes in the olfactory bulb related to learning

In addition to visible changes in the activity of the bulb related to learning, there are physiological changes occurring in the neonatal olfactory bulb following early olfactory learning. For instance, pairing an odor with a US decreases habituation of mitral cell firing in response to the odor during training (Wilson and Sullivan, 1992). In addition, the pairing produces an odor-specific long-term change in spatio-temporal output patterns of the olfactory bulb, mainly expressed as increased inhibitory responses to the learned odor,

i.e., this classical conditioning selectively modified the response patterns of mitral/tufted cells associated with regions of focal 2-DG uptake for that odor and the modified response pattern occurred on the first inhalation of the learned odor (Wilson and Leon, 1988b). It was proposed that changes in olfactory bulb output are due to changes at the mitral cell and granule cell dendrodendritic reciprocal synapse. Those changes can occur either on the mitral cell or granule cell side of the synapse to cause odor-specific, localized changes in feedback inhibition of mitral cells (Wilson and Sullivan, 1994). Further evidence has shown that systemic injection of 2 mg/kg isoproterenol (learning dose) increased nerve-evoked field potentials (ON-EFPs) in normal rat pups, compared to saline or higher (6 mg/kg) isoproterenol dose. In contrast, in bulbar 5-HT depleted rat pups, 6 mg/kg isoproterenol (nonlearning dose) increased ON-EFPs, suggesting norepinephrine (NE) can overcome serotonin (5-HT) deficits and promote specific electrophysiological changes that critically underlie odor preference learning (Yuan et al., 2000).

3.2.4 Learned responses are intrinsic to the olfactory bulb

Changes in bulb neural firing patterns only take place in response to the odor that has been learned. However, one can not distinguish the changes in the bulb by different types of learning, i.e., appetitive or avoidance. Both olfactory preference learning and avoidance learning are capable of inducing 2-DG uptake increases, and suppressing the neural activity in the bulb. Specifically, pairing odor with either an unpleasant US

(footshock) or a favorable US (tactile stimulation) modifies bulb responses in similar ways. All these response patterns differ significantly from responses in control pups, but are not different among themselves. In addition, the neural changes caused by preference learning and avoidance learning in the trained pups can be reversed with extinction (Sullivan and Wilson, 1991a).

3.3 Molecular mechanisms of olfactory learning

3.3.1 Neurotransmitters involved in olfactory learning

As mentioned above, there are learned odor responses intrinsic to the olfactory bulb. Thus, CS and US pathways must converge within the bulb. Information about the CS coming from olfactory receptor cells in the olfactory epithelium is conveyed by the olfactory nerve to glomeruli of the olfactory bulb. Information about the US is assumed to be conveyed by ascending central inputs to the bulb, e.g. noradrenergic inputs from the locus coeruleus. Therefore, there are several neurotransmitters participating in either the CS or US pathways that mediate or modulate the bulb response during learning.

3.3.1.1 Noradrenergic input is necessary and sufficient in olfactory learning

The olfactory bulb receives dense noradrenergic inputs from the locus coeruleus. These noradrenergic fibers project to all layers of the olfactory bulb, primarily to the internal plexiform and granule cell layers, to a lesser degree to the external plexiform layer, and sparsely to the glomerular layer (McLean and Shipley, 1991). These inputs are present at birth (McLean and Shipley, 1991) and functional during the first postnatal week (Wilson and Leon, 1988a).

Norepinephrine inputs to the olfactory bulb are necessary for early odor preference learning. Systemic injections of the β-adrenoceptor antagonists propranolol or timolol prior to training inhibit acquisition of conditioned odor preference (Sullivan et al., 1989; Sullivan et al., 1991). Propranolol infused directly into the olfactory bulb during training also disrupts early olfactory learning (Sullivan et al., 1992). The impairment in learning is dose dependent effects of β-adrenoceptor antagonist and is not associated with any detectable change in sensitivity to either odor or US (Sullivan et al., 1991). Furthermore, in the adult, NE depletion from the olfactory bulb with 6-OHDA does not impair olfactory detection or discrimination (Doty et al., 1988). Additionally, bilateral infusion of 6-OHDA into the locus coeruleus of PND4 pups significantly reduces olfactory bulb NE content, and impairs acquisition of trained odor preference (Sullivan et al., 1994).

Pairing of an odor with a noradrenergic activator is sufficient for the acquisition of odor preference in neonate pups. Combining an odor with a systemic injection of the β-adrenoceptor agonist isoproterenol results in a preferential behavioral response to that odor (Langdon et al., 1997; Price et al., 1998; Sullivan et al., 1989; Sullivan et al., 1991). The dose related effect on the learned response is an inverted U shaped, or parabola. Understimulation (low dose of agonist) or overstimulation (high dose of agonist) fails to induce the learned preferential response. Experimental manipulation of NE levels is consistent with in vivo effects, since direct measurement of NE in the olfactory bulbs of PND 3 rat pups indicates that tactile stimulation and oral infusion of milk increase olfactory bulb NE levels (Rangel and Leon, 1995). Firing of locus coeruleus neurons in the developing brain is intimately related to input from peripheral sensory sources, e.g. stroking (Nakamura et al., 1987).

The effects of systemic injection of isoproterenol are cumulative when paired with a regular US (stroking) in producing early olfactory learning (Sullivan et al., 1991). A suboptimal dose of isoproterenol combined with a US reduced in strength, such as less frequent stroking, produces a significant learned odor preference. However, either of them alone does not. Combination of an optimal dose (2 mg/kg) of isoproterenol with a normal frequency of stroking causes overstimulation and no learning. These results indicate that both the isoproterenol and tactile US are mediated through a common NE linked pathway in early olfactory learning. There may be pathways other than the locus coeruleus / NE to bulb input that mediate the tactile US and such redundancy in having

more than one US pathway may be ethologically advantageous to the animal. In fact, there appear to be other US mechanisms (see 3.3.1.3 and 3.3.1.4) but the neuroanatomical pathways involved are not known.

Norepinephrine is also important for consolidation of early memories in neonates. Injecting propranolol up to 1 hour after the end of the training session blocks memory for the learned odor preference acquired through odor-milk conditioning (Sullivan and Wilson, 1994). However, NE is not required in the retrieval of the acquired memory. Injecting propranolol or timolol before testing (24 h after training) has no effect on expression of the learned odor preference (Sullivan and Wilson, 1991b). This dependence on NE for acquisition and consolidation, but not expression, is similar to the role of NE in dentate gyrus long-term potentiation (LTP). Several labs have reported that NE antagonists impair induction of LTP, but do not disrupt expression of previously established LTP (Bliss et al., 1983; Dahl and Sarvey, 1989; Harley et al., 1989; Robinson and Racine, 1985).

Norepinephrine has been proposed to determine the sensitive postnatal period for acquisition of odor preference learning. Newborn rats have an enhanced ability to learn odor preferences produced by pairing an odor with tactile stimulation (stroking) during the first postnatal week (Woo and Leon, 1987). Within this period, all unconditioned stimuli serve to induce odor preference, i.e., pups learn to prefer odors paired with either tailpinch or mild footshock, just as they do odors associated with a standard reward

(Camp and Rudy, 1988). This sensitive period is attributed to the unusual physiological properties of the locus coeruleus in newborn rats. During the sensitive period, the noradrenergic locus coeruleus neuron projections are present and functional (McLean and Shipley, 1991; Shipley, 1985; Sullivan et al., 1989). There is greatly reduced autoinhibition of locus coeruleus α_2 receptors and enhancement in autoexcitation through the locus coeruleus α_1 receptor (Marshall et al., 1991). Thus, noradrenergic locus coeruleus neurons are readily and tonically activated by sensory input, e.g. tactile stimulation, during that period (Nakamura et al., 1987). Additionally, locus coeruleus neurons are equally responsive to noxious and innocuous stimuli in anesthetized pups, whereas innocuous stimuli are ineffective at inducing the activity of neurons in the locus coeruleus in mature anesthetized rats. Also, during the sensitive period, locus coeruleus neurons are electrotonically coupled; this appears to decline with age (Christie et al., 1989). Therefore, during the sensitive period, locus coeruleus neurons are more "excitable" and more locus coeruleus neurons are likely to respond due to their electrotonic coupling.

The lack of distinction between unpleasant and pleasant stimuli disappears in the second and/or third postnatal week, when the mild footshock starts to produce an aversive learning response (Camp and Rudy, 1988). During the post sensitive period, NE released in the olfactory bulb during olfactory learning is dramatically reduced, compared to that released during the sensitive period (Rangel and Leon, 1995). This reduction in NE release is ascribed to the increase of locus coeruleus α_2 receptor autoinhibition and the

decrease of α_1 receptor autoexcitation. Manipulating the neonatal locus coeruleus autoreceptors by using an α_2 antagonist and an α_1 agonist, pups were again able to demonstrate odor preference learning during the post sensitive period (Moriceau and Sullivan, 2004). This confirms the contribution of NE to the sensitive-period for preference learning in neonates.

3.3.1.2 Serotonergic input modulates noradrenergic input in olfactory learning

Another neurotransmitter involved in early olfactory learning is 5-HT. Serotonergic axons arise from the raphe nuclei to arrive in the olfactory bulb postnatally (McLean and Shipley, 1987a). They terminate extensively in the glomerular layer and less so in the external plexiform, internal plexiform and granule cell layers of the bulb (McLean and Shipley, 1987b). By localized injection of the neurotoxin 5,7-dHT into the anterior olfactory nucleus of PND 0 or PND 1 pups, serotonergic fibers that reach the olfactory bulb can be depleted. This depletion impairs odor preference learning induced by odor-tactile stimulation training on PND6 (McLean et al., 1993). However, the 5-$HT_{2A/2C}$ receptor agonist 2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) paired with odor during training on PND 6 did not induce learning and memory on PND7 (Price et al., 1998). When DOI was combined with isoproterenol, it decreased the effective US dose. That is, a suboptimal isoproterenol dose (1 mg/kg) is effective, but a previously optimal isoproterenol dose (2 mg/kg) is ineffective (Price et al., 1998). Depletion of 5-HT within the olfactory bulb shifted the isoproterenol dose curve such that a higher

isoproterenol dose (odor + stroking + 4 mg/kg or odor + 6 mg/kg) is needed to produce learning (Langdon et al., 1997). Thus, serotonin plays a modulatory role, but not a necessary role, in early olfactory learning in rats.

3.3.1.3 Glutamatergic inputs in olfactory learning

Mitral and tufted cells are glutamatergic. Glutamate is also the neurotransmitter mediating the CS odor in the bulb (Berkowicz et al., 1994; Yuan et al., 2000). Activation of glutamate receptors appears to facilitate olfactory learning. Blockade of N-methyl-D-aspartate (NMDA) receptors by using AP5 (NMDA receptor antagonist) before olfactory preference training suppresses behavioral preference and the enhanced olfactory bulb 2-DG uptake to the learned odor (Lincoln et al., 1988). However, NMDA antagonists may also impair normal bulb function, (Wilson et al., 1996), because NMDA receptors are required for transmitting olfactory information. Thus, pups may not respond to olfactory stimuli properly. The precise role of the NMDA receptor in olfactory learning remains to be identified. Additionally, other kinds of glutamate receptors also show a facilitating role in olfactory learning. Infusion of (2S, 2'R, 3'R)-2-(2'3-dicarboxycycloproplyl) glycine (DCG-IV, a type 2 metabotropic glutamate receptor agonist) in olfactory bulb induces olfactory preference learning in rat pups (Rumsey et al., 2001). In this case, DCG-IV acts as a US. This result suggests a mechanism that is different from the NE input into the olfactory bulb as the US for the induction of early olfactory preference learning. The

potential of more than one transmitter system mediating the US may be advantageous to the pup (if one system fails another could still provide an important US input).

3.3.1.4 GABAergic disinhibition of mitral cells is critical in olfactory learning

Olfactory learning in young rats involves noradrenergic modulation of reciprocal dendrodendritic synapses between mitral cells and GABAergic granule cells. By using an aversive olfactory learning model in young rats, that are trained on PND 11 and tested on PND 12, Okutani and coworkers identified a role for GABA receptors in the olfactory bulb in olfactory learning (Okutani et al., 1999; Okutani et al., 2002; Okutani et al., 2003). Infusion of the GABA(A) receptor agonist muscimol into olfactory bulbs during training impaired aversive learning in a dose-dependent manner. Infusion of low or high doses of the GABA(A) receptor antagonist bicuculline produced odor preference or aversion learning, respectively. These results indicate that disinhibition of mitral cells in the olfactory bulb has a role in olfactory learning, and suggest that the degree of disinhibition may determine the acquisition of either preference or aversion for the trained odor (Okutani et al., 1999). Bicuculline-induced aversive responses lack odor specificity. Specificity of preference responses was not tested. Bicuculline infusion resulted in aversion not only for the trained odor, but also for an odor never presented. Moreover, bicuculline infusion alone is sufficient to produce dose-dependent aversion to strange odors. Therefore, disinhibition of mitral/tufted cells from granule cells by blocking GABA(A) receptor induces aversion to normal odors non-specifically (Okutani

et al., 2002). Infusion of baclofen [a GABA(B) receptor agonist] into the olfactory bulb also prevents aversive olfactory learning in a dose-dependent manner, while infusion of saclofen [a GABA(B) receptor antagonist] induced aversive responses not only to the trained odor, but also to normal odors not presented previously. Olfactory aversion was also seen even if saclofen was infused without odor exposure (Okutani et al., 2003). Thus, olfactory preference learning in young rats is modulated through GABA(A) receptors. Aversive olfactory learning in pups is mediated by both GABA(A) and GABA(B) receptors. The aversion appears to be nonspecific to the odor. In summary, stimulation of bulb GABA (A, B) receptors blocks the aversive olfactory learning, and inhibition of bulb GABA (A, B) receptors induces nonspecific aversive olfactory learning.

3.3.1.5 Dopaminergic influence on olfactory learning

During olfactory preference learning, extracellular DA increases significantly. On PND3, odor alone or tactile stimulation alone in a 10-min training session increases extracellular DA levels by approximately 200%. This increase lasts for more than 50 minutes. Combining odor with stroking, a condition that induces olfactory preference learning, increases DA levels by approximately 400%. This change also persists for 50 minutes (Coopersmith et al., 1991).

Depletion of DA impairs early olfactory learning. On PND 5, pups were either injected with saline or treated with desmethylimipramine/6-hydroxydopamine to selectively

deplete DA from the brain. On PND 7, these pups were trained with odor-milk pairings. They were tested for odor preference on PND 8. Dopamine depleted pups did not learn an odor preference. This deficit can be reversed by amphetamine (Weldon et al., 1982; Wool et al., 1987).

Dopamine's role in early learning appears to be limited to the post-training consolidation period. Systemic injections of the DA-D1 receptor antagonist SKF 83566 immediately before or after odor-tactile stimulation training blocks odor preference learning. However, the effects of pretraining injections of SKF 83566 can be blocked by post-training injections of the DA agonist apomorphine (Weldon et al., 1991).

The role of specific DA receptors in the olfactory bulb requires clarification. Injecting a DA-D1 receptor blocker impairs odor preference after odor-tactile pairing, but a DA-D2 receptor blocker does not. Since the DA-D2 receptor is the dominant receptor subtype in the olfactory bulb (Bouthenet et al., 1987; Guthrie et al., 1991), DA effects may not be mediated through the olfactory bulb circuitry. The 2-DG uptake pattern shows several areas in the brains of pups given odor-milk pairings that differ from those of controls (Hall and Oppenheim, 1987). These areas include the olfactory tubercle, the hippocampus, and the central amygdaloid nucleus. The effects of systemic DA manipulation on odor learning may occur within the olfactory tubercle, an area rich in DA-D1 receptors (Wamsley et al., 1989). Nonetheless, (1) there are dopaminergic cells in the olfactory bulb glomerular layer, (2) there is a significant increase of DA levels in the

olfactory bulb after odor-tactile stimulation training (Coopersmith et al., 1991) and (3) DA expression in the olfactory bulb is notably sensitive to olfactory experience (Baker, 1990; Wilson and Wood, 1992). Thus, it is anticipated that olfactory bulb DA has a role in olfactory function and plasticity.

3.3.1.6 Opioid influence on olfactory learning

Intracerebral infusions or systemic injections of morphine were shown to support early olfactory preference learning (Kehoe and Blass, 1986a; Kehoe and Blass, 1986b). Recently, it was also shown that the endogenous opioid system is important in early olfactory learning (Roth and Sullivan, 2001; Roth and Sullivan, 2003). The endogenous opioid system is functional in pups during the first postnatal week. Blocking opioid receptors during training by systemic injection of naltrexone (a nonspecific opioid antagonist) disrupted acquisition of odor preference on PND 8, but not odor aversion on PND 12. Posttraining injection of naltrexone not only blocked consolidation of odor preference on PND 8, but yielded an odor aversion. Thus, the opioid system has a critical role in both acquisition and consolidation of odor preference during the sensitive period (Roth and Sullivan, 2001). Naltrexone disrupted expression of odor preference, but not the learned odor aversion in older pups. Therefore, opioids are also important in the expression of early olfactory preference (Roth and Sullivan, 2003). Since prenatal drug exposure is known to change the endogenous opioid system (Smotherman and Robinson,

1994), these results imply that human prenatal opiate exposure could disrupt early learning and maternal attachment in infants.

3.3.2 Disinhibition model of olfactory learning in neonatal rats

Wilson and Sullivan, (1994) proposed an intercellular mechanism for early olfactory learning. As mentioned previously, CS odor inputs are conveyed by the axons of olfactory sensory neurons (OSNs) that terminate on primary dendrites of mitral/tufted cells. The secondary dendrites of mitral/tufted cells form reciprocal dendrodendritic synapses with granule cells. To which, excited mitral/tufted cells release excitatory glutamate. Excited granule cells then release GABA onto mitral/tufted cells to inhibit them. Granule cells are the primary target of inputs from other areas of the brain to the olfactory bulb, including NE release from locus coeruleus (Macrides and Davis, 1983) induced by the US. US induced NE input is distributed widely in the olfactory bulb. Conditioned changes are likely to occur at areas where NE inputs overlap with CS evoked odor-specific glomeruli within the olfactory bulb. Before conditioning, the majority of mitral/tufted cells respond to a given odor in an excitatory manner, but habituate rapidly, probably because of the feedback inhibition by granule cells. During CS-US conditioning, NE inputs activated by the US attenuate the inhibitory synapse from granule cells to mitral/tufted cells (Wilson and Leon, 1988a), and block habituation to the CS. This enhanced responsiveness to CS odor during training in some way potentiates the mitral/tufted cells synaptic efficacy onto granule cells. During CS-odor-only testing, the increased synaptic efficacy is expressed as

enhanced feedback inhibition, and observed as increased suppressive responses of mitral/tufted cells to the CS odor (Wilson and Sullivan, 1994).

3.3.3 The CS-US convergence on mitral cell model and its intracellular mechanisms

Yuan et al., (2003b) proposed a new intercellular and intracellular model for early olfactory learning in the olfactory bulb. The model proposes that the synergistic action of β-noradrenergic and serotonergic 5-$HT_{2A/C}$ input takes place within the mitral cells of the olfactory bulb. By using confocal imaging, they demonstrated that β-adrenoceptors and 5-$HT_{2A/C}$ receptors colocalize mainly on mitral cells. Additionally, both β-adrenoceptor and 5-$HT_{2A/C}$ receptor activation can work through the cyclic adenosine monophosphate (cAMP) related pathway in mitral cells in the olfactory bulb, as they do in neocortex (Morin et al., 1992). β-adrenoceptor activation induced by isoproterenol significantly increases cAMP in the olfactory bulb, at either the optimal learning dose or higher doses. Both stroking with odor and stroking alone are capable of increasing cAMP. Serotonin depletion of the olfactory bulb does not influence basal level of cAMP but inhibits the cAMP elevation produced by isoproterenol. Finally, the US and the CS synergistically influence cAMP response element-binding protein (CREB) phosphorylation. Unlike the cAMP increase that is influenced by the US pathway and can be induced under both learning conditions (optimal isoproterenol dose + odor, stroking + odor), and non-

learning conditions (higher isoproterenol dose + odor, stroking only), CREB phosphorylation is related to the learning condition. Only the learned pairing (optimal isoproterenol dose + odor, stroking + odor) increases CREB phosphorylation. Neither the higher isoproterenol dose + odor, nor stroking alone increases CREB phosphorylation. Thus, CREB phosphorylation is a critical step that requires convergence of both US and CS intracellular pathways.

The new learning model can be described in the following way: On the surface of mitral cells in the olfactory bulb, the US is mediated by NE activation of β-adrenoceptors. In turn, β-adrenoceptors activate the cAMP cascade and the cyclic amp-dependent protein kinase A (PKA) pathway. The CS mediated by glutamate activates alpha-amino-3-hydroxy-5-methyl-4-iso-xazole-propionic acid (AMPA)/NMDA receptors. NMDA receptors recruit the Ca^{2+}/Calmodulin (CaM) pathway. Finally, these two pathways converge to promote CREB phosphorylation. The CREB phosphorylation triggers memory-related structure changes required for long-term memory. (Figure 1.2.)

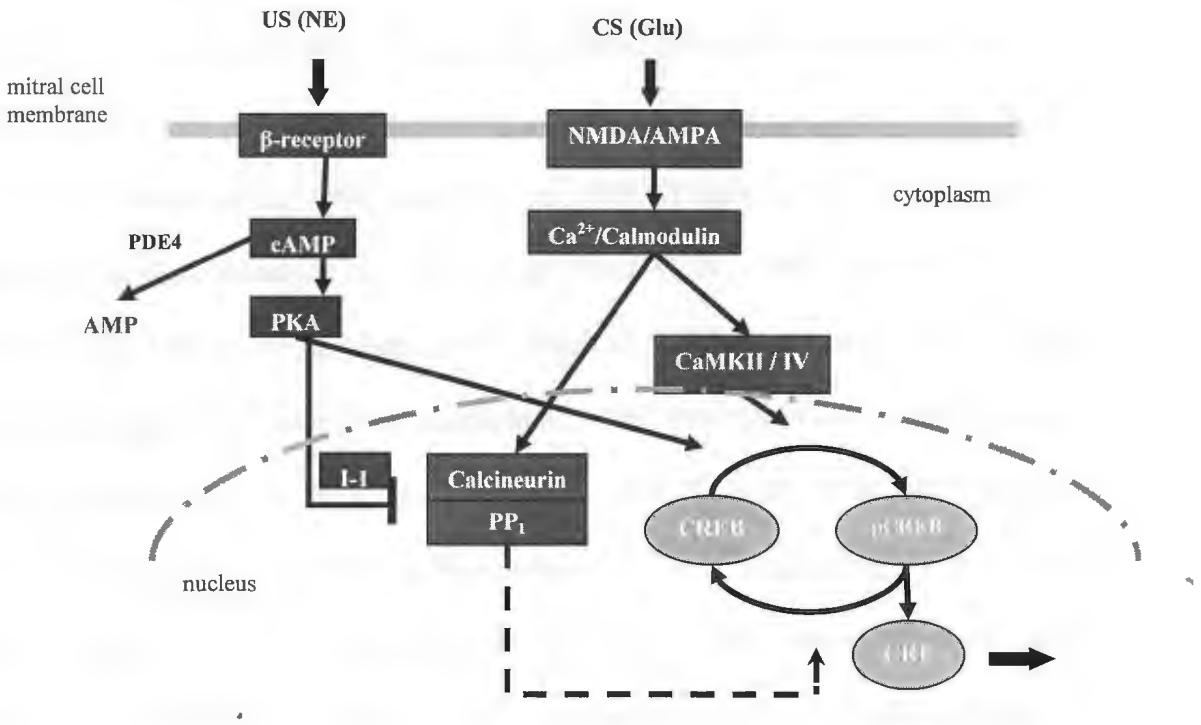

Figure 1.2 Proposed intercellular and intracellular pathways in the olfactory bulb activated by β-adrenoceptors and 5-HT2A receptors

4 cAMP/PKA/CREB cascade in learning and memory

The cAMP / PKA / CREB cascade has been implicated in invertebrate learning, e.g. 5-HT induced sensitization in *Aplysia* (Bacskai et al., 1993; Bailey and Kandel, 1993; Bartsch et al., 1995; Bartsch et al., 1998; Bernier et al., 1982; Brunelli et al., 1976; Byrne and Kandel, 1996; Castellucci et al., 1982; Castellucci et al., 1989; Dash et al., 1990; Goldsmith and Abrams, 1992; Hedge, 1997; Hu et al., 1993; Kaang et al., 1993; Kandel, 2001; MacDougald and Lane, 1995; Martin et al., 1997; Montarolo et al., 1986), olfactory learning in *Drosophila* (de Belle and Heisenberg, 1994; Goodwin et al., 1997; Hammer and Menzel, 1995; Han et al., 1992; Heisenberg et al., 1985; Heisenberg, 1998; Li et al., 1996; Nighorn et al., 1991; Skoulakis et al., 1993; Tully et al., 1990; Tully et al., 1994; Wolf et al., 1998; Xia and Storm, 1997); in mammals, cAMP is also required for conversion of early LTP to late LTP (Frey et al., 1993; Huang et al., 1994; Kandel et al., 2000). All of these models can help us to understand the possible role of the cAMP/PKA/CREB cascade in learning and memory in mammals.

4.1 cAMP/PKA/CREB cascade in *Aplysia*

Eric Kandel won the 2000 Nobel Prize in Medicine for work on the cAMP/PKA/CREB cascade in learning and memory in *Aplysia*. His studies focused on nonassociative learning—sensitization at the sensory-motor neuron connection—that supports

withdrawal reflexes (Bacskai et al., 1993; Bailey and Kandel, 1993; Bartsch et al., 1995; Bartsch et al., 1998; Bernier et al., 1982; Brunelli et al., 1976; Byrne and Kandel, 1996; Castellucci et al., 1982; Castellucci et al., 1989; Dash et al., 1990; Goldsmith and Abrams, 1992; Hedge, 1997; Hu et al., 1993; Kaang et al., 1993; Kandel, 2001; MacDougald and Lane, 1995; Martin et al., 1997; Montarolo et al., 1986). His work identified the role of cAMP/PKA/CREB cascade in both short-term and long-term memory in *Aplysia*.

Short-term memory induced by a single training session (a single tail shock or a single application of 5-HT to the sensory neurons) lasts only minutes and does not require new protein synthesis. Long-term memory induced by five spaced training sessions [five tail shocks or five applications of 5-HT (5 minutes each at 15 minutes interval) to the sensory neurons] lasts several days and requires new protein synthesis (Montarolo et al., 1986). Additional spaced training sessions produce sensitization that lasts for weeks (Castellucci et al., 1989; Kandel, 2001). These two forms of memory overlap. Both short-term and long-term memories require changes in signal strength at several synaptic connections, such as the connection between sensory and motor neurons. This increased synaptic signal strength is caused by 5-HT release onto the sensory neurons.

Intracellular second-messenger pathways, e.g., the cAMP cascade, critical in forming short-term memory, are also involved in long-term memory. In short-term sensitization, a single tail shock or one pulse of 5-HT produces one-time 5-HT release and leads to the

utilization of pre-existing proteins. Serotonin acts on G-protein coupled receptors, on the sensory neuron, to activate adenylyl cyclase (AC), leading to increases in cAMP (Bernier et al., 1982), resulting in activation of PKA (Castellucci et al., 1982). Sequentially, PKA phosphorylates a number of target substrates and enhances transmitter release by closing K^+ channels, leading to an increase in the excitability of sensory neurons. Excitability increases the Ca^{2+} influx upon subsequent stimulation resulting in increased transmitter release. The duration of these modifications is identical to that of the short-term memory (Byrne and Kandel, 1996; Goldsmith and Abrams, 1992).

Repeated tail shock or repeated exposure to 5-HT converts the short-term form of memory into a long-term form. This process requires gene expression, new protein synthesis, and the growth of synaptic connections. Repeated training sessions lead to prolonged high PKA activity. The catalytic subunits separated from the regulatory subunit of PKA have sufficient time to translocate into the nucleus (Bacskai et al., 1993). There, they phosphorylate and activate a transcription factor called CREB-1 (Kaang et al., 1993). The catalytic subunit of PKA also recruits mitogen-activated protein kinase to translocate to the nucleus (Martin et al., 1997) to remove the suppressive action of CREB-2 on CREB-1 (Bartsch et al., 1995). Once CREB-1 is activated, it triggers the expression of a number of downstream genes (Bartsch et al., 1998; Dash et al., 1990). There are two especially important downstream genes. One encodes the enzyme ubiquitin hydrolase, a specific ubiquitin protease regulating proteolysis of the regulatory subunits of PKA (Hedge, 1997). Cleavage of the regulatory subunit prolongs the activity of PKA, causing

persistent phosphorylation of PKA substrate proteins, e.g. CREB and K^+ channels. The second gene encodes CCAAT/enhancer binding protein (C/EBP), one of the factors in the gene cascade necessary for the growth of new synapses (Bailey and Kandel, 1993; Hu et al., 1993; MacDougald and Lane, 1995).

4.2 cAMP /PKA/CREB cascade in *Drosophila*

The fruit fly *Drosophila* is particularly suited to genetic manipulation. Four mutations in single genes that lead to learning deficits have been isolated: *dnc*, *rut*, *amn*, and *PKA-R1*. All of these genes are expressed in the same brain region-the mushroom bodies (MBs) (Han et al., 1992; Nighorn et al., 1991). The MBs are not directly necessary for olfactory perception (de Belle and Heisenberg, 1994; Heisenberg et al., 1985), or for simple forms of visual, tactile or motor learning (Wolf et al., 1998), but are required for olfactory learning and memory (Hammer and Menzel, 1995; Heisenberg, 1998).

Learning studies with *Drosophila* mutants suggest that there are tight constraints on the level of cAMP and PKA activation for optimal learning and memory. All four mutants fail to show either classical conditioning or sensitization. All have a defect in the cAMP cascade (de Belle and Heisenberg, 1994; Heisenberg et al., 1985). Flies with ***dnc*** mutation lack phosphodiesterase, an enzyme that degrades cAMP, resulting in unusually high levels of cAMP. Mutation in ***rut*** results in deficient Ca^{2+}/CaM-dependent AC

(Ca^{2+}/CaM AC). Ca^{2+}/CaM AC is activated by simultaneous Ca^{2+} entry from electrical activity and activation of G-proteins through a transmitter receptor-coupled system (Xia and Storm, 1997). Thus, *rut* mutants lack the protein necessary for the convergence of pathways from odor (CS) and electric shock (US). Restoring *rut* gene expression limited to the MBs rescues the normal ability for olfactory learning in *rut* mutant flies. Flies containing the ***amn*** mutation lack a peptide transmitter that acts on AC (Tully et al., 1990). This neuropeptide is most abundant in two modulatory neurons that project to the MBs. When the *amn* neuropeptide is released onto the MB lobes, it can trigger a prolonged activation of the cAMP cascade. This prolonged activation is required for the consolidation to transform short-term memory into long-term memory. Mutants of *amn* are defective in mid-term memory and long-term memory (Tully et al., 1994). Mutant ***PKA-R1*** flies are defective in PKA. PKA is the primary downstream target of cAMP. The duration of PKA activation is believed to determine whether short-, medium- or long-term memory is formed. Thus, *PKA-R1* mutants lacking PKA have learning deficits (Goodwin et al., 1997; Li et al., 1996; Skoulakis et al., 1993).

4.3 cAMP/PKA/CREB cascade in rats/mammals

There are few studies that have made direct measurements of cAMP changes in mammalian models. However, pharmacological studies in rats and mice, and genetic studies in mice provide evidence that the cAMP/PKA/CREB pathway plays an important

role in learning and memory in mammals. A series of elegant studies of transgenic mice expressing R(AB), a dominant negative form of PKA regulatory subunit, indicates impressive evidence for the role of PKA in learning (Abel et al., 1997). When tested for fear learning, R(AB) mice showed deficits in long-term, but not short-term, contextual fear conditioning. Cue learning was unaffected in these mice. Both cued and contextual fear conditioning have been shown to depend on the amygdala, whereas contextual fear conditioning involves the hippocampus (Kim et al., 1993; Phillips and LeDoux, 1992), suggesting PKA acts in the consolidation of hippocampus-dependent memories (Abel et al., 1997).

Schafe and colleagues tested the role of PKA in cued fear conditioning. They administered Rp-cAMP (a PKA inhibitor) either intraventricularly prior to training (Schafe et al., 1999), or directly into the lateral nucleus of the amygdala immediately after training (Schafe and LeDoux, 2000), to examine the effect. In both experiments, infusion of Rp-cAMP impaired long-term (24 hours after conditioning), but not short-term (0.5 and 4 hours after conditioning) fear memory trained with the conditioned auditory cue. Administration of the protein synthesis inhibitor anisomycin in each of these pharmacologic studies showed a very similar effect to that of Rp-cAMP. This suggests PKA is also involved in protein synthesis dependent learning consolidation in the amygdala, the process by which short-term memory is converted into long-term memory (Selcher et al., 2002). Differences between the R(AB) mice and normal drug treated mice with respect to cue fear conditioning may relate to the activation of PKA in transgenic

mice, i.e., amygdala PKA activity in R(AB) transgenic mice may not be inhibited as completely as in drug treated mice.

Further examination of the R(AB) transgenic mice with additional pharmacological studies show a time course for PKA-dependent contextual fear conditioning. Bourtchouladze et al., (1998) identified that an impairment in contextual memory for the R(AB) transgenic mice formed between 1 and 3 h after behavioral training. In wild-type mice, intraventricular injection of Rp-cAMPS, an inhibitor of PKA, that acts on the regulatory subunit, produced contextual learning impairments that imitated the amnesia observed in the R(AB) mice. Injection of Rp-cAMPS at various time points after training outlined different PKA-dependent consolidation periods. In a strong contextual fear training paradigm, three CS-US pairings resulted in one PKA dependent period immediately after training (< 1 h), whereas a single pairing produced two critical periods, one immediately after training and another 4 hours later. Mice injected with Rp-cAMPS at all other time points displayed normal contextual learning. These experiments suggest PKA activation is required during the consolidation period to develop long-term memory (Bourtchouladze et al., 1998).

Studies with CREB-deficient mice indicate normal CREB activity is necessary for learning (Bourtchouladze et al., 1998; Kogan et al., 1997). In studies with CRE-LacZ reporter mice, CREB-mediated gene transcription was assessed in response to contextual or auditory cued fear learning (Impey et al., 1998). In contextual fear conditioning, which

requires both amygdala and hippocampus, CRE-dependent gene expression significantly increased in areas CA1 and CA3 of the hippocampus. In auditory cue fear conditioning, which requires amygdala but not hippocampus, CRE-dependent gene expression increased in the amygdala, but not the hippocampus. Therefore, learning activates the CREB-CRE transcriptional pathway in specific areas of brain during different behavioral conditioning paradigms.

4.4 cAMP/PKA/CREB cascade in hippocampal LTP

Explicit memory in mammals is thought to involve LTP in the hippocampus (Kandel et al., 2000). In the hippocampus, there are three major pathways: the perforant pathway, the mossy fiber pathway and the Schaffer collateral pathway. A brief high-frequency train of stimuli (a tetanus) to any of these three major synaptic pathways increases the amplitude of the excitatory postsynaptic potentials in the target hippocampal neurons. This facilitation is called LTP (Kandel et al., 2000).

LTP has two phases. One stimulus train produces an early LTP that lasts 1-3 hours and does not require new protein synthesis. Four or more stimulus trains induce a late LTP that lasts at least 24 hours and requires new protein and RNA synthesis (Kandel et al., 2000). The transformation from early to late phase LTP was shown to depend on cAMP cascade activation in Schaffer collateral and mossy fiber pathways in the hippocampus

(Huang et al., 1994) and in hippocampal perforant pathway (Nguyen and Kandel, 1996). Frey et al., (1993) also provided evidence that formation of late phase LTP in hippocampal CA1 required cAMP activation. This late phase LTP could be prevented by impairing PKA or promoted by activating the cAMP cascade (Frey et al., 1993).

The process of conversion from early to late phase NMDA-dependent LTP has been described in the following way: A single train of action potentials activates NMDA receptors in postsynaptic cells, leading to Ca^{2+} influx and activation of a set of second messengers inducing early LTP. When the action potential trains are repeated, PKA will be activated for a sufficient time to translocate into the nucleus and phosphorylate CREB. Phosphorylated CREB (pCREB), in turn, activates downstream target genes, leading to structural changes at the synapse. Mutations in mice that block PKA or CREB activation decrease or abolish the late phase of LTP (Abel et al., 1997; Bourtchuladze et al., 1994).

4.5 cAMP/PKA/CREB cascade in odor preference learning in rat pups

McLean et al. (1999), observed that the phosphorylation of CREB increased significantly at 10 minutes after training in the olfactory bulbs of pups trained by odor + stroking, compared to control pups (odor only or stroking only), by using Western blot analysis. Additionally, by using immunocytochemistry, they showed pCREB in mitral cells within the dorsolateral quadrant of the bulb to be significantly elevated in pups trained by the

odor-stroke pairing. No significant differences were detected among control groups (naive, odor only, or stroking only) or in the granule or periglomerular cells of the dorsolateral region among any trained groups (McLean et al., 1999). The location of the pCREB changes is consistent with the results of 2-DG uptake and optical imaging studies, which show similar localized changes in the bulb when pups learned a preference for the same odor (Sullivan and Leon, 1987; Yuan et al., 2002). These data suggest that pCREB could have a role in memory formation for olfactory preference learning. Increases in pCREB occur as an early step following conditioning that usually induces long-term olfactory memories in rat pups.

The phosphorylation of CREB was examined during NE-induced odor preference learning in rat pups with either normal or 5-HT-depleted olfactory bulbs (Yuan et al., 2000). Systemic injection of 2 mg/kg isoproterenol, the optimal dose inducing odor preference learning, increases pCREB expression in the olfactory bulbs at 10 min after conditioning, in normal rat pups, but not in bulbar 5-HT-depleted rat pups. The dose of 6 mg/kg isoproterenol, which is ineffective in inducing odor preference learning in normal rat pups, but facilitates learning in 5-HT depleted pups (Langdon et al., 1997), enhanced pCREB expression in rat pups with bulbar 5-HT depletion. These results again suggest that there is a link between pCREB and olfactory preference memory formation. Yuan et al., (2003b) also demonstrated that 5-HT depletion reduces the optimal isoproterenol (2 mg/kg)-induced cAMP elevation. More β-adrenoceptor activation (6 mg/kg dose of

isoproterenol) overcomes the 5-HT induced deficit. This reinforced the hypothesis that NE and 5-HT activation work synergistically through the cAMP pathway.

The role for CREB and pCREB in early odor preference learning in rats was directly examined by infecting the olfactory bulb with herpes simplex virus overexpressing wild type CREB (HSV-wt-CREB) and expressing dominant-negative mutant CREB (HSV-dn-CREB) (Yuan et al., 2003a). Infection of HSV-LacZ was used as a control to determine whether virus injection itself would affect olfactory learning. Overexpression of dn-CREB prevented the learning induced by stroking + odor, and shifted the inverted U curve (isoproterenol dose - response curve) rightwards, such that a higher dose was necessary to induce learning. Overexpression of wt-CREB also impaired the learning induced by stroking + odor, but it shifted the dose-response curve to the left, i.e., the lower dose could produce learning, but the normally optimal dose did not. Control virus expressing LacZ did not affect learning. Once learning occurred, with either overexpressed wt-CREB or dn-CREB, pCREB was elevated compared to the nonlearning LacZ control groups. However, in the overexpressed wt-CREB group, in which no learning occurred, pCREB also increased higher than LacZ control group that did learn. These data demonstrate that CREB and pCREB play a causal role in early mammalian odor preference learning. Since overexpressing wt-CREB interferes with learning, there may be an optimal pCREB window for learning under normal conditions.

cAMP expression at the end of odor exposure during the odor preference training was examined (Yuan et al., 2003b). The result shows cAMP increases under the two effective learning conditions, odor paired with stroking and odor paired with 2 mg/kg isoproterenol, compared with the naive and saline groups. However, the two ineffective learning conditions, stroking alone and odor + a higher dose (4 mg/kg) of isoproterenol, also significantly increased cAMP level over naive and saline groups. There were no differences observed between either odor + stroking and stroking only, or odor + a 2 mg/kg dose of isoproterenol and odor + a 4 mg/kg dose of isoproterenol. This contrasted to the results that only effective learning conditions increase the levels of pCREB. That is, pCREB increases more with the odor + stroking than with the stroking-only condition (McLean et al., 1999) and a 2 mg/kg dose of isoproterenol induces a higher level of pCREB than the odor + a 6 mg/kg dose of isoproterenol (Yuan et al., 2000). Therefore, unlike the *Aplysia,* in which the CS and the US pathways appear to converge on AC and where higher levels of cAMP determine the occurrence of learning (Abrams et al., 1998), in the olfactory learning of rat pup, US and CS pathways appear to converge at a later stage, e.g. CREB phosphorylation. (Figure 1.2.)

5 Rationale and hypothesis

Isoproterenol-induced learning and CREB phosphorylation both show correspondent inverted U or parabolic shape with isoproterenol dose. Normally a 2 mg/kg dose of

isoproterenol is optimal to induce early olfactory learning and also elevates pCREB level. The higher (4 mg/kg) dose is ineffective as a US in normal pups (Langdon et al., 1997). The dose of 6 mg/kg has also been shown to be ineffective in learning and in producing pCREB in normal pups (Yuan et al., 2000). A similar inverted U shaped curve was described for stroking-induced learning, when stroking was combined with isoproterenol (Sullivan et al., 1991). Thus, the inverted U shaped dose-response curve might be a basic property of the learning system.

The observed cAMP increases with increasing isoproterenol dose is inconsistent with the hypothesis that cAMP levels account for the relationship seen between isoproterenol dose and behavior, and pCREB levels. The depletion of 5-HT that causes impairment of learning which can be overcome by providing a higher than normal level of NE suggests that a critical cAMP level is required for learning (Yuan et al., 2000), and that lower levels of cAMP are insufficient to produce the intracellular signals required by learning. Why then does a higher level of cAMP associate with learning failure? Yuan et al., (2003b) proposed three hypotheses to explain the lack of learning with higher isoproterenol doses: (1) Higher levels of cAMP increase Ca^{2+} entry, which, in turn, aids calcineurin-induced CREB dephosphorylation and counteracts PKA effects; (2) Higher levels of cAMP promote increased rate of cAMP hydrolysis by phosphodiesterase 4 (PDE4) activation through PKA (Ang and Antoni, 2002). This would decrease the level of the cAMP expression and alter its spatial-temporal pattern; (3) Elevated cAMP levels promote faster cAMP extrusion, another regulatory mechanism to maintain a low level of

free intracellular cAMP (Wiemer et al., 1982), thus, would decrease cAMP levels and alter its spatial-temporal pattern.

To evaluate these last two hypotheses, the present study examines in more detail the temporal relation of cAMP expression with optimal and higher doses of isoproterenol, or saline, all paired with odor. Five time points were chosen for determining cAMP levels: initiation of training (odor presentation), 5 min after initiation of training, immediately after the training session, 5 and 10 min after training finished. Because CREB phosphorylation appears to peak 10 min after training has finished under learning conditions (Yuan et al., 2000), the influence on CREB phosphorylation by cAMP should happen within this period.

To examine the role of 2 mg/kg isoproterenol alone (optimal learning associated dose) on cAMP levels, a second experiment was carried out, in which cAMP levels were tested with 3 training conditions: saline without odor (without either CS or US input), 2 mg/kg isoproterenol without odor (US input only), and 2 mg/kg isoproterenol with odor (both CS and US inputs). Two time points were chosen: immediately after training and 5 minutes after training.

I hypothesized that there was an optimal cAMP level relative to the conditioning. In that scenario, saline plus odor would not be strong enough to elevate cAMP to an optimal level. Only an optimal isoproterenol dose (2 mg/kg) paired with odor would maintain

cAMP within the optimal level. As for the 6 mg/kg dose of isoproterenol (nonlearning dose) plus odor, cAMP would increase higher than 2 mg/kg dose of isoproterenol plus odor at the end of conditioning. But afterwards, the high level of isoproterenol might either decrease cAMP very quickly below the optimal level, or keep cAMP higher than the optimal level.

CHAPTER II METHOD

1 Subjects

In Experiment I, 115 Sprague-Dawley rats, both male (n=60) and female (n=55), from 10 litters were used. Fifteen groups were included: 3 dosages (saline, 2 mg/kg of isoproterenol, 6 mg/kg of isoproterenol), at 5 time points, immediately prior to 10 minutes odorized bedding training, 5 minutes after odor training started, immediately after removal from odorized bedding, 5 minutes and 10 minutes after odor training finished.

In Experiment II, 46 Sprague-Dawley rats, both male (n=22) and female (n=24), from 4 litters were used. Six groups were included: 1 dosage of isoproterenol (2 mg/kg) at 2 time points, immediately after 10 minutes of odorized bedding training and 5 minutes after odorized bedding training, 2 dosages (saline or 2 mg/kg isoproterenol) at the same 2 time points, with respect to training, but without odor presentation (pups were sacrificed immediately after removal from 10 minutes on fresh bedding or 5 minutes post removal from fresh bedding).

Litters were culled to 12 pups per litter on PND 1 (the day of birth is considered PND 0). The dams were maintained under a 12-hour light-dark cycle at 22°C in polycarbonate cages containing hardwood chips at the Health Sciences Centre of Memorial University of Newfoundland animal care facility. Food and water were accessible ad libitum. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee, and conformed to the standards set by the Canadian Council on Animal Care.

2 Odor conditioning, drug injection and sample collection

Rat pups were distributed among groups. A maximum of one male and female pup from the same litter was used for each training group. According to the procedure described in Yuan et al., (2003b), on PND 6, saline or isoproterenol (2 mg/kg, or 6 mg/kg, Sigma Chemical, St. Louis, USA) was injected subcutaneously 40 minutes prior to odor preference training. Thirty minutes following injection, the pups were removed from the dam and placed on fresh bedding. Ten minutes later, pups in all of the groups of experiment I, or in groups trained on odor scented bedding in experiment II, were placed on peppermint scented bedding (0.3 ml peppermint extract in 500 ml bedding). In experiment II, pups in groups trained without odor were placed on fresh bedding for the training period.

All training sessions were done in an experimental room where the temperature was kept constant at 28°C. This allowed pups to maintain body temperature in the absence of the dam.

The pups in the present experiments were subjected to training procedures, but were not tested for recall, in order to examine the level of cAMP specifically during acquisition of odor preference learning. Previous studies in our laboratory (Langdon et al., 1997; McLean et al., 1993; Price et al., 1998), as well as in others (Sullivan et al., 1989; Sullivan et al., 1991; Sullivan and Leon, 1986; Sullivan and Leon, 1987), have shown that pups subjected to these training procedures show predictable behavioral outcomes, i.e., 2 mg/kg isoproterenol plus odor induce odor preference for the conditioned odor, whereas either saline or 6 mg/kg isoproterenol plus odor does not.

Samples were collected according to the method described in Yuan et al., (2000, 2003). At the appropriate time points, the pups in the relevant groups were sacrificed by decapitation. Both olfactory bulbs were removed from the skull and flash frozen on dry ice. All samples were subsequently stored at -70°C in microcentrifuge tubes until assayed for cAMP content. All groups were assayed at the same time.

3 cAMP assay and protein determination

3.1 cAMP assay

The cylic AMP Enzyme Immunoassay (cAMP EIA) Kit from Cayman Chemical (Ann Arbor, USA), was used to assay for cAMP levels. This assay is based on the competition between free cAMP and a cAMP-acetylchotinesterase (AChE) conjugate (the cAMP tracer) for a limited number of cAMP-specific rabbit antibody binding sites. Because the concentration of the cAMP tracer is held constant while the concentration of free cAMP varies between samples, the amount of cAMP tracer that is able to bind to the rabbit antibody will be inversely proportional to the concentration of cAMP contained in the sample.

3.1.1 Reagents preparation

The day before the assay was to be performed, all buffers and reagents were prepared according to the instructions provided by the Cayman Chemical (Ann Arbor, USA). Briefly, EIA buffer was made by diluting the contents in the EIA buffer concentrate vial with 90 ml of ultrapure water. Wash buffer was made by diluting 2.5 ml wash buffer concentrate to a total volume of 1 liter with ultrapure water and adding 0.5 ml of Tween 20. Phosphate buffer was made by diluting the phosphate buffer concentrate to a final

volume of 200 ml with ultrapure water. The cAMP AChE tracer was reconstituted by 6 ml EIA buffer. The cAMP antiserum was reconstituted by 6 ml EIA buffer. The cAMP standard was reconstituted by 1 ml of phosphate buffer.

3.1.2 Sample preparation

Frozen olfactory bulbs were homogenized in 250 µl phosphate buffer, containing 5% trichloroacetic acid (TCA) on ice using a hand held pestle homogenizer. Precipitates were removed by centrifuging at 1,500 x G for 10 minutes at 4°C. The supernatant solution was transferred carefully to a clean test tube, while the pellet was kept for protein determination.

TCA was extracted from the supernatant with 5 volume water-saturated ether. [To make the water-saturated ether, water was added to ether until layers formed; these were mixed and the top layer (ether layer) was used]. The residual ether was removed by heating the supernatant to 70°C for 5 minutes. The 50 µl supernatant was diluted in 150 µl phosphate buffer.

3.1.3 Performing the assay

The reagents were added in the 96-well plate provided with the kit. In each well, there were 50 µl cAMP standard or sample, 50 µl cAMP tracer, and 50 µl cAMP antiserum. Each standard or sample was run in duplicate. Afterwards, the plate was covered with plastic film and incubated for 18 hours at 4°C.

When incubation was terminated, the wells were emptied and rinsed five times with wash buffer to remove any unbound reagents. Ellman's reagent, containing the substrate for AChE and reconstituted with ultrapure water, was added to each well. After developing the plate on an orbital shaker in the dark for 120 minutes at room temperature, the enzymatic reaction in each well produced a distinct yellow color. The plate was read at a wavelength of 415 nm, by using a BIO-RAD Model 3550 Microplate Reader. The total cAMP content was calculated in pmol accounting for dilution and total volume, after comparing sample values in each well to those of the standard curve which consists of eight points from 0.115 to 15 pmol.

3.2 Protein determination

The TCA protein pellet was reconstituted with 800 µl dH_2O and then the protein concentration was determined by a Bicinchoninic Acid (BCA) protein assay Kit (Pierce,

Rockford, USA). The procedure included adding the samples, standards and reagents to a 96-well plate, incubating the plate for 30 minutes at 37°C, and reading the plate at 595 nm in the BIO-RAD Model 3550 Microplate Reader. The total protein amount was calculated in mg accounting for dilution and total volume, after comparing sample values in each well to those of the standard curve which consists of eight points from 0.005 to 0.05 mg. The final cAMP value in each animal was then calculated as pmol/mg protein.

4 Statistical analysis

In experiment I, a two-way ANOVA was used to statistically compare the drug dose (saline, 2 mg/kg isoproterenol, or 6 mg/kg isoproterenol), the time, and drug dose over time interaction effects. The least significant differences (LSD) test was used for post hoc comparisons.

In experiment II, a one-way ANOVA was used for statistical comparison of the treatment groups (saline without odor, 2 mg/kg isoproterenol without odor, and 2 mg/kg isoproterenol with odor). The Student-Newman-Keuls test was used for post hoc comparisons. A Student t-test was used for the comparison of times (immediately after training and 5 minutes after training) within the same treatment group.

CHAPTER III RESULTS

1 Experiment I

Before I started the experiment, I hypothesized that there was an optimal cAMP level relative to the conditioning. In that case, saline plus odor would not be strong enough to elevate cAMP to an optimal level. Only an optimal isoproterenol dose (2 mg/kg) paired with odor would maintain cAMP within the optimal level. As for 6 mg/kg dose of isoproterenol (nonlearning dose) plus odor, cAMP would increase higher than 2 mg/kg dose of isoproterenol plus odor at the end of conditioning. But afterwards, the high level of isoproterenol might either decrease cAMP very quickly below the optimal level, or keep cAMP higher than the optimal level. However, the results I got are unexpected.

In the 2-way ANOVA drug (3) over time (5), there was a significant drug by time interaction [$F(8,100)=2.925$, $P=0.0056$]. See Figure 3.1. There was also a significant drug effect [$F(2,100)=14.056$, $P<0.0001$] which showed that the cAMP expression of the 6 mg/kg isoproterenol group was higher than that of the saline and 2 mg/kg isoproterenol groups.

1.1 cAMP expression at different time points under the three drug conditions

At the start of training (0 min in Figure 3.1), cAMP expression was not significantly different among the three dosage groups: saline, 2 mg/kg dose of isoproterenol and 6 mg/kg dose of isoproterenol, although the mean level was lowest in the saline group [Mean ± SEM = 83 pmol/mg ± 7.6 (saline); 94 pmol/mg ± 3.9 (2 mg/kg iso); 98 pmol/mg ± 11.6 (6 mg/kg iso)].

Five minutes after the pups were placed on the peppermint scented bedding (5 min in Figure 3.1), cAMP expression in the 2 mg/kg isoproterenol group decreased significantly (Mean ± SEM = 73 pmol/mg ± 6.3), compared with the 6 mg/kg isoproterenol group (Mean ± SEM = 106 pmol/mg ± 9.4; 2 mg/kg iso vs. 6 mg/kg iso, $P<0.05$). There were no differences between the 2 mg/kg isoproterenol group and the saline group (Mean ± SEM = 100 pmol/mg ± 8.6), or between the 6 mg/kg isoproterenol group and the saline group.

After the 10 minute odor presentation (10 min in Figure 3.1), cAMP levels were significantly higher in both the 2 mg/kg isoproterenol group (Mean ± SEM = 114 pmol/mg ± 11; $P<0.05$) and the 6 mg/kg isoproterenol group (Mean ± SEM = 118 pmol/mg ± 9.3; $P<0.05$) than in the saline group (Mean ± SEM = 81 pmol/mg ± 8.2). There was no difference in cAMP levels between the isoproterenol treated groups.

Five minutes after ending odor presentation (15 min in Figure 3.1), cAMP levels in the 2 mg/kg isoproterenol group decreased (Mean ± SEM = 74 pmol/mg ± 8.2), and was significantly lower ($P<0.05$) than the 6 mg/kg isoproterenol group (Mean ± SEM = 127 pmol/mg ± 9.2). There was no difference between the saline group (Mean ± SEM = 101 pmol/mg ± 7.6) and either isoproterenol group.

Ten minutes after ending odor presentation (20 min in Figure 3.1), cAMP expression was highest in the 6 mg/kg isoproterenol group (Mean ± SEM = 139 pmol/mg ± 8.7) and lowest in the saline group (Mean ± SEM = 80 pmol/mg ± 7.7). cAMP levels in the 2 mg/kg isoproterenol group (Mean ± SEM = 112 pmol/mg ± 7.4) was also higher than in the saline group ($P<0.05$), but significantly lower than in the 6 mg/kg isoproterenol group ($P<0.05$).

1.2 cAMP expression within treatment groups

Every treatment group shows a different cAMP pattern. See Figure 3.1. There was no difference in cAMP levels among the five time points in the saline group, although a weak oscillatory pattern is suggested in the means [(Mean ± SEM = 83 pmol/mg ± 7.6 (0 min); 100 pmol/mg ± 8.6 (5 min); 81 pmol/mg ± 8.2 (10 min); 101 pmol/mg ± 7.6(15 min); 80 pmol/mg ± 7.7 (20 min)], such that cAMP expression was higher at five minutes

after training started and at five minutes after training finished. The 2 mg/kg isoproterenol group exhibited a significant oscillatory pattern of cAMP level. The cAMP levels were lowest 5 minutes after the start of odor exposure (Mean ± SEM = 73 pmol/mg ± 6.3) and highest at 10 minutes after the start of odor exposure (Mean ± SEM = 114 pmol/mg ± 11; $P<0.05$). Level of cAMP then decreased and was significantly lower 5 minutes after the end of odor exposure (Mean ± SEM = 74 pmol/mg ± 8.2) than at the end of odor exposure ($P<0.05$). But by 10 minutes after the end of odor exposure, it was significantly elevated (Mean ± SEM = 112 pmol/mg ± 7.4; $P<0.05$) over the previous time point. In the 6 mg/kg isoproterenol group, cAMP levels increased linearly with time. Some of the time points showed the increase was significant: 5 min (Mean ± SEM = 106 pmol/mg ± 9.4) vs. 10 min (Mean ± SEM = 118 pmol/mg ± 9.3; $P<0.05$, increase); 10 min vs. 15 minutes (Mean ± SEM = 127 pmol/mg ± 9.2; $P<0.05$, increase); 15 min vs. 20 minutes (Mean ± SEM = 139 pmol/mg ± 8.7; $P<0.05$, increase).

Therefore, the condition previously shown to produce learning, the 2 mg/kg dose of isoproterenol paired with odor, produces a significant oscillatory pattern of cAMP levels in 5 minute intervals. The nonlearning condition, 6 mg/kg isoproterenol paired with odor, has much higher cAMP levels and cAMP increases in a temporal linear pattern across the whole period; saline plus odor shows a weak oscillation pattern, but there is no difference among those time points.

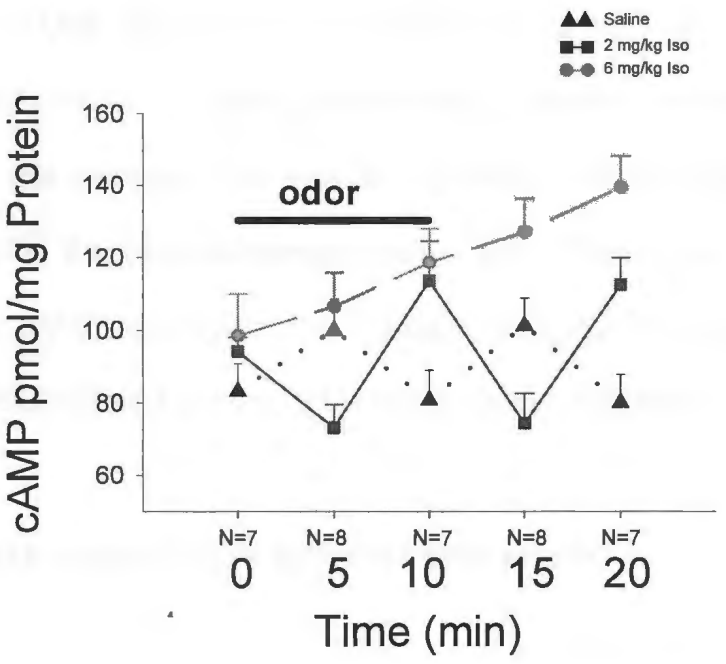

Figure 3.1. cAMP shows an oscillatory pattern with learning.

CS (odor) paired with an optimal US (2 mg/kg iso) produces 5 min peaks and troughs of cAMP expression. With training by odor plus saline (Saline), cAMP levels show a non-significant weak pattern of oscillations. Odor paired with a supraoptimal non-learning isoproterenol dose (6 mg/kg iso) produces a linearly increasing cAMP pattern.

2 Experiment II

It was not clear if 2 mg/kg dose of isoproterenol itself could produce the peak and trough pattern of cAMP activation, or that pattern requires both odor and 2 mg/kg dose of isoproterenol. Is the oscillation pattern learning specific? Experiment II was set up to addresses that question. There were three groups in different training conditions: saline without odor, 2 mg/kg isoproterenol without odor, or pairing of 2 mg/kg isoproterenol with odor. cAMP measurements were taken at the end of training and 5 minutes later, a time of the cAMP peak and the cAMP trough seen in Experiment I.

2.1 cAMP expression at different time points

At the end of training (10 minutes in Figure 3.2), there were significant differences in cAMP expression among saline (no odor) group, 2 mg/kg isoproterenol (no odor) group, and 2 mg/kg isoproterenol + odor group, [one-way ANOVA, $P=0.0123$, $F(2,20)=5.528$]. See Figure 3.2. cAMP levels in the 2 mg/kg isoproterenol (no odor) group (Mean ± SEM = 119 pmol/mg ± 8.4) and the 2 mg/kg isoproterenol + odor group (Mean ± SEM = 127 pmol/mg ± 10.3) were significantly higher than the saline (no odor) group (Mean ± SEM = 88 pmol/mg ± 5.1; $P<0.05$). Five minutes after training finished (15 min in Figure 3.2), there were again significant differences in cAMP levels between the three groups [one-way ANOVA, $P = 0.0038$, $F(2,20) = 7.469$]. cAMP level in the 2 mg/kg isoproterenol +

odor group (Mean ± SEM = 75 pmol/mg ± 6.3) were significantly lower than the 2 mg/kg isoproterenol (no odor) group (Mean ± SEM = 114 pmol/mg ± 9.1; $P<0.01$). The saline (no odor) group (Mean ± SEM = 86 pmol/mg ± 6.9) was significantly lower than the 2 mg/kg isoproterenol (no odor) group ($P<0.05$), but did not differ from the 2 mg/kg isoproterenol + odor group.

2.2 cAMP expression within treatment groups

The saline (no odor) group expressed a similar cAMP level at the end of training (Mean ± SEM = 88 pmol/mg ± 5.1), and five minutes after training finished (Mean ± SEM = 86 pmol/mg ± 6.9). The 2 mg/kg isoproterenol (no odor) group showed a higher level of cAMP both when the training had just finished (Mean ± SEM = 119 pmol/mg ± 8.4) and five minutes later (Mean ± SEM = 114 pmol/mg ± 9.1). There was no difference between these two time points. cAMP expression in the 2 mg/kg isoproterenol + odor group decreased significantly at five minutes after training finished (Mean ± SEM = 75 pmol/mg ± 6.3), compared to the point when training had just finished (Mean ± SEM = 127 pmol/mg ± 10.3; $P<0.01$).

In summary, when there was neither odor input nor isoproterenol input, cAMP remained constant in olfactory bulbs (Figure 3.2A). A 2 mg/kg dose of isoproterenol alone (US input alone) elevated cAMP levels relative to the saline alone group at both time points

(Figure 3.2B). Only pups that received odor paired with 2 mg/kg dose of isoproterenol showed the peak/trough pattern (Figure 3.2C), a significant peak at the end of pairing and a significant trough 5minutes later. That is, this peak/trough pattern is specifically associated with the pairing of the CS (odor) and the US (2 mg/kg dose of isoproterenol). It does not occur with the US alone, CS alone, or without either US nor CS. These data suggest that the oscillation of cAMP which was shown in Experiment I is specific to conditioning.

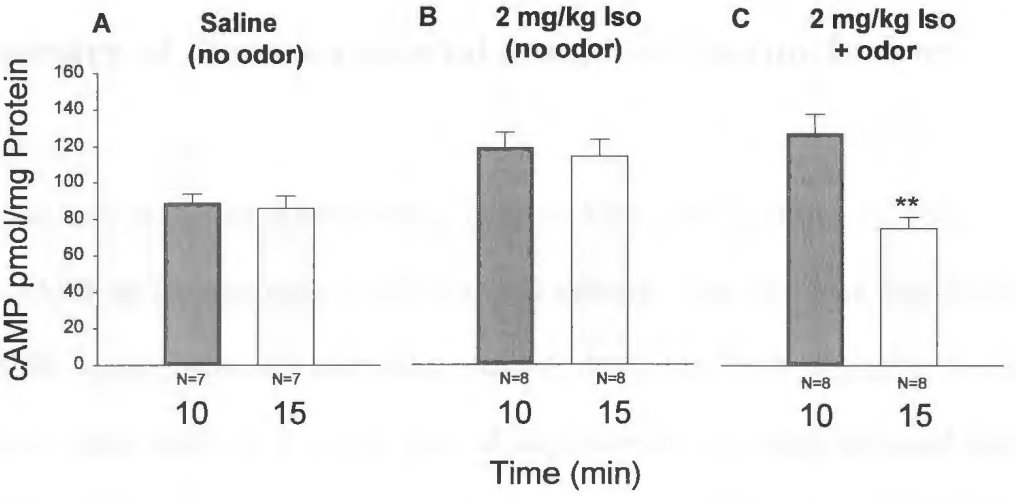

Figure 3.2. The oscillatory pattern of cAMP requires CS-US pairing.

cAMP levels were measured at two time points: the end of training (10 min) and 5 min later (15 min), in three training groups: saline without odor, 2 mg/kg isoproterenol without odor, and 2 mg/kg isoproterenol with odor. **A**. Without either odor (CS) or isoproterenol (US) input, cAMP levels remained constant in the bulb. **B**. 2 mg/kg isoproterenol input only (without odor) increases cAMP levels at both time points relative to saline. **C**. Pairing of odor with 2 mg/kg isoproterenol produced a significant peak at 10 min relative to saline and a significant trough at 15 min relative to the 2 mg/kg isoproterenol alone (t-test. ** $p < 0.01$).

CHAPTER IV DISCUSSION

1 Summary of the experimental design and major findings

This project was an extension of the study done by Yuan et al., (2003b). In Yuan et al., (2003b), cAMP levels were determined following training. They identified that olfactory bulb cAMP levels increased with isoproterenol dose, i.e., both 2 mg/kg dose of isoproterenol plus odor and 4 mg/kg dose of isoproterenol plus odor increased cAMP significantly, compared with saline plus odor; 1 mg/kg isoproterenol plus odor group showed intermediate cAMP level. In addition, cAMP was also increased by stroking with or without odor. Therefore, olfactory bulb cAMP levels increased in response to β-adrenergic activation (the US), under both learning and nonlearning conditions. Consequently, there arose a question: what is the pattern of cAMP activation related to learning? Testing cAMP levels at one time point did not provide enough information to answer this question. The objective of this study was to characterize the temporal changes in cAMP levels in a learning and nonlearning environment relative to the β-adrenergic agonist isoproterenol dose at several time points during and after training.

When I first began my study, I hypothesized that there was an optimal cAMP level relative to the learning. In that scenario, saline plus odor would not be strong enough to

elevate cAMP to an optimal level. Only an optimal isoproterenol dose (2 mg/kg) paired with odor would maintain cAMP within the optimal level. As for 6 mg/kg dose of isoproterenol (nonlearning dose) plus odor, cAMP levels would be higher than 2 mg/kg dose of isoproterenol plus odor at the end of conditioning. Afterwards, the high level of isoproterenol would either decrease cAMP very quickly below the optimal level, or keep cAMP above the optimal level.

After the study, I found the temporal change of cAMP shows an oscillatory pattern with learning condition and the oscillations are specific to conditioning. The present results that 2 mg/kg and higher doses of isoproterenol paired with odor produce significantly higher cAMP in the olfactory bulb than saline paired with odor at the end of the 10 minute training period, replicate the previous finding in Yuan et al., (2003b). The peak/trough pattern of cAMP expression seen immediately after training relative to 5 minutes after training in the 2 mg/kg dose of isoproterenol + odor pairing group in Experiment I was replicated in Experiment II.

2 Reports exemplifying cAMP oscillations and their time course

The finding of cAMP peaks and troughs when odor is paired with a learning-effective dose of isoproterenol was unexpected when I first formulated the hypothesis of the study.

However, there is increasing evidence showing that oscillatory patterns of cAMP are likely to be a more important pattern for intracellular signaling than a simple cAMP increase. Reports exemplifying this pattern in slime mold, secretory cells, LTP and long-term facilitation are discussed below.

Periodic synthesis and release of cAMP in aggregation of the cellular slime mold *Dictyostelium discoideum* was reported by Konijn and Shaffer (Konijn, 1972; Shaffer, 1975). During their aggregation on a surface, one or a few cells at the center of an aggregation territory autonomously emits pulses of cAMP with a period of about five minutes. These pulses are detected by adjacent cells that respond by chemotaxing towards the source, and also by emitting their own pulses of cAMP. Hence, cAMP travels as a wave out from the aggregation center due to two processes: a triggering by autonomous cells and signal transmission by relay-adjacent cells (Raman et al., 1976).

More closely related to olfactory cAMP oscillations are cAMP oscillations found in gonadotropin-releasing hormone (GnRH) secretion. GnRH secretion is controlled by a variety of regulatory mechanisms intrinsic to individual neurons or networks of GnRH-secreting neurons and by extrinsic regulatory mechanisms regulated by neurotransmitters released onto GnRH neurons. The development of the highly differentiated GT1 GnRH-secreting neuronal cell lines has provided a model to study the signaling mechanisms involved in the complex regulation of GnRH secretion (Mellon et al., 1990). The pulsatile release of GnRH approximately every 15 - 20 minutes appears to be an intrinsic property

of individual or networks of GnRH neuron(s) (Martinez et al., 1992b). In *vivo*, numerous neurotransmitters are released onto GnRH neurons to stimulate GnRH secretion (Kordon et al., 1994). In *vitro*, neurotransmitters stimulating GnRH release from GT1 cells include DA (Martinez et al., 1992c) and NE (Martinez et al., 1992a). Treatment of GT1 cells with DA and NE increase intracellular cAMP levels and GnRH secretion in a dose-dependent manner (Martinez et al., 1992a; Martinez et al., 1992c). The level of secretion of GnRH was proportional to the activation of AC and increases in cAMP levels (Martinez et al., 1995).

There are studies focusing on the role of the cAMP signaling pathway in regulating the pulsatile GnRH secretion (Vitalis et al., 2000). First, they showed treatment of GT1 cells with DA increased cAMP levels, and also stimulated GnRH secretion. Second, they found GT1 cells expressed and had functional cAMP-gated cation channels. Third, PKA did not increase GnRH release, but conversely inhibited the cAMP-induced GnRH release. Forth, GT1 cells expressed AC V, which was inactivated by PKA. Based on these data, they proposed signaling pathways to represent potential timing mechanisms for the pulsatile release of GnRH. The stimulation of GT1 cells increased cAMP formation through activated AC. In turn, cAMP activated cAMP-gated cation channels increased the excitability of GT1 cells, resulting in the stimulation of GnRH secretion. On the other hand, increased cAMP activated PKA. The activated PKA decreased cAMP levels by inactivating AC V, thereby constituting a negative-feedback mechanism for GnRH

secretion. This mechanism results in oscillation in cAMP levels, which provides a biochemical basis for timing the pulsatile release of GnRH.

Similar to the cAMP oscillation in GnRH secreting cells is regulation of secretory activity in prolactin cells. Haisenleder et al., (1992) examined whether pulsatile changes in cAMP are more effective than a continuous stimulation in increasing pituitary hormone gene expression. They found prolactin and alpha thyrotropin subunit mRNAs were increased by cAMP analogs, 8-bromo cAMP or Bt cAMP, only in a pulsatile manner (given every 60 minutes for 24 hours). Continuous Bt cAMP was ineffective. These data suggest that pulsatile changes in intracellular cAMP are essential for maximal expression of the prolactin and alpha genes. Thus, pulsatile changes in intracellular second messengers may be necessary for signal transduction from the plasma membrane to the nucleus (Haisenleder et al., 1992).

Oscillatory patterns of cAMP are also found outside the areas of the brain controlling reproduction (pituitary/hypothalamus). Oscillations have also been found in the hippocampus. A PKA - dependent LTP in hippocampus is induced by the stimulation of temporally spaced trains of high frequency every 5 minutes (Duffy and Nguyen, 2003). Continuous trains do not produce PKA-dependent LTP (Woo et al., 2003). More specifically, stimulation to the CA1 Schaffer collateral pathway with multiple trains of 100 Hz, which are spaced at 5-10 minute intervals, induces long-lasting LTP. This long-lasting LTP requires activation of the cAMP - PKA pathway for its full expression and

continuance (Abel et al., 1997; Blitzer et al., 1995; Huang and Kandel, 1994; Nguyen et al., 2000; Wong et al., 1999; Woo et al., 2003). Genetic and pharmacological manipulations show that only spaced stimulation preferentially induces PKA-dependent, long-lasting LTP in area CA1 (Woo et al., 2003). LTP induced by temporally spaced stimulation was impaired in the Schaeffer collateral pathway in hippocampal slices from PKA mutant mice. On the contrary, LTP induced by temporally compressed or continuous stimulation was normal in slices from PKA mutant mice (Woo et al., 2003). In addition, LTP induced by spaced stimulation was significantly suppressed by KT-5720, an inhibitor of catalytic subunits of PKA, in brain slices from wildtype mice. In contrast, the long-term maintenance of the LTP induced by temporally compressed stimulation in brain slices from PKA mutant mice is not significantly influenced by application of KT-5720 (Woo et al., 2003). Therefore, LTP induced by these compressed patterns of stimulation does not require PKA activation, but PKA-dependent LTP is selectively induced by temporally spaced, multitrain stimulation. Changing the temporal spacing of stimulation critically modulates the PKA-dependence of hippocampal LTP.

Duffy and Nguyen, (2003) used intracellular perfusion of various PKA modulators to examine the role of postsynaptic PKA in long-lasting LTP in hippocampal area CA1 (Duffy and Nguyen, 2003). They found that four 100 Hz trains (4 × 100 Hz), separated by 5 minutes, evoked a large, non-decaying postsynaptic potentiation for up to 2 hours in the Schaeffer collateral pathway. This postsynaptic potentiation decayed significantly within 1.5 hour by postsynaptic infusion of PKA inhibitor, either Rp-cAMPS or PKI_{6-22} (6-22

amide peptide). In contrast, postsynaptic infusion of PKI_{6-22} did not change the postsynaptic potentiation induced by a single 100 Hz train. These findings reinforce the view that long-lasting LTP induced by temporally spaced stimulation requires activation of postsynaptic PKA. The cAMP-PKA – dependent LTP is strongly correlated with hippocampal long-term memory in mice (Abel et al., 1997). The coincidence of its stimulation required for 5-minute intervals suggests cAMP oscillations may be involved in PKA-dependent LTP.

In addition to cAMP being associated with LTP, cAMP oscillations have been found in long-term memory of *Aplysia.* Repeated spaced 5 minutes pulses of 5-HT induce long-term sensitization in *Aplysia* (Montarolo et al., 1986). Facilitation of the monosynaptic connections between the sensory and motor neurons contributes to both long-term and short-term sensitization of the gill and siphon withdrawal reflex in *Aplysia.* To analyze the relationship between these two forms of sensitization at the cellular and molecular level, this monosynaptic sensorimotor component in *Aplysia* can be reconstituted in dissociated cell culture. Montarolo et al., (1986) exposed the cultures to either a single 5 minute application of 5-HT or to five applications of 5-HT (5 minutes each) at 15 minutes intervals. Then the treated cells and connections were reexamined 24 hours later. The cultures treated with five applications of 5-HT showed a significant increase in the strength of the connections. In contrast, untreated controls or cultures receiving a single 5-HT application showed no significant change in their synaptic strength (Montarolo et al., 1986). 5-HT application could activate the cAMP pathway which is required for the

formation of both short-term and long-term sensitization (Kandel et al., 2000). Repeated stimulation by 5-HT may induce cAMP changes in an oscillatory pattern given the close temporal coupling between 5-HT application and cAMP production seen in other studies (Huang et al., 1971). Although it still needs to be determined whether these various observations reflect a basic temporal requirement of intracellular cAMP signaling, they suggest that cAMP oscillations are a highly conserved phenomenon used to regulate cellular functions.

3 Mechanisms underlying cAMP oscillations

Changes in concentrations of intracellular messengers are now known to be only one of the means used to encode transduction information. Specific responses can also be attained through changes in the speed, amplitude and spatial–temporal pattern of signaling messengers (Hunter, 2000). Different pathways interact with one another to shape the final biological response. The resulting signaling networks provide unique cellular responses to induce complex behaviors (Bhalla and Iyengar, 1999). Therefore, I speculate the mechanism underlying cAMP oscillations is likely to be based on interactions between diverse intracellular signals.

3.1 Interaction between intracellular Ca^{2+} and cAMP signaling pathways

As mentioned previously, olfactory learning in neonatal rats is a classical conditioning paradigm. Classical conditioning involves learning a relationship between the CS and US (Kandel et al., 2000). According to the intracellular model for this early olfactory learning paradigm, proposed in Yuan et al., (2003b), US and CS co-activate mitral cells in the olfactory bulb. Inside mitral cells, the cAMP - PKA pathway is activated by US activation of the β-adrenoceptors. The CS odor signal, conveyed by olfactory sensory axons, activates NMDA/AMPA receptors on the mitral cells. Activated NMDA receptors lead to Ca^{2+} influx, which, in turn, activates intracellular Ca^{2+}/CaM – protein kinase C (PKC) pathways. There have been many reports showing that Ca^{2+} and cAMP signaling pathways interconnect with each other, both at the level of second messenger generation and at the level of their intracellular target processes.

An important issue related to learning surrounds the identification of substrates where both the CS and US interact within cells to specifically induce learning. The cAMP signaling pathway is initiated by the binding of transmitters with G protein coupled receptors on the cell membrane (Kandel et al., 2000). The binding activates the receptor coupled G protein, which in turn activates AC, the enzyme producing cAMP from adenosine triphosphate (ATP) (Kandel et al., 2000). AC is a large polypeptide located on the plasma membrane in a transmembrane manner. There is a Ca^{2+}/CaM binding site

believed to be in its cytosolic domain (Vorherr et al., 1993; Wu et al., 1993). Stimulation of AC by Ca^{2+} is mediated by CaM (Bakalyar and Reed, 1991; Tang and Gilman, 1992). There are several isoforms of AC. Ca^{2+} can activate some of them, but inhibits others, e.g. AC1 and AC8 are Ca^{2+}/CaM-stimulated enzymes, whereas Ca^{2+} inhibits AC3.

Hippocampal mossy fiber LTP requires an increase in presynaptic Ca^{2+} (Johnston, 1992; Zalutsky and Nicoll, 1990). Coupling of Ca^{2+} to activation of AC1 is crucial for mossy fiber LTP (Villacres et al., 1998; Weisskopf et al., 1994). Mossy fiber LTP is significantly impaired in AC1 knockout mice. High concentrations of forskolin, an AC activator, induces mossy fiber LTP to comparable levels in wild type and AC1 mutant mice, indicating that signaling components downstream from AC, including PKA, are not affected in AC1 knockout mice. Cerebellar parallel fibers exhibit an LTP with similar properties to hippocampal mossy fiber LTP (Salin et al., 1996). It is also dependent on extracellular Ca^{2+} and AC activation. AC1 knockout mice show a complete lack of parallel fiber/Purkinje cell LTP (Lev-Ram et al., 2002; Storm et al., 1998). This blockade is bypassed by application of an exogenous cAMP analog, suggesting that it results specifically from deletion of AC1. AC1 knockout mice do not show normal spatial memory when examined in the Morris water task, a test that measures the ability of a mouse to navigate by means of direct and indirect visual cues (Wu et al., 1995). This suggests that AC1 may be important for spatial memory. Both AC1 knockout or AC8 knockout mice exhibit normal long-term memory for contextual and passive avoidance learning and long-lasting LTP (L-LTP), but AC1 X AC8 mice do not (Wong et al., 1999).

Administration of forskolin to area CA1 of the hippocampus restores normal memory for passive avoidance learning in AC1 X AC8 mice. The defect in L-LTP is also reversed by application of forskolin to AC1 knockout X AC8 knockout hippocampal slices. These data indicate that Ca^{2+} -stimulated AC activity is essential for L-LTP as well as long-term memory. L-LTP and long-term memory are initiated by activation of NMDA receptors and postsynaptic Ca^{2+} increases. Both of these processes depend on cAMP signaling and gene transcription. The increased cAMP signal is hypothesized to arise from CaM-stimulated AC (Wang and Storm, 2003).

AC3 has been identified to be critical for detection of odorants in the main olfactory epithelium (Wong et al., 2000). The mechanism recruits inhibition of AC3 by Ca^{2+}/CaM kinase II, which in turn causes cAMP decreases associated with odorant-stimulated cAMP transients (Wei et al., 1998). Therefore, AC3 is ideally suited to provide a mechanism that contributes to rapid decline in intracellular cAMP.

PDE is another effector on the cAMP signaling pathway (the intracellular US pathway), which is also acted on by intracellular Ca^{2+} (the intracellular CS pathway). PDE is the enzyme that controls the intracellular concentrations of cyclic nucleotides by catalyzing their hydrolysis (Beavo et al., 1994). Several PDE isoforms have been identified. There are Ca^{2+} -dependent isoforms, e.g. PDE1, and Ca^{2+}-independent isoforms, e.g. PDE4. Each PDE1 enzyme has two CaM-binding domains, which allow their activity to be stimulated by Ca^{2+} (Rybalkin and Beavo, 1996). Various PDE1 isoforms respond to Ca^{2+}

stimulation differently. In addition, they show different sensitivities to the CaM-dependent phosphodiesterase inhibitors (Yan et al., 1996).

PDEs are targets of complex regulation by various intracellular signaling pathways (Beavo, 1995; Conti, 2000; Houslay and Milligan, 1997). In regulating cardiac adrenergic responses, the increase in Ca^{2+} triggered by the α-adrenoceptor activation attenuates cAMP increase levels by activating PDE1 (Houslay and Milligan, 1997). Modulation of cAMP hydrolysis by Ca^{2+} and protein phosphorylation is important in the generation of second messenger signals in AtT20 cells (Ang and Antoni, 2002). [The pituitary corticotroph tumour cell line AtT20 has been extensively studied as a model system for the mechanism of action of neuropeptides (Antoni, 1996; Antoni, 2000; Axelrod and Reisine, 1984). PDE1, and PDE4 enzymes are the main cAMP hydrolyzing PDEs in AtT20 cells (Ang and Antoni, 2002).]

The cAMP pathway provides feedback influences on both Ca^{2+} channels and Ca^{2+} pumps to regulate the intracellular Ca^{2+} level. cAMP can act directly on the cyclic nucleotide-gated (CNG) channels to regulate Ca^{2+} entry. CNG channels were first identified in retinal photoreceptors and OSNs. Although their activity shows very little voltage dependence, they are classified in the voltage-gated ion channels superfamily (Kaupp and Seifert, 2002).

The cyclic nucleotides, cAMP and cGMP, directly activate CNG channels by binding to a site on the channel protein. The channel activation depends on the ligand concentration. All CNG channels respond to some extent to both cAMP and cGMP (Kaupp et al., 1989). In hair cells, specific activation by cAMP and not by cGMP has been reported (Kolesnikov et al., 1991). CNG channels are nonselective cation channels. They can not discriminate well between alkali ions and allow the pass of divalent cations evenly, in particular Ca^{2+} (Finn et al., 1997; Hackos and Korenbrot, 1997; Picones and Korenbrot, 1995). Unlike ligand-gated neurotransmitter receptors, CNG channels do not desensitize in the continuous presence of the ligand. But, their activity is modulated by the Ca^{2+}-binding protein calmodulin and by phosphorylation (Gordon et al., 1992).

As a second messenger, cAMP can act on channels located throughout the cell through the cAMP/PKA pathway. Target channels can be located on the cell soma, dendrites, axons, and even presynaptic terminals. Therefore, different types of channels can be affected, including resting channels, voltage-gated channels, that form the action potential and provide Ca^{2+} influx for neurotransmitter release, and ligand-gated channels, such as the NMDA receptor which is permeable to Ca^{2+} when opened by ligand binding (Kandel et al., 2000). As a result, Ca^{2+} enters through a variety of pathways, many of which can be regulated by the cAMP pathway.

The internal Ca^{2+} stores are in the smooth endoplasmic reticulum. The release into the cytoplasm of the high concentration of Ca^{2+} in the lumen and is controlled by a Ca^{2+}

channel in the membrane (Kandel et al., 2000). That Ca^{2+} channel is a large protein that contains a receptor on the cytoplasmic surface for inositol 1,4,5-trisphosphate (IP3). Binding of IP3 to that receptor leads to opening the Ca^{2+} channel and releasing Ca^{2+} into the cytoplasm (Kandel et al., 2000). IP_3 is a second messenger on the inositol-lipid pathway. When binding of transmitter to a receptor activates the G protein, the G protein, in turn, activates phospholipase C (PLC). This phospholipase cleaves phosphatidylinositol 1,4,5-bisphosphate (PIP_2) into two second messengers, IP3 and diacylglycerol (DAG; Kandel et al., 2000). cAMP can exert its influence on Ca^{2+} release through phosphorylation of IP3 receptors by PKA to change their characteristics. Phosphorylation by PKA has been identified for several types of IP3 receptors (Mignery et al., 1990; Sudhof et al., 1991). Phosphorylation of purified type I IP3 receptor from mouse cerebellum increases Ca^{2+} flux in reconstituted lipid vesicles (Nakade et al., 1994). In permeabilized hepatocytes Ca^{2+} release is enhanced after the addition of catalytic subunits of PKA (Bird et al., 1993).

Plasma membrane Ca^{2+} -ATPase is important because it removes Ca^{2+} from the cytosol to maintain resting Ca^{2+} concentrations in the cytosol (Haynes, 1993). Sarco-endoplasmic reticulum Ca^{2+} -ATPase performs the essential function of removing Ca^{2+} from the cytosol to the smooth reticulum (Pogwizd et al., 2001). Both are key points for the regulation of Ca^{2+} metabolism, Ca^{2+} extrusion and Ca^{2+} sequestration, respectively. The activity of Ca^{2+} -ATPase has been shown to be influenced by several mechanisms, including Ca^{2+}/CaM, PKA and PKC (Carafoli, 1994). cAMP was indirectly observed to

increase Ca^{2+} extrusion in platelets (Johansson et al., 1992). Therefore, cAMP could regulate Ca^{2+} metabolism by acting on these Ca^{2+} pumps.

3.2 Evidence for intracellular Ca^{2+} oscillations

Wayman et al., (1995) reported that 4 minute oscillations of cAMP and intracellular Ca^{2+} occur in Human Embryonic Kidney-293 (HEK-293) cells, when the cells are exposed to isoproterenol. Incubation of HEK-293 cells expressing AC3 with isoproterenol causes Ca^{2+} oscillations. These oscillations were not dependent on cAMP increases, because dibutyryl cAMP or (S)-cAMP was not able to stimulate them (Wayman et al., 1995). On the contrary, they were dependent upon the interaction between cAMP and Ca^{2+}/calmodulin-dependent intracellular cascades. Among these interactions, AC3 is a key factor, which is stimulated by hormones and inhibited by elevated Ca^{2+}.

Wayman et al., (1995) and others (Cooper et al., 1995; Rapp and Berridge, 1977) have proposed a mechanism for these hormone-stimulated cAMP and Ca^{2+} oscillations in AC3 cells. When isoproterenol activates AC3, AC3 catalyzes ATP to produce cAMP. In turn, cAMP stimulates PKA, which phosphorylates and activates IP_3 receptors, inducing internal stored free Ca^{2+} to release to the cytoplasm. As cytoplasmic Ca^{2+} rises, AC3 activity is inhibited and the production of cAMP decreases. Together with degradation of cAMP by phosphodiesterases, intracellular cAMP levels decrease. When cAMP levels

drops below a threshold point, the IP_3 receptors are inactivated and endoplasmic reticulum Ca^{2+} ATPase pumps remove cytoplasmic Ca^{2+} to re-establish an equilibrium state. If AC3 is continuously exposed to an activator such as isoproterenol, this cycle repeats.

Ca^{2+} oscillations with a similar time course have also been reported for Kenyon cells in the MBs of *Drosophila* by Rosay et al., (2001). By expressing a transgene of Ca^{2+} sensitive luminescent protein, they showed Kenyon cells had an intrinsic intracellular Ca^{2+} oscillation with an average period of five minutes. These Ca^{2+} oscillations can be strongly modulated by pharmacological agents either in amplitude and/or frequency. Both verapamil and diltiazem, that can block vertebrate L-type Ca^{2+} channels, reduce oscillation amplitude (Gielow et al., 1995; Gu and Singh, 1995). Neither of them has an effect on oscillation frequency (Rosay et al., 2001). Tetrodotoxin (TTX), a potent blocker of voltage-gated Na^{+} channels (O'Dowd and Aldrich, 1988), inhibits oscillations in a concentration-dependent manner (Rosay et al., 2001). An inhibitor of fast transient voltage-gated potassium channels 4-aminopyridine (4-AP) in cultured *Drosophila* larval CNS neurons (Solc and Aldrich, 1988), reduces Ca^{2+} oscillation amplitude and frequecy in a dose-dependent manner (Rosay et al., 2001). Kenyon cells express acetylcholine (ACh) sensitive receptors (Bertrand et al., 1994; Buckingham et al., 1997). ACh reduced oscillation amplitude in a dose-dependent and reversible manner (Rosay et al., 2001). Kenyon cell dendrites also receive GABAergic input (Yamazaki et al., 1998). GABAergic input increases both the amplitude and period of Ca^{2+} oscillations (Rosay et

al., 2001). Thus, Ca^{2+} oscillations can also be mediated through the plasma membrane, to be co-acting with electrical activity, and to be affected by excitatory and inhibitory inputs.

The amplitude of Kenyon cell Ca^{2+} oscillations is reduced in the mutant *amn*. Mutations of *amn* impair olfactory consolidation in *Drosophila* (Feany and Quinn, 1995; Quinn et al., 1979). This information and the following 3 findings imply that the Ca^{2+} oscillations have a role in learning and memory: 1) the mushroom bodies are key structures in this insect brain for associative learning, especially when the learning is aroused by olfactory cues. Kenyon cells are the intrinsic neurons in the MBs. 2) Memory consolidation in the honeybee appears to be particularly sensitive to interference within a 3-5 minute window after a conditioning trial, i.e., intertrial intervals of 1 or 20 minutes result in stable long-term retention, whereas 3 minutes intervals result in reduced retention (Gerber et al., 1998). 3) A spaced training procedure in which the CS and the US are repeatedly paired at intervals of up to 15 minutes induces long-term olfactory memory in *Drosophila* (Tully et al., 1994; Yin et al., 1994). Thus, Ca^{2+} oscillations act upon both the capability and specificity of gene expression (Dolmetsch et al., 1998; Li et al., 1998). Rosay et al., (2001) proposed that Ca^{2+} oscillations in the *Drosophila* MBs have a role in affecting the gene expressions that will induce synaptic modifications relevant to memory consolidation.

3.3 Interactions of cAMP transients and spontaneous Ca^{2+} spikes

Gorbunova and Spitzer, (2002) showed that there were interactions between cAMP transients and spontaneous Ca^{2+} spikes in *Xenopus* spinal neurons. They found spontaneous Ca^{2+} spikes produce increases in the concentration of cAMP and changes in cAMP level modulate Ca^{2+} spike frequency. In turn, Ca^{2+} spikes are necessary to induce changes in cAMP levels. Elimination of natural Ca^{2+} spikes by the removal of extracellular Ca^{2+} or by the addition of Ca^{2+} and Na^{+} channel blockers in the presence of Ca^{2+}, blocked production of cAMP transient spikes, although AC remained active. Further, Gorbunova and Spitzer, (2002) demonstrated that the pattern of Ca^{2+} spikes strongly influenced the amplitude and duration of cAMP responses. That suggests that specific patterns of Ca^{2+} spikes are selectively required by cAMP transients, whereas other patterns are not.

They proposed a model to describe the reciprocal influence between Ca^{2+} and cAMP. In their model they assumed that (1) Ca^{2+} oscillations are generated by IP_3-receptor-activated Ca^{2+} release from internal stores and Ca^{2+} extrusion–sequestration by plasma membrane and intracellular Ca^{2+} pumps, respectively, (2) cAMP is generated by a Ca^{2+}-activated AC and (3) cAMP is degraded by cAMP-activated PDE (Gorbunova and Spitzer, 2002). This model produces spontaneous patterns of Ca^{2+} spike and cAMP oscillations at about 5-10 minutes that closely resemble those observed experimentally.

First, cAMP transients are generated by Ca^{2+} increases and terminated by self-activated inhibition. Second, wavelet analysis of Ca^{2+} transients showed frequencies produced by the model were similar to those from the experimental records. Third, increases of cAMP increased the frequency of Ca^{2+} transients. Fourth, different Ca^{2+} spike patterns are differentially effective in stimulating increases of cAMP. Fifth, selective increases of cAMP appear to result from the match of AC and PDE with the time course of Ca^{2+} oscillations. Elimination of any of the feedback loops abolishes the patterns of transients generated by the model (Gorbunova and Spitzer, 2002).

4 Conclusion and future directions

Gorbunova & Spitzer's model suggests a way for me to interpret the cAMP oscillations I observed in the early olfactory preference learning model. As mentioned before, the CS and US pathways in mitral cells were proposed to be the odor associated Ca^{2+} signals and NE associated cAMP signals, respectively. I further hypothesize intracellular feedback between these two pathways. Odor (CS) activates the NMDA receptor and induces Ca^{2+} entry. Increased Ca^{2+} initiates the feedback loop. Initially a positive feedbacks occurs: 1) Increased Ca^{2+} activates AC to generate cAMP. 2) Activation of β-adrenoceptor enhances the AC activation. 3) Increased cAMP activates PKA and, in turn, PKA activates IP3 receptors on smooth endoplasmic reticulum to induce Ca^{2+} release from Ca^{2+} internal stores. This is followed by negative feedback: 1) Activated PKA may activate Ca^{2+}

pumps on either plasmic membrane or endoplasmic reticulum to increase Ca^{2+} extrusion and sequestration, to decrease Ca^{2+} in cytosol. 2) Increased Ca^{2+} activates PDE to degrade cAMP. 3) Increased Ca^{2+} inhibits AC3 to decrease cAMP generation, completing one cycle of cAMP oscillation.

From these feedback loops, we can see that if cAMP production is too strong, which may occur with higher doses of isoproterenol (e.g. 6 mg/kg), the oscillatory interaction with Ca^{2+} may be suppressed. Similarly if cAMP activation is too weak, as seen with the suboptimal dose of isoproterenol, or with saline, the necessary levels of peaks and troughs may not occur. This argues that receptor recruitment of G protein coupled AC is an important component in associative learning. The NMDA receptor that is activated by olfactory input provides an opportunity to activate AC, but odor alone is insufficient to trigger associative learning. As seen in the present experiments, weak oscillations occur in the odor plus saline condition, but the effect was not significant and, directly out of phase with the CS-US combination that produces cAMP oscillations.

cAMP oscillations can provide an explanation for the inverted U curve of US signaling relative to learning. As mentioned in the Introduction, there is an inverted U curve effect between US level and learning. Only the 2 mg/kg dose of isoproterenol plus odor produces learning. When either a suboptimal dose (1 mg/kg) or a supraoptimal dose (4 mg/kg or 6 mg/kg) of isoproterenol is employed as the US, no learning occurs (Langdon et al., 1997; Yuan et al., 2000). Also, when an optimal dose of isoproterenol is used

together with an optimal stroking, the US becomes ineffective (Sullivan et al., 1991). Alternatively, a suboptimal dose of isoproterenol and a weak stroking synergized to promote learning whereas neither alone could (Sullivan et al., 1991). Thus, there appears to be a narrow window for US activation to induce learning.

An inverted U curve effect also exists between the isoproterenol dose and CREB phosphorylation. Only the 2 mg/kg dose of isoproterenol plus odor, the learning condition, increased pCREB significantly 10 minutes after training finished. Neither saline plus odor, nor the 6 mg/kg dose of isoproterenol plus odor could increase pCREB after training (Yuan et al., 2000). Thus, there is a relatively narrow window for US activation to increase CREB phosphorylation.

According to the intracellular model proposed in Yuan et al., (2003b), the US mediated by β-adrenoceptors on the mitral cell in the olfactory bulb activates CREB phosphorylation through the cAMP-PKA pathway. The US activation level influences cAMP. In turn, the cAMP influences pCREB. Thus, relatively narrow and specific windows exist for the intracellular US signaling pathway (the cAMP/PKA/CREB pathway) to produce learning. The oscillatory pattern of cAMP expression seen here may provide an explanation for this narrow and specific signaling window.

Finally, cAMP levels acting in a frequency-encoding mode, in addition to the familiar amplitude-encoded mode, offers several advantages: large energetically wasteful

transitions in signals are spared; insignificant fluctuations in signals can be ignored; cooperatively effectors only respond to peak concentrations; desensitization to a signal can be avoided and diffusion of the signal within the cell can be controlled. Thus, the signaling capability of cAMP is greatly increased (Cooper et al., 1995). Taken together, the present results, which show that a learning dose of isoproterenol plus odor produces peaks and troughs of cAMP occurring at 5 minute intervals; that a higher and nonlearning dose of isoproterenol plus odor produces only a linear increase in cAMP; that the oscillatory pattern only occurs when the CS and the US are paired, suggest that an oscillatory pattern of cAMP is associated with the induction of cAMP-dependent classically conditioned odor preference learning in the rat pup.

Of course, it remains to be seen whether these observations reflect a fundamental temporal property of intracellular cAMP signaling that optimally drives all types of learning. Doing more experiments with more time points may help to determine whether the weak oscillations in saline plus odor group were only due to random variation of data or whether they were caused by the odor. If odor itself does induce cAMP oscillations, it is possible that there is phase shift in the saline plus odor group, compared to the 2 mg/kg isoproterenol plus odor group. 2 mg/kg isoproterenol plus odor may increase cAMP sooner than odor alone and, in turn, induce cAMP decrease earlier than odor alone. However, doing more experiments was not feasible in the context of this thesis. In the future, it will be of interest to test the universality of cAMP oscillations in other learning procedures or models. Other techniques, such as immunocytochemistry will reveal

whether there is localization of cAMP oscillatory patterns in odor-encoding mitral cells, or fluorescence resonance energy transfer will show directly cAMP temporal pattern in vivo. On the other hand, Ca^{2+} oscillations as proposed by the interaction models are clearly predicted and should be investigated as a strong test of the machinery of the cellular model which mediates the intracellular CS-US signaling pairing in mitral cells in rat pup odor preference learning. Optical imaging could permit a direct examination of Ca^{2+} patterns. Thus, by measuring both cAMP and Ca^{2+} oscillations in odorant-responding mitral cells involved in CS odor encoding and in mitral cells responding to a control odor may allow us to identify the temporal patterns associated with learning in further detail and to localize their occurrence to specific cells involved in learning and memory.

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