Brief Communication

\(\beta_1\)-Adrenoceptor or \(\alpha_1\)-adrenoceptor activation initiates early odor preference learning in rat pups: Support for the mitral cell/cAMP model of odor preference learning

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We proposed that mitral cell \(\beta_1\)-adrenoceptor activation mediates rat pup odor preference learning. Here we evaluate \(\beta_1, \beta_2, \alpha_1,\) and \(\alpha_2\)-adrenoceptor agonist effects in learning. The \(\beta_1\)-adrenoceptor agonist, dobutamine, and the \(\alpha_1\)-adrenoceptor agonist, phenylephrine, induced learning, and both exhibited an inverted U-curve dose-response relationship to odor preference learning. Phenylephrine-induced learning occurred in the presence of propranolol to prevent indirect activation of \(\beta_2\)-adrenoceptors. \(\alpha_2\)-Adrenoceptor mediation may represent a novel mechanism inducing learning or may increase cAMP in mitral cells via indirect activation of \(GABA\)_A receptors. Neither the \(\beta_2\)-adrenoceptor agonist, salbutamol, nor the \(\alpha_2\)-adrenoceptor agonist, clonidine, induced learning.

Since initial groundbreaking studies in *Aplysia* (for a review, see Pittenger and Kandel 2003) and later *Drosophila* (Yin and Tully 1996), there has been interest in the hypothesis that the cyclic adenosine monophosphate/protein kinase A/cyclic adenosine monophosphate response element binding protein (cAMP/PKA/CREB) cascade might be a universal mechanism underlying learning and memory. In mammalian models there is also supporting evidence for such an assertion, but in adult rat models the associative pathways involved in recruiting cAMP activation in learning are difficult to identify (Alberini 1999). The rat pup odor preference learning model offers an advantage in that respect as the role of \(\beta_1\)-adrenoceptor activation as an unconditioned stimulus is well established (Sullivan et al. 1989, 1991a; Sullivan and Wilson 1991; Langdon et al. 1997), and more recently, a causal role for cAMP in this form of associative learning has been demonstrated (McLean and Harley 2004).

In 2003, we proposed that early odor preference learning in the week-old rat pup induced by pairing a novel odor with tactile stimulation (stroking), or with injection of the nonselective \(\beta\)-agonist isoproterenol, was mediated by the activation of \(\beta_1\)-adrenoceptors on mitral cells that interacted with glutamatergic odor input to the same cells (Yuan et al. 2003b). This interaction was proposed to produce alterations in mitral cell connectivity that altered the pup’s memory of, and preference for, the novel odor. We showed that \(\beta_1\)-adrenoceptors are predominantly localized on mitral cells and that they are colocalized with 5-HT_1B receptors (Yuan et al. 2003b), which facilitate learning by potentiating the cAMP response initiated by \(\beta_1\)-adrenoceptors. We have also found that cAMP has a causal role in odor preference learning consistent with our hypothesis (Yuan et al. 2003a,b).

All previous work with odor preference learning and \(\beta_1\)-adrenoceptor activation has used the nonselective \(\beta_1\)-adrenoceptor agonist isoproterenol. It has not been demonstrated that selective \(\beta_1\)-adrenoceptor activation produces learning. Isoproterenol, infused directly into the olfactory bulb (Sullivan et al. 2000) or given systemically (Sullivan et al. 1991a; Langdon et al. 1997), induces odor preference learning when paired with a novel odor. The nonspecific \(\beta\)-antagonist propranolol, infused directly into the olfactory bulb (Sullivan et al. 1992) or given systemically (Sullivan et al. 1991a), prevents odor preference learning induced by stroking. This is consistent with the hypothesis that \(\beta_1\)-adrenoceptors in the olfactory bulb are the critical substrate of the unconditioned stimulus in odor preference learning. Together these results have been taken as evidence that \(\beta_1\)-adrenoceptors are necessary and sufficient for early odor preference learning (Sullivan et al. 1989, 2000).

Odor preference learning induced by the naturalistic pairing of odor and stroking is proposed to be mediated by the pairing of odor and norepinephrine release in the olfactory bulb (Sullivan et al. 1991b). Stroking produces sustained locus coeruleus activation in week-old pups (Nakamura et al. 1987), and increased norepinephrine release in the olfactory bulb during the odor and stroking paradigm has been demonstrated (Rangel and Leon 1995).

This odor plus locus coeruleus activation model of odor preference learning is confirmed by the work of Moriceau and Sullivan (2004). They proposed that the restricted period for odor preference learning was governed by the development of \(\alpha_2\)-adrenoceptor autoinhibition in locus coeruleus. By pharmacologically blocking \(\alpha_2\)-adrenoceptor inhibition and by directly activating the locus coeruleus with a combination of the \(\alpha_1\)-adrenoceptor agonist phenylephrine and a cholinergic agonist, they extended the critical period for odor preference learning to older pups. Direct activation of bulbar \(\beta_1\)-adrenoceptors using isoproterenol was also effective in inducing odor preference learning in the older pups that normally do not display such learning.

Our work has focused on the cellular events underlying memory formation in the olfactory bulb. We have shown that effective odor and reward pairings increase cAMP (Yuan et al. 2003b; McLean et al. 2004) and increase CREB phosphorylation (McLean et al. 1999) in olfactory bulb mitral cells. Enhancing or interfering with normal CREB levels by using a viral vector shifts the inverted U dose-response curve for isoproterenol-induced...
odor preference learning to the left or right, respectively. This is consistent with a critical role for the PKA/CREB cascade in this form of learning. We have also shown that 5-HT facilitates the action of isoproterenol in elevating cAMP (Yuan et al. 2003a) and in producing odor preference learning (Price et al. 1998). These data led to the hypothesis that β1-adrenoceptors on mitral cells, with support from the colocalized 5-HT2A/C receptors (Yuan et al. 2003b), interact with odor input to provide the cellular signals for learning and memory (McLean and Harley 2004).

However, the roles of specific adrenoceptor agonists have not been tested in pup odor preference learning. Norepinephrine released by locus coeruleus activation would interact with all adrenoceptor subtypes present in the olfactory bulb. Both β1- and β2-adrenoceptor subtypes are present in the olfactory bulb (Nicholas et al. 1993; Woo and Leon 1995) and would be activated by isoproterenol. α-Adrenoceptor subtypes also occur in the olfactory bulb (McCune et al. 1993). The α1-adrenoceptor subtype enhances odor learning in adult rat (Spreng et al. 2001), while the α2-adrenoceptor subtype plays a role in memory in other brain regions (Sara and Devauges 1989; Amstern and Leslie 1991; Carlson et al. 1992; Coull et al. 1996; Birnbaum et al. 2000; Gibbs and Summers 2003). Thus, to evaluate our hypothesis, we examine here the effectiveness of β1-, β2-, α1-, and α2-adrenoceptor agonists as unconditioned stimuli for early odor preference learning.

Sprague-Dawley rat pups of both sexes were used in this study. Litters were culled to 12 pups/litter on postnatal day (PND) 1 (the day of birth is considered PND0). The dams were maintained under a 12-h light/12-h dark cycle, with ad libitum access to food and water. All experimental procedures were approved by the Memorial University Institutional Animal Care Committee, which abides by the standards set by the Canadian Council on Animal Care. No more than one male and one female pup were used for each condition per litter.

All experiments followed the same basic design. Each adrenoceptor was tested with a separate group of litters. Pups were removed from the dam on PND6 10 min before training and placed on terpinene-scented bedding (0.25 mL α-terpinene, Aldrich Chemical Company/500 mL cedar chip bedding) in a manner described previously (Langdon et al. 1997). On the day following training (PND7), pups were removed individually from the dam and tested for odor preference by using a two odor choice test as described previously (Sulivan and Leon 1987; Sullivan et al. 1989; Langdon et al. 1997; Price et al. 1998; McLean et al. 1999). Briefly, each pup was placed in a neutral zone and given five 1-min tests. The amount of time over terpinene was calculated as the percentage of time the pup spent over that odor divided by the total time over either odor.

Drugs were obtained from Sigma Canada. As a positive learning control, isoproterenol (2 mg/kg in 50 µL saline), which is known to induce learning by activating β-adrenoceptors (Sulivan et al. 1989; Sullivan and Wilson 1994; Langdon et al. 1997; Price et al. 1998), was injected subcutaneously (s.c.) 40 min before odor pairing. For a nonlearning control, saline (50 µL) was injected in other pups.

The adrenoceptor agonists tested were dobutamine, a β1-adrenoceptor agonist, at 0.1, 1, and 4 mg/kg, intraperitoneally (i.p.) (five litters, 34 pups); salbutamol, a β2-adrenoceptor agonist at 0.1, 1, and 3 mg/kg, i.p. (seven litters, 68 pups); phenylephrine, an α1-adrenoceptor agonist at 0.5, 1, and 2 mg/kg, i.p. (six litters, 32 pups); and clonidine, an α2-adrenoceptor agonist at 5, 10, 20, 50, 100, and 200 µg/kg s.c. (10 litters, 59 pups). All agonists were given 30 min prior to odor exposure, and the pup was returned to the dam until training. An additional condition was added after the initial α1-adrenoceptor agonist results were obtained; propranolol (20 mg/kg, i.p.) + phenylephrine (at 1 or 2 mg/kg, i.p.). Propranolol, a β-adrenoceptor antagonist was given 15 min prior to phenylephrine. These groups were tested against the learning effective dose of phenylephrine (1 mg/kg) as well as the usual controls (six litters, 46 pups). This added condition was required because α1-adrenoceptors are present in both the locus coeruleus (McCune et al. 1993) and olfactory bulb (McCune et al. 1993; Pieribone et al. 1994), while β-adrenoceptors are present in the olfactory bulb (Woo and Leon 1995) but not in the locus coeruleus (Nicholas et al. 1993). Thus, blocking the β-adrenoceptors in the olfactory bulb, while activating α1-adrenoceptors, would rule out β-adrenoceptor involvement in learning in the presence of α1-adrenoceptor activation.

Dobutamine (β1-adrenoceptor agonist) displayed an inverted U-curve effect (Fig. 1), being effective at a dose of 1 mg/kg, and ineffective at a lower, or higher, dose (ANOVA F(4,27) = 5.642, P < 0.01). Post hoc analysis using the Dunnett multiple comparison test confirmed that the positive control (isoproterenol) group was significantly different from the odor-saline control (P < 0.05). With isoproterenol, pups showed a preference for the odor CS spending 64.9 ± 11.3 (SEM)% of time over the terpinene compared with saline-odor nonlearning control at 26.7 ± 7.1%. With dobutamine (1 mg/kg), pups averaged 71.0 ± 9.1% of the time over terpinene, and this was also significantly different (P < 0.05) from saline-odor control. Neither the 0.2 nor 4 mg/kg dobutamine dose produced preferences significantly different from the saline nonlearning control.

Salbutamol (β2-adrenoceptor agonist) did not differ signifi-
cantly from either the saline (nonlearning) control or the isoproterenol (learning) control (Fig. 2). In a planned Student t-test, the latter two groups differed significantly from each other (non-learning control, 34.7 ± 5.2%; learning control 58.6 ± 6.3%; \( P < 0.01 \)).

Phenylephrine (\( \alpha_1 \)-adrenoceptor agonist) also displayed an inverted U-curve effect (Fig. 3), being effective at a dose of 1 mg/kg and ineffective at a lower, or higher, dose (ANOVA, \( F_{(1,21)} = 6.724, P < 0.01 \)). Post hoc analysis using the Dunnett multiple comparison test confirmed that the positive control (isoproterenol) group was significantly different from the odor-saline control (\( P < 0.05 \)). With isoproterenol, the pups showed preference for the odor CS spending 67.0 ± 9.0% of the time over the terpine compared with the saline-odor nonlearning control at 33.6 ± 4.6%. With phenylephrine (1 mg/kg), pups averaged 64.2 ± 12.1% of the time over terpine, and this was also significantly different (\( P < 0.05 \)) from the saline-odor control. Neither the 0.5 nor 2 mg/kg phenylephrine dose was significantly different from the saline (nonlearning) control.

In the presence of the \( \beta_2 \)-adrenoceptor antagonist propranolol (Fig. 4), 1 mg/kg phenylephrine was still an effective unconditioned stimulus (\( F_{(1,12)} = 9.804, P < 0.0001 \)). In post hoc analysis using the Dunnett multiple comparison test, phenylephrine (1 mg/kg) with saline (odor preference of 49.8 ± 7.4%) or with propranolol (odor preference of 58.9 ± 5.4%) was significantly different from saline only (odor preference of 23.9 ± 4.1%). Phenylephrine with propranolol was not significantly different from phenylephrine with saline in inducing a preference. The 2 mg/kg dose of phenylephrine was still ineffective (odor preference of 26.0 ± 4.2%) in the presence of propranolol and did not differ from the nonlearning saline alone group.

Clonidine (\( \alpha_2 \)-adrenoceptor agonist) failed to produce an odor preference at any concentration (Fig. 5). The nonlearning and learning controls performed as expected, spending 25.0 ± 4.9% and 66.3 ± 8.2% of the time, respectively, over the odor CS. A one-way ANOVA was highly significant (\( F_{(4,51)} = 5.893, P < 0.0001 \)). However, the Dunnett multiple comparison post hoc tests showed only the isoproterenol group differed from the saline control group (\( P < 0.01 \)).

In any study using systemic drugs, there are concerns about site of action and selectivity of action. While the present study builds on earlier evidence for intrabulbar effectiveness of a \( \beta_2 \)-adrenoceptor agonist (Sullivan et al. 2000) and antagonist (Sullivan et al. 1992) and on intrabulbar 5-HT depletion (McLean et al. 1993, 1996) to argue that the critical necessary and sufficient learning targets are intrabulbar, this is not examined directly here. Each of the drugs used would also have effects at other sites and on other receptors, e.g., clonidine (Eglan et al. 1998), phenylephrine (Ishac et al. 1987; Gadeck-Michalska et al. 1990; Watkins et al. 1990; Quigley et al. 2005), and salbutamol (Waldmeier 1981; Murugaiah and O’Donnell 1995; Garnier et al. 1997).

To estimate the intrabulbar drug concentrations of the systemic drugs that produced learning in the present study, we assumed pups were 70% water (Mullins 1983) and lacked an effective blood-brain barrier for these drugs, in contrast to adults (Conway et al. 1987). Pups were previously shown to learn an odor preference with 50 µM intrabulbar isoproterenol (Moriceau and Sullivan 2004). Our estimated systemic isoproterenol dose was 83 µM. In previous studies, halving or doubling this dose did not induce learning (Sullivan et al. 1989; Langdon et al. 1997). Dobutamine, the \( \beta_1 \)-adrenoceptor agonist, was effective at 56 µM. Phenylephrine was effective at 34 µM. Salbutamol was ineffective when tested up to an estimated concentration of 120 µM. While highly speculative, these estimates likely represent maximal levels that could occur in the olfactory bulb; thus, they are useful for addressing questions of selectivity. Since isoproterenol, dobutamine, and phenylephrine all exhibit inverted U-curves in their dose/response relationship to learning, their nonspecific effects, more likely to occur at higher doses, are less likely to account for the observed induction of learning seen here.

The effectiveness of dobutamine in the present study in inducing odor preference learning, supports our hypothesis of a critical interaction between the odor input to mitral cells and \( \beta_1 \)-adrenoceptor activation of the same cells in the mediation of odor preference learning. This result is also consistent with evidence that \( \beta \)-adrenoceptor-mediated increases in cAMP and activation of the PKA phosphorylation cascade (Yuan et al. 2003a; McLean et al. 2005) participate causally in the acquisition of early odor preference learning.

Isoproterenol, the most commonly employed pharmacological agent as an unconditioned stimulus for odor preference learning, has a consistent inverted U dose-response curve in terms of its role in learning. In the present study, for the \( \beta_2 \)-adrenoceptor agonists, only dobutamine replicated the inverted U-curve pattern, further supporting the hypothesis that \( \beta_1 \)-adrenoceptors mediate the effect of isoproterenol as an unconditioned stimulus. The effective dose of dobutamine was half that of the \( \beta_1/\beta_2 \)-adrenoceptor agonist isoproterenol, which is also consistent with mediation by \( \beta_1 \)-adrenoceptors since the tissue...
concentrations of the two drugs are similar. Further studies might examine whether activation of β2-adrenoceptors by salbutamol can shift the inverted U-curve of either isoproterenol or dobutamine leftward, testing a supportive role for β2-adrenoceptors in early odor preference learning.

The density of α1-adrenoceptors in the olfactory bulb is among the highest in the mammalian brain (McCune et al. 1993; Pieribone et al. 1994). They have been implicated in enhancing the ability of peri-threshold odor nerve stimulation to excite mitral cells but have no effect on suprathreshold stimulation (Ciombor et al. 1999). However, terpinene, in the present study, is unlikely to have been peri-threshold for olfactory nerve activation.

The present study is the first report of α1-adrenoceptor activation acting as an unconditioned stimulus for odor preference learning in the rat pup. α1-Adrenoceptor activation also exhibited an inverted U-curve profile similar to that seen with the β-adrenoceptor agonists isoproterenol and dobutamine. In other studies, α1-adrenoceptor activation in the olfactory bulb increases feedback inhibition and GABA release (Mouly et al. 1995). Consistent with these effects, the component of the olfactory nerve evoked potential is inhibited by locus coeruleus activation and antagonized by an α1-adrenoceptor blocker (Perez et al. 1987). This late component may represent feedback inhibition of granule cells onto mitral cells.

An α1-adrenoceptor-mediated increase in GABA release suggests a possible mechanism for odor preference learning consistent with our current model, which proposes that odor input interacts with increases in mitral cell cAMP to induce circuitry changes underlying odor preference. In the olfactory bulb, GABA_A receptors can activate cAMP in granule cells and in the external plexiform layer by a pathway independent of β-adrenoceptor activation (Olianas and Onali 1999). The external plexiform layer contains the dendrodendritic synaptic connections between granule and mitral cells. Mitral cells possess GABA_A receptors (Panzanelli et al. 2004), and therefore, the cAMP elevation observed by Olianas and Onali (1999) in the external plexiform layer may have occurred in mitral cells, although it was implied that the elevation was within granule cell dendrites. Thus, the enhanced GABA feedback initiated by α1-adrenoceptors might, in this view, provide another route to increase cAMP in mitral cell dendrites (Fig. 6). α1-Adrenoceptor activation in the olfactory bulb also enhances β-adrenoceptor-mediated cAMP two to three times more effectively than in any other brain area (Stone and Herrera 1986) and may support the increases in cAMP induced by isoproterenol, just as 5-HT_2A receptors activation has been postulated to do (Price et al. 1998; Yuan et al. 2003b; McLean and Harley 2004). However, the effectiveness of α1-adrenoceptor activation, in the presence of propranolol, argues for an action separate from β-adrenoceptors in the present study. Phenylephrine itself has recently been reported to increase cAMP in two systems (Saeed et al. 2004; Gallego et al. 2005) and to couple to CREB phosphorylation through the PKA pathway in another system (Markou et al. 2004). We indicate this possibility in Figure 6. Investigating cAMP levels in the phenylephrine learning condition would test cAMP involvement in the phenylephrine-induced learning. The use of GABA_A antagonists would assess whether any cAMP increase was directly or indirectly mediated. Alternatively, α1-adrenoceptor activation may act via a mechanism unrelated to cAMP. For example, α1-adrenoceptors most often act through second messenger systems via protein kinase C or MAP kinases (for a review, see Koshimizu et al. 2002). In this respect, α1-adrenoceptors could act directly on mitral cells (Hayar et al. 2001) and recruit intracellular signaling pathways unrelated to cAMP signaling to promote learning.

With stroking, β-adrenoceptor blockade is sufficient to prevent odor preference learning, suggesting that the α1-adrenoceptor stimulation produced by locus coeruleus norepinephrine release is not sufficient, on its own, to induce learning. If α1-adrenoceptor activation supports β-adrenoceptor-driven cAMP, however, it is likely that an α1-adrenoceptor antagonist might also prevent odor learning.

α2-Adrenoceptor activity has been shown previously in the olfactory bulb. Clonidine produces presynaptic disinhibition in the olfactory bulb by reducing glutamatergic drive to the granule cells and by reducing GABA feedback on the mitral cells (Trombly and Shepherd 1992; Trombly 1994). Mitral cell disinhibition by bicuculline produces odor preference learning in older pups when infused into the olfactory bulb at low doses (Okutani et al. 1999), but clonidine was ineffective at any dose tried in this study. Possibly the degree of disinhibition induced by clonidine in vivo is intermediate between the low level that produces preferences and the high level that produces aversions (Okutani et al. 1999), or disinhibition may be less effective in week-old pups. However, we have previously shown that a metabotropic gluta-

![Figure 5](Image)

**Figure 5.** The α2-adrenoceptor agonist, clonidine, does not appear to act as an unconditioned stimulus because no dose used induced learning. The β-adrenoceptor agonist, isoproterenol, however, was effective as an unconditioned stimulus vs. the nonlearning saline group. **P < 0.01. N values ranged from six to 10 pups for each group.

![Figure 6](Image)

**Figure 6.** A simplified schematic showing the proposed direct and indirect influence of β1- and α1-adrenoceptors on mitral and granule cells in the olfactory bulb. The potential influence of the adrenoceptors as unconditioned stimuli on cAMP cascades in mitral cells is also illustrated. β1-Adrenoceptors act directly on mitral cells to increase cAMP, while α1-adrenoceptors may act indirectly via granule cells and GABA_A activation. α1-Adrenoceptors may also act directly on mitral cells to activate cAMP (see arrow), but the second messenger cascades are more likely through PKC or MAPK pathways.


Arnsten, A.F. and Leslie, F.M. 1991. Behavioral and receptor binding lus. These results also demonstrate that α1-, but not α2-, adrenoceptor activation may mediate a noradrenergic learning signal in the olfactory bulb.

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