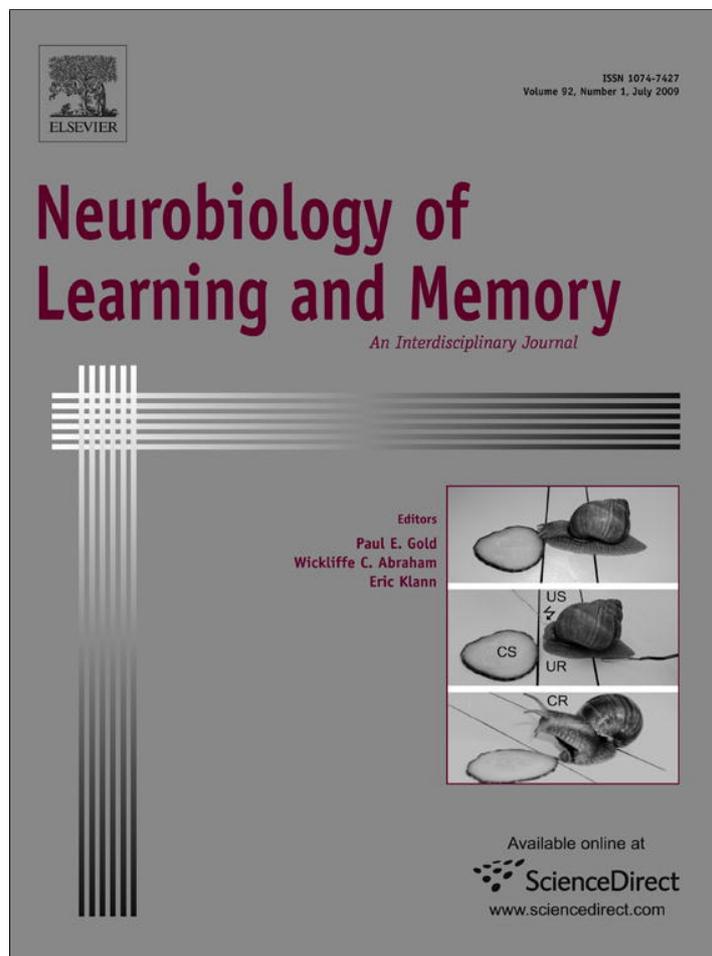


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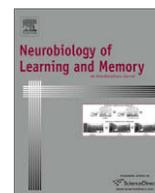
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A phosphodiesterase inhibitor, cilomilast, enhances cAMP activity to restore conditioned odor preference memory after serotonergic depletion in the neonate rat

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ABSTRACT

In various learning and memory models, preventing the breakdown of cyclic adenosine monophosphate (cAMP) by using a phosphodiesterase (PDE) inhibitor promotes memory. In the rat pup odor preference learning model serotonin, acting through 5-HT_{2A/C} receptors, has been shown to influence cAMP levels in the olfactory bulb initiated by β-adrenoceptor activation, as also seen in the neocortex. Since depletion of olfactory bulb serotonin prevents learning in the rat pup odor preference model, we ask whether a PDE inhibitor could restore that learning and also examined the influence of these manipulations on the temporal bulbar cAMP signal associated with successful learning. In this study, we found that a PDE4 inhibitor overcame learning deficits seen 24 h after a 10 min training trial on postnatal day 6 using the β-adrenoceptor agonist, isoproterenol as the unconditioned stimulus. We found in a previous study, that use of a PDE4 inhibitor during learning in normal pups extended memory to more than 48 h. However, in the present study the PDE4 treatment did not enable this memory extension in 5-HT depleted pups. An increase in the cAMP signal at the end of the 10 min training trial occurred in the presence of the PDE4 inhibitor. Such a cAMP increase has been associated with successful learning and is normally absent with bulbar 5-HT depletion. These results suggest PDE4 inhibitors may be useful therapeutically in disorders associated with reductions in serotonergic function.

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1. Introduction

Serotonin (5-HT) is involved in a wide variety of functions in the brain ranging from mood, to pain, to memory modulation. Loss of cortical 5-HT axons in development has been hypothesized to underlie autism (Whitaker-Azmitia, 2001, 2005). A better understanding of the role of 5-HT in developing animal models could lead to novel therapeutic approaches to clinical conditions associated with serotonin disruption.

Odor preference learning in the rat pup is disrupted by the loss of serotonin axons in the olfactory bulb, a simple cortical tissue. This paradigm provides a tool to assess behavioral deficits associated with early 5-HT loss and to explore mechanisms that remediate 5-HT loss-induced deficits. The model is relevant to neocortex because the olfactory bulb, although relatively simple, contains many of the same elements as the neocortex including glutamatergic output neurons (Liu, Crandes, Matute, Cuenod, & Streit, 1989) and noradrenergic (McLean & Shipley, 1991; McLean, Shipley, Nickell, Aston-Jones, & Reyher, 1989), serotonergic (Gomez et al.,

2007; McLean & Shipley, 1987a, 1987b) and cholinergic (Gomez et al., 2006; Le Jeune, Aubert, Jourdan, & Quirion, 1995) modulatory inputs.

In early odor preference learning, an unconditioned stimulus (US, e.g. tactile stimulation) is paired with a conditioned stimulus (CS, odor). Normally, pups learn to associate the odor with the tactile US (stroking the hind area which mimics the dam licking the pup) and 24 h later demonstrate a preference for the conditioned odor. Stroking activates noradrenergic neurons in the locus coeruleus (Nakamura, Kimura, & Sakaguchi, 1987). Excitation of these neurons leads to noradrenergic release in the olfactory bulb (Rangel & Leon, 1995) which, in turn, activates adrenoceptors, most notably via β-adrenoceptors (Harley, Darby-King, McCann, & McLean, 2006; Sullivan, Stackenwalt, Nasr, Lemon, & Wilson, 2000). Tactile stimulation of the pup, as the US, may be substituted by systemic (Sullivan, McLaugh, & Leon, 1991) or intrabulbar (Sullivan et al., 2000) injections of isoproterenol (ISO, β-adrenoceptor agonist) which has the benefit of titrating critical US levels. Pairing odor with 2 mg/kg ISO for 10 min during the first postnatal week, but not 1 mg/kg or 4 mg/kg doses, induces 24 h memory, thus displaying an inverted-U curve (Sullivan et al., 1991). We found, in the neonate rat, that bulbar 5-HT depletion (McLean, Darby-King, Sul-

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livan, & King, 1993) or intrabulbar infusion of a 5-HT₂ receptor antagonist (McLean, Darby-King, & Hodge, 1996) prevents normal early odor preference learning (McLean et al., 1993). The serotonergic deficit can be overcome by giving supra-optimal (6 mg/kg) ISO. Serotonin is not effective as an US on its own but appears to function by promoting and modulating learning induced by norepinephrine (Price, Darby-King, Harley, & McLean, 1998).

We have since found there is a specific relationship between 5-HT₂ receptors and β -adrenoceptors in bulbar mitral cells (Yuan, Harley, & McLean, 2003). A similar relationship has been shown in cells of other cortical systems since both 5-HT₂ receptors (Cornea-Hébert, Riad, Wu, Singh, & Descarries, 1999) and β -adrenoceptors (Gereau & Conn, 1994; Liu, Jia, Strosberg, & Cynader, 1993; Moudy & Schwartzkroin, 1992) have been localized in pyramidal cells. While β 1-adrenoceptors are directly coupled to cyclic adenosine monophosphate (cAMP) activation through adenylate cyclase (De Blasi, 1989), 5-HT_{2A} receptors do not directly engage the cAMP system (Mobley & Sulser, 1979) but enhance the β -adrenoceptors effect on cAMP in cortical slices (Morin, Sapena, Zini, & Tillement, 1992). We found that depletion of 5-HT input to the bulb, by itself, has no effect on basal cAMP levels but the presence of 5-HT increases the levels of cAMP generated upon activation of β 1-adrenoceptors (Yuan et al., 2003). The exact intracellular mechanism for this interaction is unknown. Despite the unknown mechanism, these findings support a priming role for 5-HT_{2A} receptor activation in cAMP mediated conditioned olfactory learning and support our memory model in which activation of the cAMP-protein kinase A (PKA)-cAMP response element binding protein (CREB) cascade in olfactory bulb mitral cells leads to long term odor preference memory (Yuan et al., 2003).

During normal learning, cAMP is degraded by phosphodiesterase 4 (PDE4) which, in effect, stops the catalytic portion of PKA from translocating to the nucleus to phosphorylate CREB and, presumably, ends further downstream transcription mediated by activated (phosphorylated) CREB (Kandel, Schwartz, & Jessel, 2000). A potential means of facilitating memory, successful in other models, is to prevent the breakdown of cAMP by using PDE4 inhibitors (Barad, Bourtchouladze, Winder, Golan, & Kandel, 1998; Bourtchouladze et al., 2003; Egawa et al., 1997; Imanishi et al., 1997; Randt, Judge, Bonnet, & Quartermain, 1982; Romano, Delorenzi, Pedreira, Tomsic, & Maldonado, 1996; Villiger & Dunn, 1981; Weishaar, Cain, & Bristol, 1985).

Given the association between 5-HT and NE in affecting cAMP levels in the olfactory bulb and neocortex, we hypothesized that deficits in 5-HT produced by the loss of 5-HT axons might be remediated by a PDE4 inhibitor that would enhance the normal effect of β 1-adrenoceptors on the cAMP temporally mediated signaling leading to learning (Cui, Smith, Darby-King, Harley, & McLean, 2007). We further hypothesize that PDE4 inhibition might prolong memory in the 5-HT depletion model as has been found with PDE4 administration in normal pups (McLean, Darby-King, & Harley, 2005). As predicted, PDE4 inhibition using cilomilast, overcomes bulbar 5-HT deficit by permitting cAMP changes in a learning-specific pattern. However, cilomilast only normalized, but did not prolong memory as it does in non-depleted pups. These results suggest PDE4 inhibition merits exploration as a therapeutic approach in disorders related to a loss of 5-HT inputs.

2. Materials and methods

2.1. Animals

Sprague-Dawley rat pups of both sexes were used in this study. The day of birth was considered to be postnatal day (PND) 0. All dams were housed and bred in 22 °C in polycarbonate cages (47 × 24 × 20 cm) containing hardwood chips at the Health Sciences

Centre of Memorial University of Newfoundland animal care facility. Food (Purina lab chow No. 2015) and water were available *ad libitum* while dams were maintained on a 12-h light/dark cycle (lights on at 8 a.m.). 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.

2.2. Serotonin depletions

On PND 1 or PND 2, pups were removed from the dam for either 5-HT depletion or sham depletion of the olfactory bulb. This approach of depleting serotonergic fibers specifically to the olfactory bulb produces no observable olfactory deficits, per se, or motor deficits while depleting 5-HT fibers specifically in the olfactory bulb (Langdon, Harley, & McLean, 1997; McLean & Darby-King, 1994; McLean et al., 1993). In brief, 40 min prior to the 5-HT depletion surgery, the norepinephrine uptake inhibitor, desipramine (0.2 mg/10 gm, Sigma Chem), was given intraperitoneally into each pup. Pups were anaesthetized for surgery by hypothermia and were kept on ice throughout the surgery. Depletion of olfactory bulb 5-HT fibers was accomplished by bilateral 5,7-dihydroxytryptamine (5,7-dHT, Sigma Chem, 150 nl in Ringer's solution with 0.2% ascorbic acid) injection into the anterior olfactory nucleus while only vehicle was injected into sham-operated pups. Toes were clipped for later identification. Surgery generally lasted 15–20 min and once the pup breathed regularly, showed healthy skin color and moved around alertly, it was returned to the dam.

2.3. Injection of drugs

Injection of drugs and training took place on PND 6. Each pup was removed from the dam individually and given ISO (1 or 2 mg/kg) or saline vehicle subcutaneously (s.c. 50 μ l) 40 min prior to odor conditioning. The animals were given ISO as a substitute for tactile stimulation (US) in order to take advantage of the inverted-U curve effect of the drug mentioned in the introduction. Pups were then returned to the nest until they were injected s.c. with cilomilast (3 mg/kg, a phosphodiesterase 4 inhibitor, kind gift from Greg Rose, Memory Pharmaceuticals) or the vehicle (5% dimethyl sulfoxide, DMSO) 30 min prior to training (see training below or as described previously) (McLean et al., 2005). The pups were once again returned to the nest with the dam until 10 min before training. At that time, pups were removed from the nest and placed alone on warm (27 °C) bedding for 10 min until training.

2.4. General training procedures

Pups were trained on PND 6. Training groups, drug administration and predicted behavior for each group are shown in Table 1. Only one male and one female were assigned to each group in order to avoid potential litter effects. In Experiments 1 and 3, training consisted of placing pups individually on peppermint-scented bedding (CS) for 10 min in a sound proof room set at 27 °C. The pups were returned to the dam immediately after training.

2.4.1. Experiment 1: 24 h memory groups

A total of 51 rat pups of both sexes, from 13 litters were used in this study. In each litter, there were four 5-HT depleted (two males and two females) and two shams (one male and female) assigned to three treatment groups (Table 1). The pups were distributed so that no more than one male and one female pup from each litter were in each training group. The control learning and non-learning groups were decided upon based upon previous studies that showed 5-HT depletions interrupt learning that can be overcome by higher than normal doses of isoproterenol (Langdon et al., 1997; Yuan, Harley, Bruce, Darby-King, & McLean, 2000).

Table 1

Timeline of training, testing and assays are presented (top) as well as the combination of training groups, drug groups and predicted behavioral results (bottom).

PND 1	PND 6			PND 7/8
	Training			Memory Testing
5-HT dep	ISO/veh 40 min	Cilo/veh 30 min	Odor ——— cAMP assay time points: 0–5–10–15–20 min	24/48 h
Investigation	5-HT dep	ISO (s.c.) dose	CILO (s.c.) dose	Expected odor memory?
Expts. 1 and 2 (24 h memory and cAMP assays)	veh 5,7-dHT 5,7-dHT	2 mg/kg 2 mg/kg 2 mg/kg	veh veh 3 mg/kg	Yes (control) No (control) ?? (Experimental group)
Expt. 3 (48 h memory)	veh veh 5,7-dHT 5,7-dHT	2 mg/kg 1 mg/kg 2 mg/kg 2 mg/kg	veh 3 mg/kg vehicle 3 mg/kg	No (control) Yes (control) No (control) ?? (Experimental group)

Abbreviations: 5-HT dep, depletion of serotonin to the olfactory bulb; 5,7-dHT, 5,7-dihydroxytryptamine; Cilo, cilomilast; ISO, isoproterenol; PND, postnatal day; s.c., subcutaneous injection; veh, vehicle.

2.4.2. Experiment 2: cAMP sampling

A total of 93 rat pups of both sexes from 12 litters were used in this study. From each litter a total of approximately 7–10 pups were used for olfactory bulb sample collection across three surgery/drug administration conditions and 5 time points. Littermates were trained and tested for 24 h memory and the results of the testing were added to the 24 h testing group of Experiment 1. The three surgery/drug administration conditions are shown in Table 1. Pups were distributed among training groups so that no two pups from the same litter were placed in the same group in order to obviate potential litter effects. The time points included: immediately prior to 10 min odorized bedding training, 5 min after odor training initiation, at cessation of odor training, and 5 min or 10 min following odor training completion. Pups were decapitated at the appropriate time point and the olfactory bulbs immediately removed and flash frozen on dry ice. Frozen paired olfactory bulbs were subsequently stored at -80°C in 1.5 ml Eppendorf tubes until cAMP assays were performed.

2.4.3. Experiment 3: 48 h memory groups

A total of 79 rat pups of both sexes, from 11 litters were used in this study. In each litter there were four 5-HT depleted (two males and two females) and four shams (two males and two females) assigned to four treatment groups (Table 1). The number of male and female pups was balanced for all groups. The groups were chosen based upon previous study that showed combinations of ISO and cilomilast that produce prolonged (>24 h) memory (McLean et al., 2005) and previous studies that showed bulbar 5-HT depletions disrupt learning (Langdon et al., 1997; McLean et al., 1993). Briefly, when cAMP breakdown is attenuated with cilomilast (1 mg/kg), learning occurs with a normally subthreshold dose of ISO (1 mg/kg) (McLean et al., 2005). Learning is prevented with a normally learning-optimal dose of ISO (2 mg/kg) paired with cilomilast (McLean et al., 2005). In addition, a 1 mg/kg dose of ISO accompanied by 3 mg/kg of cilomilast has been observed to lead not only to memory formation, but to prolongation of memory from 24 h to at least 96 h (McLean et al., 2005). It is important to note the necessity of $\beta 1$ -adrenoceptor activation of cAMP in this early odor preference learning as cilomilast administration without ISO fails to result in learning; cilomilast cannot function as an independent unconditioned stimulus (McLean et al., 2005). Following drug administration, 10 min odor conditioning was performed as described for 24 h training.

2.5. Testing procedures

On PND 7 or 8, pups were given an odor preference test in a quiet room at 27°C as described previously (McLean et al., 2005;

Price et al., 1998). Briefly, a stainless steel testing box ($36 \times 20 \times 18$ cm) with a mesh bottom was centered over two trays. The trays were 2 cm apart, creating a neutral zone in the center. One tray contained 500 ml of fresh bedding while the other contained 500 ml of peppermint-scented bedding prepared at the same concentration used during training. The tester was blind to the previous training procedure given to the pup.

Each pup underwent five 1-min trials, starting in the neutral zone and alternately facing towards or away from the tester with each subsequent trial. When the pup's snout and one paw moved from the neutral zone to either the peppermint or control zone, a timer for that side was started. Pups were given thirty second rest periods between trials. Summation of the time spent over the peppermint side divided by the total time active (time spent over peppermint plus time spent over control) gave the percent time over peppermint. Thus, preference for the conditioned odor was measured by the percent of time spent by the pup over the peppermint odor. We note that pups normally do not like the smell of peppermint odor so non-learning controls generally spend 30–40% of the time over the conditioned peppermint odor.

2.6. cAMP assays

Procedures for cAMP assays were described previously (Cui et al., 2007). In brief, olfactory bulbs were homogenized in 250 μl 5% trichloroacetic acid (TCA) on ice using a tissue grinder. Precipitates were removed by centrifuging at 1500g for 10 min at 4°C . The supernatant solution was transferred to a clean centrifuge tube, while the pellet was kept for protein determination. The TCA was extracted from the supernatant with 5 volumes of water-saturated ether. The residual ether was removed by heating the supernatant to 70°C for 5 min. The cAMP in the supernatant was measured using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The protein pellet was reconstituted with 800 μl of dH_2O and the protein content was assayed by Bicinchoninic Acid protein assay Kit (Pierce, Rockford, IL). The cAMP content was normalized by protein content in each sample and expressed as pmole/mg protein.

2.7. Immunohistochemistry

Immunohistochemistry of 5-HT in the olfactory bulb was used to confirm 5-HT depletions as described previously (Langdon et al., 1997; McLean et al., 1993). The immunohistochemistry was performed on pups following 24 h or 48 h testing and on littermates of pups sacrificed for cAMP assays. Briefly, pups were decap-

itated following behavior testing and the brains were removed from the skull and fixed immediately in 4% paraformaldehyde. The brains were then left overnight (4 °C) in 20% sucrose/0.1 M phosphate buffer solution. The following day, the olfactory bulbs and anterior olfactory nuclei were cut frozen at 30 μ m using a cryostat. Antibodies to 5-HT (1/1500 dilution, DiaSorin, Stillwater, MN) and tyrosine hydroxylase (1/2500 dilution, CalBiochem, San Diego, CA) were put onto the slides overnight. The tyrosine hydroxylase antibody was utilized in order to determine if putative noradrenergic axons (from the locus coeruleus) and intrinsic dopaminergic periglomerular cells were affected by 5-HT depletions. Visualization of the antibodies utilized the Vectastain ABC technique (Vector Labs, Mississauga, Ont) and diaminobenzidine.

2.8. Statistics

For analysis of behavior, one-way analysis of variance (ANOVA) was used followed by post-hoc Dunnett's test. For cAMP assay analysis, two-way ANOVA was used. Least significant difference was used for two-way ANOVA post-hoc analysis

3. Results

3.1. Experiment 1. PDE4 inhibition overcomes 5-HT depletion to preserve 24 h memory

Testing results for the three training groups at 24 h memory are shown in Fig. 1. One-way ANOVA statistics showed a significant group effect ($F_{(2,48)} = 8.051$; $p = 0.001$). Dunnett's post-hoc analysis revealed that the 5-HT depleted + 2 mg/kg ISO group was significantly different ($p < 0.01$) from the learning control group that showed a preference for the conditioned odor. Thus, the 5-HT depleted group did not show a preference for the peppermint odor which was in keeping with results from a previous study that showed a 5-HT depletion induced learning deficit specifically related to the olfactory bulb (Langdon et al., 1997). Finally, and importantly, post-hoc analysis showed that the experimental group (5-HT depletion + 2 mg/kg ISO + 3 mg/kg cilomilast) was not significantly different from the learning control group, indicat-

ing that the PDE4 inhibitor was able to overcome or remediate the bulbar 5-HT depletion effect.

3.2. Experiment 2. cAMP changes associated with PDE4 inhibition and rescue of memory

The present study investigated the temporal pattern of cAMP activity in subjects depleted of serotonergic input to the olfactory bulb and administered a 2 mg/kg dose of ISO in addition to a 3 mg/kg dose of the phosphodiesterase inhibitor cilomilast. As demonstrated above (Fig. 1), this combination of surgery/drug administration leads to conditioned odor preference learning at 24 h in 5-HT depleted pups. Littermates of pups used for cAMP assays in this portion of the study showed odor preference learning and those results were included with the behavioral data described above. The cAMP activity pattern (Fig. 2) of the experimental group in this study (5-HT depletion + 2 mg/kg ISO + 3 mg/kg cilomilast) was studied in comparison to that of two other groups: a learning control (sham depletion + 2 mg/kg ISO) and a non-learning control (5-HT depletion + 2 mg/kg ISO) group (Langdon et al., 1997; McLean et al., 1993). A two-way ANOVA showed a significant drug effect ($F_{(2,78)} = 6.91$). Post-hoc least significant difference analysis showed, at 15 min, the cilomilast-treated pups were significantly different from both the sham ($p = 0.03$) and 5-HT depleted pups ($p = 0.02$). The cAMP activity pattern observed in the learning control (Fig. 2) was quite similar to that observed in previous learning conditions (Cui et al., 2007) which showed a peak in cAMP expression at the end of the 10 min odor exposure. However, in the present study, this peak at 10 min was not quite significant for the cilomilast treated ($p = 0.09$) or the sham treated ($p = 0.18$) groups versus the 5-HT depleted non-learning group.

3.3. Experiment 3. PDE4 inhibition does not facilitate prolonged 48 h memory in 5-HT depleted pups

Groupings of pups for observation of odor preference memory at 48 h were slightly different from groupings chosen at 24 h mem-

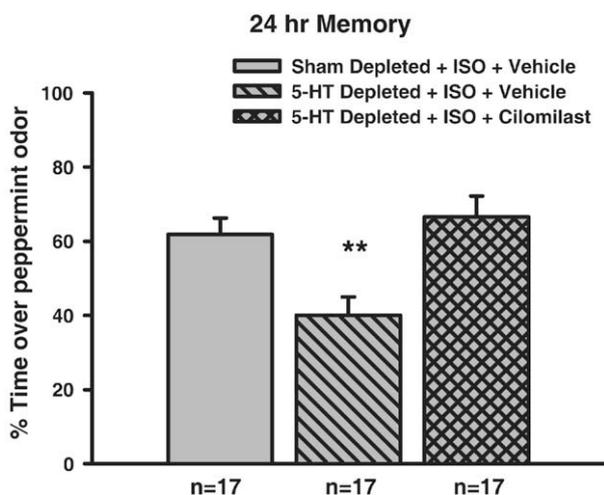


Fig. 1. 24 h memory test showing the mean percentage of time spent over odor by subjects in each of three surgery/drug conditions. Rat pups that underwent sham surgery + saline administration on PND 6, and subjects which underwent 5-HT depletion + 3 mg/kg cilomilast administration on PND 6 showed an odor preference on PND 7, while 5-HT depleted + saline subjects failed to do so. All subjects received 2 mg/kg of isoproterenol (ISO). Abbreviations: Vehicle = 5% dimethyl sulfoxide. * $p < 0.05$ and ** $p < 0.01$.

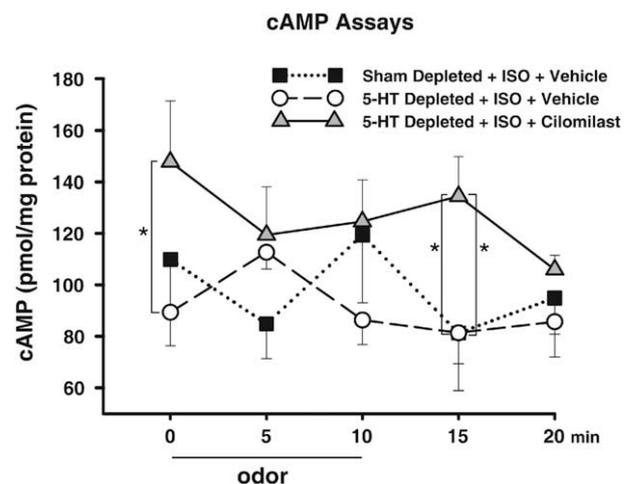


Fig. 2. cAMP levels were measured by enzyme immunoassay. Sham surgery followed by odor exposure paired with a 2 mg/kg dose of isoproterenol (ISO) showed a general trend toward a transient peak in cAMP activity at 10 min. 5-HT depleted subjects that subsequently received a 2 mg/kg dose of ISO showed a trend toward no difference in cAMP levels from baseline throughout and following the training procedure. 5-HT depleted subjects that received 2 mg/kg of ISO in addition to 3 mg/kg of cilomilast exhibited a trend toward an initially elevated level of cAMP which lowered and remained stable from the 5 to 15 min time points and declined once more at the 20 min time point. Each point represents values from five to seven pups. Error bars are standard error of the mean. * $p < 0.5$

ory because past research indicated a need for different combinations of PDE4 inhibitor and ISO in order to prolong memory past 24 h. Specifically a normally subthreshold ISO dose gives rise to prolonged memory when paired with a PDE4 inhibitor. Since 5-HT depletion results in 2 mg/kg ISO dose being subthreshold, the appropriate comparisons involve 1 mg/kg ISO dose in normal pups and 2 mg/kg ISO dose in depleted pups. Fig. 3 shows the results of the different groupings. One-way ANOVA of the various training groups analyzed for memory at 48 h showed a significant group effect ($F_{(3,75)} = 3.354$; $p = 0.0233$). Dunnett's post-hoc test showed that only the learning control group (sham depleted + 1 mg/kg ISO + 3 mg/kg cilomilast) was significantly different from the non-learning control of 2 mg/kg ISO ($p < 0.05$). The 2 mg/kg ISO group was non-learning consistent with previous research showing that 2 mg/kg ISO 10 min training results in 24 but not 48 h memory (McLean et al., 2005). At 48 h post-training, neither the 5-HT depleted group nor the 5-HT depleted/cilomilast-treated group was different from the non-learning control (Fig. 3).

3.4. Verification of 5-HT depletion specificity

Previous studies have shown by quantitative analysis that the 5,7-dHT treatment consistently depletes the olfactory bulbs of about 85% 5-HT axons in the bulb without affecting 5-HT in other areas or other relevant transmitters such as norepinephrine (McLean & Darby-King, 1994; McLean et al., 1993). The present study utilized qualitative analysis to confirm 5-HT depletion and the lack of effect on noradrenergic/dopaminergic fibers. The olfactory bulbs of pups given the 5,7-dHT treatment were shown to have consistent 5-HT depletion while intrinsic dopaminergic periglomerular cells or putative noradrenergic fibers were at levels equal to sham controls (Supplemental Fig. S1) which replicated our previous findings (Langdon et al., 1997; McLean & Darby-King, 1994; McLean et al., 1993).

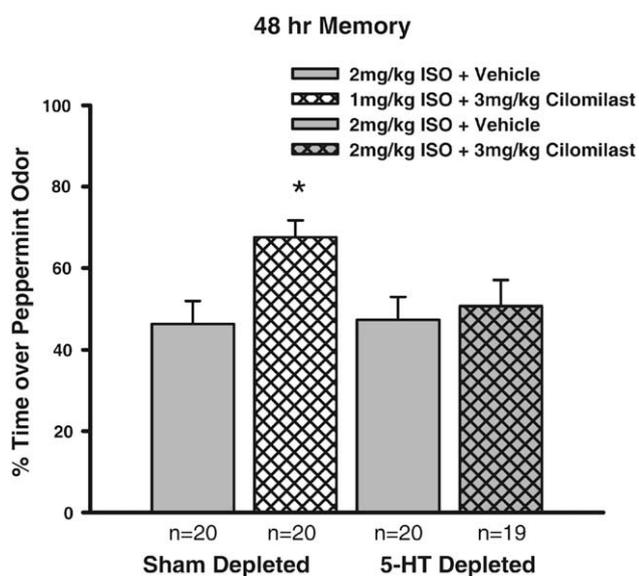


Fig. 3. 48 h memory test showing the amount of time spent over the peppermint odor compared to amount of time over normal scented bedding for the different treatment groups. By 48 h, 5-HT sham depleted pups given the 2 mg/kg ISO paired with odor at training no longer display memory that was observed at 24 h (Fig. 1) while sham depleted pups given PDE4 inhibition (cilomilast) at training do show preference memory. The 5-HT depleted experimental group given cilomilast did not show retention of odor preference memory, in contrast to memory observed under the same conditions when tested at 24 h (Fig. 1).

4. Discussion

In the present study, the effect of a PDE4 inhibitor, cilomilast, was investigated in an animal model with a selective surgically-induced loss of serotonergic innervation in the olfactory bulb. In the presence of cilomilast, a dose of ISO (2 mg/kg) that was shown previously (Langdon et al., 1997) to be insufficient for learning in the absence of 5-HT innervation, became sufficient. Through its inhibition of PDE4, cilomilast reduces the breakdown of cAMP and thereby increases either the level (Cui et al., 2007), timing, or duration of the cAMP signal produced upon β -adrenoceptor activation.

The present study showed a trend, although not significant, toward a spike in cAMP activity at the 10 min time point after commencement of training. This is in keeping with previous experiments in which cAMP levels peaked in learning groups 10 min after training commencement (Cui et al., 2007). The PDE4 inhibition by cilomilast in the present study seemed to have caused a general increase in cAMP level up to 15 min after training commencement before levels decreased again. In addition, the sustained low-level pattern for cAMP observed for the non-learning control group (5-HT depletion + 2 mg/kg ISO) was similar to other low-level stimulation, non-learning conditions, including, previously, a 1 mg/kg ISO only condition (Cui et al., 2007).

The importance of cAMP activity at the 10 or 15 min time point has been hypothesized to be related to the timing of cAMP increases in relation to other intracellular events (Cui et al., 2007). The timing of cAMP peaks appears critical for the recruitment of downstream substrate phosphorylation, which in this model includes the phosphorylation of CREB. Maximal CREB activation generally occurs at the 20 min time point (i.e. 10 min after odor training) making cAMP activity at the 20 min point less likely to exert a critical effect on long term memory. At this time, we do not know what variation in the effects of cilomilast account for the prolonged memory in normal pups.

4.1. 5-HT modulation of cAMP

Of importance for the elucidation of serotonergic influence in olfactory learning is a mechanistic understanding of the 5-HT_{2A} receptor, and the potential way(s) in which 5-HT modulates cAMP concentrations. The β -adrenoceptor is coupled, through a stimulatory G-protein (Gs), to adenylate cyclase (De Blasi, 1989) and, thus, stimulation of the receptor should result in increased cAMP concentrations. Studies have shown that there is considerable "cross-talk" between the phospholipase C (PLC) transduction system, known to be coupled to the 5-HT₂ receptor, and the adenylate cyclase signal transduction systems (Bell & Brunton, 1987; Katada, Gilman, Watanabe, Bauer, & Jakobs, 1985; Sibley, Jeffs, Daniel, Nambi, & Lefkowitz, 1986; Sibley, Nambi, Peters, & Lefkowitz, 1984; Yoshimasa, Sibley, Bouvier, Lefkowitz, & Caron, 1987a; Zifa & Fillion, 1992).

How is such cross-talk mediated? Phospholipase C acts by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. IP₃ releases Ca²⁺ from intracellular stores while diacylglycerol mediates the activation of protein kinase C (PKC). A recent study using mice has shown abundant labeling of the PKC isozyme PKC η 2 in specific brain regions including the olfactory bulb (Nakahara et al., 2005). In experiments using a cell line derived from the rat cortical neuron, which shows a high density of 5-HT_{2A} receptors, 5-HT was found to increase hydrolysis of phosphatidylinositol (PI), increase intracellular Ca²⁺ concentrations, and increase the activity of PKC (Berg et al., 1994). Additionally, all of the above were eliminated by low concentrations of ketanserin, a high-affinity antagonist to the 5-HT_{2A} receptor, indi-

cating that the observed effects were due to 5-HT_{2A} binding and not 5-HT_{2B} or 5-HT_{2C} (Berg et al., 1994).

Serotonin receptors in the mitral cell of the olfactory bulb coupled to PLC could potentiate cAMP production by β -adrenoceptor activation in a number of ways. Protein kinase C phosphorylation of adenylyl cyclase could directly increase adenylyl cyclase activity when stimulated by β -adrenoceptor activation as proposed in the frog erythrocyte membrane (Yoshimasa, Sibley, Bouvier, Lefkowitz, & Caron, 1987b). Elevated intracellular Ca²⁺ activation of calmodulin and subsequent binding of Ca²⁺/calmodulin to adenylyl cyclase could prime the enzyme for cAMP production. Studies on olfactory type III AC have shown stimulation of that enzyme by Ca²⁺/calmodulin when it was concomitantly activated by another effector (Choi, Xia, & Storm, 1992). Protein kinase C may also phosphorylate the G-protein of the β -adrenoceptor, causing an increase in its affinity for adenylyl cyclase. In keeping with observations to date (Morin et al., 1992; Price et al., 1998), these mechanisms would not activate adenylyl cyclase production of cAMP in the absence of β -adrenoceptor activation, but would increase β -adrenoceptor mediated cAMP levels.

While the 5-HT_{2A}/ β -adrenoceptor mediated changes in cAMP levels are given above as possible mechanisms leading to learning, the case is not proven. For example, the alterations in cAMP induced by the PDE inhibitor may have compensated for a cAMP-independent effect of 5-HT_{2A} mediated via its more commonly recognized target PKC (Kramer, Poblete, & Azmitia, 1997; Sossin & Schwartz, 1992).

4.2. PDE4 inhibition in other models of memory disruption

The present study, showing a beneficial effect of PDE4 inhibition on memory in a 5-HT interrupted system, is similar to the results described recently in which the PDE4 inhibitor Rolipram overcame object memory impairments in a tryptophan depletion model (Rutten, Lieben, Smits, & Blokland, 2007). A difference between that study and ours was that our associative odor memory study required at least some noradrenergic activation while in the Rutten study, there was no direct manipulation of the noradrenergic system. Nonetheless, novelty is known to enhance norepinephrine release and locus coeruleus activation (Sara, Vankov, & Hervé, 1994) and it is possible that a similar mechanism to that revealed here mediate these recent effects. The authors however suggest that the PDE4 inhibition effect in their study was due to increased 5-HT release. This is unlikely to be the case in the present study due to the virtual total loss of 5-HT axons.

Although PDE4 inhibition has been used successfully in animal models to enhance certain types of memory even in aged animals (Barad et al., 1998), there are caveats. For example, PDE4 inhibition may disrupt working memory mediated in the prefrontal cortex, possibly by producing too much PKA activity in the cAMP-PKA-CREB cascade (Ramos et al., 2003). Results may vary with PDE4 inhibition depending upon the US in some models of learning (Thompson, Sachs, Kantak, & Cherry, 2004). Therefore, use of PDE4 inhibitors appears to be a valuable memory enhancer depending upon the type of memory examined.

5. Conclusions

If PDE4 inhibition is to be a successful clinical treatment for cognitive dysfunction, the complex intracellular pathways involved in learning and the multiplicity of influences upon cAMP must be elucidated. Without a complete understanding of the possible implications, administration of PDE4 inhibitors could have unforeseen complications. The 5-HT depletion model of learning disruption

used here lends itself to many experimental analyses. The present study demonstrates that, in the 5-HT depleted neonatal rat olfactory bulb, the PDE4 inhibitor cilomilast allows learning in conjunction with β -adrenoceptor activation by ISO and restores evoked cAMP signaling to more normal levels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nlm.2009.02.003.

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