PKA increases in the olfactory bulb act as unconditioned stimuli and provide evidence for parallel memory systems: Pairing odor with increased PKA creates intermediate- and long-term, but not short-term, memories

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Neonatal odor-preference memory in rat pups is a well-defined associative mammalian memory model dependent on cAMP. Previous work from this laboratory demonstrates three phases of neonatal odor-preference memory: short-term (translation-independent), intermediate-term (translation-dependent), and long-term (transcription- and translation-dependent). Here, we use neonatal odor-preference learning to explore the role of olfactory bulb PKA in these three phases of mammalian memory. PKA activity increased normally in learning animals 10 min after a single training trial. Inhibition of PKA by Rp-cAMPs blocked intermediate-term and long-term memory, with no effect on short-term memory. PKA inhibition also prevented learning-associated CREB phosphorylation, a transcription factor implicated in long-term memory. When long-term memory was rescued through increased β-adrenoceptor activation, CREB phosphorylation was restored. Intermediate-term and long-term, but not short-term odor-preference memories were generated by pairing odor with direct PKA activation using intrabulbar Sp-cAMPs, which bypasses β-adrenoceptor activation. Higher levels of Sp-cAMPs enhanced memory by extending normal 24-h retention to 48–72 h. These results suggest that increased bulbar PKA is necessary and sufficient for the induction of intermediate-term and long-term odor-preference memory, and suggest that PKA activation levels also modulate memory duration. However, short-term memory appears to use molecular mechanisms other than the PKA/CREB pathway. These mechanisms, which are also recruited by β-adrenoceptor activation, must operate in parallel with PKA activation.

Rat pup odor-preference learning is an adaptive form of learning critical for organisms that require their mother for protection, warmth, and food during early life. Within the adult mammalian brain, odor pathways are extensive and multiple areas are recruited to facilitate odor learning (Staubli et al. 1995; Tronel and Sara 2002; Rolls et al. 2003). However, the advantage of rat pup odor-preference learning is that many components of the odor pathway are immature, resulting in the elements of odor learning being localized to the olfactory bulbs (Sullivan et al. 2000b).

Odor-preference learning can be induced in a single trial of a 10-min exposure to novel odor (conditioned stimulus, CS) paired with β-adrenoceptor activation in the olfactory bulb (unconditioned stimulus, UCS) (Sullivan et al. 1991; Langdon et al. 1997; Price et al. 1998). The glutamatergic odor input, which permits NMDA receptor activation and calcium entry, primes the β-adrenoceptor stimulation of coupled G-proteins (Yovell and Abrams 1992) to create a wave of cAMP GluA1 (Cui et al. 2011) critical for associative learning (Yovell and Abrams 1992; Rosenberg and Li 1995; Yuan et al. 2003b). The primary action of cAMP is to activate protein kinase A (PKA) (Taylor et al. 1990; Dell’Acqua and Scott 1997).

PKA is involved in long-term memory formation in both invertebrates (Barco et al. 2006) and vertebrates (Abel and Nguyen 2008). The catalytic subunits of PKA phosphorylate multiple learning-relevant substrates such as serine 845 of the AMPA receptor GluA1 subunit (Banke et al. 2000; Ahn and Choe 2009) and serine 133 of the transcription-factor cAMP response element binding protein (CREB) (Arias et al. 1994; Tao et al. 1998; Delgandi et al. 2005).

The phosphorylation of CREB (pCREB) is implicated as a mediator of learning and memory in fruit flies (Tully and Quinn 1985; Yin et al. 1991; Tully et al. 2003), Aplysia (Abel and Kandel 1998), and rodents (Silva et al. 1998; Colombo et al. 2003; Kudo et al. 2005; Brightwell et al. 2007). Previous research from our laboratory demonstrated the requirement for an increase in pCREB within the mitral cells of the olfactory bulb 10 min after learning for the formation of long-term odor-preference memory (McLean et al. 1999; Yuan et al. 2000, 2003a). Since odor-preference learning has been shown to require a cAMP increase (McLean et al. 2005) and CREB phosphorylation (McLean et al. 1999; Yuan et al. 2003a), in the present study we sought to assess the requirement for PKA increases as mediators of the sequence of biochemical events underlying odor-preference memory.

We found that we could generate both intermediate-term and long-term memory with direct PKA activation as the UCS. These memories were formed independently of short-term odor-preference memory, suggesting a PKA-independent mechanism for short-term memory and supporting the hypothesis of parallel
memory pathways. We found that long-term memory blocked by PKA inhibition could be rescued by additional pharmacological activation of β-adrenoceptors and produce a memory formed apparently independent of PKA, suggesting a compensating molecular pathway. This compensating pathway was associated with CREB phosphorylation solidifying previous results, demonstrating a requirement of pCREB for long-term odor-preference memory (Yuan et al. 2003a).

Results

PKA activity increases 10 min following odor-preference training

Single-trial odor-preference training induces an oscillatory peak in olfactory bulb cAMP levels at the end of the 10-min odor presentation (Cui et al. 2007). Phosphorylation of PKA substrates CREB (McLean et al. 1999; Yuan et al. 2000, 2003b), NMDA receptor subunit N1, and AMPA receptor subunit GluA1 (Cui et al. 2011) are highest 10 min later, following the end of odor presentation and the cAMP peak (Cui et al. 2007). In the present study, PKA activity was assessed using a multissubstrate phosphorylation assay in groups sacrificed immediately prior to odor presentation and at 5-min intervals during and up to 10 min following odor presentation. Animals receiving odor + saline served as the baseline standard in each assay run. PKA activity was significantly elevated 10 min following odor presentation in the odor + 2 mg/kg isoproterenol group (the learning condition, 36% increase) relative to odor + 6 mg/kg isoproterenol (nonlearning condition, 6% increase), with PKA activity normalized to the nonlearning saline control. No other time points differed (Fig. 1). The result replicates the temporal pattern of PKA activity indexed by the PKA substrate phosphorylation measures already described.

Inhibition of PKA disrupts intermediate-term and long-term odor-preference memory with no effect on short-term memory

Work from this laboratory has found three different phases of neonatal odor-preference memory (Grimes et al. 2011). Short-term memory up to 3 h is translation independent, while intermediate-term memory occurs around 5 h and is translation dependent and transcription independent. Long-term memory at 24 h is both translation and transcription dependent. To examine the causal role of PKA in these distinct phases of odor-preference memory, Rp-cAMPs, a competitive inhibitor of PKA, was infused into the olfactory bulbs 10 min after the s.c. injection of isoproterenol and 30 min before odor presentation. PKA inhibition disrupted normal odor preference (2 mg/kg isoproterenol as the UCS) 24 h after training (Fig. 2A) and 5 h after training (Fig. 2B), while pups tested 3 h after training displayed odor-preference memory with a normal inverted U curve relationship across isoproterenol dosages (Fig. 2C).

In rat pups given 6 mg/kg of isoproterenol as the UCS, which normally do not learn (Langdon et al. 1997), Rp-cAMPs unexpectedly produced a 24-h odor preference (Fig. 2A). In the 3-h memory group (Fig. 2C) and 5-h memory group (Fig. 2B), no odor preference occurred in the 6-mg/kg isoproterenol group, even with prior intrabulbar Rp-cAMPs (Fig. 2C). The inhibition of intermediate-term and long-term odor-preference memory by Rp-cAMPs suggests that intermediate-term and long-term memories require PKA activation, while short-term memory uses alternate molecular pathways. The ability to generate long-term, but not intermediate-term memory, with a higher dose of isoproterenol in the presence of Rp-cAMPs, suggests that these are also separable memory streams, and long-term memory can occur without the prior appearance of intermediate-term memory. This is the first test of the role of the cAMP/PKA/CREB cascade in short- and intermediate-term mammalian odor-preference memory.

PKA activity in the olfactory bulb under training conditions is strongest in the glomerular and mitral cell layers and is significantly inhibited by infusion of Rp-cAMPs

PKA activity patterns have not been previously described in the olfactory bulb. We examined the localization of bulbar PKA activity using a multiple phosphorylated substrates antibody (Sindreu et al. 2007) and immunohistochemistry in animals sacrificed 10 min after training (the period of peak PKA activity) (Fig. 1). As seen in Figure 3C, PKA activity was highest in the glomeruli (gl) and mitral cells (mcl). Mitral cell dendrites also exhibited reactivity in the region of lateral dendritic spread (inner external plexiform layer, epl). Reactivity was lower in the granule cell region (gcl). High levels of AKAP-150, a PKA anchoring protein, have been described in the glomeruli and in the lateral mitral cell dendritic area in rats (Glanitz et al. 1992) and in the mitral cell bodies in mice (Ostroveanu et al. 2007), which is consistent with the picture here for PKA. A high level of PKA in the granule cell layer reported for AKAP-150 in rats (Glanitz et al. 1992), but not mice (Ostroveanu et al. 2007), was not observed. MAP2, which may be the main neuronal PKA anchoring protein (Zhong et al. 2009), has a similar distribution to that seen here for PKA (Philpot et al. 1997) and is located in glomerular mitral cell dendrites at the EM level (Kasowski et al. 1999). This localization is consistent with our model, in which learning is initiated in and supported by changes in olfactory nerve-mitral cell connections (McLean and Harley 2004).

To assess the effectiveness of Rp-cAMPs in inhibiting PKA activity, a unilateral infusion of Rp-cAMPs into one bulb was compared with the opposite bulb receiving a saline infusion. Pups trained with a 2-mg/kg learning dose of isoproterenol demonstrated significantly lower PKA activity in all regions of the Rp-cAMPs infused bulb (Fig. 3A) than in the saline-infused bulb. In a second set of comparisons, pups received a nonlearning dose of 6 mg/kg isoproterenol. The higher dose of isoproterenol also revealed that Rp-cAMPs-infused bulbs had lower levels of PKA.
PKA activity is required for 5-h intermediate-term and 24-h long-term memory, but not 3-h short-term memory, while 24-h long-term memory is rescued through increased β-adrenoreceptor activity. Pups received an olfactory bulb infusion of Rp-cAMPs 10 min after the s.c. injection of isoproterenol. (A) Pups were tested 24 h after learning for long-term odor-preference memory. A one-way ANOVA \( F_{(4,43)} = 10.399, \quad P < 0.0001; \quad n = 8–9 \) per group and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that inhibiting PKA activity in the olfactory bulbs disrupts long-term memory formation. However, long-term memory was restored with increasing β-adrenoreceptor activation (6 mg/kg Iso). (B) Five-hour intermediate-term memory is also reliant on PKA activity. A one-way ANOVA \( F_{(4,13)} = 24.255, \quad P < 0.0001; \quad n = 4 \) and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that PKA inhibition in the olfactory bulb disrupts intermediate-term memory. However, the memory was not rescued through increased β-adrenoreceptor activity. (C) Three-hour short-term memory does not rely on PKA activity. A one-way ANOVA \( F_{(4,21)} = 34.398, \quad P < 0.0001; \quad n = 5–6 \) and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that inhibiting PKA had no effect on short-term memory formation, and the experimental groups demonstrated an inverted U-curve response to β-adrenoreceptor activation, which has been demonstrated in previous work without olfactory bulb manipulations (Grimes et al. 2011). (*) \( P < 0.05, \quad ** \) \( P < 0.01 \). (Iso) Isoproterenol. Error bars are SEM.

Figure 2. PKA activity is required for 5-h intermediate-term and 24-h long-term memory, but not 3-h short-term memory, while 24-h long-term memory is rescued through increased β-adrenoreceptor activity. Pups received an olfactory bulb infusion of Rp-cAMPs 10 min after the s.c. injection of isoproterenol. (A) Pups were tested 24 h after learning for long-term odor-preference memory. A one-way ANOVA \( F_{(4,43)} = 10.399, \quad P < 0.0001; \quad n = 8–9 \) per group and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that inhibiting PKA activity in the olfactory bulbs disrupts long-term memory formation. However, long-term memory was restored with increasing β-adrenoreceptor activation (6 mg/kg Iso). (B) Five-hour intermediate-term memory is also reliant on PKA activity. A one-way ANOVA \( F_{(4,13)} = 24.255, \quad P < 0.0001; \quad n = 4 \) and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that PKA inhibition in the olfactory bulb disrupts intermediate-term memory. However, the memory was not rescued through increased β-adrenoreceptor activity. (C) Three-hour short-term memory does not rely on PKA activity. A one-way ANOVA \( F_{(4,21)} = 34.398, \quad P < 0.0001; \quad n = 5–6 \) and Dunnett’s post hoc test comparing experimental groups with the saline/saline nonlearning control revealed that inhibiting PKA had no effect on short-term memory formation, and the experimental groups demonstrated an inverted U-curve response to β-adrenoreceptor activation, which has been demonstrated in previous work without olfactory bulb manipulations (Grimes et al. 2011). (*) \( P < 0.05, \quad ** \) \( P < 0.01 \). (Iso) Isoproterenol. Error bars are SEM.

Olfactory bulb infusion of Rp-cAMPs decreases CREB phosphorylation, but increased β-adrenoreceptor activation rescues CREB phosphorylation

Since the phosphorylation of CREB on serine 133 is required for odor-preference learning (McLean et al. 1999; Yuan et al. 2003a), it was hypothesized that PKA inhibition would be associated with lower levels of CREB phosphorylation in nonlearning groups. This was assessed by using phosphorylated CREB antibodies in immunohistochemistry on olfactory bulbs taken 10 min after training, which is the time point of the significant increase in the phosphorylation of CREB in neonatal odor-preference learning (McLean et al. 1999; Yuan et al. 2000, 2003a). Pups given odor + 2 mg/kg isoproterenol revealed lower levels of pCREB in the Rp-cAMPs-infused bulb relative to the saline-infused bulb, as predicted (Fig. 4A). Experiments conducted with odor + 6 mg/kg of isoproterenol (nonleaming dose) revealed elevated pCREB staining density in the Rp-cAMPs-infused bulb relative to the saline-infused bulb (Fig. 4B), demonstrating the association of the restoration of learning in the 6-mg/kg condition, and elevated phosphorylation of CREB.

Activating PKA generates intermediate-term and long-term memory without short-term memory

The blocking effect of PKA inhibition on odor-preference memory at 24 h supports a causal role for PKA in odor-preference learning. A causal role was further probed by attempting to induce learning with PKA activation. The PKA activator, Sp-cAMPs, was infused at varying concentrations into the olfactory bulbs 20 min before odor exposure. Three hours after training there was no evidence of odor-preference memory at any concentration of Sp-cAMPs paired with odor (Fig. 5A). However, 5 h after training and 24 h after training, all groups of pups given a concentration of 9 μg/0.5 μL or higher, showed odor-preference memory (Fig. 5B,C). The lowest concentration of Sp-cAMPs used (4.5 μg/0.5 μL) was ineffective. This pattern of results is consistent with the inactivation data. Both inhibition and activation manipulations suggest that PKA has a causal role in intermediate-term and long-term odor-preference memory. PKA is neither necessary, nor sufficient for short-term odor-preference memory. These results support a model of independent and parallel memory mechanisms for short-term and longer-term odor-preference learning.

Increasing PKA activity extends the duration of odor-preference memory

Increases in cAMP levels using phosphodiesterase inhibition have previously been shown to extend the duration of single-trial odor-preference memory in the rat pup, which is normally limited to 24 h (McLean et al. 2005). In the present study, extended memory durations were assessed when we directly activated PKA with varying concentrations of Sp-cAMPs as the UCS 20 min before odor exposure. The two highest concentrations (18 μg/0.5 μL and 36 μg/0.5 μL) were associated with significant odor preference for peppermint 48 h (Fig. 6A) and 72 h (Fig. 6B) after training. There was no evidence of odor preference at 96 h (Fig. 6C) with the concentrations used. Enhanced activation of PKA alone is sufficient to extend memory duration for odor preference.

Discussion

PKA activation can act as an UCS

PKA activity is required for long-term memory formation across many mammalian models (Abel and Nguyen 2008). In typical associative memory, the activation of PKA has been shown to promote long-term memory when CS + UCS pairings are given, but not to create it (Muller 2000; Viola et al. 2000; Ma et al. 2009). Here, we were able to generate associative mammalian memory with direct PKA activation as the sole UCS. This underscores the requirement of cAMP signaling through PKA within the olfactory bulbs for the formation of odor-preference memory. Rat pups are primed for odor memory formation and retrieval for their survival. Special features of early locus coeruleus (Rangel and Leon 1993; Sullivan et al. 2000b; Moriceau and Sullivan 2004) and amygdala (Sullivan et al. 2000a; Moriceau et al. 2006; Thompson et al. 2008) functioning result in locus coeruleus noradrenaline/ or noradrenaline acting as the UCS for early odor-preference learning. Isoproterenol in the olfactory bulbs will produce preference learning in older pups beyond the critical period (Moriceau and Sullivan 2004). It will be interesting to examine direct PKA activation within the olfactory bulbs of older rats in this context.
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This could provide a memory model and tool to define downstream mechanisms of memory over the lifespan.

PKA activation does not exhibit an inverted U-curve response and can lead to memory extension

When PKA activity was increased with higher activator doses, an inverted U-curve response did not occur. An inverted U-curve is found with β-adrenergic receptor activation (Langdon et al. 1997). Consistent with the inverted U-curve for learning was the finding here that PKA activation 10 min following training did not occur with 6 mg/kg isoproterenol as the UCS. However, higher doses of our PKA activator, instead of being less effective, both initiated and extended memory. Memory enhancement through increasing PKA activity has been found in other mammalian memory models (Bernabeu et al. 1997; Izquierdo et al. 2000; Vianna et al. 2000; Jentsch et al. 2002; Ma et al. 2009), but these also required an additional UCS and memory extension was not probed. With punishment, memory can be so robust that extension is hard to measure (Izquierdo et al. 2000). This is an advantage of our model, where single-trial training just produces 24 h memory, while multiple trials lead to longer memory (Woo and Leon 1987), permitting us to relate memory extension to altered molecular events within the olfactory bulb.

We hypothesize that these changes in molecular activity would alter synaptic strength in order to cause memory formation and extension. Activating PKA can deliver AMPA receptors to the synapse (Zheng and Keifer 2009), increase excitability of existing synapses (Banke et al. 2000; Bocchio et al. 2003; Han and Whelan 2009), and activate silent synapses (Ma et al. 1999). Investigation of synaptic changes between the olfactory nerve and mitral cells within odor-learning areas (Sullivan and Leon 1987; Sullivan et al. 1989, 1991; McLean et al. 1999) could illuminate the cellular modifications required for memory formation and extension.

Intermediate-term and long-term memories, but not short-term memory, depend on PKA activation

PKA activation within the olfactory bulbs did not appear to be sufficient or necessary for short-term odor-preference memory. Inhibiting PKA activity inhibited intermediate-term and long-term memory, but not short-term memory. It is possible that if we had given a higher concentration of the PKA inhibitor, there might have been an effect on 3-h memory. However, activating PKA reinforced the results of inhibiting PKA as it generated intermediate-term and long-term memory without short-term memory. Work in both invertebrate (Romano et al. 1996; Muller and Carew 1998; Fiala et al. 1999; Michel et al. 2008) and vertebrate (Abel et al. 1997; Bernabeu et al. 1997; Izquierdo et al. 2000; Jentsch et al. 2002; Locatelli et al. 2002; Sharizfazadeh et al. 2005; Ma et al. 2009) memory models have shown that PKA activity is implicated selectively in long-term memory. Some forms of intermediate-term memory are PKA dependent within invertebrates (Sutton and Carew 2000; Sutton et al. 2001), but this is the first demonstration in an associative mammalian memory model. Knowing our intermediate-term memory is translation dependent (Grimes et al. 2011) suggests that PKA could be an important kinase for regulating translation within the olfactory bulb. PKA has been found to regulate local translation in the hippocampus (Nayak et al. 1998), possibly through its regulation of elongation factors (Sutton and Schuman 2005).

Other short-term mammalian memory models reinforce the present pattern of results in demonstrating that PKA activity is required for short-term memory (Abel et al. 1997; Goosens et al. 2000; Schafe and LeDoux 2000; Koh et al. 2003), but there is some support for PKA in short-term memory (Viana et al. 2000; Runyan and Dash 2005) An alternative mechanism to covalent modification of existing proteins by PKA is the activation of calcium pathways. Ca2+/calmodulin-dependent kinase 2 (CaMK2) has autophosphorylating capabilities (Miller and Kennedy 1986; Rich and Schulman 1998; Yang and Schulman 1999; Listman et al. 2002) and can cause receptor insertion (Hayashi et al. 2000; Listman and Zhabotinsky 2001), trafficking (Hayashi et al. 2000; Listman et al. 2002; Malinow and Malenka 2002), and phosphorylation at Ser831 of the AMPA receptor (Barria et al. 1997a,b; Mammel et al. 1997; Derkach et al. 1999). Unpublished data from our laboratory demonstrate...
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**The role of CREB**

Mammalian memory models have demonstrated the requirement for the phosphorylation of CREB, for long-term but not short-term memory (Bourtchuladze et al. 1994; Guzowski and McGaugh 1997; Kogan et al. 1997; Lamprecht et al. 1997; Josselyn et al. 2001; Pittenger et al. 2002). The examination of CREB phosphorylation in the olfactory bulbs here also suggests that this transcription factor is required for long-term, but not short-term memory formation. The rescuing of long-term memory in the presence of bulbular Rp-cAMPs through increased β-adrenoceptor activation by 6 mg/kg of isoproterenol is accompanied by increased CREB phosphorylation 10 min after learning, when PKA activity is inhibited. Increased CREB phosphorylation does not occur with the higher dose of isoproterenol in the absence of PKA inhibition (Yuan et al. 2000). Two explanations might be considered. We had previously suggested that excessive PKA activation might preferentially recruit phosphatases, leading to a net dephosphorylation of critical substrates like pCREB (Yuan et al. 2003a). Limiting PKA increases might reduce this effect. While Rp-cAMPs inhibits the PKA activity produced relative to saline, we did not compare the levels of PKA relative to 2-mg/kg infusions. Alternatively, another molecular pathway could be responsible for the CREB phosphorylation observed. We know CAMP levels are linearly increased over time with 6 mg/kg of isoproterenol (Cui et al. 2007). It is possible that this could increase Epac activity and stimulate MAPK. Such a parallel pathway could restore the phosphorylation of CREB and long-term memory while PKA is inhibited. It would not suffice, however, if phosphatases were active as hypothesized in our normal 6-mg/kg isoproterenol nonlearning condition. Since 5-h intermediate-term memory was not restored by increased β-adrenoceptor activation, Epac would not be hypothesized to have a role in initiating intermediate-term memory under this hypothesis. CREB phosphorylation is likely driving intermediate-term memory, consistent with its dependence on translation, not transcription.

This study is the first to show that PKA itself can act as an unconditioned stimulus. PKA-triggered learning is strongly predicted by our assumptions about the CAMP/PKA/initiates learning at a critical threshold and extends memory. Somewhat unexpectedly, there was no suggestion of an inverted U curve at the doses of the unconditioned stimulus, isoproterenol, that we used.

The pattern of expressed memories in our experiments supports the view that memory is supported by parallel processes. Sequential "consolidation" formulations cannot account for the independence of memory expression seen here. Each memory phase could be separately elicited in the absence of the others. This pattern of outcomes argues that there is still considerable progress to be made in our understanding of memory creation.

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**Figure 4.** Olfactory bulb infusion of Rp-cAMPs decreases the phosphorylation of CREB (pCREB) immunohistochemically expression across multiple layers of the olfactory bulb (A,C). However, pCREB is elevated when long-term memory is rescued by increased β-adrenoceptor activation (B,D). (A,C) Pups received an olfactory bulb infusion of saline in one bulb and Rp-cAMPs in the other bulb 10 min after a s.c. injection of 2 mg/kg isoproterenol. Pups were sacrificed 10 min after training, which is the time point of significant bulbar pCREB increase (McLean et al. 1999). Olfactory bulb sections were subsequently processed for pCREB immunohistochemistry. A repeated measures ANOVA $F_{(5,4)}$ = 15.036, $P < 0.0001$ and Bonferroni post hoc test comparing the Rp-cAMPs-infused olfactory bulb with the saline-infused olfactory bulb revealed a significant decrease in pCREB expression in the glomerular layer (gl, $n = 5$, [*] $P < 0.05$), mitral cell layer (mcl, $n = 5$, [*] $P < 0.01$), and granule cell layer (gcl, $n = 5$, [*] $P < 0.01$). (B,D) When we examined increased β-adrenoceptor activation that rescued long-term memory, there was an elevation in the phosphorylation of CREB. Pups underwent the same procedure as described above (A), except they received a s.c. injection of 6 mg/kg of isoproterenol instead of 2 mg/kg. A repeated measures ANOVA $F_{(5,4)}$ = 18.498, $P < 0.0001$ and Bonferroni post hoc test comparing the Rp-cAMPs-infused olfactory bulb to the saline-infused olfactory bulb revealed a significant increase in CREB phosphorylation in the glomerular layer (gl, $n = 5$, [*] $P < 0.01$), mitral cell layer (mcl, $n = 5$, [*] $P < 0.01$), and granule cell layer (gcl, $n = 5$, [*] $P < 0.01$). Error bars are SEM. Scale bar, 200 μM.

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a causal role for this kinase in odor-preference memory and is activated earlier than PKA. The involvement of autophosphorylated CaMK2 in short-term memory has been shown in other memory models (Zhao et al. 1999; Takahashi et al. 2009; Antonov et al. 2010).

Another possibility for short-term memory support and/or for the long-term memory seen with higher doses of isoproterenol, both occurring in the absence of PKA inhibition, is the activation of Epac (exchange protein activated by cAMP). Epac is activated by cAMP increases (de Rooij et al. 1998; Kawasaki et al. 1998) and activates RAP-1 and increases the phosphorylation of mitogen-activated protein kinase (MAPK) (de Rooij et al. 1998; Morozov et al. 2003; Gelinas et al. 2008; Ma et al. 2009). Epac can initiate a cellular model of short-term memory, early phase LTP (Gelinas et al. 2008), and MAPK, and is also implicated in some forms of short-term mammalian memory (Igaz et al. 2006). Thus, an increase in the activities of the MAPK pathway through β-adrenoceptor activation, and the calcium pathway through novel odor exposure, could be responsible for memories occurring in the presence of PKA inhibition.
Materials and Methods

Animals
Sprague-Dawley rat pups of both sexes were used. The dams were maintained under a 12-h light/dark cycle, with ad libitum access to food and water. The litters were culled to 12 pups on postnatal day 1 (PND1). No more than one male and one female pup were used for each condition per litter. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee.

Experimental design

Cannula surgery
On PND5, pups underwent guide cannula placement surgery. The guide cannula was constructed of two 23-gauge/6-mm long stainless-steel tubes (Small Parts, Inc.) set in dental acrylic (Lyn Dental) 2 mm apart. Each pup was anesthetized by hypothermia and then placed in an ice bath in a stereotaxic apparatus (Rumsey et al. 2001). A sagittal incision of the scalp was made from the snout to approximately the level of the lamboid suture. Two holes were drilled through the skull over the olfactory bulbs and the dura was removed gently with fine forceps. The guide cannula was lowered into place as each olfactory bulb had a stainless-steel cannula resting on the top of it. The guide cannula was held in place while it was secured to an upside plastic screw glued to the skull using dental acrylic. The animal was removed from the ice bath and placed on a heating pad, where its scalp was sutured around the cannula. When the pup was resuscitated, bitter apple biting deterrent (Grannick’s Bitter Apple Company) was applied to the sutures and cannula to prevent the dam from biting at the area. The pup was then placed back with the dam.

Odor–preference learning
Experiments were completed in a sound-proof dimly lit room with a temperature of 27°C. On PND6, the pups underwent odor-preference training. The training started with the pup receiving a subcutaneous (s.c.) injection of either saline or isoproterenol (2 mg/kg or 6 mg/kg, Sigma). Thirty minutes after the s.c. injection of saline or isoproterenol, the pup was removed from the dam and placed in a clean weigh boat for 10 min. The pup was then placed on peppermint-scented bedding (500 mL of bedding containing 300 µL of peppermint extract, and allowed to air out for 3–5 min for the evaporation of alcohol) for 10 min, where it was allowed to freely move. After the odor exposure, the pup was placed back with the dam.

Inhibiting PKA during odor–preference learning
Pups underwent odor-preference learning as described above, except that 10 min after the isoproterenol or saline injection, the animal received a 1-µL injection of either saline or Rp-cAMPs (22 mM = 10 µg/µL, dissolved in saline, Sigma) into both olfactory bulbs. This concentration of the PKA inhibitor was chosen as it was in the mid-range of effective concentrations used in previous studies investigating fear learning (Schafe and LeDoux 2000; Roozendaal et al. 2002). The infusion was administered by lowering a 30-gauge/7-mm long stainless-steel infusion cannula into each of the guide cannula. The infusion cannula was attached to polyethylene tubing, which was, in turn, connected to a 10-µL Hamilton syringe (Hamilton Company). One micro-liter of solution was infused into each olfactory bulb over a 4-min period. The infusion cannula was left in place as the cold stimulant solution for an additional 2 min to allow the solution to diffuse throughout the olfactory bulb. The animal was placed back with the dam after the olfactory bulb infusion.

Activating PKA to generate odor–preference learning
On PND6, odor-preference training started with the pups receiving an olfactory bulb infusion (described above) of either saline or Sp-cAMPs (4.5, 9, 18, or 36 µg/0.5 µL, dissolved in saline, Alexis Biochemicals). Twenty minutes later, the pup was placed on peppermint-scented bedding (described above) for 10 min, Figure 6. Increased PKA activity as an UCS generated memory extension that lasted 72 h after learning. Pups received an olfactory bulb infusion of Sp-cAMPs 20 min before odor exposure. (A) Pups tested 48 h after training exhibited an odor preference if given the two highest doses of Sp-cAMPs. A one-way ANOVA \( F(4,28) = 22.143, P < 0.0001; n = 4–6 \) and Dunnett’s post hoc test comparing experimental groups to the saline nonlearning control revealed that increased PKA activity in the olfactory bulbs extended normal 24-h long-term memory. (B) Pups tested 72 h after training also demonstrated memory extension at the two highest doses of Sp-cAMPs. Again, a one-way ANOVA \( F(4,28) = 21.947, P < 0.0001; n = 5–7 \) and Dunnett’s post hoc test comparing experimental groups to the saline nonlearning control revealed a significant odor-preference. (C) However, memory extension was not seen 96 h after training with these doses. A one-way ANOVA \( F(4,28) = 0.3751, P = 0.8241; n = 5–8 \) per group) was not significant. (*) \( P < 0.01 \). Error bars are SEM.
where it was allowed to freely move. After odor exposure, the pup was returned to the dam.

Odor–preference testing
For PKA inhibition studies, pups were tested 3, 5, or 24 h after odor exposure for short, intermediate, and long-term odor-preference memory, respectively. With PKA activation studies, pups were tested 3, 5, 24, 48, 72, or 96 h after odor exposure for short, intermediate, long-term, and extension of odor-preference memory, respectively. Each pup was tested at one time point only. The pup was removed from the dam and placed in a clean weanling boat and transferred to a separate testing room that was dimly lit, 27°C, and sound proof. The animal was lowered into the testing chamber, which consisted of a stainless-steel box with a small grid floor that was covered in a 1000-μm polypropylene mesh (Small Parts Inc.) that was separated into two halves by a small neutral zone (no bedding underneath). One-half of the floor lay over natural bedding, while the other half lay over peppermint-scented bedding. The animal was placed into the neutral zone and the examiner recorded the time the animal spent over the peppermint-scented bedding compared with the natural bedding for a total of 1 min. The animal was given five 1-min trials, switching the direction (away or toward the examiner) the animal faced for each trial. After the final trial, the animal was given a 2-min intertrial interval before being returned to the dam.

Sample collection / PKA activity assay
The pups were given the odor-preference learning procedure. The animals were then killed by decapitation at five different time points (immediately before odor exposure, 5 min into odor exposure, immediately after odor exposure, 5 min after odor exposure, and 10 min after odor exposure). The olfactory bulbs were removed from the skull within 2 min of decapitation, frozen on dry ice, and stored at −80°C in microcentrifuge tubes until they were assayed for PKA activity. Olfactory bulbs were homogenized in a microcentrifuge tube with 100 μL of ice-cold extraction buffer (PBS containing 1 mM EGTA, 1 mM EDTA, 0.01% Triton X-100, 5 mM DTT, 0.5 mM PMSE, 10 mg/mL Chymostatin, 10 mg/mL Leupeptin, 10 mg/mL Antipain, 10 mg/mL Pepstatin, and 10 mg/mL Aprotinin) using a tissue grinder. The tubes were quickly vortexed and stored on ice, then transferred to a rotator at 4°C for 30 min. The tubes were then centrifuged at 14,000 RPM for 15 min. The supernatant solution was carefully transferred to a clean centrifuge tube and stored on ice, while the pellet was discarded. The protein content of the supernatant solutions was assayed using a Biocinchoninic Acid protein assay kit (Pierce). PKA content was normalized by protein content in each sample. PKA activity in the supernatant was measured by using a PKA activity assay kit (Millipore/Upstate). Briefly, 20 μg of protein from the supernatant was mixed in a microcentrifuge tube with 30 μL of the ATP cocktail containing C⁶-PATP cocktail, pairing each sample with its own control with no ATP. Background controls contained everything except the supernatant, while negative samples contained everything plus PKA inhibitor cocktail (PKI). Each sample was incubated at 30°C for 5 min and assayed in duplicate by blotting the mixture on PQI paper squares (Cheng et al. 1998). The paper squares were washed 5 × 2 min in 0.75% phosphoric acid and allowed to air dry for 90 min. The squares were then submerged in scintillation fluid in scintillation tubes and read in a scintillation counter for the optical density (OD). PKA activity (expressed as pmol [C⁶-PATP] ATP/min/μg of protein) was calculated relative to the background activity without added supernatant (Issegia et al. 2006).

Immunohistochemistry
Pups underwent cannula surgery and the odor-preference learning (2 mg/kg, 6 mg/kg Ido or saline) procedures as described above. However, during the olfactory bulb infusion, the pup received Rp-cAMPs (22 mM) in one olfactory bulb, while being given saline in the other olfactory bulb. Each pup was perfused 10 min after the peppermint odor exposure, as this was determined to be a critical time point for the phosphorylation of CREB (McLean et al. 1999; Yuan et al. 2000) and the increase in PKA activity. The perfusion procedure was completed as previously reported by this lab (McLean et al. 1999). Immunohistochemical procedures followed a standard protocol for this laboratory as published previously (McLean et al. 1999; Yuan et al. 2003). Briefly, 30-μm sections were molted on slides with two alternating slide sets collected. Rabbit phosphorylated CREB (Ser133, Cell Signaling Technology) and phosphorylated PKA substrate (Cell Signaling Technology) antibodies were diluted to 1:500 or 1:1000 in 0.1 M phosphate buffered saline with 0.2% Triton X-100, 0.02% sodium azide, and 2% normal goat serum and placed on the tissue for 48 h in a humidified chamber at 4°C. The tissue was removed from the cold room and processed further using the avidin–biotin complex technique (Vectorstain Elite, Vector Labs). All sections from the same experiment had identical incubation times in the antibodies and identical exposure to chromogen solutions.

Image analysis
The density of pCREB staining was analyzed using a Bioquant image analysis system (R&M Biometrics). Slides were coded so that the observer was blind to the experiment. The glomerular, mitral cell, and granule cell layers were encircled by visualizing sections of the olfactory bulb on a computer screen using a 4X objective on a Leitz microscope connected to a CCD camera. Light intensity from the microscope was always set at an optical density (OD) of 220. Background correction was performed to provide an even field of illumination. Relative optical density was determined by the optical density of the background region (readings taken from the olfactory nerve layer), less the area of interest on the tissue (i.e., glomerular, mitral cell, or granule cell layer), divided by the optical density of the background tissue. The relative optical density was obtained from all regions of the main olfactory bulb.

Statistical analysis
A one-way analysis of variance (ANOVA) was used in the animal behavior analysis to compare drug effects. A Dunnett’s post hoc analysis was used to compare the various behavioral training groups with the nonlearning saline control group. For the PKA activity assay, the isoproterenol experimental groups were normalized to the saline control within the same time point and analyzed using a one sample t-test. For the relative optical density analysis of immunohistochemistry, a repeated measures ANOVA was used, followed by Bonferroni post hoc analysis. Statistical significance was taken as P < 0.05 for all tests.

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PKA increases in the olfactory bulb act as unconditioned stimuli and provide evidence for parallel memory systems: Pairing odor with increased PKA creates intermediate- and long-term, but not short-term, memories

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