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POTENTIATION AND PROLONGATION OF LONG-TERM ODOR MEMORY IN NEONATE RATS USING A PHOSPHODIESTERASE INHIBITOR

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Abstract—Cyclic AMP has been shown to have a critical role in learning and memory in invertebrates. Here we use the rat pup odor preference learning model in which odor acts as a conditioned stimulus and β-adrenoceptor stimulation acts as an unconditioned stimulus to test the role of cyclic AMP in an associative mammalian paradigm. A phosphodiesterase inhibitor that prevents cyclic AMP breakdown (cilomilast) makes a low, learning-ineffective dose of a β-adrenoceptor agonist (isoproterenol, 1 mg/kg) an effective unconditioned stimulus in pup odor preference learning. A dose of the phosphodiesterase inhibitor (cilomilast, 1 mg/kg) that induces learning with a weak unconditioned stimulus interferes with learning using a normally optimal unconditioned stimulus (isoproterenol, 2 mg/kg). Cilomilast (3 mg/kg) paired with peppermint odor during learning, prolonged memory at least four times longer than without the drug (24 h vs. 96 h). These data demonstrate a causal role for cyclic AMP in the acquisition and duration of odor preference learning in the rat pup.

Key words: cilomilast, conditioned learning, prolong memory, olfactory bulb, isoproterenol, norepinephrine.

The neonate rat rapidly acquires preferences for odors associated with maternal care (Sullivan and Leon, 1987), as do human neonates (Sullivan et al., 1991b). In rat pups, stroking simulates maternal care, activating the locus coeruleus of the pons (Nakamura et al., 1987) to increase olfactory bulb norepinephrine (Rangel and Leon, 1995). A 10 min pairing of odor and stroking produces odor preference 24 h later (McLean et al., 1993; Sullivan and Leon, 1987). Stroking-induced norepinephrine release activates cyclic AMP (cAMP)-coupled β-adrenoceptors (Yuan et al., 2003b) in the olfactory bulb. Odor-paired β-adrenoceptor activation in the olfactory bulb is both necessary and sufficient for preference learning (Sullivan et al., 1989). However, an inverted U-curve effect is observed such that too much or too little β-adrenoceptor activation interferes with learning (Langdon et al., 1997; Sullivan et al., 1989).

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Abbreviations: cAMP, cyclic AMP; DMSO, dimethylsulfoxide; CREB, cyclic AMP response element binding protein; PDE, phosphodiesterase; PDE4, phosphodiesterase type IV; PKA, protein kinase A; PND, postnatal day; US, unconditioned stimulus.

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cAMP has been regarded for some time as a pivotal intracellular signal for short-term and long-term memory formation in *Aplysia*. For example, short-term memory is often associated with phosphorylation of pre-existing intracellular targets downstream of cAMP which leads to temporary changes lasting minutes to hours (Castellucci et al., 1980, 1982; Schacher et al., 1988; Yovell et al., 1987). cAMP activation is also critical in long-term memory formation by inducing genomic changes and subsequent changes in protein and structure (Brunelli et al., 1976; Byers et al., 1981; Dale et al., 1987; Dash et al., 1990; Montarolo et al., 1986; Schacher et al., 1988; Yovell et al., 1987) via phosphorylation of the transcription factor cyclic AMP response element binding protein (pCREB). In mammals, cAMP mediates the conversion from early long-term potentiation to late long-term potentiation (Blitzer et al., 1995; Frey et al., 1993). The difference between short- and long-term memory may be in the length of time ubiquitin hydrolase is available to degrade the regulatory subunit of protein kinase A (PKA) which normally regulates the catalytic subunit of PKA (Chain et al., 1999). Catalytic PKA is the agent that phosphorylates CREB and many other pre-existing proteins.

From these observations, one might predict that prevention of the breakdown of cAMP may help memory formation and even prolong memory, if duration of cAMP activation is an important factor in memory formation. Phosphodiesterase (PDE) inhibitors can be used to produce such an effect. In this study, we have utilized a mammalian conditioned odor preference model to address the hypothesis that a phosphodiesterase type IV (PDE4) inhibitor will enhance learning in the neonate rat. We show that the PDE4 inhibitor cilomilast (Ariflo) enhances both memory formation and memory duration following a suboptimal unconditioned stimulus (US).

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley (Charles River, Saint-Constant, Quebec, Canada) rat pups of both sexes were used in this study. A total of 34 litters were used in these experiments. Litters were culled to 12 pups/litter on postnatal day 1 (PND1, the day of birth is considered PND0). No more than one male and one female pup were used for each condition per litter. The dams were maintained under a 12-h light/dark cycle, with *ad libitum* access to food and water. 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. The authors

endeavored to minimize animal use and suffering in these experiments.

Injection of drugs

Briefly, on PND6, saline or (±) isoproterenol (Sigma Chemical Co., St. Louis, MO, USA, 1 mg/kg, 2 mg/kg, or 6 mg/kg) was injected s.c. into pups 40 min before their exposure to odor conditioning. Some of the pups received s.c. injection of 5% dimethylsulfoxide (DMSO, Sigma Chemical Co., in saline, vehicle) or of a PDE4 inhibitor (cilomilast, gift from Greg Rose, 0.001–3.0 mg/kg or rolipram, ICN Biomedicals Inc., Ohio, 0.01–1.0 mg/kg, in 5% DMSO) 30 min before odor conditioning.

Odor conditioning

On PND6, pups were removed from the dam 10 min before odor exposure (training). In the odor+stroking group, pups were placed on peppermint-scented bedding (0.3 ml peppermint/500 ml normal bedding) and stroked vigorously on the hind region using a sable brush every other 30 s for 30 s over a 10 min period. In the odor only group or odor+drug groups, pups were simply exposed to the peppermint scented-bedding for 10 min. Immediately after this training period, the pups were returned to the dam.

Odor preference test

Pups had odor preference testing 3, 24, 48, 96 h or 7 days following training. A stainless steel test box with a mesh bottom (30×20×18 cm) was placed on two training boxes which were separated by a 2–4 cm neutral zone (separation was greater for older pups). One training box contained fresh bedding; the other contained peppermint-scented bedding. Each pup was removed from the dam and placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint-scented bedding or normal bedding was recorded for five 1-min trials (McLean et al., 1996). The percentage of time the pup spent on peppermint-scented bedding over the 5 min period was calculated.

RESULTS

Inhibition of cAMP breakdown induces odor preference learning when paired with a weak/suboptimal US

A common test for the causal role of cAMP in synaptic plasticity has been to administer PDE inhibitors that reduce the breakdown of cAMP to increase the level and/or duration of the cAMP signal. The most commonly employed inhibitor for that purpose has been rolipram, a PDE4 inhibitor, that crosses the blood–brain barrier, but has unwanted side effects at high doses (Heaslip and Evans, 1995; Robichaud et al., 1999). We used rolipram to test the causal role of cAMP in odor preference learning, but we were also able to use cilomilast (Ariflo), a more effective PDE4 inhibitor with fewer problematic side effects. Cilomilast does not readily cross the blood–brain barrier (Giembycz, 2001), but as the barrier is weakly developed in neonatal pups, cilomilast can be used effectively at PND6. To test the causal role of cAMP in learning we gave rat pups a low, learning-ineffective 1 mg/kg dose of isoproterenol paired with odor. In littermates, we gave the same dose together with increasing doses of cilomilast to prevent the breakdown of cAMP. Isoproterenol (1 mg/kg) paired with odor, together with cilomilast doses higher than the lowest dose of 0.001 mg/kg, resulted in odor preference 24 h after training (Fig. 1A). Similar results were obtained with rolipram, another PDE4 inhibitor (Fig. 1B).

Ten minutes of odor preference training produces 3 h and 24 h odor preference memory

Previous work on the duration of odor preference memory with repeated training trials (once a day for 18 days) had provided evidence that such a preference lasted through weaning and that effects of the training could be lifelong (Coopersmith and Leon, 1986). Adult male rats had enhanced sexual responses to females odorized with the odorant on which the males were preference trained as neonates (Fillion and Blass, 1986). Adult female rats showed more interest in pups odorized with the same odor the dams had learned to prefer as neonates (Shah et al., 2002). With a single 10 min odor preference trial, odor preference had been demonstrated at 24 h in numerous studies (Langdon et al., 1997; McLean et al., 1993, 1999; Sullivan et al., 1991a; Sullivan and Leon, 1987; Yuan et al., 2003a) but longer intervals have not been tested. We paired odor and stroking and gave the odor preference test at intervals of 3 h to 7 days after the training trial. We observed that the single 10 min trial of odor and tactile stimulation produced robust memory 3 h and 24 h after training, but odor preference memory was not observed at 48 h or 7 days (Fig. 2A–D) suggesting that the single training period induces memory duration of about a day. It might be noted that control pups show some avoidance of the peppermint odor and, thus, reducing odor aversion, as well as promoting preference, may contribute to the behavioral changes induced by this conditioning.

Inhibition of cAMP breakdown extends odor preference memory duration

To examine memory duration with enhanced cAMP we gave pups a normally ineffective dose of isoproterenol (1 mg/kg) and either 1 mg/kg or 3 mg/kg of the PDE4 inhibitor cilomilast and tested separate groups at 48 and 96 h following training. Pups given odor paired with the optimal dose of 2 mg/kg isoproterenol did not show significant odor preference at 48 h (Fig. 2E) which was similar to the results of pups given odor-tactile stimulation pairing (Fig. 2C). However, pups given 3 mg/kg of cilomilast paired with 1 mg/kg of isoproterenol showed significant odor preference learning at 48 h (Fig. 2E) and at 96 h (Fig. 2F) after pairing compared with non-learning controls. Pups given 1 mg/kg cilomilast and 1 mg/kg of isoproterenol showed intermediate effects at 48 h; not differing from non-learning or learning groups. Thus, the PDE4 inhibitor potentiated the initiation of memory and, in a dose dependent fashion, prolongation of memory.

Inhibition of cAMP breakdown, alone, is ineffective in inducing odor preference memory and can disrupt normal odor preference learning

Recent evidence has suggested that activation of the cAMP cascade is enhanced in aging rats and that, for working memory, PDE inhibition can be detrimental

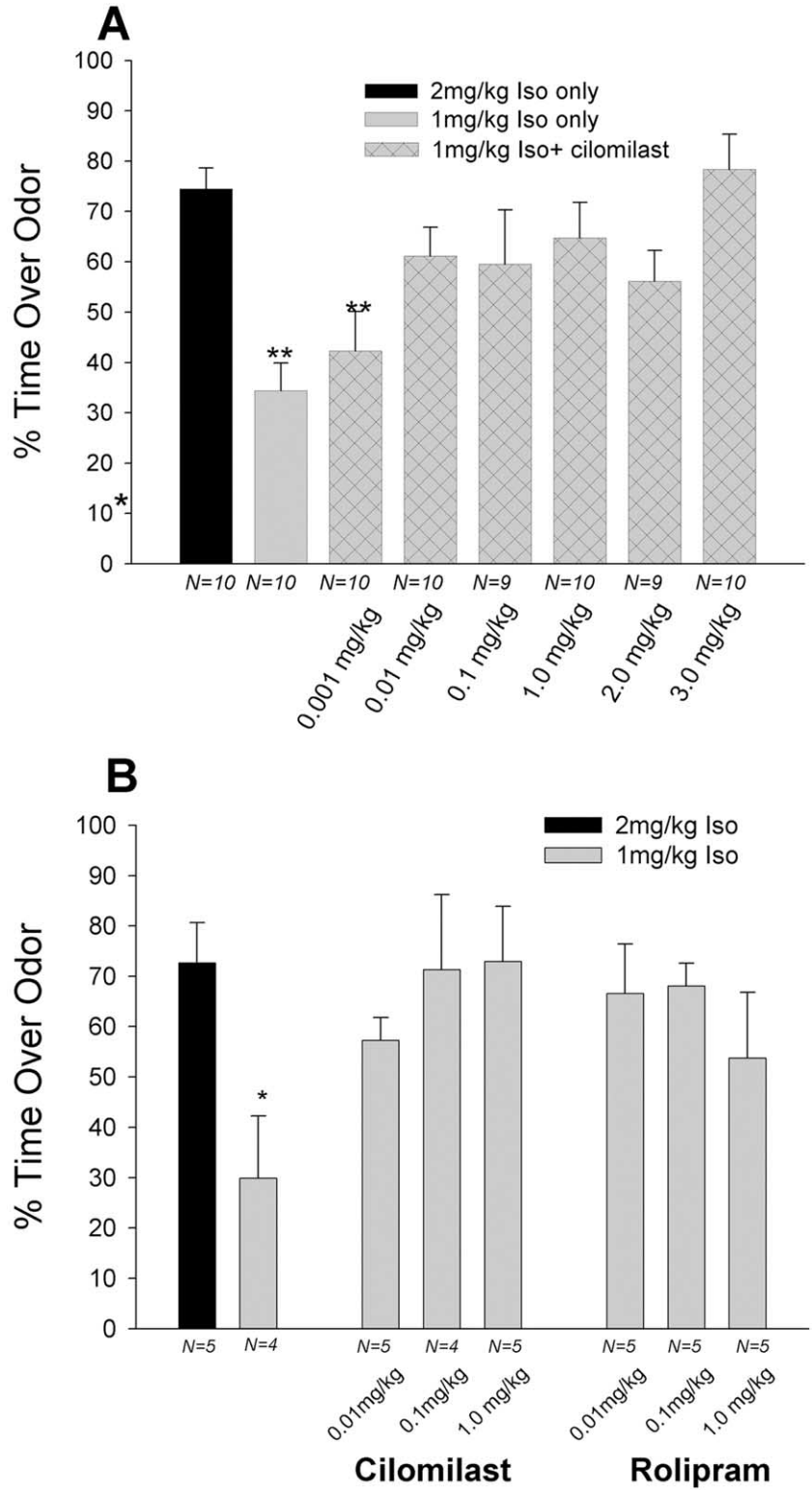

Fig. 1. (A) Cilomilast, a PDE4 inhibitor, facilitates learning when given with a suboptimal dose of the US, isoproterenol (Iso), and paired with peppermint odor for 10 min on PND6. Pups were tested 24 h after training. Pups given a suboptimal dose of Iso (1 mg/kg) did not show preference for the paired odor compared with those trained with an optimal dose of Iso (2 mg/kg). Only the lowest concentration of cilomilast was ineffective in producing learning when paired with suboptimal Iso. One-way ANOVA, $P=0.003$, $F(7, 69)=4.569$ followed by Dunnett multiple comparisons post hoc test was used for statistical analysis. (B) Comparison of two PDE4 inhibitors, cilomilast and rolipram on memory. Both inhibitors gave similar profiles, however, rolipram results did not quite reach significance (one-way ANOVA, $P=0.056$, $F[4, 19]=2.791$).

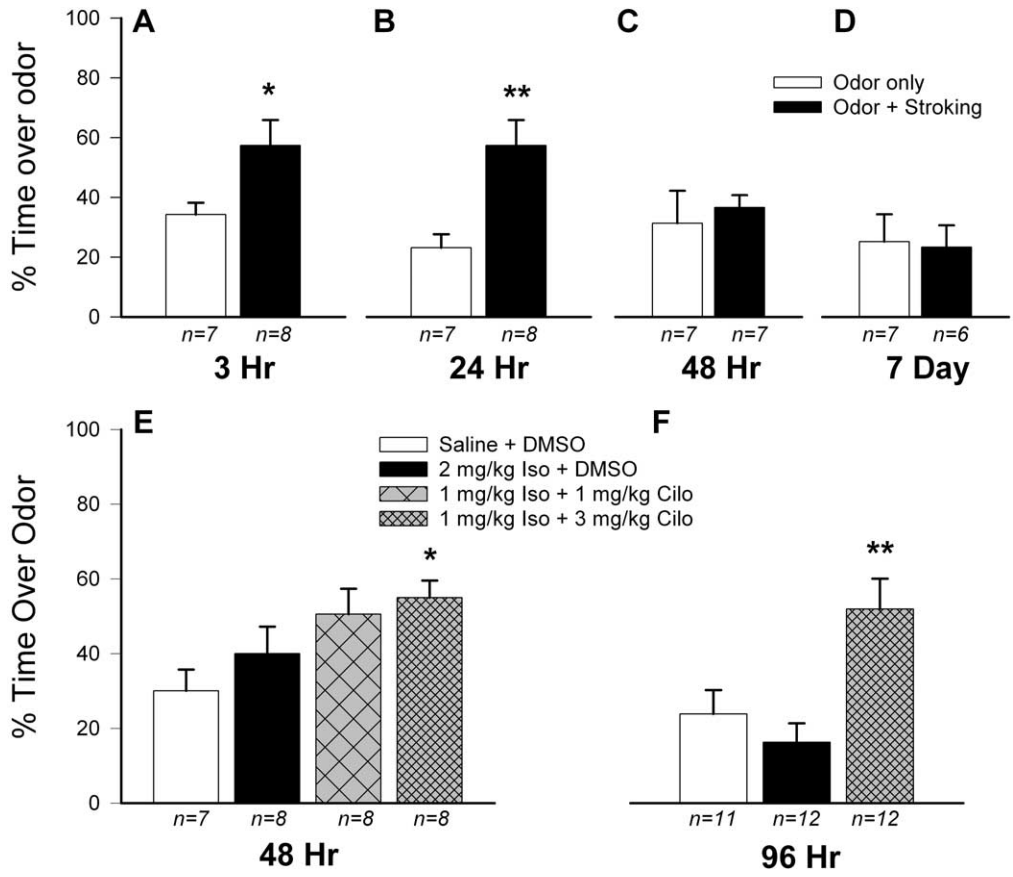

Fig. 2. A PDE4 inhibitor prolongs memory. (A–D) When 6 day old pups are given 10 min of stroking paired with peppermint odor, they show preference for peppermint when tested 3 h (A) or 24 h (B) later compared with unpaired pups (odor only). By 48 h (C) and 7 days (D) odor preference is no longer demonstrated. Student's *t*-test (two tailed) was used for each pair at each time point. (A) $P=0.035$, $t=2.352$ (d.f.=13) (B) $P=0.0014$, $t=4.055$ (d.f.=13). (C, D) N.s. *t*-tests, * $P<0.05$; ** $P<0.01$. (E) Pups given 2 mg/kg Iso paired with peppermint do not display memory for peppermint 48 h later compared with non-learning control pups (odor only). In contrast, pups given suboptimal Iso (1 mg/kg) paired with cilomilast (Cilo, 3 mg/kg) show a preference for the odor. Pups given a lower Cilo dose (1 mg/kg) were intermediate. One-way ANOVA, $P=0.0423$, $F(3, 27)=3.125$. Student-Newman-Keuls was used for the post hoc test. (F) Pups given suboptimal Iso and 3 mg/kg Cilo also show significant preference at 96 h. One-way ANOVA, $P=0.0014$, $F(2, 32)=8.171$. Student-Newman-Keuls was used as the post hoc.

(Ramos et al., 2003). Detrimental effects of PDE inhibition have been reported in normal adults for reference memory also, but this has been linked to higher doses of rolipram and ascribed to side effects (Barad et al., 1998). Thus, we first gave a dose of cilomilast (1 mg/kg) that was shown to be effective for learning (Fig. 1A) paired with a dose of isoproterenol (2 mg/kg) that is normally effective alone in promoting odor preference learning (Fig. 3). However, this combination interfered with learning (Fig. 3). This result is consistent with the inverted U curve seen with isoproterenol (Langdon et al., 1997; Sullivan et al., 1991a) and suggests there is some optimal spatiotemporal pattern for cAMP. We also asked if enhancing basal cAMP by pairing odor and PDE inhibition that promotes low dose-isoproterenol-induced learning would, on its own, produce learning. However, cilomilast alone (1 mg/kg) paired with odor did not produce learning (Fig. 3).

DISCUSSION

Here we have shown that cAMP has a causal role in a mammalian associative learning model, odor preference learning. Preventing cAMP breakdown produced learning when the US paired with odor was a weak dose of β-adrenoceptor agonist (1 mg/kg isoproterenol) that, in previous studies, did not elevate cAMP significantly more than saline (Yuan et al., 2003b). This outcome is consistent with other evidence arguing for a role of the cAMP/PKA/CREB cascade in rat pup odor preference learning. Viral-transfected mutant CREB prevents normal odor preference learning to an optimal dose of isoproterenol, although it permits learning when the dose of isoproterenol is higher than optimal (Yuan et al., 2003a). This observation argues for an imbalance in kinase/phosphatase signaling with higher doses of isoproterenol. Normal viral-transfected CREB lowers the threshold for learning, such that a 1 mg/kg dose of isoproterenol is effective, while preventing normal learning with a 2 mg/kg dose (Yuan et al., 2003a). In this latter instance CREB phosphorylation is significantly higher than that seen with normal learning arguing that too much pCREB can be deleterious also. All other instances of ineffective learning conditions that we have explored are associated with a failure of significant CREB phosphoryla-

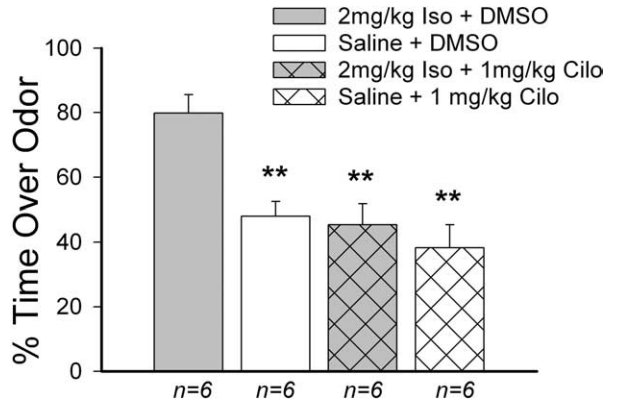

Fig. 3. Inhibition of cAMP breakdown is ineffective alone or with a normal US. Pups given Iso (2 mg/kg) paired with odor at training on PND6 showed significant preference for the odor 24 h later while pups given saline at training did not show an odor preference. The PDE4 inhibitor, cilomilast (Cilo, 1 mg/kg) paired with optimal Iso (2 mg/kg), interfered with learning of the paired odor. Cilo (1 mg/kg), paired alone with odor, did not facilitate learning of the odor. One-way ANOVA, $P=0.0004$, $F(3, 20)=9.44$ followed by Dunnett multiple comparisons post hoc test was used. ** $P<0.01$.

tion (McLean et al., 1999; Yuan et al., 2000). Our previous data and the present data suggest there are relatively narrow and specific requirements for intracellular signaling in the cAMP/PKA/CREB pathway to produce odor preference learning and memory.

Consistent with a specific intracellular signaling requirement, we find here that a moderate level of PDE inhibition can also disrupt normal learning when the conditioned stimulus–US pairing is optimal. This argues that dosages may be critical when employing PDE inhibitors to enhance memory in normal or dysfunctional memory conditions. Learning induction was seen with doses of cilomilast as low as 0.01 mg/kg. The PDE4 interference effect, seen here, is consistent with shifting the inverted U curve observed when stroking is used in combination with isoproterenol as the US (Sullivan et al., 1991a). Thus, two effective unconditioned stimuli become ineffective. On the other hand, a weak dose of isoproterenol and a weak stroking stimulus sum to promote learning that neither alone can initiate (Sullivan et al., 1991a). These outcomes reinforce the hypothesis that there are specific requirements for learning-effective cAMP cascade activation.

Recent observations in our laboratory suggest temporal patterns of cAMP are pivotal for effective learning (McLean et al., 2004), thus we suggest cAMP patterns may be disrupted by preventing cAMP breakdown when the US is optimal. In contrast, a suboptimal US, in this instance, 1 mg/kg isoproterenol, initiates an effective pattern, if cAMP breakdown is reduced by either 1 mg/kg or 3 mg/kg cilomilast. It is likely that we are closer to exceeding a cAMP range that can be temporally modulated when 2 mg/kg isoproterenol is the initiating dose than when 1 mg/kg isoproterenol is the initiating dose. Studies that directly measure cAMP modulation are needed to address this hypothesis.

These are the first experiments to assess the duration of odor preference memory in rat pups after a single training trial. The finding that PDE enhancement of cAMP can prolong memory duration is consistent with other data supporting a role for the level and/or duration of activation of the cAMP/PKA/CREB cascade in promoting longer memories in invertebrates (Feany, 1990; Yin et al., 1995). It will be of interest to see whether these changes in memory duration are linked to longer activation of CREB.

The failure of PDE inhibition alone to be an effective US argues that receptor recruitment of G protein-coupled adenyl cyclase is an important component of associative signaling. There is evidence that NMDA receptor activation can recruit adenyl cyclase activation through intracellular signaling pathways (Chetkovich and Sweatt, 1993). The NMDA receptor is activated by olfactory nerve input in rat pups (Yuan et al., 2000) providing an opportunity for such activation, but we have not found significant increases in cAMP with odor alone (Yuan et al., 2003b) and the synergy with PDE inhibition, if any, was insufficient here to trigger associative learning.

Taken together these studies demonstrate a causal role for norepinephrine activation of cAMP as a US in a mammalian associative learning paradigm and reveal that inhibiting the breakdown of cAMP can enhance the duration of the associative memory.

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