Research

Mitral Cell β_1 and 5-HT_{2A} Receptor Colocalization and cAMP Coregulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb

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In the present study we assess a new model for classical conditioning of odor preference learning in rat pups. In preference learning β_1-adrenoceptors activated by the locus coeruleus mediate the unconditioned stimulus, whereas olfactory nerve input mediates the conditioned stimulus, odor. Serotonin (5-HT) depletion prevents odor learning, with 5-$HT_{2A/2C}$ agonists correcting the deficit. Our new model proposes that the interaction of noradrenergic and serotonergic input with odor occurs in the mitral cells of the olfactory bulb through activation of cyclic adenosine monophosphate (cAMP). Here, using selective antibodies and immunofluorescence examined with confocal microscopy, we demonstrate that β_1-adrenoceptors and 5-HT_{2A} receptors colocalize primarily on mitral cells. Using a cAMP assay and cAMP immunocytochemistry, we find that β-adrenoceptor activation by isoproterenol, at learning-effective and higher doses, significantly increases bulbar cAMP, as does stroking. As predicted by our model, the cAMP increases are localized to mitral cells. 5-HT depletion of the olfactory bulb does not affect basal levels of cAMP but prevents isoproterenol-induced cAMP elevation. These results support the model. We suggest the mitral-cell cAMP cascade converges with a Ca^{2+} pathway activated by odor to recruit CREB phosphorylation and memory-associated changes in the olfactory bulb. The dose-related increase in cAMP with isoproterenol implies a critical cAMP window because the highest dose of isoproterenol does not produce learning.

The olfactory bulb is an excellent preparation for demonstrating classical conditioning. Odor preferences can be produced in rat pups as young as 1 wk when an odor (conditioned stimulus, or CS) is paired with any of several unconditioned stimuli (UCS) including milk, stroking, or even mild footshock (Sullivan and Wilson 1994; Sullivan et al. 2000a). The learning is localized to the olfactory bulb (Sullivan et al. 2000b), and modifications of the electrical (Wilson et al. 1987; Wilson and Leon 1988) and metabolic (Sullivan and Leon 1987; Sullivan et al. 1991) activity of the olfactory bulb are observable after conditioning. Olfactory bulb norepinephrine (NE), acting through β-adrenoceptors, is both necessary and sufficient as a neural substrate for the UCS (Sullivan et al. 2000b).

Based on these data and others, Sullivan and Wilson (1994) suggested that learning results from the disinhibition of mitral cells, which permits activation of NMDA receptors and could promote long-term potentiation-like changes in the olfactory-granule-cell–mitral-cell connection. In this scenario, NE input from the locus coeruleus to the olfactory bulb acts as the UCS by inhibiting granule cell interneurons in the bulb through β-adrenoceptors to produce disinhibition.

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

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

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

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

In the present study, we pursue three lines of evidence in support of a direct action of NE on mitral cells as the neural substrate for early olfactory learning. First, selective antibodies for the β_1-adrenoceptors and the 5-HT_{2A} receptor are used to examine the localization and colocalization of these two receptors in the olfactory bulb. Second, the expression of cAMP following odor preference conditioning is examined with a cAMP assay. The dependence of β-adrenoceptor activation of cAMP signaling on normal levels of 5-HT input was also tested using 5-HT depletion. Third, the localization of cAMP increases associated with odor preference conditioning is examined immunocytochemically in the olfactory bulb.

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

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

RESULTS

Experiment 1: 5-HT_{2A} Receptor and β_1-Adrenoceptor Localization

Microwave Irradiation and β_1-Adrenoceptor Labeling

We observed substantially improved immunocytochemical labeling of β_1-adrenoceptors in olfactory bulb sections by using the microwave procedure described in Materials and Methods (Fig. 1, A vs. B). Strong immunocytochemical staining was observed in the mitral cells and tufted cells. Label was mainly confined to the cytoplasm of the somata. Fainter label was observed in periglomerular cells and small subsets of granule cells. Without microwave irradiation, only faint, punctate labeling of cells was observed (Fig. 1A). Thus, microwave treatment produced enhanced visualization of cells immunoreactive for β_1-adrenoceptors within the bulb. To control for possible nonspecific staining resulting from microwave irradiation, some sections were incubated without the presence of the primary antibody. This procedure served as a

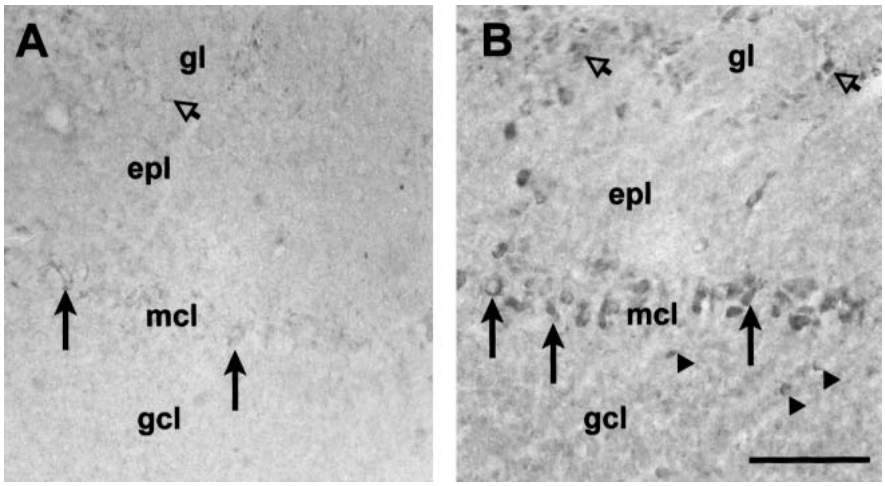

Figure 1 Localization of the β_1-adrenoceptor in the olfactory bulb by immunocytochemistry. (*A*) Visualization of the receptor without the use of microwave heating. (*B*) Visualization of the receptor after the use of microwave heating. Note the faint label of mitral cells in *A* and the clear labeling of mitral and tufted cells and a small number of granule and periglomerular cells in *B*. (epl) External plexiform layer; (gcl) granule cell layer; (gl) glomerular layer; (mcl) mitral cell layer; (solid arrows) mitral cells; (hollow arrows) periglomerular cells; (arrow heads) granule cells. Bar, 100 μm.

negative control for β_1-adrenoceptor immunocytochemistry and produced no cellular label in the olfactory bulb, although nonspecific label of fiber bundles within the deep granule cell layer was present (data not shown).

Immunofluorescence Double Label

To investigate the targets of NE and 5-HT action, immunofluorescence double labeling of β_1-adrenoceptor and 5-HT_{2A} receptors was performed. Consistent with previous studies (Pompeiano et al. 1994; McLean et al. 1995; Hamada et al. 1998; Cornea-Hébert et al. 1999), the mitral cell and external plexiform layers were intensely labeled by the 5-HT_{2A}-receptor antibody (Fig. 2D). Labeled tufted cells were also found in the main olfactory bulb. Figure 3 shows CY3 immunofluorescence label of mitral cells in a postnatal day 35 (PND35) rat. In a few cells, both cell bodies and their dendrites were clearly labeled for the 5-HT_{2A} receptor.

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

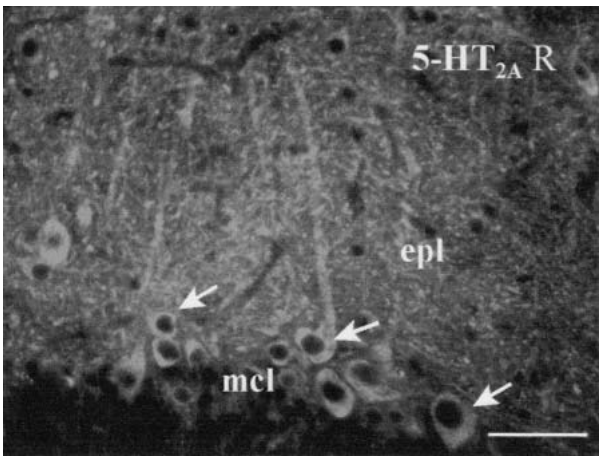

Figure 3 Immunofluorescence label of mitral cells in a PND35 rat using an antibody to the 5-HT_{2A} receptor. Note the mitral cell body (arrows) and dendritic label. Bar, 50 µm.

Experiments 2A and 2B: cAMP Expression Following Odor Preference Training

cAMP expression in the olfactory bulb is increased by effective odor preference training protocols (Fig. 4A). It is also increased by protocols that do not produce odor preference learning. The groups receiving 2 mg/kg isoproterenol and 4 mg/kg isoproterenol paired with odor had significantly more cAMP than the odor-only control group (Repeated measures ANOVA, $F_3 = 3.20$, $p < 0.05$; Least significant differences tests, $p < 0.05$). The 1 mg/kg isoproterenol group was intermediate. From the histogram (Fig. 4A) it also appears that isoproterenol increases cAMP in a dose-related manner.

Pups receiving stroking paired with odor also had significantly more cAMP than the naive control group ($p < 0.01$, paired $t_{1\text{-tailed}}$-test). Pups with stroking alone had the same mean cAMP levels as those with odor pairing and were also different from the naive control group ($p < 0.05$, paired $t_{1\text{-tailed}}$-test). The mean cAMP levels of the odor-only pups also did not differ from that of naive pups. This indicates that stroking, acting as the UCS, is sufficient to activate the cAMP cascade, but the CS (peppermint odor) appears to have no further influence on the level of cAMP expression during odor preference learning.

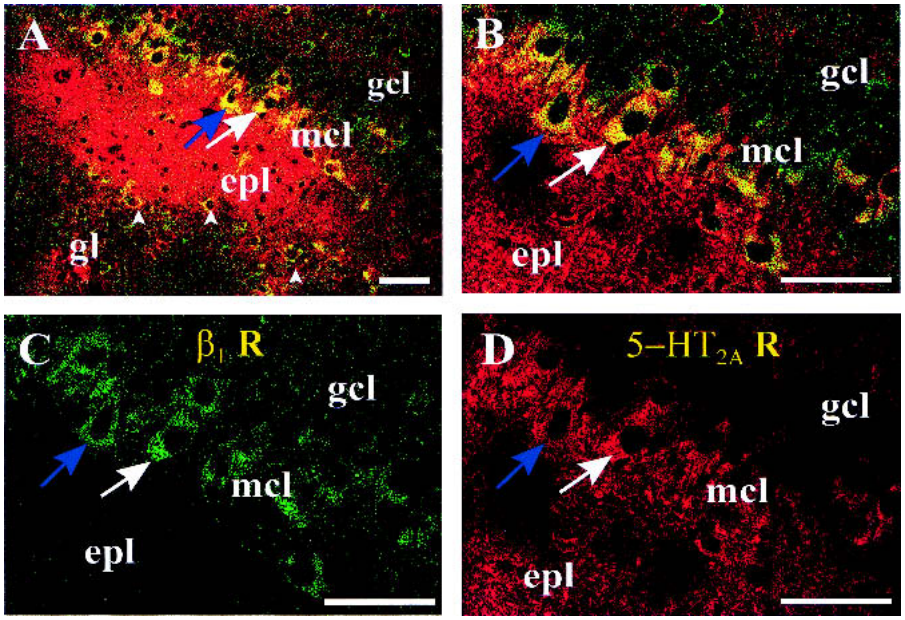

Figure 2 Confocal images of the olfactory bulb from a PND10 pup. Note the double-labeled mitral cells (e.g., at white and blue arrows) at low (*A*) and higher magnification (*B–D*). (*A,B*) The combined label of β_1-adrenoceptors and 5-HT_{2A} receptors. Double label was also observed in tufted cells (arrowheads) near the glomerular layer (*A*). Bars, 50 µm.

In 5-HT-depleted olfactory bulbs, the level of cAMP was significantly ($p < 0.05$, $t_{2\text{-tailed}}$-test) reduced compared with nondepleted sides in both the 2-mg/kg and 6-mg/kg isoproterenol groups (Fig. 4B). This indicates that 5-HT depletion reduces the ability of norepinephrine to activate cAMP during odor preference learning.

Experiments 3A and 3B: cAMP Immunocytochemistry Following Unilateral 5-HT Depletion and Isoproterenol Injection

Consistent with β_1-adrenoceptor and 5-HT_{2A} immunocytochemical localization, strong cAMP immunocytochemical staining was observed mainly in the mitral cells (Fig. 5) and tufted cells. Only a few periglomerular cells and granule cells were stained. This indicates that NE action through the β_1-adrenoceptor observed here increases cAMP mainly in the output cells of the olfactory bulb. To support our hypothesis that NE and 5-HT act synergistically to enhance cAMP signaling in the CREB phosphorylation pathway, quantitative analysis of the relative optical density of the medial regions of mitral cell layers was performed on both the 5-HT-depleted bulbs and the control sides. Figure 6A shows that, after the pairing of isoproterenol and odor exposure, in 5-HT-depleted olfactory bulbs there was significantly ($p < 0.01$, paired $t_{2\text{-tailed}}$-test, $n = 7$) less cAMP staining in the mitral cell layer (reflected by relative optical density in medial regions) compared with that in the control side of the same animals. To determine if unilateral 5-HT depletion reduces the basal level of cAMP expression in mitral cells, cAMP immunocytochemistry was also performed on the olfactory bulbs of non-isoproterenol-injected pups. Figure 6B shows that there is no significant difference in the relative optical density of cAMP immunocytochemical staining in mitral cells in the 5-HT-depleted sides and the control sides. Comparisons were only made within animals to avoid variability arising from differences in the overall immunocytochemical reaction.

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

DISCUSSION

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

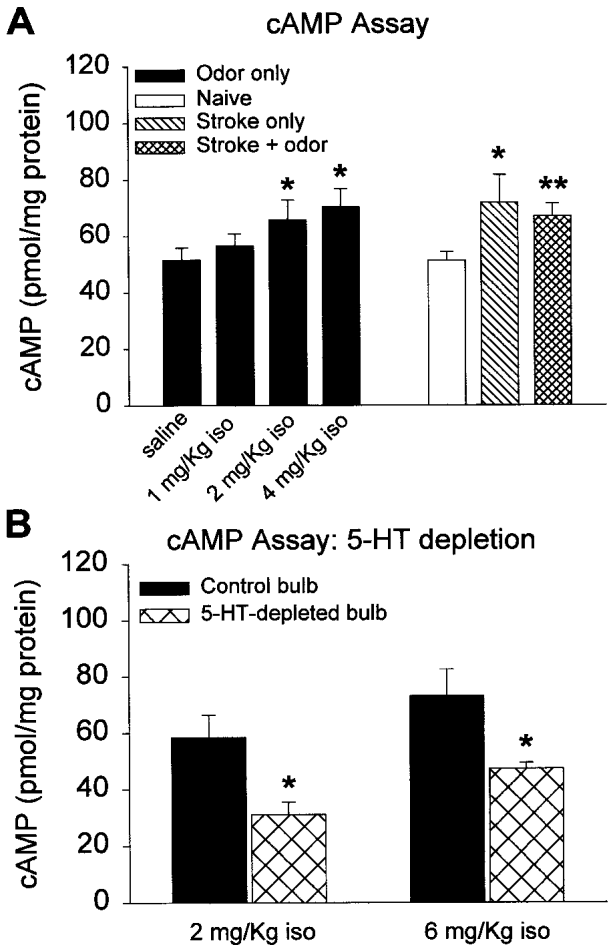

Figure 4 cAMP expression in the olfactory bulb of PND6 pups immediately after various training sessions. (*A*) An increase in cAMP expression is observed with increasing β-adrenoceptor activation (isoproterenol) compared with saline-injected controls exposed to odor only at the time of training ($N = 9$; *: $p < 0.05$ compared with saline group). The act of stroking the pup appears to increase cAMP to levels equivalent to pups given stroking plus odor ($N = 9$; *: $p < 0.05$; **: $p < 0.01$). (*B*) 5-HT depletion reduces the isoproterenol-induced cAMP expression, which can be partially overcome by inducing more activation of β-adrenoceptors (via isoproterenol). *: $p < 0.05$; 5-HT-depleted bulb compared with normal control bulb within subjects. $N = 4$ for 6-mg/kg iso control group; $N = 5$ for the other three groups. (iso) Isoproterenol.

Cellular Localization of the β_1-adrenoceptor and the 5-HT_{2A} Receptor

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

With the increased sensitivity of the present heat-induced antigen retrieval method for immunocytochemistry, we are the first to report substantial neuronal localization of the β_1-adrenoceptors in the olfactory bulb output cells. The

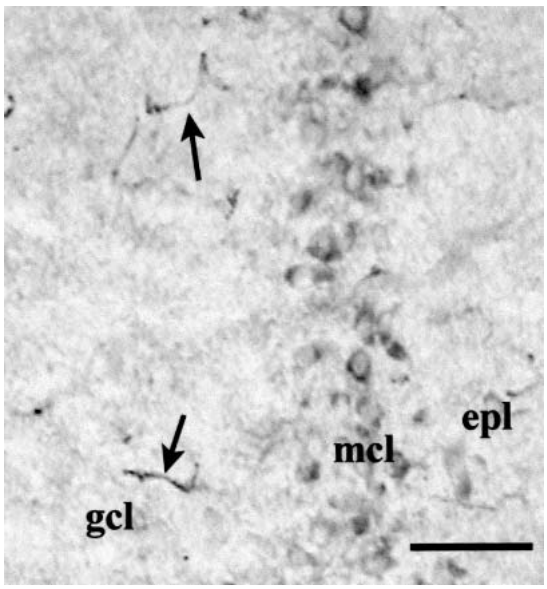

Figure 5 Immunocytochemistry shows the mitral cell location of cAMP in the olfactory bulb. The immunocytochemical methods used here showed selective cAMP expression in mitral cells of the mitral cell layer. Arrows indicate artifact labeling of blood vessels. (epl) External plexiform layer; (gcl) granule cell layer; (mcl) mitral cell layer. Bar, 50 μm.

finding that the receptor is localized primarily in the output cells of the bulb (mitral and tufted) and colocalized with 5-HT_{2A} receptors is consistent with the demonstrated functional interaction of these receptors in the olfactory bulb of the neonate rat and supports the present model of critical neural substrates.

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

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

The reasons for the failure of the binding studies to identify mitral cells as important sites of β_1-adrenoceptors are unclear. As noted by Woo and Leon (1995), because iodopindolol is biased toward the detection of β_2-adrenoceptors, even examining radiolabeling in the presence of a β_2-adrenoceptor antagonist may not be sufficient to eliminate some binding of β_2-adrenoceptors, which are numerous in the olfactory bulb. The profiles of the two receptors seen in the binding studies were identical except for additional external plexiform labeling for β_2-adrenoceptors. Mitral cells also have prominent internalized β_1-adrenoceptor labeling in the present study. Internalized receptors have low binding affinity (Flugge et al. 1997), and their demonstration may depend critically on ligand concentration. Finally, Leon's laboratory, in a brief report (Ivins et al. 1993) using in situ hybridization methodology, identified β_1-adrenoceptor mRNA in mitral cells, consistent with the present observations.

Isoproterenol interacts with β_2-, as well as β_1-adrenoceptors, in the olfactory bulb. The present study does not rule out a role for the β_2-adrenoceptors in olfactory preference learning. In situ hybridization study demonstrates that β_2-adrenoceptors are more widely expressed than β_1-adrenoceptors in the olfactory bulb, being identified primarily in the granule cell and glomerular layers (Nicholas et al. 1993). Their specific contribution to early olfactory learning remains to be identified.

Functional Significance of cAMP Activation via β_1-Adrenoceptors and 5-HT_{2A} Receptors in Output Cells of the Olfactory Bulb

cAMP increases accompanied both odor paired with stroking and odor paired with 2 mg/kg isoproterenol, the two effective learning conditions, as predicted by the present hypothesis. However, odor + stroking did not produce increases greater than those of stroking alone, although the odor + stroking group was less variable. In contrast to the present failure to detect an additional odor-induced increase in cAMP with odor pairing, the studies of pCREB using Western blots showed higher levels of pCREB in the odor + stroking than the stroking-only condition (McLean et al. 1999). With 10% peppermint as the odorant, cells involved in the odor representation appear widespread and were sufficient to demonstrate the effect of pairing on pCREB. Thus, we would have expected sufficient sensitivity in the present design. If cAMP is not increased by odor pairing, it suggests that, unlike the *Aplysia* model in which sensory input and a monoaminergic input converge on adenylyl cyclase activation, in the rat pup olfactory bulb the sensory input influences learning by convergence on the pCREB pathway (see Fig. 7).

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

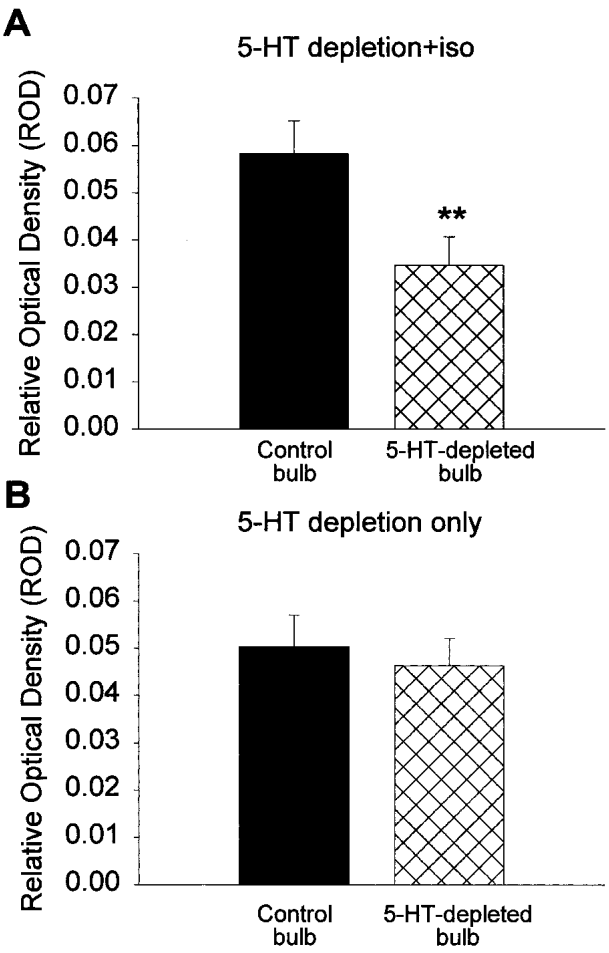

Figure 6 Relative optical density (ROD) measures of cAMP showing the influence of isoproterenol (iso) and/or unilateral bulbar 5-HT depletion on cAMP expression in the mitral cell layer. (*A*) ROD of cAMP immunocytochemical staining in the 5-HT-depleted olfactory bulbs and the control bulbs of the same animals after pairing of 2-mg/kg isoproterenol injections with odor exposure. Significantly less cAMP level is seen in 5-HT-depleted olfactory bulbs compared with the control sides ($N = 7$, $p < 0.01$). (*B*) ROD of cAMP immunocytochemical staining in the 5-HT-depleted olfactory bulbs and the control bulbs of the same animals without isoproterenol injections or odor exposure. Note that there is no difference between these two groups ($N = 5$, $p > 0.05$), which indicates that 5-HT depletion by itself does not affect basal cAMP levels.

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

The dose-dependent increase in cAMP with increasing isoproterenol did not support the initial hypothesis that biphasic agonist control of cAMP explains the inverted U-curve seen behaviorally, electrophysiologically, and biochemically. Although biphasic cAMP control of behavior has been reported in other models (Ozacmak et al. 2002), the present data indicate instead that there is an optimal level of cAMP activation, which can be exceeded. In the present model three possibilities suggest themselves: (1) Higher levels of cAMP recruit increased calcium entry, which might favor calcineurin-induced dephosphorylation; (2) higher levels of cAMP promote greater phosphodiesterase 4 (PDE4) activation through protein kinase A (PKA; Ang and Antoni 2002), and this may critically shorten the duration of the cAMP signal; and (3) elevated cAMP levels promote faster cAMP extrusion (Wiemer et al. 1982), which, again, would shorten the signal duration. CREB phosphorylation has been shown to be enhanced by longer durations of cAMP signaling (Barad et al. 1998). Manipulation of calcineurin and measurements of the time course of cAMP elevation with varying doses of isoproterenol would test these hypotheses.

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

The New Model of Noradrenergic-Mediated Early Olfactory Preference Learning in the Rat Pup

In the present study, we present the hypothesis that mitral cells, the main output cells in the olfactory bulb, are the postsynaptic cellular substrate for olfactory preference learning. Immunocytochemistry demonstrated the colocal-

ization of β_1-adrenoceptors and 5-HT$_{2A}$ receptors in mitral cells. Manipulation of β-adrenoceptor activation and depletion of 5-HT affected cAMP levels in mitral cells in a predictable pattern. This profile of results supports the hypothesis that the β_1-adrenoceptor and 5-HT$_{2A/2C}$ receptors interact in early odor preference learning via a synergistic promotion of cAMP in mitral cells in the olfactory bulb, as they do in neocortex (Morin et al. 1992), and that the critical learning change occurs in the mitral cell processing of olfactory nerve input.

This new model of olfactory preference learning mediated by β_1-adrenoceptor activation is illustrated at the circuit and intracellular level in Figure 7. This model accounts for several aspects of what we have seen.

Isoproterenol paired at a learning-effective dose with olfactory nerve input potentiates the olfactory nerve EPSP including both NMDA and non-NMDA components (Yuan et al. 2000). Such potentiation may be mediated by cAMP-initiated phosphorylation of NMDA and AMPA channels. Phosphorylation of L-Ca^{2+} channels could also contribute to membrane depolarization and potentiation of NMDA currents. Closing of K^+ channels following isoproterenol, which has been reported in other systems (Karle et al. 2002), is another mechanism by which depolarization might occur. The failure of higher levels of isoproterenol to produce the electrophysiological potentiation would again be related to an excess of dephosphorylation activity or a shortening of the elevation of cAMP.

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

The approach behavior of rat pups 24 h following odor–UCS pairing is thought to be linked to these initiating events. Optical imaging experiments in trained pups indicate increased activation in the olfactory bulb to the conditioned odor, but not to a control odor, 24 h after training (Yuan et al. 2002). Pairing odor with local infusion of isoproterenol in the rat pup is sufficient to produce the approach response (Sullivan et al. 2000b). It appears that a change in the representation of the odor in the olfactory bulb determines the response. However, we do not even know why some odors are inherently attractive. A study of the bulbar representation of such odors might be helpful. Our data indicate that the odor representation potentiates, both NMDA and AMPA components of olfactory nerve input, are strengthened during acquisition procedures (Yuan et al. 2000), whereas activation probed by optical imaging is stronger 24 h later (Yuan et al. 2002). How this increased activation is coupled to approach behavior is unknown.

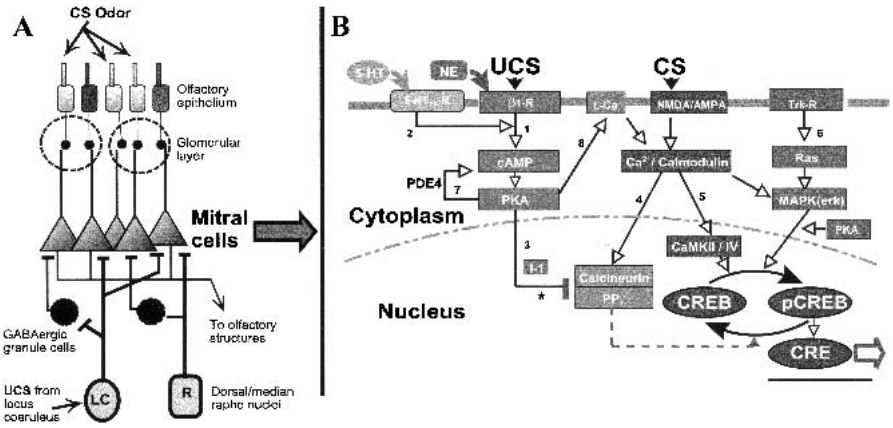

Figure 7 Proposed intercellular and intracellular pathways in the olfactory bulb activated by β_1-adrenoceptors and 5-HT$_{2A}$ receptors. (*A*) Schematic diagram indicating the major circuitry in the olfactory bulb as it relates to the present odor learning model and identifying the convergence of odor input (via olfactory receptor cells), and noradrenergic and serotonergic input onto mitral cells. (*B*) Intracellular circuitry of the mitral cell. β_1-Adrenoceptors mediate the unconditioned stimulus (UCS) via either tactile stimulation or a β-adrenoceptor agonist. The conditioned stimulus (CS) is provided by odors that stimulate glutamate receptors on mitral cells. When adequate stimulation occurs, the pathways induce phosphorylation of CREB and learning. (*) cAMP/PKA "gating" of the calcineurin dephosphorylation pathway of CREB (see Discussion for further details of the pathways; (1) Madison and Nicoll 1986; (2) Morin et al. 1992; (8) Yu et al. 1993; (3,4) Blitzer et al. 1995; (5) Thompson et al. 1995; (4) Bito et al. 1996; (5) Deisseroth et al. 1996; (3) Liu and Graybiel 1996; (6) English and Sweatt 1997; (6) Impey et al. 1998; (5) Shieh et al. 1998; (3,4) Winder et al. 1998; (6) Xing et al. 1998; (1) Werstiuk and Lee 2000; (7) Ang and Antoni 2002).

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

Finally, the present model is closely related to the learning

model well-described for *Aplysia*. Indeed, the Kandel group originally suggested that norepinephrine in mammals might play the role of 5-HT in *Aplysia* (Brunelli et al. 1976). The present model follows that suggestion. A key difference between the two models would appear to be in the coincidence detection mechanism. In *Aplysia*, adenylyl cyclase itself is the coincidence detector for the CS and UCS, and higher levels of cAMP determine the occurrence of learning. In the present model, high levels of cAMP alone do not consistently produce learning, and the CS pathway appears to interact with the UCS pathway at a later stage.

MATERIALS AND METHODS

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

Experiment 1

Animals and Sacrifice

Both young (PND6–PND12) and older (PND30–PND50) Sprague-Dawley rats totaling 26 rats from 22 litters were used in this study. The rats were anesthetized with an overdose of sodium pentobarbital and perfused as described previously (McLean et al. 1999).

Immunocytochemistry/Immunofluorescence

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

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

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

To improve the results of immunocytochemistry and immunofluorescence, a heat-induced antigen retrieval protocol was used. Heat assists in unmasking the epitopes or antigens that are hidden as a result of protein cross-linking induced by formaldehyde fixation (Shi et al. 1991; Cattoretti et al. 1993; Jiao et al. 1999). In this study, microwave irradiation was used before the commencement of immunostaining. Briefly, both slide-mounted sections and free-floating sections were placed in a microwave in containers containing 0.1 M PBS solution (pH 7.4). Irradiation at the maximum setting for 1–2 min raised the temperature of the PBS solution to 90°–95°C, after which the power setting was adjusted to keep the solution at a constant temperature of 90°–95°C for 10 min. The sections were kept in the PBS for another 20 min to cool down. Standard immunocytochemical staining as described above was performed after the microwave irradiation. To exclude the possible nonspecific staining resulting from microwave irradiation, sections with no primary antibody incubation were also included in the experiment.

Image Processing

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

Experiments 2A and 2B

In Experiment 2A, 63 Sprague-Dawley rat pups of both sexes from nine litters were used. Seven training groups were included in this experiment. In Experiment 2B, 10 rat pups from five litters were subjected to unilateral 5-HT depletions of the olfactory bulbs on PND1 and given either 2 mg/kg or 6 mg/kg isoproterenol (β_1-adrenoceptor agonist; Sigma) injections before training. All litters were culled to 12 pups/litter. No more than one pup of either sex from each litter was assigned to each training group.

Odor Conditioning and Drug Injection

The procedure for conditioning has been described in detail before (Langdon et al. 1997; Price et al. 1998; McLean et al. 1999; Yuan et al. 2000). Briefly, on PND6, saline or isoproterenol (1 mg/kg, 2 mg/kg, and 4 mg/kg for Experiment 2A; 2 mg/kg and 6 mg/kg for Experiment 2B) was injected subcutaneously into normal pups (Experiment 2A) or pups with unilateral 5-HT depletion of olfactory bulbs (Experiment 2B) 40 min before their exposure to odor conditioning. The odor conditioning was performed by placing the pups on peppermint-scented bedding for a period of 10 min (0.3 mL of peppermint extract in 500 mL of fresh wood-chip bedding). Also, in this study, serotonergic fiber depletion was performed unilaterally in the olfactory bulbs. Either the low (2 mg/kg) or the high (6 mg/kg) dose of isoproterenol was injected systemically into the pups to investigate β-adrenergic interaction with serotonergic depletion in inducing cAMP cascade activation (Experiment 2B).

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

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

5-HT Depletion

Unilateral 5-HT depletions of olfactory bulbs were performed to provide intraanimal controls for the effect of 5-HT on isoproterenol-induced cAMP expression. The procedure of 5-HT depletion has been previously described in detail (McLean et al. 1993). Briefly, PND1 pups were removed from the dams, pretreated with 10 mg/kg desipramine by intraperitoneal injection, and placed on fresh bedding. Then, 45 min later, after being anesthetized by hypothermia on ice, the pups were placed in a modified stereotaxic instrument, and 150 nL of 5,7-dihydroxytryptamine (5,7-dHT) in Ringer's solution plus 0.02% ascorbic acid was injected bilaterally into the anterior olfactory nucleus. Immunocytochemistry performed on the olfactory bulbs of some 5-HT-depleted pups confirmed 5-HT fiber depletion.

cAMP Assay

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

Experiments 3A and 3B

Animal Preparation

In these experiments, 12 Sprague-Dawley rat pups of both sexes from four litters were used. All pups were given 5-HT depletions of left olfactory bulbs on PND1 as described in Experiment 2. On PND6, in Experiment 3A, seven pups were subjected to 2-mg/kg isoproterenol injections (s.c.) 40 min before being placed on peppermint-scented bedding for 10 min. The pairing of 2 mg/kg isoproterenol and odor on PND6 normally induces odor preference in pups (Sullivan et al. 1989; Langdon et al. 1997). Immediately after odor exposure, pups were killed by decapitation. The brains were removed from the skulls, fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h, then kept in 20% sucrose and 1.5% paraformaldehyde in 0.1 M phosphate buffer overnight. The next day, the brains were transferred to a 20% sucrose solution for 1 h and then cut frozen at 30 µm using a cryostat. In Experiment 3B, 5 pups were killed directly on PND6, to test whether unilateral 5-HT depletion itself affects the basal level of cAMP in the olfactory bulb. Immediately after death, the brains were removed from the skull and processed as described in Experiment 3A.

Immunocytochemistry

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

Image Processing and Analysis

The 5-HT depletions were confirmed under bright-field microscopy. Consistent with previous results (McLean et al. 1993), the process produced >80% depletion of the 5-HT fibers of the left olfactory bulb as shown by immunocytochemistry. For cAMP immunocytochemistry, the relative amount of cAMP expression was quantified systematically by comparing the optical density (darkness) of label in the mitral cell layer from both bulbs. In each bulb, five sections at even intervals through the entire olfactory bulb were examined.

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

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REFERENCES

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., and Bourtchouladze, R. 1997. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* **88:** 615–626.

Ang, K.L. and Antoni, F.A. 2002. Reciprocal regulation of calcium dependent and calcium independent cyclic AMP hydrolysis by protein phosphorylation. *J. Neurochem.* **81:** 422–433.

Barad, M., Bourtchouladze, R., Winder, D.G., Golan, H., and Kandel, E. 1998. Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. *Proc. Natl. Acad. Sci.* **95:** 15020–15025.

Bito, H., Deisseroth, K., and Tsien, R.W. 1996. CREB phosphorylation and dephosphorylation: A Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression. *Cell* **87:** 1203–1214.

Blitzer, R.D., Wong, T., Nouranifar, R., Iyengar, R., and Landau, E.M. 1995. Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. *Neuron* **15:** 1403–1414.

Brunelli, M., Castellucci, V., and Kandel, E.R. 1976. Presynaptic facilitation as a mechanism for behavioral sensitization in *Aplysia*. *Science* **194:** 1176–1180.

Byers, D., Davis, R.L., and Kiger Jr., J.A. 1981. Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in *Drosophila melanogaster*. *Nature* **289:** 79–81.

Cattoretti, C., Pileri, S., Parravicini, C., Becker, M.H., Poggi, S., Bifulco, C., Key, G., D'Amato, L., Sabattini, E., Feudale, E., et al. 1993. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J. Pathol.* **171:** 83–98.

Chakraborti, S., Chakraborti, T., and Shaw, G. 2000. β-Adrenergic mechanisms in cardiac diseases: A perspective. *Cell. Signal.* **12:** 499–513.

Cornea-Hébert, V., Riad, M., Wu, C., Singh, S.K., and Descarries, L. 1999. Cellular and subcellular distribution of the serotonin 5-HT$_{2A}$ receptor

in the central nervous system of adult rat. *J. Comp. Neurol.* **409:** 187–209.

Czesnik, D., Nezlin, L., Rabba, J., Muller, B., and Schild, D. 2002. Noradrenergic modulation of calcium currents and synaptic transmission in the olfactory bulb of *Xenopus laevis* tadpoles. *Eur. J. Neurosci.* **13:** 1093–1100.

Davis, R.L., Cherry, J., Dauwalder, B., Han, P.L., and Skoulakis, E. 1995. The cyclic AMP system and *Drosophila* learning. *Mol. Cell. Biochem.* **149–150:** 271–278.

Deisseroth, K., Bito, H., and Tsien, R.W. 1996. Signaling from synapse to nucleus: Postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16:** 89–101.

English, J.D. and Sweatt, J.D. 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J. Biol. Chem.* **272:** 19103–19106.

Flugge, G., Ahrens, O., and Fuchs, E. 1997. β-Adrenoceptors in the tree shrew brain. I. Distribution and characterization of [^{125}I]iodocyanopindolol binding sites. *Cell. Mol. Neurobiol.* **17:** 401–415.

Hamada, S., Senzaki, K., Hamaguchi Hamada, K., Tabuchi, K., Yamamoto, H., Yamamoto, T., Yoshikawa, S., Okano, H., and Okado, N. 1998. Localization of 5-HT$_{2A}$ receptor in rat cerebral cortex and olfactory system revealed by immunohistochemistry using two antibodies raised in rabbit and chicken. *Mol. Brain. Res.* **54:** 199–211.

Impey, S., Obrietan, K., Wong, S.T., Poser, S., Yano, S., Wayman, G., Deloulme, J.C., Chan, G., and Storm, D.R. 1998. Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* **21:** 869–883.

Ivins, K.J., Ibrahim, E., and Leon, M. 1993. Localization of β-adrenergic receptor mRNA in the olfactory bulb of neonatal rats. *Soc. Neurosci. Abst.* **19:** 124.

Jiao, Y., Sun, Z., Lee, T., Fusco, F.R., Kimble, T.D., Meade, C.A., Cuthbertson, S., and Reiner, A. 1999. A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *J. Neurosci. Methods* **93:** 149–162.

Kandel, E.R., Schwartz, J.H., and Jessel, T.M. 2000. Cellular mechanisms of learning and the biological basis of individuality. In *Principles of neural science* (eds. E.R. Kandel et al.), pp. 1252–1254. McGraw-Hill, New York.

Karle, C.A., Zitron, E., Zhang, W., Kathofer, S., Schoels, W., and Kiehn, J. 2002. Rapid component I(Kr) of the guinea-pig cardiac delayed rectifier K(+) current is inhibited by β(1)-adrenoreceptor activation, via cAMP/protein kinase A-dependent pathways. *Cardiovasc. Res.* **53:** 355–362.

Langdon, P.E., Harley, C.W., and McLean, J.H. 1997. Increased β adrenoceptor activation overcomes conditioned olfactory learning deficits induced by serotonin depletion. *Dev. Brain. Res.* **102:** 291–293.

Liu, F.-C. and Graybiel, A.M. 1996. Spatiotemporal dynamics of CREB phosphorylation: Transient versus sustained phosphorylation in the developing striatum. *Neuron* **17:** 1133–1144.

Madison, D.V. and Nicoll, R.A. 1986. Cyclic adenosine 3′,5′-monophosphate mediates β-receptor actions of noradrenaline in rat hippocampal pyramidal cells. *J. Physiol. (Lond.)* **372:** 245–259.

McLean, J.H., Darby-King, A., Sullivan, R.M., and King, S.R. 1993. Serotonergic influence on olfactory learning in the neonate rat. *Behav. Neural. Biol.* **60:** 152–162.

McLean, J.H., Darby-King, A., and Paterno, G. 1995. Localization of 5-HT$_{2A}$ receptor mRNA by in situ hybridization in the olfactory bulb of the postnatal rat. *J. Comp. Neurol.* **353:** 371–378.

McLean, J.H., Darby-King, A., and Hodge, E. 1996. 5-HT$_2$ receptor involvement in conditioned olfactory learning in the neonate rat pup. *Behav. Neurosci.* **110:** 1426–1434.

McLean, J.H., Harley, C.W., Darby-King, A., and Yuan, Q. 1999. pCREB in the neonate rat olfactory bulb is selectively and transiently increased by odor preference-conditioned training. *Learn. Mem.* **6:** 608–618.

Mons, N., Guillou, J.L., and Jaffard, R. 1999. The role of Ca^{2+}/calmodulin-stimulable adenylyl cyclases as molecular coincidence detectors in memory formation. *Cell. Mol. Life. Sci.* **55:** 525–533.

Morin, D., Sapena, R., Zini, R., and Tillement, J.-P. 1992. Serotonin enhances the β-adrenergic response in rat brain cortical slices. *Eur. J. Pharmacol.* **225:** 273–274.

Mouly, A.M., Elaagouby, A., and Ravel, N. 1995. A study of the effects of noradrenaline in the rat olfactory bulb using evoked field potential response. *Brain. Res.* **681:** 47–57.

Nicholas, A.P., Pieribone, V.A., and Hokfelt, T. 1993. Cellular localization of messenger RNA for β-1 and β-2 adrenergic receptors in rat brain: An in situ hybridization study. *Neuroscience* **56:** 1023–1039.

Okutani, F., Kaba, H., Takahashi, S., and Seto, K. 1998. The biphasic effects of locus coeruleus noradrenergic activation on dendrodendritic inhibition in the rat olfactory bulb. *Brain. Res.* **783:** 272–279.

Ozacmak, V.H., Thorington, G.U., Fletcher, W.H., and Hessinger, D.A. 2002. *N*-Acetylneuraminic acid (NANA) stimulates in situ cyclic AMP production in tentacles of sea anemone (*Aiptasia pallida*): Possible role in chemosensitization of nematocyst discharge. *J. Exp. Biol.* **204:** 2011–2020.

Pompeiano, M., Palacios, J.M., and Mengod, G. 1994. Distribution of the serotonin 5-HT$_2$ receptor family mRNAs: Comparison between 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. *Mol. Brain. Res.* **23:** 163–178.

Poser, S. and Storm, D.R. 2001. Role of Ca^{2+}-stimulated adenylyl cyclases in LTP and memory formation. *Int. J. Dev. Neurosci.* **19:** 387–394.

Price, T.L., Darby-King, A., Harley, C.W., and McLean, J.H. 1998. Serotonin plays a permissive role in conditioned olfactory learning induced by norepinephrine in the neonate rat. *Behav. Neurosci.* **112:** 1430–1437.

Rangel, S. and Leon, M. 1995. Early odor preference training increases olfactory bulb norepinephrine. *Dev. Brain. Res.* **85:** 187–191.

Rovescalli, A.C., Brunello, N., Perez, J., Vitali, S., Steardo, L., and Racagni, G. 1993. Heterologous sensitization of adenylate cyclase activity by serotonin in the rat cerebral cortex. *Eur. Neuropsychopharmacol.* **3:** 463–475.

Shi, S.R., Key, M.E., and Kalra, K.L. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* **39:** 741–748.

Shieh, P.B., Hu, S.C., Bobb, K., Timmusk, T., and Ghosh, A. 1998. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* **20:** 727–740.

Sullivan, R.M. and Leon, M. 1987. One-trial olfactory learning enhances olfactory bulb responses to an appetitive conditioned odor in 7-day-old rats. *Dev. Brain. Res.* **35:** 307–311.

Sullivan, R.M. and Wilson, D.A. 1994. The locus coeruleus, norepinephrine, and memory in newborns. *Brain. Res. Bull.* **35:** 467–472.

Sullivan, R.M., Wilson, D.A., and Leon, M. 1989. Norepinephrine and learning-induced plasticity in infant rat olfactory sytem. *J. Neurosci.* **9:** 3998–4006.

Sullivan, R.M., McGaugh, J.L., and Leon, M. 1991. Norepinephrine-induced plasticity and one-trial olfactory learning in neonatal rats. *Dev. Brain. Res.* **60:** 219–228.

Sullivan, R.M., Landers, M., Yeaman, B., and Wilson, D.A. 2000a. Good memories of bad events in infancy. *Nature* **407:** 38–39.

Sullivan, R.M., Stackenwalt, G., Nasr, F., Lemon, C., and Wilson, D.A. 2000b. Association of an odor with activation of olfactory bulb noradrenergic β-receptors or locus coeruleus stimulation is sufficient to produce learned approach responses to that odor in neonatal rats. *Behav. Neurosci.* **114:** 957–962.

Tang, Y., Hu, L.A., Miller, W.E., Ringstad, N., Hall, R.A., Pitcher, J.A., DeCamilli, P., and Lefkowitz, R.J. 1999. Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the β$_1$-adrenergic receptor. *Proc. Natl. Acad. Sci.* **96:** 12559–12564.

Thompson, M.A., Ginty, D.D., Bonni, A., and Greenberg, M.E. 1995. L-type voltage-sensitive Ca^{2+} channel activation regulates c-fos transcription at multiple levels. *J. Biol. Chem.* **270:** 4224–4235.

Trombley, P.Q. 1992. Norepinephrine inhibits calcium currents and EPSPs

via a G-protein-coupled mechanism in olfactory bulb neurons. *J. Neurosci.* **12:** 3992–3998.

Trombley, P.Q. 1994. Noradrenergic modulation of synaptic transmission between olfactory bulb neurons in culture: Implications to olfactory learning. *Brain. Res. Bull.* **35:** 473–484.

Trombley, P.Q. and Shepherd, G.M. 1992. Noradrenergic inhibition of synaptic transmission between mitral and granule cells in mammalian olfactory bulb cultures. *J. Neurosci.* **12:** 3985–3991.

Wanaka, A., Kiyama, H., Murakami, T., Matsumoto, M., Kamada, T., Malbon, C.C., and Tohyama, M. 1989. Immunocytochemical localization of β-adrenergic receptors in the rat brain. *Brain. Res.* **485:** 125–140.

Werstiuk, E.S. and Lee, R.M. 2000. Vascular β-adrenoceptor function in hypertension and in ageing. *Can. J. Physiol. Pharmacol.* **78:** 433–452.

Wiemer, G., Hellwich, U., Wellstein, A., Dietz, J., Hellwich, M., and Palm, D. 1982. Energy-dependent extrusion of cyclic 3′,5′-adenosine-monophosphate. A drug-sensitive regulatory mechanism for the intracellular nucleotide concentration in rat erythrocytes. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **321:** 239–246.

Wilson, D.A. and Leon, M. 1988. Noradrenergic modulation of olfactory bulb excitability in the postnatal rat. *Dev. Brain. Res.* **42:** 69–75.

Wilson, D.A., Sullivan, R.M., and Leon, M. 1987. Single-unit analysis of postnatal olfactory learning: Modified olfactory bulb output response patterns to learned attractive odors. *J. Neurosci.* **7:** 3154–3162.

Winder, D.G., Mansuy, I.M., Osman, M., Moallem, T.M., and Kandel, E.R. 1998. Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. *Cell* **92:** 25–37.

Wong, S.T., Athos, J., Figueroa, X.A., Pineda, V.V., Schaefer, M.L., Chavkin, C.C., Muglia, L.J., and Storm, D.R. 1999. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron* **23:** 787–798.

Woo, C.C. and Leon, M. 1995. Distribution and development of β-adrenergic receptors in the rat olfactory bulb. *J. Comp. Neurol.* **352:** 1–10.

Woo, C.C., Wilson, D.A., Sullivan, R.M., and Leon, M. 1996. Early locus coeruleus lesions increase the density of β-adrenergic receptors in the main olfactory bulb of rats. *Int. J. Dev. Neurosci.* **14:** 913–919.

Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A., and Greenberg, M.E. 1998. Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol. Cell. Biol.* **18:** 1946–1955.

Yu, H.J., Ma, H., and Green, R.D. 1993. Calcium entry via L-type calcium channels acts as a negative regulator of adenylyl cyclase activity and cyclic AMP levels in cardiac myocytes. *Mol. Pharmacol.* **44:** 689–693.

Yuan, Q., Harley, C.W., Bruce, A.J., Darby-King, A., and McLean, J.H. 2000. Isoproterenol increases CREB phosphorylation and olfactory nerve-evoked potentials in normal and 5-HT-depleted olfactory bulbs in rat pups only at doses that produce odor preference learning. *Learn. Mem.* **7:** 413–421.

Yuan, Q., Harley, C.W., McLean, J.H., and Knopfel, T. 2002. Optical imaging of odor preference memory in the rat olfactory bulb. *J. Neurophysiol.* **87:** 3156–3159.

Zhong, Y., Budnik, V., and Wu, C. 1992. Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: Role of cAMP cascade. *J. Neurosci.* **12:** 644–651.

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Mitral Cell β_1 and 5-HT$_{2A}$ Receptor Colocalization and cAMP Coregulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb

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