A role for the anterior piriform cortex in early odor preference learning: evidence for multiple olfactory learning structures in the rat pup

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Morrison GL, Fontaine CJ, Harley CW, Yuan Q. A role for the anterior piriform cortex in early odor preference learning: evidence for multiple olfactory learning structures in the rat pup. J Neurophysiol 110: 141–152, 2013. First published April 10, 2013; doi:10.1152/jn.00072.2013.—cFos activation in the anterior piriform cortex (aPC) occurs in early odor preference learning in rat pups (Roth and Sullivan 2005). Here we provide evidence that the pairing of odor as a conditioned stimulus and β-adrenergic activation in the aPC as an unconditioned stimulus generates early odor preference learning. Enhancement of aPC cAMP response element-binding protein (CREB) phosphorylation in trained hemispheres is consistent with a role for this cascade in early odor preference learning in the aPC. In vitro experiments suggested theta-burst-mediated long-term potentiation (LTP) at the lateral olfactory tract (LOT) to aPC synapse depends on N-methyl-D-aspartate (NMDA) receptors and can be significantly enhanced by β-adrenoceptor activation, which causes increased glutamate release from LOT synapses during LTP induction. NMDA receptors in aPC are also shown to be critical for the acquisition, but not expression, of odor preference learning, as would be predicted if they mediate initial β-adrenoceptor-promoted aPC plasticity. Ex vivo experiments 3 and 24 h after odor preference training reveal an enhanced LOT-aPC field excitatory postsynaptic potential (EPSP). At 3 h both presynaptic and postsynaptic potentiations support EPSP enhancement while at 24 h only postsynaptic potentiation is seen. LOT-LTP in aPC is excluded by odor preference training. Taken together with earlier work on the role of the olfactory bulb in early odor preference learning, these outcomes suggest early odor preference learning is normally supported by and requires multiple plastic changes at least at two levels of olfactory circuitry.

RAT PUPS BORN BLIND AND DEAF readily acquire a preference for an odor paired with unconditioned stimuli (US) that mimic maternal care (Sullivan and Hall 1988) or that activate the noradrenergic locus coeruleus system (Sullivan et al. 2000). A single 10-min pairing of a novel odor (to-be-conditioned stimulus or CS) and a US produces an odor preference memory lasting at least 24 h (Sullivan and Leon 1987; Sullivan and Wilson 2003). Such a preference assists the pup in maintaining proximity to the dam and should enhance survival. Human infants demonstrate similar early odor preference learning (Delaunay-El Allam et al. 2010).

The mechanisms underlying this form of learning have been extensively studied in the olfactory bulb. Blocking β-adrenoceptors in the bulb (Sullivan et al. 2000) or blocking N-methyl-D-aspartate (NMDA) receptors (NMDARs) in the bulb (Lethbridge et al. 2012) before training prevents learning but does not prevent expression of an already acquired preference. The β-adrenoceptor agonist isoproterenol infused directly into the bulbs before novel odor exposure produces learning (Lethbridge et al. 2012; Sullivan et al. 2000). These pharmacological results suggest plasticity of the odor representation in the bulb is both necessary and sufficient for early odor preference learning. The bulbar cellular changes associated with learning suggest that increased cAMP response element-binding protein (CREB) phosphorylation in mitral cells (McLean et al. 1999) and synaptic changes in mitral cell connections (Lethbridge et al. 2012), such as an increase in AMPA receptor (AMPAR) responses in the area of the odor representation (Yuan and Harley 2012), are among the modifications that support early odor preference learning.

Odor information from the olfactory bulb is transmitted over the lateral olfactory tract (LOT) to the anterior piriform cortex (aPC), among other sites (Isaacson 2010). The aPC has spatially diffused odor representations (Ilg and Haberly 2003; Poo and Isaacson 2009; Stettler and Axel 2009), in contrast to those seen in the olfactory bulb (Takahashi et al. 2004; Wachowiak and Cohen 2001). The more diffused piriform representation is driven by LOT activity from olfactory bulb “hot spots.” Roth and Sullivan (2005) have shown increased cFos activation in both the olfactory bulb and the aPC, following odor preference training in rat pups, but a mechanistic study of the role of aPC in early odor preference learning has not been carried out.

In the present study we target the aPC using the technique of bilateral local infusions to assess its role in early odor preference learning. Follow-up experiments examine in vitro analogs of learning in an LOT-aPC slice preparation. In the last set of experiments, unilateral nostril occlusion during training is used to confine learning to one hemisphere (Kucharski and Hall 1987; Kucharski et al. 1986; Yuan and Harley 2012) and ex vivo studies of aPC slices examine the changes in LOT-evoked field excitatory postsynaptic potentials (EPSPs) induced at 3 or at 24 h following training when odor preferences are readily demonstrable (Grimes et al. 2011).

MATERIALS AND METHODS

Animals and Ethics Statement

All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland.
with adherence to the guidelines set by the Canadian Council on Animal Care. Male and female Sprague-Dawley rat pups (Charles River) were used in this study. Animals were bred and pups were born onsite at the research facility. Litters were culled to 12 pups with equal numbers of males and females on postnatal day 1 (PD1; day of birth is designated PD0). Dams were maintained under a 12-h reverse light-dark cycle with ad libitum access to food and water.

**Behavioral Studies**

Behavioral experiments were carried out in a temperature controlled room at ~28°C and followed the standard protocols previously established for early odor preference learning (McLean et al. 1999; Sullivan and Leon 1987) as described below. One-way ANOVAs and post hoc Fisher tests were used to determine statistical significance throughout the experiments.

**Odor preference training and testing.** On PD7, pups were assigned to an odor plus stroking (O/S) or an odor only (O/S-1) condition. Pups were removed from the nest and placed on normal, unscented bedding for 10 min. After habituation, pups receiving conditioning training (O/S+) were placed on peppermint-scented bedding (0.3 ml of peppermint in 500 ml of bedding) and vigorously stroked with a paintbrush for 30 s, followed by a 30 s rest, for a total of 10 min. Pups in the nonlearning condition (O/S-) were placed in peppermint-scented bedding for 10 min following the habituation period. These pups were not stroked. Once odor conditioning was complete, pups were returned to the dam.

On PD8, pups were tested for odor preference memory using a two-choice odor preference procedure. The testing apparatus was a stainless steel chamber (30 x 20 x 18 cm) placed over two testing boxes. One box contained peppermint-scented bedding and the other contained normal, unscented bedding. Testing boxes were separated by a 2-cm neutral zone. For testing, pups were removed from the dam and placed in the neutral zone. Pups moved freely for 1 min, while the time spent over each testing box was measured, and were then removed from the test chamber for 1 min. This was repeated for a total of five trials. The average time spent over peppermint bedding was calculated for each pup. Direction preference was eliminated by alternating the orientation of the starting position of the pups in the neutral zone.

**Cannula implantation surgery.** On PD6 pups were anesthetized via hypothermia and placed in a stereotaxic apparatus in a skull flat starting position. A horizontal incision was made just posterior to the eyes so that bregma was visible. Two small holes were drilled ~2.5 mm anterior and 3.0–3.25 mm bilateral with respect to bregma. Two guide cannulae (Vita Needle, MA) with insect pins were lowered from the home cage. The cannulae, which were cleared of any debris from the home cage, were removed from the dam, and insect pins were removed from the skull with dental acrylic (Lang Dental). Two sutures were made on either side of the cannula, and pups were placed on warm bedding to recover before being placed back with the dam.

**Intracerebral infusions.** Twenty-four hours following surgical cannula implantation, pups underwent odor training. Pups receiving infusions were removed from the dam, and insect pins were removed from the cannulae, which were cleared of any debris from the home cage. The pups were then placed on plastic hexagonal weighing dishes where they were infused with 1 μl of the desired solution at a rate of 0.25 μl/min. Infusions were followed by 6 min of rest to allow for diffusion of the solution into the brain. Pups were then returned to the dam for 10 min before training or testing. Following testing, pups received intracranial infusions of 4% methylene blue dye (Fisher Scientific) and were killed, and the brains were collected and sliced to confirm cannula placement.

Pups with misplaced cannula were excluded from data analysis.

**Pharmacological agents.** Lidocaine-hydrochloride (4%, dissolved in phosphate buffered saline; Sigma-Aldrich), a reversible sodium channel blocker; muscimol (50 mM, dissolved in saline; Sigma-Aldrich), a GABA<sub>A</sub> agonist; propranolol hydrochloride (100 μM, dissolved in saline; Sigma-Aldrich), a β-adrenoceptor antagonist; various concentrations of the β-adrenoceptor agonist isoproterenol (5 μM, 50 μM, or 500 μM; dissolved in saline; Sigma-Aldrich); and D-(-)-2-amino-5-phosphonopentanoic acid (D-APV), an NMDAR antagonist (100 μM, dissolved in saline; Tocris), were infused into the aPC at various time points as described in Results. Saline infusions were used in control animals.

**Reversible nostril occlusion for ex vivo experiments.** Nose plugs were constructed using polyethylene 20 (PE 20) tubing, silk surgical thread, and human hair, as per procedures described previously (Cummings et al. 1997). To insert the plug, a small dab of a sterile local anesthetic, 2% Xylocaine (AstraZeneca), a lidocaine hydrochloride jelly, was applied to the left nostril. Pups were given one min to rest before the plug was gently inserted in the left nostril. After nostril plug insertion pups were placed on unscented bedding to habituate to the nose plug, followed by appropriate odor conditioning training.

**pCREB Immunohistochemistry**

pCREB immunohistochemistry was performed in two sets of experiments: first, following unilateral infusion of lidocaine into the aPC to test the silencing effect of lidocaine on the pyramidal cells (Fig. 1, A and D); second, following odor preference training to test the activation level of the pyramidal cells in the aPC (Fig. 2).

**Brain tissue collections.** **TRANSIENT SILENCING USING LIDOCAINE.** On PD6 or 7, following hypothermia and stereotaxic surgeries, pups were infused with methylene blue dye (4%, Fisher Scientific) in one hemisphere and lidocaine (4%, dissolved in methylene blue solution, Fisher Scientific) in the other hemisphere. One microliter of dye solution was slowly administered into one hemisphere by gently pushing the syringe, while 1 μl of lidocaine solution was administered into the opposite hemisphere. After each application, the syringe was held in place for 30 s to ensure solution diffusion into the tissue. Pups were placed on warm bedding to recover for 20–30 min from the time of drug infusion. Animals were placed in a plastic container with pure peppermint oil soaked in a piece of tissue for 10 min. Pups were anaesthetized with chloral hydrate intraperitoneally (400 mg/kg; Fisher Scientific) and perfused transcardially with saline (0.9%, 300 ml) followed by paraformaldehyde (4%, dissolved in 0.1 M PBS). Brains were collected and placed in paraformaldehyde overnight at 4°C. The following day, the tissue was transferred to a sucrose solution (20%) for an additional 24 h.

**PYRAMIDAL CELL ACTIVATION FOLLOWING ODOR PREFERENCE LEARNING.** Pups with a single nostril occluded underwent either O/S+ or O/S- training. After training, the pups were returned to normal bedding for an additional 10 min before perfusion for brain collection as described above.

**Immunohistochemistry.** For slicing, brains were mounted using Cryomatrix fixative (Thermo Scientific) and immediately placed in a cryostat at ~20°C until fully frozen. Thirty-micrometer coronal sections were cut and two slices were collected every 200 μm and were mounted on chrome-gelatin coated slides in an alternating fashion, allowing for Nissl comparison staining. Following slicing, slides were kept at 4°C for 10 min before being brought to room temperature to dry. A pCREB antibody (1:100; Cell Signaling) was applied to the alternate slides. The antibody was dissolved in phosphate buffered saline with 0.2% Triton-X-100, 0.02% sodium azide, and 2% normal goat serum and left on sections overnight at 4°C in a humidified chamber. The following day, the slices were washed with PBS and a biotinylated anti-rabbit secondary antibody solution (Vectastain) was applied to the slides, followed by an avidin and biotinylated (A+B) enzyme amplification step. Finally, sections were stained using a diaminobenzidine tetrahydrochloride (DAB) reaction (50 mg DAB (Amresco); dissolved in 50 ml 0.1 M PBS, 50 ml dH<sub>2</sub>O), and 30 μl 30% H<sub>2</sub>O<sub>2</sub>), which was monitored for completion under an upright light microscope (Olympus). Slides were then dehydrated and coverslipped. Hemispheric sections on each slide were compared under the microscope to confirm there was less pCREB expression in the lidocaine-injected hemisphere.
Tissue preparation and extracellular recording. Animals were anesthetized with halothane inhalation and quickly decapitated. Brain tissue was extracted and placed in an ice-cold high glucose artificial cerebrospinal fluid (aCSF; in mM: 83 NaCl, 2.5 KCl, 0.5 CaCl₂, 3.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, and 72 sucrose, equilibrated with 95% O₂-5% CO₂) for 10 min. Sagittal slices (400 μm) were cut using a vibrating blade (Vibratome 1000P; Leica Microsystems) and were incubated in an Isotemp 205 chamber (Fisher Scientific) at 34°C in the aforementioned solution for at least 60 min before use. Tissue slices were transferred to an RC-40 open bath recording chamber (Warner Instruments) which was continuously perfused with aCSF (in mML 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, equilibrated with 95% O₂-5% CO₂) at 30–32°C and viewed with an upright microscope (Olympus BX51). Extracellular field potentials were recorded with glass pipettes filled with aCSF and placed in layer Ia of the aPC. A concentric bipolar stimulating electrode (FHC) was lowered into the pyramidal cell layer where less pCREB staining is evident following lidocaine (C) or muscimol (D) injection compared with saline injection. Higher magnification is shown of a portion of the pyramidal cell layer. Scale bars = 500 μm for low magnification and 100 μm for high magnification.

Image analysis. To obtain a quantitative comparison between occluded and spared hemispheres in O/S⁺ or O/S⁻ conditions, image analysis was performed using a Bioquant system (R&M Biometrics). Images were obtained using a CCD camera and viewed with a Leitz microscope with a consistent light intensity. Slices were analyzed in several rostral to caudal. An optical density (OD) reading of the background was taken near the midline of each slice to use for staining comparisons. A region of interest (ROI) was manually traced in both a lateral and a medial position in the pyramidal cell layer of the piriform cortex for both hemispheres. The relative OD (ROD) was calculated using the following formula:

\[ \text{ROD} = \frac{\text{OD of ROI} - \text{OD of background}}{\text{OD of background}} \]

The medial and lateral ROD measurements were compared in the spared and occluded hemispheres of each animal and were also compared between experimental conditions (O/S⁺ vs. O/S⁻). To account for individual differences between animals, measurements were normalized (ratio of the spared hemisphere to the occluded hemisphere), and the percentage differences were calculated using the formula: %difference = 100% × (spared ROD/occluded ROD).

Values displayed indicate the percentage differences between medial and lateral regions of the spared and occluded hemispheres and between O/S⁺ and O/S⁻ animals. Student’s t-tests were used to determine statistical significance.

Electrophysiology

Tissue preparation and extracellular recording. Animals were anesthetized with halothane inhalation and quickly decapitated. Brain Electrophysiology was performed using a Bioquant system (R&M Biometrics). Tissue was extracted and placed in an ice-cold high glucose artificial cerebrospinal fluid (aCSF; in mM: 83 NaCl, 2.5 KCl, 0.5 CaCl₂, 3.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, and 72 sucrose, equilibrated with 95% O₂-5% CO₂) for 10 min. Sagittal slices (400 μm) were cut using a vibrating blade (Vibratome 1000P; Leica Microsystems) and were incubated in an Isotemp 205 chamber (Fisher Scientific) at 34°C in the aforementioned solution for at least 60 min before use. Tissue slices were transferred to an RC-40 open bath recording chamber (Warner Instruments) which was continuously perfused with aCSF (in mML 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, equilibrated with 95% O₂-5% CO₂) at 30–32°C and viewed with an upright microscope (Olympus BX51). Extracellular field potentials were recorded with glass pipettes filled with aCSF and placed in layer Ia of the aPC. A concentric bipolar stimulating electrode (FHC) was lowered into the LOT and delivered single test pulses, ranging from 10 to 80 μA.

Electrophysiological data were recorded with Multiclamp 700B (Molecular Devices), filtered at 2 kHz, and digitized at 10 kHz. Data acquisition and analysis were performed with pClamp10 and Clamp-Fit 2.10 (Molecular Devices) and Igor Pro 6.10A (WaveMetrics). Student’s t-tests were used to determine statistical significance.

In vitro electrophysiology. Naïve PD7–11 animals were used for recording. A baseline of fEPSPs evoked by single pulse test stimulation was recorded at 0.05 Hz until the last 10 min were consistent and was then followed by theta-burst stimulation (TBS; 10 times 5 Hz trains, each train contains 5 pulses at 100 Hz). Protocols varied among
one, four, or eight TBSs, with each TBS separated by 30 s. TBS was followed by the same single test pulse recording for 30–60 min. The stimulation intensity for recording and stimulation was determined as that at which 50% of the maximum response was evoked. The recordings were analyzed to determine the amount of long-term potentiation (LTP) at the LOT to aPC synapses. In subsets of experiments, the fEPSP input-output (I/O) relationship was measured as the ratio of the size of the presynaptic fiber volley (FV) to the slope of the fEPSP; the paired pulse ratio (PPR; 2nd EPSP amplitude/1st EPSP amplitude) was measured in two consecutive stimulations with an interval of 50 ms, using mid-range stimulation strength.

d-APV (50 μM; Tocris) and isoproterenol (20 μM; Sigma) were used in bath application in subsets of experiments. Drugs were washed into the recording chamber 5 min before the TBS and washed out following the TBS. The sizes of fEPSPs were measured, and PPRs during isoproterenol wash-in were compared before and immediately following the TBS.

Ex vivo electrophysiology. Pups undergoing ex vivo electrophysiological analysis were subjected to odor conditioning with one nostril occluded as described earlier. Following a 5-min habituation period, pups underwent O/S+ or O/S− training. Upon completion, the plugs were removed and pups were returned to the dam. Pups were killed 3 or 24 h following training, and brain slices were prepared. The hemispheres of the brain were kept separately within the incubation chamber to achieve intra-animal control. I/O relationships and PPRs were measured as with in vitro experiments and compared between the spared and occluded slices. To reduce heterogeneities of the inputs in slices, two or three pairs of slices from the two hemispheres at the same cutting planes were used for recording and the results were averaged for each hemisphere. The stimulation and recording configurations were kept the same for each pair of slices. The slices from two hemispheres were recorded alternately. In another set of experiments, one and eight TBSs were used to induce LTPs at the LOT synapses in the nostril occluded and spared slices.

RESULTS

aPC is Critically Involved in Early Odor Preference Learning in Rats

Rainiki et al. (2009) showed that with strong shock and illness-related odor aversion learning, rat pups from 7–24 days of age increase 2-deoxy-D-glucose (2-DG) uptake in the posterior, but not the anterior, piriform cortex. At 7–8 days, however, mild shock pairings with odor induce odor preference and selective 2-DG uptake in both the olfactory bulb and aPC. To confirm the role of the aPC in early odor preference learning with a stroking US, two aPC silencing experiments were performed. The first employed lidocaine. Lidocaine is a transient sodium channel blocker, which silences neurons for up to 30–60 min (Martin 1991). On PD7, lidocaine was infused into the aPC 10 min before odor training or was infused on PD8 10 min before odor preference testing. To obtain a more specific silencing effect on neurons within the aPC, muscimol, a GABAa receptor agonist, was also used in the same protocols. Figure 1A shows that animals infused with lidocaine or muscimol before training spent less time over peppermint-scented bedding (lidocaine, 40.73 ± 4.23%, n = 17; muscimol, 20.34 ± 4.79%, n = 7) compared with saline-infused counterparts (saline O/S+, 54.81 ± 3.26%, n = 19). Saline-infused odor only (O/S−) animals (39.02 ± 2.70%, n = 18) were comparable to lidocaine pups. A one-way ANOVA reveals a significant difference among groups \( F(3,57) = 10.77, P = 1.041 \times 10^{-6} \). Post hoc Fisher least significant difference testing demonstrates significant differences between saline O/S+ and saline O/S− groups (\( P = 0.001 \)), saline O/S+ and lidocaine O/S+ animals (\( P = 0.005 \)), and saline O/S+ and muscimol O/S+ groups (\( P = 1.074 \times 10^{-6} \)). Figure 1B shows that animals that underwent O/S+ training on PD7, followed by lidocaine or muscimol infusions before testing on PD8, demonstrated no preference for peppermint (lidocaine, 36.86 ± 7.92%, n = 6; muscimol, 34.92 ± 5.32, n = 10) compared with saline-infused counterparts (57.08 ± 4.69%, n = 7). Animals who received O/S− training and saline infusions also showed no preference for peppermint (28.98 ± 4.29%, n = 8). A one-way ANOVA shows significant between-groups differences \( F(3,31) = 5.25, P = 0.005 \). Post hoc testing demonstrates significant differences between saline O/S− and saline O/S+ animals (\( P = 5.675 \times 10^{-4} \)), saline O/S+ and lidocaine O/S+ animals (\( P = 0.024 \)), and saline O/S− and muscimol O/S+ animals (\( P = 0.006 \)). These results show that the aPC is required...
for memory encoding and recall processes in odor preference learning: either the aPC directly participates in memory encoding or, as part of the olfactory circuitry, is necessary for odor perception during learning and memory.

To verify that pyramidal cells were silenced in response to lidocaine infusion, pCREB immunohistochemistry was performed following a unilateral lidocaine or muscimol infusion and peppermint odor exposure. Figure 1, C (lidocaine) and D (muscimol), shows pCREB expression in the aPC at low (middle) and high (bottom) magnifications. Hemispheres injected with lidocaine or muscimol show much less staining than saline-injected hemispheres (e.g., lidocaine ROD: 69.8 ± 12.3% of the saline-injected side, t = 6.50, P = 6.333 E−4, n = 7). Nissl staining (Fig. 1, top) confirms that pyramidal cell bodies are present and homogeneous in the aPC in both hemispheres. No spread to the olfactory bulb or the posterior thalamic nuclei was no silencing of adjacent areas. However, as we note elsewhere, feedforward and feedback electrophysiological interactions should be altered. There were also no long-lasting effects of lidocaine and muscimol at 24 h, the time of testing, judged by pCREB immunohistochemistry in aPC itself (n = 4, data not shown).

Both 2-DG (Raineki et al. 2009) and cFos (Roth and Sullivan 2005) increases in the aPC have been described accompanying odor preference training in 7- to 8-day-old rat pups. In the olfactory bulb, increased pCREB staining in the peppermint-encoding region is selectively associated with odor preference learning (McLean et al. 1999). In the present study, pyramidal neurons in the aPC were assessed for a role in odor preference memory encoding by measuring changes in pCREB levels in these cells shortly after odor training. To eliminate potential differences due to heterogeneity in background odors and immunohistochemical processing among different animals, unilateral nostril occlusions were employed. This allowed for intra-animal comparisons, where the occluded hemispheres were compared with the spared hemispheres. Pups underwent O/S− or O/S+ training and differences in pCREB expression in the aPC were observed (Fig. 2, A and B). RODs were measured in a lateral and a medial region in the aPC in both occluded and spared hemispheres. Data were normalized to account for individual differences and expressed as a percent difference [100% − (spared ROD/occluded ROD); Fig. 2B]. With the use of a paired sample t-test, O/S− animals showed no significant differences in normalized pCREB expression between medial (1.81 ± 0.70%) and lateral (2.10 ± 0.42%) regions of the aPC (n = 7, t = 0.36, P = 0.730), while O/S+ animals showed much higher staining in the lateral region (3.60 ± 0.76%) of the aPC than in the medial region (1.74 ± 0.43%, n = 5; t = 5.15, P = 0.007). Comparing medial and lateral regions between training conditions, a two sample t-test found no significant differences in the medial regions of the aPC between O/S− and O/S+ animals (t = 0.080, P = 0.939). However, there were significant differences in the lateral regions of the aPC between O/S− and O/S+ animals, with stronger staining in the O/S+ animals (t = 1.87, P = 0.046). These results suggest that O/S+ training activates pyramidal neurons more than O/S− training, specifically in the lateral regions of the aPC. This pattern of results also suggests that, as in olfactory bulb, pCREB-directed transcription may play a causal role in odor preference memory.

Odor Preference Learning is Dependent on NMDARs in the aPC

Since reversible lesions and CREB activation data were both consistent with a role for the aPC in early odor preference learning, the next series of experiments examined plasticity mechanisms in aPC that might support such learning. NMDA plasticity in the LOT input from the olfactory bulb to the piriform cortex is age dependent and most evident in young rat pups (Poo and Isaacson 2007). Such plasticity has been implicated in many associative learning models (Debanne 1996). NMDARs in the aPC were blocked using d-APV infusions 10 min before O/S+ training. When animals were tested the following day, it was found that blocking NMDARs in the aPC prevented early odor preference learning (Fig. 3A). Such animals spent significantly less time over peppermint-scented bedding compared with saline-infused counterparts (d-APV, 38.58 ± 4.27%, n = 15; saline O/S+, 54.81 ± 3.22%, n = 19). In addition, the saline-infused O/S− animals (39.02 ± 2.70%, n = 18) were comparable to d-APV-infused animals. A oneway ANOVA shows that there were significant differences among groups [F(2,51) = 7.85, P = 0.001]. Post hoc testing further shows that saline O/S− animals spent much less time over peppermint compared with saline O/S+ animals (P = 0.001) and d-APV infusion before O/S+ training significantly reduced the time that animals spent over peppermint bedding compared with saline O/S+ animals (P = 0.001). These results suggest that NMDAR activation in the aPC is required for early odor preference learning.

To test whether d-APV infusion into the aPC alters odor perception instead of preventing learning, animals were trained with the O/S+ protocol and d-APV was infused before odor preference testing the next day. If blocking NMDARs in the
aPC alters odor perception, it would be expected that D-APV infusion before testing would abolish the preference for peppermint in O/S\(^+\) animals. However, Fig. 3B showed that D-APV infusion before testing did not affect the preference for peppermint that was formed in O/S\(^+\) animals. When these animals were compared with learning (O/S\(^+\) with saline infusion) and nonlearning (O/S\(^-\) with saline infusion) controls, a one-way ANOVA showed significant group effects \(F_{2,30} = 8.08, P = 0.002\). Pups infused with D-APV before testing showed similar results (51.80 ± 5.91%, \(n = 12\)) to those of saline-infused O/S\(^+\) animals (57.08 ± 4.70, \(n = 8\), \(P = 0.496\)). Both groups spent significantly more time on the peppermint side than the control O/S\(^-\) animals (28.98 ± 4.29%; \(P < 0.05\)).

Together, these results suggest that D-APV infusion did not affect odor perception, but rather the encoding of memory.

**β-Adrenoceptor Activation in the aPC Can Mediate Early Odor Preference Learning**

Previously it has been shown that direct pharmacological activation of β-adrenoceptors in the olfactory bulb is sufficient for early odor preference learning to occur (Lethbridge et al. 2012; Sullivan et al. 2000). However, it is not known to what extent natural release of norepinephrine during O/S\(^+\) learning engages the piriform cortex. We tested whether β-adrenoceptors in the aPC are involved in early odor preference learning. First, β-adrenoceptors were blocked by infusing propranolol directly into the aPC 10 min before O/S\(^+\) training. Propranolol-infused animals (39.59 ± 4.82%, \(n = 8\)) spent significantly less time on peppermint bedding than saline-infused O/S\(^+\) animals (54.81 ± 3.23%, \(n = 19\)) and were comparable to nonlearning saline-infused O/S\(^-\) animals (39.02 ± 2.70%, \(n = 18\); Fig. 4). Next, stroking was replaced with an intracranial infusion of varying concentrations of the β-adrenoceptor agonist isoproterenol during odor exposure. Animals infused with 5 µM isoproterenol (38.86 ± 4.29%, \(n = 6\)) or 50 µM isoproterenol (38.06 ± 8.68%, \(n = 6\)) did not learn to prefer peppermint; however, animals infused with 500 µM isoproterenol (58.41 ± 10.98%, \(n = 6\)) did show a preference for peppermint. The learning effect of 500 µM isoproterenol was blocked when confounded with propranolol (27.38 ± 11.45%, \(n = 4\)). A one-way ANOVA shows significant differences among groups \(F_{6,60} = 3.66, P = 0.004\). Post hoc testing reveals significant differences between saline O/S\(^+\) and propranolol O/S\(^-\) animals \((P = 0.023)\). In addition, there was a significant difference between saline O/S\(^-\) animals and 500 µM isoproterenol animals \((P = 0.010)\), and 500 µM isoproterenol and propranolol coinfusion groups \((P = 0.004)\). These results suggest that early odor preference learning engages β-adrenoceptors in the aPC and that β-adrenoceptor activation in the aPC paired with a novel odor is sufficient to induce odor preference learning independent of the olfactory bulb β-adrenoceptor-induced learning effect.

**Induction of LTP at the LOT Synapse In Vitro Supports Learning Acquisition Mechanism**

We next set out, using in vitro experiments, to characterize synaptic mechanisms occurring in the aPC that could support odor preference learning. We first characterized LTP at the LOT to aPC synapses. By stimulating LOT afferent inputs, and recording from the pyramidal cell dendritic layer Ia, LTP of pyramidal cell fEPSPs was induced by TBS protocols (Fig. 5). TBS protocols capture, to some extent, the natural sniffing-mediated input rhythms from olfactory bulb to aPC (Kepcecs et al. 2006). Figure 5A shows LTP of fEPSPs following 1 TBS. A paired sample \(t\)-test comparing the normalized fEPSP slope (mV/ms) of the baseline to that 30 min post-TBS recording reveals that they are significantly different (post-LTP: 108.8 ± 3.8% of the baseline; \(n = 15, t = 2.31, P = 0.037\)). Furthermore, an eight TBS protocol induces a larger LTP (post-LTP: 116.5 ± 3.3%; \(n = 14, t = 4.93, P = 2.772 E^{-4}\); Fig. 5B).

Therefore, consistent with previous reports (Franks and Isaacson 2005; Kanter and Haberly 1990), the LOT to aPC synapse is plastic at these ages and is capable of strengthening synaptic communication, a change that is proposed to underlie learning.

We then investigated the influence of β-adrenoceptor activation on TBS-associated LOT LTP. Isoproterenol was bath applied to the slices, and eight TBSs were administered. Isoproterenol application increased the LTP magnitude compared with that induced by 8 TBSs alone (8 TBS + ISO: 129.9 ± 5.2%, \(n = 9\); 8 TBS: 116.5 ± 3.3%, \(n = 14\); \(t = 2.28, P = 0.033\); Fig. 5C). Our behavioral results revealed that NMDARs were critical for early odor preference learning. LTP induced in the presence of isoproterenol was also blocked by D-APV (93.2 ± 5.1%, \(n = 6, t = 1.32, P = 0.245\); Fig. 5D). What is the acute effect of isoproterenol on the LOT synapses that could lead to an enhanced LTP induction? To test if isoproterenol affects presynaptic release from LOT, we measured PPRs of two consecutive fEPSPs before and immediately following TBS induction. LOT synapses exhibited paired pulse facilitation (Fig. 5E). The PPRs in the ACSF perfused slices did not change following TBS (pre-TBS, 1.29 ± 0.03; post-TBS, 1.25 ± 0.024, \(n = 6, t = 1.97, P = 0.106\)). Conversely, for isoproterenol perfused slices, the PPRs were significantly lower post-TBS compared with pre-TBS (pre-TBS, 1.37 ± 0.078; post-TBS, 1.22 ± 0.055, \(n = 5, t = 6.32, P = 0.003\); Fig. 5E), implying that there was an increase in presynaptic release following TBS in the presence of isoproterenol. While we cannot exclude a direct effect of isoproterenol on postsynaptic pyramidal cells during TBS induction, an increase in presynaptic release by isoproterenol could account for the larger LTP change. These results are consistent with the critical roles of NMDARs and β-adrenoceptors in vivo in early odor preference learning.
LOT to aPC LTP Is Expressed as Enhanced Postsynaptic Responses

We then explored the expression mechanisms of the LOT to aPC LTP. fEPSP I/O relationships and PPRs were measured to indicate post- and presynaptic changes correspondingly. The slope of the fEPSP was used for the I/O measurement and is largely composed of an AMPAR component (Franks and Isaacson 2005). We measured the fEPSP slope in the presence of NMDAR and AMPAR blockers. D-APV did not significantly change the fEPSP slope (95.1 ± 3.5% of the baseline, n = 5, t = 1.37, P = 0.244); however, the AMPAR antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX; 20 μM) abolished the fEPSP in the presence of D-APV (n = 3, data not shown). Figure 6A shows a typical example of evoked fEPSPs in response to different stimulation intensities (top traces) and the changes following LTP (bottom traces). To characterize the postsynaptic changes relative to the LOT release that occur following LTP, the fEPSP I/O relationships were determined by comparing the presynaptic FV with the EPSL slope. A paired t-test reveals that the I/O was greater post-LTP compared with pre-LTP (pre-LTP: 1.26 ± 0.37; post-LTP: 1.57 ± 0.49, n = 15, t = 1.87, P = 0.041; Fig. 6A). To test whether there were presynaptic changes resulting from LTP, PPRs were measured pre- and 30 min post-LTP, following isoproterenol washout, either when TBS was applied during aCSF alone (pre-LTP, 1.20 ± 0.080; post-LTP, 1.23 ± 0.055, n = 6) or in the presence of isoproterenol (pre-LTP, 1.34 ± 0.047, post-LTP, 1.34 ± 0.047, n = 7; Fig. 6B). A paired sample t-test yielded no significant differences in the PPR before and after LTP, either in aCSF (t = 1.06, P = 0.336), or in the presence of ISO (t = 0.35, P = 0.738). Significant changes in the fEPSP I/O but not PPR suggest a postsynaptic expression of the LOT LTP.

Early Odor Preference Learning Induces LTP-like Changes at the LOT to aPC Synapse

Finally, we examined whether natural odor preference learning induces long-term synaptic changes at the LOT synapse in the aPC. Ex vivo electrophysiology was conducted following behavioral training. Such training involved unilateral nostril occlusion paired with O/S− or O/S+ training, followed by electrophysiological recordings at 3 or 24 h posttraining. At this age there is no contralateral communication between the two aPC areas (Kucharski and Hall 1987; Kucharski et al. 1986) so that learning can be confined unilaterally with single nostril occlusion (Yuan and Harley, 2012). We focused on recording from the sagittal slices containing the lateral portion of the aPC since the pCREB data demonstrated significant changes in that area following O/S+ training. We measured the fEPSP I/O relationship and PPRs from occluded and spared slices from the same animals. Because we compare fEPSP responses in different slices (spared and occluded), it is impossible to compare fEPSP slope with a single stimulation.
Instead, the I/O relationship measuring the ratio of fEPSP slope to the presynaptic FV allows us to directly compare the relative number of functional AMPARs at LOT synapses from spared and occluded hemispheres of the same animals (Franks and Isaacson 2005). For example, a bigger I/O slope indicates a greater number of functional AMPARs at LOT synapses from spared (0.99 ± 0.04) hemisphere compared to the occluded (1.19 ± 0.11) hemisphere (n = 5, t = 0.432, P = 0.694; Fig. 7A1) and occluded hemispheres (1.18 ± 0.17; n = 5, t = 0.04, P = 0.974; Fig. 7C2). These results suggest that postsynaptic LTP at the LOT synapse was sustained for up to 24 h following odor preference learning, and at a shorter period (3 h posttraining), there was also an increase in presynaptic release, most likely reflecting enhanced mitral cell output from the olfactory bulb (Lethbridge et al. 2012).

To further test if learning-induced LTP shares the same properties as those induced by TBS of the LOT, we performed LTP recording in spared vs. occluded slices 3–24 h posttraining. Figure 8 shows that in occluded slices, one TBS generated LTP of fEPSPs comparable to those generated in naïve slices in Fig. 5A (post-LTP: 109.9 ± 4.5% of the baseline; n = 7 slices from 4 animals, t = 2.21, P = 0.035), while eight TBSs further potentiated fEPSPs (post-LTP: 116.8 ± 7.4% of the baseline; t = 2.27, P = 0.032). In contrast, in spared slices, there was no potentiation of fEPSPs either by 1 TBS (post-LTP: 94.0 ± 10.4% of the baseline; n = 6 slices from 4 animals, t = 0.57, P = 0.705) or by a following set of 8 TBSs (post-LTP: 91.5 ± 10.1% of the baseline; t = 0.85, P = 0.436).

These results suggest early odor preference learning results in LTP changes at the LOT synapses that are exclusive of further LTP induction at the same synapses.

**DISCUSSION**

As reviewed in the Introduction, pairing of a CS (odor) and a US (β-adrenocceptor activation) in the olfactory bulb is sufficient to produce early odor preference learning (Lethbridge et al. 2012; Sullivan et al. 2000). Blocking β-adrenocceptors in the olfactory bulb demonstrates that β-adrenergic activation is necessary for normal early olfactory learning (Sullivan et al. 2000). While this leads to the suggestion that plasticity in the rat pup olfactory bulb is both necessary and sufficient for early odor preference learning, there remains the possibility that other structures in the forebrain olfactory circuit play a role in supporting, or even in generating, this form of learning. The present experiments provide evidence that the aPC is also both necessary and sufficient for early odor preference learning. The evidence argues that early odor preference learning is normally supported by, and generated in, multiple forebrain memory circuits. We propose that normally, changes in the olfactory bulb drive changes in aPC. Thus earlier experiments that locally induced olfactory bulb circuitry enhancement to peppermint in turn entrained aPC, while blocking such enhancement prevented the recruitment of aPC changes. Here we show that bypassing the olfactory bulb change and inducing aPC change directly permits memory expression, but if aPC circuitry is prevented from altering then olfactory bulb change alone is not able to drive odor preference behavior.

**Evidence for aPC as a Critical Node in Early Olfactory Forebrain Learning Circuitry**

Previous experiments using cFos activation to examine forebrain olfactory structures exhibiting immediate early gene activation following odor preference learning in rat pups of the same age as those used in the present experiments found only two structures with enhanced cFos activation, the olfactory bulb and the aPC (Roth and Sullivan 2005). Similar results were seen with 2-DG uptake (Raineki et al. 2009). Aversive odor training recruited the olfactory bulb, the posterior piri-
form cortex, but not the aPC, and the central amygdala (Raineki et al. 2009; Roth et al. 2006). In the present studies, with the use of pCREB activation as a marker of early transcriptional activation implicated in learning, the results confirm that the aPC is activated by peppermint odor. More compellingly, pairing a peppermint odor CS with stroking, a maternal mimic of US, produces increased pCREB activation in the aPC as it does in the olfactory bulb (McLean et al. 1999). This pCREB learning effect is selective to the lateral aspect of the aPC in the present study, but the possibility remains of more general involvement below the threshold of our optical density measurements. An earlier cFos developmental study of aPC activation by odor has shown that there is major spatial reorganization of odorant representation occurring at PD10 (Illig 2007) just at the end of the critical period for early odor preference learning. It is possible that the effects seen here are specific to the critical window period. LOT afferent LTP is also more readily elicited at this early age (Poo and Isaacson 2007). It is also the case that NMDA-dependent NE release is selectively elevated in cortex at this age (Brown 1993). In future studies, pharmacological manipulations could be used to alter the nature of odor learning (e.g., appetitive vs. aversive) or to modulate the critical period window (Moriceau and Sullivan 2004; Moriceau et al. 2006; Roth et al. 2006). The spatial correlates of pCREB activation with different forms of learning at the same age and with the same learning at different ages may be informative. Consistent with the gene activation evidence for a role of aPC in early odor preference learning, lidocaine or muscimol infusions, which prevented aPC participation either during training or during testing, also prevented the acquisition and expression of early odor preference learning. This result is consistent with a role for aPC in preference learning of the type of early odor learning studied here.

Fig. 7. Learning induced LTP changes at the LOT to pyramidal cell synapse. A1 and A2: I/O relationships and PPRs at the LOT synapses in the piriform cortex 3 h postodor only (O/S\textsuperscript{−}) training. Recordings from occluded and spared slices of the same animals were compared. A1, top: examples of fEPSP traces at various stimulation intensities in an occluded and a spared slice from the same animal. Middle: I/O relationship of the slopes of the fEPSPs and the sizes of the presynaptic FV from these 2 slices (occluded: broken line with solid circles; spared: solid line with open circles). A1, bottom: average I/O from the 2 groups. A2, top: example traces of paired pulse recordings from an occluded and a spared slice from the same animal. A2, bottom: average PPRs of the 2 groups. B1 and B2: I/O and PPRs of fEPSPs in the piriform cortex 3 h post-O/S\textsuperscript{−} training. C1 and C2: I/O and PPRs of fEPSPs in the piriform cortex 24 h post-O/S\textsuperscript{−} training. Scale bars = 0.2 mV/5 ms. Error bars are means ± SE. *P < 0.05; **P < 0.01.
learning, but the effect could be indirect, for example, piriform cortex feedback to the olfactory bulb may modulate the bulbar representation (Boyd et al. 2012; Strowbridge 2009) to affect learning or relaying olfactory information per se through the aPC may be critical. Additional experiments here, however, provided evidence for a direct role of aPC mechanisms in the generation of early odor preferences.

Evidence for aPC Plasticity in the Generation of Early Odor Preference Learning

The NMDAR antagonist d-APV infused locally in the aPC before training prevented early odor preference learning in vivo. Again it was possible that this was an indirect effect mediated by a change in the modulation of sensory input either in the piriform cortex, or in the olfactory bulb. However, the failure of the NMDA antagonist to alter the expression of odor preference learning 24 h after normal training argues that sensory distortion is not an explanation for the ability of NMDA infusions to prevent learning.

In vitro experiments revealed a role for NMDARs in theta-burst LTP at the LOT to aPC synapse, as has been previously reported (Kanter and Haberly 1990). Of particular interest was the ability of β-adrenoceptor activation, via isoproterenol, to significantly strengthen theta-burst LTP at this synapse. During the application of isoproterenol, a reduction in the PPR suggested that one way in which isoproterenol enhanced LTP effects was through increasing the levels of glutamate released during the theta burst. Such an effect is consistent with the report that isoproterenol directly prevents rapid presynaptic habituation at the LOT to aPC synapse by modifying the phosphorylation of the metabotropic III receptor that is responsible for reduced transmitter release (Best and Wilson 2004). In vivo studies demonstrated that blocking β-adrenoceptors in the aPC, as in the olfactory bulb, prevented early odor preference learning. Finally, infusion of isoproterenol in aPC paired with peppermint produced learning. Taken together these data argue that β-adrenergic support of novel theta-burst odor input to the aPC leads to learning modifications at LOT to aPC synapses that can support the acquisition and expression of an olfactory preference.

Synaptic Changes Supporting Early Odor Preference Learning and Expression in the aPC

Ex vivo experiments carried out at 3 and 24 h after training in slices from paired trained and untrained hemispheres revealed the LTP predicted at the LOT to aPC synapses based on the in vitro experiments. Paired pulse evidence suggested that at 3 h posttraining there was enhanced presynaptic release as well as an increase in postsynaptic responses. At 24 h, increased presynaptic release was no longer in evidence but enhanced postsynaptic responsiveness remained. These data argue that memory effects in the aPC may depend primarily on increased AMPA receptor currents or increased AMPA receptor insertion as seen at olfactory nerve to mitral cell synapses in the olfactory bulb (Cui et al. 2011; Lethbridge et al. 2012; Yuan and Harley 2012). Under natural learning conditions norepinephrine release in the aPC would enhance NMDAR currents and postsynaptic calcium events evoked by LOT glutamate release. Additionally, local LOT glutamate release could engage NMDA receptors on norepinephrine terminals to increase norepinephrine output in a positive feedback effect that would increase plasticity locally (Brown 1993; Wang et al. 1992). Local plasticity effects would support a synergistic recruitment of long-term synaptic potentiation and of the CREB cascade in pyramidal cells of the aPC. Another set of effects seen in the olfactory bulb, but not evaluated here, could include changes in the synchrony of neuronal firing (de Almeida et al. 2012; Doucette et al. 2011). Norepinephrine is known to suppress intracortical input relative to afferent LOT input in aPC and subsequently enhance the signal-to-noise ratio (Hasselmo et al. 1997).

Piriform Cortex and Olfactory Bulb Interactions in the Support of Odor Learning

Local infusions of the US isoproterenol in either the olfactory bulb (Lethbridge et al. 2012; Sullivan et al. 2000) or the aPC (Fig. 4), paired with odor can generate early odor preference learning and memory. Blockade of β-adrenoceptors at either site prevents natural odor preference acquisition. Consistent with a model in which noradrenergic input normally supplies the US at this developmental stage, both structures receive extensive innervation from the noradrenergic locus coeruleus nucleus (Fallon and Moore 1978; McLean et al. 1989; Shipley and Ennis 1996). LTP of olfactory nerve input to mitral cells (Lethbridge et al. 2012; Yuan and Harley 2012) and, here, of LOT input to aPC pyramidal cells accompanies early odor preference learning and may be the major mechanism underlying its expression. The two structures interact directly via feed forward input from mitral and tufted cell axons to aPC layers II and III, and via feedback input from pyramidal cells in layers II/III of aPC to the granule cells of the olfactory bulb (Boyd et al. 2012; Isaacson 2010; Strowbridge 2009), which have been shown to modulate rhythmic activity and synchronization in the olfactory bulb (Neville and Haberly 2003; Poo and Isaacson 2009). Recent data (Doucette et al. 2011) suggest the frequency of rhythmic activity in olfactory bulb odor representations (and possibly those of aPC) provides the signature for appetitive stimulus encoding in the olfactory...
Developmental alterations in N-methyl-D-aspartate stimulated best AR, Wilson DA.

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