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**Supplemental Information**

**Acetylcholine Regulates Olfactory  
Perceptual Learning through Effects  
on Adult Neurogenesis**

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## Transparent Methods

### *Subjects*

A total of 77, 8 to 12 week old, male WT and ChAT ChR2 EYFP mice [B6.Cg-Tg(ChAT-COP4\*H134R/EYFP,Slc18a3) 6Gfng/J; RRID: IMSR\_JAX:014546;(Zhao et al., 2011)] on a C57Bl6/N background (Charles River Laboratories, Wilmington, MA) were used for this study. All mice were bred in-house. Mice were housed on a 12 h light/ 12 h dark cycle with food and water provided *ad libitum*. Testing occurred during the first half of the light phase of the light/dark cycle.

### *Odorants Preparation*

For olfactory perceptual learning (cross-habituation and enrichment), stimulus odorants were diluted in the odorless carrier, mineral oil, to 0.1 Pascal (Pa) vapor pressure. For spontaneous olfactory discrimination, stimulus odorants were diluted in mineral oil to 0.01 Pa. Dilution volumes were acquired through analysis of vapor pressures using ACD ChemSketch software (Advanced Chemistry Development, Toronto, Ontario, Canada). For olfactory perceptual learning tasks, + limonene (habituation) and – limonene (test) odorants were used. For spontaneous olfactory discrimination tasks, five sets of odorants were used:

1. N-hexyl acetate (Hab), N-amyl acetate (S1), N-butyl acetate (S2), Anisole (D)
2. Propanoic acid (C3) (Hab), Butanoic acid (C4) (S1), Pentanoic acid (C5) (S2), 5-methylfurfural (D)
3. Octanal (Hab), Heptanal (S1), Hexanal (S2), Anisole (D)
4. Octanoic acid (C8) (Hab), Heptanoic acid (C7) (S1), Hexanoic acid (C6) (S2), (R)-(+)-limonene (D)
5. Pentanol (Hab), Hexanol (S1), Heptanol (S2), Citronellal (D)

### *Cross-Habituation Task: Olfactory Perceptual Learning*

Mice were individually positioned in polycarbonate cages before testing. For each trial, odorants were introduced by placing 100  $\mu$ l of an odorant onto a piece of filter paper (Whatman, Florham Park, NJ) and suspending the filter paper in each mouse's cage by enclosing the paper in a tea ball hanging in the

upper corner of the cage. During the four habituation trial, mice were exposed to 0.1 Pa of a habituation odorant. For the test trials, the enantiomer to the habituation odorant was presented in lieu of the habituation odorant. Each trial was one minute in length followed by a five-minute inter-trial interval. Fresh odorants and filter paper were used in each trial. All trials were videotaped using Ethovision XT 8.5 tracking software (Noldus), after which investigation time was manually scored with the rater blind to drug group or genotype and odorant identity.

Enantiomer pairs were chosen for their perceptual similarity and the inability of our mice to behaviorally distinguish them in our pretest data (Linster et al., 2001b). Enantiomer pairs (one habituation odorant and its enantiomer) were used in each behavioral experiment. For consistency, the + form of the enantiomer pair was always used in the habituation trial and the – form was always used in the enantiomer trial.

#### *Cross-Habituation Task: Spontaneous Olfactory Discrimination*

Each mouse was placed individually into a polycarbonate cage. For each trial, the odorants were presented by introducing 100  $\mu$ L of 0.01 Pa diluted odorant onto a piece of filter paper (Whatman, Florham Park, NJ), and placing the filter paper inside a tea ball (Table S2). The tea ball was subsequently draped inside the upper corner of the cage. The mice were presented with a single aliphatic acid odorant on four consecutive trials (habituation). Three novel odorant trials followed, where three odorants were introduced in a counter-balanced randomized order: a highly similar (S1 – differing by a single unbranched hydrocarbon from the habituation odorant), moderately similar (S2 – differing by two unbranched hydrocarbons from the habituation odorant) and dissimilar odorant (D – structurally and perceptually unique odorant). All trials were one minute long with a five-minute inter-trial interval. This procedure was repeated with five different odorant sets over the course of five days. Following manual scoring, the five days of testing were averaged, and the data was pooled by trial type and drug group or genotype to assess the effects of drug group or genotype on odorant detection, habituation and discrimination.

### *Bromodeoxyuridine Injections*

To test if any of our manipulations altered the density of surviving newly born cells, we injected mice with BrdU (IP: 50 mg/kg) three times at two-hour intervals on the day following the final pretest. We allowed labeled cells eight days to migrate from their birthplace in the SVZ to the OB (Winner et al., 2002) before beginning olfactory manipulation. Thirty-nine days post-BrdU injection, mice were sacrificed, and we used immunohistochemistry and stereological counting to test for the effects of enrichment on newborn cell survival in the OB granule cell layer. To quantify the density of BrdU-positive cells in the granule cell layer of the olfactory bulb, an optical fractionator method was used. Every third serially obtained section (80  $\mu\text{m}$  interval) was mounted and processed immunohistochemically to detect BrdU. Sections were counterstained with Nissl and a reference volume of the granule cell layer was traced using a stereology system (Stereoinvestigator; MicroBrightField, Williston, VT). BrdU-positive nuclei were counted within a 20 x 20 x 30  $\mu\text{m}$  counting frame, which randomly sampled within a 122.8 x 68.9  $\mu\text{m}$  randomized counting grid using a meander scanning technique. BrdU-positive cells throughout the z-plane were counted; those that contacted the lateral or upper exclusion plane remained uncoun ted. The total number of cells counted were divided into the number of counting frames sampled multiplied by the size of the counting frame to obtain an estimate of the density of BrdU-positive cells within this structure.

### *Drug Administration*

To determine if the activity of cholinergic receptors during olfactory enrichment is necessary for olfactory perceptual learning, mice were administered Cogentin (25 mg/kg in saline; Enzo Life Sciences, Farmingdale, NY), a selective M1 muscarinic acetylcholine receptor antagonist, or saline, intraperitoneally 20 minutes prior to each enrichment session (10 injections total over 10 days).

### *Optogenetic Surgery*

For the optogenetics experiments, we used eight to 12 week old ChAT ChR2 EYFP male mice. Transgenic and control (C57Bl6/N) mice were injected intraperitoneally with the analgesic buprenorphine hydrochloride 20 minutes before surgery [0.1 mg/kg, Buprenex Injectable, Reckitt Benckiser Healthcare

(UK) Ltd., Hull, England]. Mice were anesthetized using an isoflurane-oxygen mixture (induction = 2-3%, maintenance = 1-2.5, O<sub>2</sub> flow rate=75 ppm), shaved and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A craniotomy was made on the skull above the coordinates for the left HDB (AP = +0.3 mm, ML = -2.0 mm, 13° lateral to medial), and an optical fiber was implanted (DV = 5.0 mm; fiber made in-house, core diameter = 200 μm, NA = 0.22). The fiber was fixed in place using C&B Metabond Quick Adhesive Cement System (Parkell Inc., Edgewood, NY) and dental acrylic (Repair Resin Quick Set Lab Pack, All 4 Dentist by Interguide Dental & Medical Supply, Burlingame, CA).

### *Osmotic pump Surgery*

For the AraC experiment, in addition to the optogenetic implant, mice were implanted subcutaneously with a micro osmotic pump (Model 1002, ALZET Osmotic Pumps, Cupertino, CA). The pump was connected via polyvinyl tubing to a cannulae aimed at the right subventricular zone (SVZ; AP = +1.2 mm, ML = +0.9 mm, DV = 3.0 mm; brain infusion kit 3, ALZET Osmotic Pumps, Cupertino, CA). The pump was filled with 100 μL cytosine-β-d-arabino-furanoside (AraC; 4% in 0.9% saline solution, Sigma Aldrich, St. Louis, MO) with a flow rate of 0.25 μL per hour (Moreno et al., 2012). Post-surgery observations were maintained for three days.

### *Optogenetic Stimulation*

Each mouse received one hour of laser stimulation every day for 10 consecutive days (in lieu of olfactory enrichment). In the first optogenetic experiment (Figure 3B), stimulation was achieved by coupling a patch cord (Doric Lenses, Quebec, Canada) to a blue light diode laser (MBL-III-473, 100mW, OptoEngine, LLC, Midvale, UT), measuring approximately 5 mW at tip of implanted fiber. In the second optogenetic experiment (Figure 4B), stimulation was achieved by coupling a patch cord (manufactured in-house) to a blue LED (PlexBright LED Module, 24.9mW, Plexon, Dallas, TX), measuring approximately 5 mW at tip of implanted fiber. In both experiments, each optogenetic stimulation session began with a 10 second habituation period, followed by eight-second trains of light pulses with 23-second intervals between

each train. For each train of light pulses, we randomly assigned a frequency (from 5-50 Hz, at 5 Hz intervals) using MATLAB coupled to an Arduino. Each pulse lasted for 15 ms (protocol adapted from Ma and Luo, 2012).

### *Statistics*

We used two-way ANOVAs to analyze the habituation sessions. When necessary, follow-up post-hoc sidak multiple comparison tests were used. For all other comparisons, two-tailed student's *t*-tests were used. All data is presented in graphs and figures as mean  $\pm$  SEM. Statistical analysis was performed using Prism Graphpad or SPSS software. Graphs were made using Prism Graphpad software.