EXTENDED EXPERIMENTAL PROCEDURES

Constructs and Viruses

Codon-optimized ChR2, VChR1 and the lentiviral backbone, from which all variants of ChR2 were expressed, were kind gifts from Dr. Karl Deisseroth. They include the following: pLenti.hSynapsin-ChR2:EYFP, pLenti.hSynapsin-ChR2:Cherry and pLenti.CaMKIIa-VChR1:EYFP. Cre-inducible double-floxed-inverse-orf (DIO) ChR2:EYFP and ChR2:Cherry were obtained from Karl Deisseroth in adenoassociated virus (AAV) backbone and cloned into the lentiviral backbone to generate pLenti.EF1alpha-doublefloxed ChR2:EYFP and pLenti.EF1alpha-doublefloxed ChR2:Cherry. Cre-inducible DIO ChR2:EYFP-IRES-nuclear Cherry was generated by inserting IRES-nuclear Cherry into pLenti.EF1alpha-doublefloxed ChR2:EYFP at the 5' end of EYFP in reverse orientation. Cre:EGFP (Le et al., 1999) was cloned into the lentiviral backbone carrying hSynapsin1 promoter to generate pLenti.hSynapsin1-Cre:EGFP. EGFP was cloned into the lentiviral DIO backbone carrying EF1alpha promoter to generate pLenti.EF1alpha-doublefloxed EGFP. For some animals injected with viruses at two anatomical locations (one in each hemisphere), one injection site received pLenti.EF1alpha-doublefloxed VChR1:EYFP. It was generated by cloning VChR1:EYFP (from pLenti.CaMKIIa-VChR1:EYFP) into the lentiviral DIO backbone carrying EF1alpha promoter. Lentiviruses for in vivo injection were produced as previously described (Zhang et al., 2010). They were replication-incompetent, and there was no indication that they were taken up by fibers of passage or by axon terminals. For animals, in which ChR2 was densely expressed using the dual virus strategy, EF1alpha-doublefloxed ChR2:EYFP-IRES-nuclear Cherry in AAV backbone was used.

Stereotaxic injection into the mouse brain

Strains of mice used for the experiments were wild-type C57 BL6/J from Jackson Laboratory, wild-type mixed 129SvEv X C57BL6/J and heterozygous Emx1-IRES-Cre (Gorski et al., 2002). The surgeries were carried out using aseptic techniques. Mice were anesthetized with intraperitoneal injections of ketamine and xylazine and stereotaxic (Kopf Instruments, Tujunga, CA) injections of viruses were made through pulled glass pipettes (Drummond, 2-000-001) at the layer 2 and 3 of the piriform cortex (AP=-0.6, ML= 3.8, DV=4.0). A guide cannula (tubing size of 22GA, Plastics One) was subsequently placed 300-500 μm above the virus injection site. The cannula was fixed in place with a small amount of dental cement and the skin was glued back with Vetbond tissue adhesive.

Tissue slice preparation and immunohistochemistry

Mice were perfused with 6 ml of PBS, followed by 7 ml of 4% paraformaldehyde. The brains were dissected out and post-fixed overnight in 4% paraformaldehyde. The brains were cut coronally at 80-120 μm. The prepared slices were labeled with the following primary antibodies: chicken anti-GFP (Abcam, ab5450), goat anti-c-Fos (Santa Cruz, sc-52-G), rabbit anti-c-Fos (Santa Cruz, sc-7270), rabbit anti-DsRed (Clontech, 632496). The following fluorophore-conjugated secondary antibodies were used: Alexa 488 goat anti-chicken (Invitrogen, A11039), Alexa 633 donkey anti-goat (Invitrogen, A21082), Alexa 633 goat anti-rabbit (Invitrogen, A21071), Alexa 568 goat anti-rabbit (Invitrogen, A11036). They were counter-stained with NeuroTrace fluorescent Nissl (Invitrogen). All images were taken using a Zeiss LSM-710 confocal microscope system. In order to

quantify the number of neurons expressing ChR2 for animals co-injected with pLenti.hSynapsin1-Cre:EGFP and pLenti.EF1alpha-doublefloxed ChR2:EYFP-IRES-nuclear Cherry, the slices were labeled for Cherry and scanned at every 15-20 μm optical section. The number of neurons expressing Cherry was manually counted from the scanned images. This number was doubled to produce total number of ChR2-expressing neurons for brains that were also processed for c-Fos expression on adjacent sections. In order to obtain the percentage of $ChR2^+$ neurons that also expressed c-Fos, animals co-injected with pLenti.hSynapsin1-Cre:EGFP and pLenti.EF1alpha-doublefloxed ChR2:EYFP-IRES-nuclear Cherry were photostimulated (30 seconds per minute for 10 minutes) an hour before they were perfused. Brain slices were double-labeled for c-Fos and Cherry. The percentage of $ChR2^+$ neurons expressing c-Fos at the center of the injection site was obtained by manual counting. As a control, the percentage of layer 2 and 3 piriform neurons expressing c-Fos in a 500 μm x 500 μm area (anterior-posterior levels equivalent to ChR2-expressing areas) at the un-injected hemisphere was quantified. For animals expressing ChR2 in dense populations of piriform neurons, numbers of $c\text{-}Fos^+$ neurons in 200 μm x 200 μm areas at the injection site and the neighboring non-injected site were manually counted and compared to quantify the fold-increase after photostimulation. Percentage of piriform neurons expressing ChR2 at a given injection site was generated by manually counting $Cherry^+$ neurons and all of the piriform neurons counter-stained with Neurotrace 640/660nm within a 500 μm x 500 μm area. The neurons were counted on at least three different anterior-posterior levels, and the counted numbers were averaged for each injection site. The experimenter carrying out the manual cell counting was not blind to experimental conditions. Averages and

standard deviations were respectively used as measure of center and variability for all quantifications. In order to map the centers of ChR2-expressing ensembles generated from the dual virus strategy, coronal slices of injected animals were labeled for Cherry. Bregma levels of coronals sections, on which Cherry+ neurons first and last appeared, were respectively taken as the anterior and posterior boundary of the ensemble. The median of this boundary was determined as the anterior-posterior center of the ensemble. Dorsoventral coordinate of the ensemble was determined by measuring the distance between the ventral border of the slice and the center of the Cherry+ ensemble.

Aversive behavioral paradigm

All animals were single-housed and kept on a reverse day-night cycle. Behavioral tests began at least 10 days post-surgery, and were carried out 2-6 hours after onset of the dark period. Behavioral conditioning took place over two training sessions spaced three days apart. Testing occurred ~24 hours after the second training session. For each training session, animals were lightly anesthetized with isoflurane to allow insertion of an optical fiber (ThorLabs, 300 µm core, 0.37 NA). The optical fiber was coupled to a 473 nm laser (Crystal Laser, or 532 nm for VChR1 activation). Laser output was maintained at 10-15 mW as measured at the end of the fiber. The conditioning apparatus was a rectangular chamber (9 cm W x 57 cm L x 16 cm H) with a stainless-steel rod floor (custom-made, MedAssociates, St. Albans VT). Each half of the apparatus was connected to an aversive stimulator (MedAssociates, 115 V, 60 Hz), allowing foot-shock to be applied independently to either side. Experimental animals were allowed to habituate to the apparatus for ~5 minutes. The conditioning paradigm consisted of 3-4 s of

photostimulation (20 Hz with 25 ms pulses) followed immediately by a 0.7 mA foot-shock. Foot-shock was applied only when the animal was in or near either end of the apparatus, forcing the animal to run to the opposite side. Photostimulation/shock pairings were spaced 3-4 min apart. Each of the two training sessions consisted of 10 photosimulation/shock pairings, for a total of 20 pairings. The testing session was identical in set-up to the training sessions, but only photostimulation (no foot-shock) was applied when the animal was located in either end of the apparatus. Photostimulation was applied 7 times over the testing session, every 3-4 minutes. The sessions were video-recorded. Distance moved (arbitrary unit, maximum per run = 70) after photostimulation and percent of photostimulations after which the animal ran (% flight behavior) were scored manually. For experiments in which shock was paired with odor, acetophenone (CS^+) and ethyl acetate (CS^-) (Sigma-Aldrich) diluted to 5% in light mineral oil (Fisher Scientific) were used. Odorant delivery was controlled by a four-valve electronic switch box. Air flowed constantly throughout the experiment; CS^+ or CS^- odors were delivered for approximately 3-4 s before foot-shock or no foot-shock, respectively. For experiments in which shock was paired with a multi-component CS, ethyl acetate and citronellol diluted to 5% or 10% in mineral oil were co-delivered with photostimulation. The trainings were carried out on two consecutive days, and the testing occurred on the same day as the last training session. Training sessions consisted of more than a total of 20 CS-US parings for some animals. Video recording and subsequent scoring protocols were the same as described above. Behavioral scoring was performed by an experimenter blind to the experiment conditions. Averages and standard deviations were respectively used as measure of center and variability for quantification of behaviors.

Appetitive go/no-go discrimination assay

Mice were adapted to a reverse 12 hr light/dark cycle and water restricted (~1-1.5ml per day to maintain 85-90% of baseline weight) for 1 week prior to training and testing. Training and testing were performed using the Slotnick operant conditioning paradigm (Bodyak and Slotnick, 1999) and a liquid-dilution, eight-channel olfactometer (Knosys, Lutz, FL). During pre-training, one odor was paired with a water reward following a 2 second delay (CS^+ odor). Another odor (or air) was not paired with a water reward (CS^- odor). The CS^+ and CS^- odors were presented in a random order, and the readout of the assay was the number of licks during the 2-second interval following the odor pulse. The data are presented in blocks of 20 trials (10 CS^+ trials and 10 CS^- trials). All odorants were purchased from Sigma-Aldrich (highest grade available) and were dissolved in light mineral oil. Once the animals were sufficiently trained to perform the task with monomolecular odorants (i.e. ethyl acetate, acetophenone), they were injected with lentivirus carrying ChR2 in the piriform cortex and cannulated. Two weeks after the surgery, they were trained to discriminate between the photostimulation of $ChR2^+$ piriform neurons as CS^+ (photostimulation = 20 or 30 Hz with 25 ms pulses) and absence of photostimulation as CS^-. Both the CS^+ and CS^- were accompanied by a pulse of air to mimic the pre-training condition. The fraction correct licks were calculated as number of licks following the CS^+ / total number of licks. Odor discrimination was considered successful when the fraction correct licks surpassed 0.7 for two consecutive blocks. The control group included animals that were co-injected with virus carrying Cre-inducible EGFP and a second virus carrying Cre and animals that were injected only with virus

carrying Cre-inducible ChR2 without the second Cre-expressing virus. The control animals underwent a minimum of 60 training blocks. The ChR2-expressing group included an Emx1-IRES-Cre transgenic animal injected with virus carrying Cre-inducible ChR2 and wild-type animals co-injected with virus carrying Cre-inducible ChR2 with the second Cre-expressing virus. In order to quantify the amount of training required to elicit conditioned behaviors using odor for the ChR2-expressing animals, a novel odor for the animals (carvone, Sigma-Aldrich) diluted to 0.5% in light mineral oil (Fisher Scientific) was used as the CS^{+}. Averages and standard deviations were respectively used as measure of center and variability for quantification of behaviors.

Social approach paradigm

Behavioral training and testing were carried out in a custom-built three-chambered arena. Each chamber (35.5 cm x 17.75 cm x 17.75 cm) was constructed from 1 cm-thick clear acrylic Plexiglas. The middle chamber included two 10-cm-wide openings in each long wall, allowing free movement between all three chambers. The day before the experiment, subject males were habituated for at least 10 minutes to the arena and to wire cages (11 cm height, 10.5 cm bottom diameter, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, Ohio) (Moy et al., 2009) that were to be used later. During training, a wire cage containing a female (wild-type mixed 129SvEv X C57BL6/J) was placed in one side chamber while an empty wire cage was placed in the opposite side chamber. The wire cages allowed direct interactions with the femals. At the beginning of each trial, subject males were placed in the middle chamber, and the latency to the male finding the cage containing the female was recorded. Photostimulation was

applied only when the male actively investigated the female and lasted for total of ~30 seconds per trial. Each female was used for 2 consecutive trials, and placement of the female-containing cage was randomly selected for each trial. A minimum of 10 trials was completed, with an inter-trial interval of ~3 minutes. During testing in the absence of a female, photostimulation was delivered when the male was in one of the arbitrarily chosen side chambers (CS^+ chamber). The sessions were video-recorded. The time spent in each chamber during a 5 minute testing period was manually scored. For experiments in which the female was paired with odor, orange or anise extracts were used as either CS^+ or CS^- odors.

Behavioral preference test

Testing was carried out in a custom-built three-chambered arena. The sessions were video-recorded. Each session consisted of a 5-min baseline period in which no photostimulation was delivered in any of the three chambers, followed immediately by 5 or 10 minutes during which photostimulation was delivered when the subject animal was in one of the arbitrarily chosen side chambers ((+)-photostimulation chamber). The time spent in each chamber during was manually scored.

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