Olfactory learning in the rat pup: A model that may permit visualization of a mammalian memory trace

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Over the past 10 years considerable insight into intracellular interactions leading to long-term memory formation have been gleaned from various neural circuits within invertebrate and vertebrate species. This review suggests that, while certain intracellular signaling pathways are commonly involved across species, it is important to analyze specific neural systems because critical differences among systems appear to exist. The olfactory bulb has been used by our group to estimate the influence of neuromodulatory systems (serotonin and norepinephrine) on intracellular processes leading to learning. We describe here how activation of noradrenergic input to mitral cells increases cAMP leading to CREB phosphorylation when paired with a conditioning stimulus, odor. CREB phosphorylation is causal in odor preference learning leading to long-term memory for the odor. However, the relationship between cAMP activation and CREB phosphorylation is not straightforward; overstimulation of cAMP pathways impedes learning and prevents CREB phosphorylation. Excessive CREB phosphorylation also interferes with learning.

Key words: CREB; Learning model; Neonatal rat; Norepinephrine; Olfaction; Second messengers

INTRODUCTION

In recent years, the mechanisms of learning at the cellular level have been increasingly elucidated, especially in invertebrates where simpler circuitry and ease of cellular manipulations facilitate such understanding. Studies in Aplysia and Drosophila led to the discovery of a pivotal role for phosphorylation of the transcription element cAMP response element binding protein (pCREB) in long-term memory formation [1,2]. pCREB has, thus, been proposed as a universal memory molecule [3] since it has been shown to be causal in many memory formation scenarios and because many genes activated by pCREB result in intracellular structural changes leading to memory.

Much of this review will focus on the intercellular and intracellular mechanisms that we propose facilitate learning in the olfactory system of the rat pup (Fig. 1). Many of the proposed intracellular cascades are based on work by others in various learning systems.

VARIOUS LEARNING MODELS

A detailed analysis of intracellular events relating changes in behavior to the strengthening of a sensory-motor synaptic connection has been undertaken in Aplysia. In that model the nature of the sensory representation and the cells mediating it have been identified; cAMP increases elicited by an unconditioned stimulus event mediated by serotonin modifies the synaptic strength of sensory neurons sensitization. A number of critical intracellular signaling pathways have been identified in this Aplysia model [4–9]. The critical role of 5-HT activation of cAMP pathways in the signaling leading to CREB phosphorylation and to conditioned learning in this organism has been well documented [2,5,10]. Studies in Drosophila have also shown the importance of CREB phosphorylation in memory formation [11] and even memory enhancement [12]. In another invertebrate, the honeybee, cAMP cascades have also been shown to be important for long-term memory formation [13].

The ability to translate these findings in invertebrates to mammalian systems is again facilitated by the study of relatively simple systems. Various mammalian models have been explored. For example, the conditioned eye blink response in rabbits has been useful in studying cerebellar-mediated learning [14,15] as well as hippocampal function [16,17]. Other mammalian models include fear conditioning [18–22] and taste aversion [23–26]. Long term potentiation, both NMDA-dependent and non-NMDA-dependent, has been used as a model for memory formation for many years. These represent only a sampling of the models available for studying learning in mammals. But one drawback of many of the mammalian models is an inability to characterize the representation being learned at the cellular level. Unlike the situation with Aplysia where the sensory representation can be identified, in most mammalian models the networks
mediating the learned representation are either diffuse or not identified.

EARLY ODOR PREFERENCE LEARNING AS A MAMMALIAN MODEL

We have utilized the olfactory bulb of the neonate rat during the first week of life as a means of understanding mammalian learning. The neuroanatomical constituents are relatively few (Fig. 1a) compared to other mammalian cortices and the learning is potent and biologically relevant. The ability of young animals to learn to prefer odors via a conditioned response to an odor stimulus has a fundamental survival consequence for rat pups because pups rely substantially on olfaction to recognize the food source, the dam, at birth. Humans also use olfaction at birth and can show conditioned preference for a smell at birth [27–29]. Some aspects of the conditioned olfactory preferences observed in neonate rats can be long lasting and changes in olfactory behavioral responses to these odors can be seen even into adulthood following preference training in the neonate rat [30–33].

During the early postnatal period, pairing an odor (using an odorant the pup does not normally encounter which provides the conditioned stimulus) with a tactile stimulus like stroking (termed the unconditioned stimulus) leads to a clear preference for the odor when the pup is given an odor preference test several hours or a day later [34,35]. Conditioning stimulus and unconditioned stimulus pairings can also occur in utero. The conditioned learning effect from such pairings may be seen postnatally; for example, maternal ethanol intoxication paired with a novel odor in utero, can produce a preference for the odor (and ethanol) after birth [36]. Exposure of fetal rats to opioids also induces a conditioned preference to odors paired with the drug [37]. The importance of such observations may lie in the potential of prenatal or perinatal environmental factors (alcohol, nicotine, chemicals in the environment) to have long-term behavioral effects on the animal.

It is of consequence to the rat pup that olfactory preference learning occurs during a critical period whether the unconditioned stimulus paired with odor is prima facie painful or pleasurable, up to around postnatal day 10 [38–42]. This response may relate to survival requirements: pups are blind, relatively immobile and do not regulate body temperature well during this period. Thus, they need the mother, which normally provides both the conditioned and unconditioned stimulus, in nature, whether the mother is abusive (e.g., bites the pups) or not [40,41]. After a certain period, around postnatal day 10, a ‘pleasurable’ unconditioned stimulus shows less ability to induce preference learning [43] while a painful unconditioned stimulus now produces an odor aversion response [38,39]. The switch is likely due to developmental changes in the amygdala.

Fig. 1. Working model of intracellular pathways involved in learning in the olfactory bulb mitral cells. This is a modification of a model presented previously [68]. For clarity, major cascades are shown while others, less significant to the proposed learning, are left out. The numbers indicate references to papers dealing with specific parts of the pathways either in the olfactory bulb, other parts of the brain or other species.

1. 5-HT + NE interact synergistically to affect cAMP levels [67,68].
2. cAMP increases via NE receptor coupled to Adenylyl cyclase [68,85–89].
3. Ca²⁺/calmodulin acts through adenylyl cyclase to increase cAMP [90–93].
4. Breakdown of cAMP by phosphodiesterase 4 [92,94–96].
5. Multiple pathways: Inhibitor 1, PPI dephosphorylates CaMKII, Ca²⁺/calmodulin activates calcineurin [9798].
6. PKA phosphorylates CREB at SER 133 [99,100].
7. PKA phosphorylates NMDA/AMPA [101–103].
8. Ca²⁺/calmodulin leads to CaMKII/IV to CREB phosphorylation at SER 133 [104].
9. Calcineurin dephosphorylates NMDA [105].
10. NT-R leads to MAPK and then to pCREB at SER 133 [106–108].
11. PKA phosphorylation of MAPK leads to pCREB [70,108,109].
and/or locus coeruleus [39,41,44] and correlates with the time period when the pups open their eyes, become mobile and thermo regulate. Nevertheless, the basic mechanisms involved in the memory formation appear to remain in the olfactory bulb, even in older pups, since learning can still be achieved by directly infusing noradrenergic receptor agonists in the bulbs of older (postnatal day 14) pups [44].

For our purposes a critical feature of olfactory preference learning is its dependence on the pairing of odor and unconditioned stimulus information in the olfactory bulb itself. This appetitive learning can be induced by localizing the unconditioned stimulus to the olfactory bulb [45–49]. Natural odor learning with stroking as the unconditioned stimulus is dependent upon noradrenergic input from the pontine locus coeruleus activating β-adrenoceptors in the bulb (Fig. 1); infusing a β-adrenoceptor agonist in the bulb can substitute for stroking. Thus activation of the cAMP-coupled β-adrenoreceptor is both necessary and sufficient to produce the odor preference learning [50,51].

The early postnatal time is a highly plastic period with tremendous growth in the bulb and other cortices. This fact may enhance our ability to produce and visualize changes relatively rapidly. However, controls, such as presenting backward pairing (stroking followed by odor), odor alone, or stroking alone serve to show that the learning is, indeed, associative in nature. The memories formed can be observed very quickly, within 1 h [42]. Such rapid memory formation may reflect phosphorylation events rather than structural changes in the bulb. On the other hand, the associative learning can produce structural changes. An interesting aspect of the learning is that a single trial does not appear to produce a permanent memory, possibly because of overwriting during developmental processes. Multiple trials can, apparently, produce permanent memory [31,53] and even contribute to growth of the olfactory bulb. The structural changes in the olfactory bulb observed following multiple conditioned trials include increased glomerular size and increased number of juxtaglomerular cells (neurons and/or glia) surrounding odor-activated glomeruli [52]. Thus, both labile and enduring changes can be examined in this system.

When aversive conditions are paired with odor, the olfactory bulb shows heightened neural activity in regions involved in the learning coding marked by 2-deoxyglucose activity [53] although there is some disagreement here [54]. As aversive learning begins to predominate in older animals (i.e. greater than postnatal day 10), other structures such as the amygdala become increasingly important in learning and/or memory related to odors [55,56]. That is not to say that the amygdala has no role in learning in younger pups; it may act to modulate the responses to the unconditioned stimulus [57].

**INTERCELLULAR MECHANISMS OF OLFACTORY LEARNING**

Despite the extensive behavioral and physiological observations concerning conditioned odor preference learning in the neonate, the mechanisms involved in mediating the learning at the cellular level are just beginning to be a focus of interest. For some time, there has been evidence to suggest that a critical site of circuit change in odor preference learning was the dendrodendritic mitral cell-granule cell-mitral cell synapse [49,58–61]. Disinhibition of granule cells by norepinephrine was thought to result in NMDA receptor activation on the granule cell side leading to strengthening of the mitral to granule cell connection and a concomitant increase in feedback inhibition. This is supported by the finding of a greater number of single cell inhibitory responses to a conditioned odor, peppermint, after learning [62].

While granule cell disinhibition, and a subsequent increase in inhibitory feedback, contribute to plasticity in this circuit, our data suggest that a major correlate of odor preference learning is an initial enhancement of the glutamatergic olfactory nerve synaptic input to the mitral cell (Fig. 1a), including both its NMDA and AMPA components [63]. Further we have demonstrated this enhanced synaptic input is likely to be an enduring feature of memory as an enhanced response to the conditioned odor, but not to a control odor, occurs 24 h after training [64].

**INTRACELLULAR MECHANISMS OF ODOR PREFERENCE LEARNING AND VISUALIZATION OF THE MEMORY**

Our focus on the mitral cell’s role in learning emerged from a consideration of the interaction between noradrenergic and serotonergic inputs to the olfactory bulb in odor preference learning. Serotonin depletion prevents odor learning [34], but this effect can be overcome with higher doses of isoproterenol, a β-adrenoceptor agonist [65]. Interestingly, higher doses of isoproterenol normally interrupt learning in normal rat pups. Isoproterenol exhibits an inverted U curve relationship to learning such that too high or too low a dose does not produce learning [51,65]. The serotonergic effect depends on input from the raphe nuclei which activates 5-HT2 receptors in the bulb [66]. While the 5-HT2A receptor does not directly engage the cAMP system believed to be critical to the unconditioned stimulus effect, in vitro data from rat neocortex showed 5-HT2A receptor activation enhances cAMP levels generated by isoproterenol activation of the β-adrenoceptor [67]. We assessed this possibility in the rat pup olfactory bulb and found, as predicted, that serotonin depletion reduces the level of cAMP produced by isoproterenol, although depletion does not, by itself, affect cAMP levels [68]. This study showed that cAMP changes in the olfactory bulb induced by isoproterenol were observed in mitral cells. We asked further if β1-adrenoceptor and 5-HT2A receptor subtypes were co-localized in the bulb and found them both on mitral cells [68]. Finally, our prior work demonstrated that mitral cell CREB phosphorylation in odor-specific areas accompanied learning in the odor preference model [48] and we now have evidence that the CREB pathway is causal in neonate odor learning [47]. We also have evidence that cAMP changes (located upstream of CREB as shown in Fig. 1b) related to learning are located in mitral cells in odor activated regions (unpublished observation). Others have shown CREB phosphorylation is also causal in odor aversion learning in older pups [69] and that the MAPK/ERK signaling pathway, which also leads to CREB phosphorylation, is required in long term aversive olfactory learning [70].

For odor preference learning in the neonate rat our data point to a new model (Fig. 1b) that highlights the noradrenergic-cAMP/PKA intracellular cascade in the mitral cell. A particularly exciting aspect of our findings is that the pCREB changes we observed, as was true for earlier
metabolic changes associated with rat pup odor learning [35,51,71], are localized to the quadrant of the olfactory bulb where the odor is represented [48]. Thus, in this model it may be possible to directly characterize the learned odor representation by taking advantage of optical imaging and other methods to highlight the population of cells involved in specific odor encoding and then to examine the changes in proteins and structure related to that population and representation in contrast to other populations and representations in the bulb. Such an approach has been attempted using an adult mouse olfactory learning paradigm but not significant structural changes were observed in mitral cell dendrites [72].

Effective control conditions are available when localization is possible. Even at a coarse level one olfactory bulb could act as a control for the other, using nasal occlusion or local transmitter depletions, to permit sensitive within-subject comparisons [68]. Other controls are possible because of the inverted U curve relationship between learning and pharmacological or natural unconditioned stimuli and the ability to shift that relationship with 5-HT depletion. Using subtly different learning conditions provides a range of controls for the exploration of proteomic and genomic changes critically related to learning. Finally, as noted above, since olfactory information is spatially coded in this simple structure the model provides a particular advantage for probing the localization of memory-associated change even within a bulb. We are currently examining phosphorylation of components shown in the proposed intracellular pathways (e.g. targets of PKA) using immunocytochemistry. These substrates alter their function when they become phosphorylated and this altered function may support short-term memory in invertebrate models. Thus, we hope to relate behavioral expression of short-term memory in the rat pup to phosphorylation sites, with a view to dissecting the nature and the location of phosphorylation events in short-term memory and to identifying likely targets for change in long-term memory.

**DOES THE RAT PUP OLFACTORY MODEL COMPARE WITH INVERTEBRATE LEARNING MODELS?**

With respect to invertebrate models and, particularly, to the *Aplysia* model, we find many similarities to that of our odor preference conditioning model. As originally suggested by Kandel’s group [73], norepinephrine appears to function in mammals as serotonin functions in *Aplysia*. However, important differences have also emerged. We have not found an additive effect of odor and the unconditioned stimulus on cAMP levels as described for *Aplysia* and, indeed, too much cAMP may be detrimental in our learning model. These observations suggest that a simple increase in cAMP levels above some threshold is unlikely to be the mediator of co-incident detection in the olfactory bulb as it appears to be in *Aplysia*. On the other hand, work in *Drosophila* suggests that a critical window of cAMP levels exists [74–77] so there may be similarities between the invertebrate model and the proposed model.

Finally, the *Aplysia* model highlighted in the past presynaptic facilitation in the sensory neuron as the primary change supporting sensitization or habituation. However, we believe postsynaptic changes will be more critical in the odor preference learning model since there is little evidence for a direct norepinephrine input to the olfactory receptor neurons. Recent work by Glanzman and colleagues, in fact, suggests that multineuronal mechanisms that involve presynaptic and postsynaptic changes are involved in classical conditioning in *Aplysia* [78,79] again highlighting similarities between pup odor learning and *Aplysia*. We have also found that too much, as well as too little, pCREB did not lead to learning [47], while in other models, both invertebrate [80] and mammalian [18], over-expression of pCREB has not been linked to memory failure.

**ADVANTAGES OF THE OLFACTORY LEARNING MODEL**

In mammalian learning models a variety of manipulations, such as enhancement or interference with glutamatergic [81] or GABAergic [82] pathways, can affect learning. Similar effects have been reported for odor preference learning in the neonate [49,58]. It is likely that other neuromodulators, as described here for 5-HT, will have critical supporting roles in memory in the olfactory bulb as well (dopamine, for example). To what extent the wide-spectrum of receptor-mediated effects will converge on common intracellular pathways is unknown. Most critically for the present review, β-adrenoceptors and/or cAMP coupled pathways have been implicated in mammalian learning models across species from mice to humans. An advantage of the olfactory bulb learning model over other mammalian models is that we believe learning representations can be localized to particular olfactory bulb cells. For example, studies of the hippocampus have been unable to specify the memory representation since, for the most part, individual brain structures are not well related to the encoding of critical information or are diffusely [83] or even questionably implicated [83,84] in learning and memory for the tasks under investigation.

In summary, the rat olfactory bulb offers a relatively simple mammalian structure to study the visualization of learning. Because the learning involved can be localized within odor specific areas of the bulb, this provides opportunities to study learning in a biologically relevant mammalian system. Odor preference learning provides a unique opportunity to improve our understanding of the cAMP/PKA cascade in a relatively simple circuit where neural change is both necessary and sufficient for learning and memory.

**ABBREVIATIONS**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>5-HT, 5-HT₂A-R, AMP, AC</td>
<td>5-hydroxytryptamine or serotonin, 5-hydroxytryptamine receptor subtype, adenosine monophosphate, adenylate cyclase</td>
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<td>AMPA, β₁-R, Ca²⁺</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-proprionic acid, β₁-adrenoceptor subtype, calcium</td>
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<td>CAMKII/IV, cAMP, CS, CRE</td>
<td>calcium calmodulin-dependent kinase II/IV, cyclic adenosine 3’,5’-monophosphate, conditioning stimulus, CREB response element</td>
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REFERENCES


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