The role of central amygdala neuronal types in drug-related and appetitive behaviors

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CHAPTER 9. MATERIALS AND METHODS

9.1 Animals

Mice were group-housed in a 12-hour light-dark cycle (lights on from 7 a.m. to 7 p.m.), with water and food available *ad libitum*. The SST-IRES-cre, SST-IRES-cre;Ai14, Prkcd-FlpO, Prkcd-FlpO;tdTomato, VIPR2-IRES-cre, GluA3$^{lox/lox}$;ACTB-cre, GluA3$^{lox/lox}$ and Glua1$^{lox/lox}$ mice were bred onto C57BL/6J background. All procedures involving animals were approved by the Institutional Animal Care and Use Committees of Cold Spring Harbor Laboratory and carried out in accordance with US National Institutes of Health standards.

9.2 Behavioral paradigms

9.2.1 Methamphetamine-induced conditioned place preference

Methamphetamine-induced conditioned place preference is a type of Pavlovian conditioning used to assess the rewarding properties of drugs. The drug-induced conditioned place preference was performed in a sound-attenuating box, which had two chambers that are clearly distinct through tactile, olfactory and visual stimuli. First, mice were habituated to the box for one 30-minute session. After this, animals underwent a pre-test of 20 min in which their preference for either chamber was assessed. This was followed by 8 days of 30 min conditioning. Sessions in which methamphetamine injections (1 mg/kg, i.p.) were paired with one chamber (drug-paired chamber, unpreferred chamber during pre-test), were alternated with sessions in which saline injections were paired with the other chamber (saline-paired chamber, preferred chamber during pre-test). A total of 4 sessions were done for both drug and saline condition. After conditioning, their preference for the chambers was evaluated in a 20 min test. Conditioning score was calculated based on *time spent in drug-paired chamber post-test* divided by *time spent in drug-paired chamber pre-test*.

9.2.2 Elevated Plus Maze

The Elevated Plus Maze (EPM) apparatus was constructed from white plexiglas and consists of two open arms without walls that are 30cm long and 5 cm wide, and two arms enclosed by 15.25 cm high non-transparant walls. The arms were extended from a central platform (5 cm x 5 cm), and were arranged such that the identical arms were opposite ot each other. The maze was raised to a height of 50 cm above the floor with an overhead light. At the start of the session, animals were placed in the center zone and allowed to explore the maze for 10 minutes in the absence of the experimenter, while their behavior was videotaped using a monochrome CCD camera (Panasonic WV-BP334) at 4 Hz. The resulting data was analyzed using Ethovision XT 5.1 (Noldus Information Technologies). Parameters assessed were total distance travelled, velocity, time spent in the open arms, number of entries to the open arms, and latency to the first entry into an open arm. The maze was thoroughly cleaned with 70% ethanol between subjects.

9.2.3 Food and water intake

Animals were switched from water bottles to hydrogels one week before the experiment. One day before the experiments animals were single-housed. On the day of the experiment, animals received 3 pieces of chow and a hydrogel that was weighed beforehand, and then left alone. After 24 hours, chow and food dust was separated

from bedding and weighed, and the hydrogel was weighed to measure the amount of food and water animals used.

9.2.4 Acute food deprivation

For acute food deprivation, animals were single-housed one day before the experiment. Food was removed from their cages for 18 hours. One hour before the start of re-feeding, animals were moved into clean cages. One piece of chow was weighed and left in the cage at timepoint 0. Chow was weighed at 30, 60, 90, 120 en 180 minute timepoints and food intake was calculated.

9.2.5 23 hour locomotion

Animals were single housed one day before the start of the experiment. At the start of the experiment, animals were put in a cage that was similar to their homecage and allowed to habituate for one hour. After habituation, animals movement was recorded with a Yi overhead camera for 23 hours. Position of the animals was analyzed with DeepLabCut and velocity during the dark and light cycle was calculated using Matlab.

9.3 Viruses

Viruses: AAV-fDIO-cre-GFP (lot# 5826, UNC), AAV9-FLEX-GFP (lot# AV5220b, UNC), AAV9-GFP-IRES-Cre (lot# AV3587, UNC), AAV9-CAG-Flex-TeLC-eGFP-WPRE.bGH (lot#V5466S, UPenn), AAV-CAG-FLEX-GFP (lot#AV5220c, UNC). All viral vectors were stored in aliquots at −80 °C until use.

9.4 Stereotaxic surgery

Animals were anesthetized with ketamine (100 mg/kg) supplemented with dexmedetomidine hydrochloride (0.4 mg/kg) intraperitoneally, or anesthetized with intranasal isoflurane (2%) and positioned in a stereotaxic apparatus connected to a computer system with a digital mouse brain atlas (Angle Two Stereotaxic System, myNeuroLab.com). Injections of virus or tracer (0.2-0.4 µl) were delivered with a glass micropipette (tip diameter approximately 1 µm) through a skull window (1-2 mm^2) by pressure application (5-12 psi, 5-20 ms at 1 Hz) controlled by a Picrospritzer III (General Valve) and a pulse generator (Agilent). The injections were performed within the following stereotaxic coordinates; CeA: -1.18 mm from bregma, 2.9 mm lateral from midline, and 4.65 mm vertical from pial surface. Mice were injected subcutaneously with meloxicam (2 mg/kg) after surgery. During procedures, animals were kept on a heating pad and they were brought back to their home cages after regaining movement. After surgery, viruses were allowed to express for 3-6 weeks to allow maximal expression before behavior or recordings were done.

For *in vivo* imaging with gradient-index (GRIN) lenses, one week after viral injection, a second surgery was performed for GRIN lens implantation into the virus infected area. To implant the GRIN lens (dimeter, 600 µm; length: 7.3 mm; Inscopix, Palo Alto, CA 94303, USA), we first enlarged the cranial window using a thrill, and then used a holder (Inscopix) to hold and carefully lower the GRIN lens through the window into the target area at a low speed (~100 µm/min). We subsequently fixed the GRIN lens in place using adhesive luting cement (Parkell Prod). The holder was released until the cement was completely cured. A metal head-bar (for head-restraint in all the mice used in the imaging and behavioral experiments) was subsequently mounted onto the skull with black dental cement (Ortho-Jet). We waited for a minimum of 6 weeks before starting the imaging experiments in these mice.

9.5 Electrophysiology

Mice were anesthetized with isoflurane and decapitated, and their brains were quickly removed and chilled in ice-cold dissection buffer (110.0 mM choline chloride, 25.0 mM NaHCO3, 1.25 mM NaH2PO4, 2.5 mM KCl, 0.5 mM CaCl2, 7.0 mM MgCl2, 25.0 mM glucose, 11.6 mM ascorbic acid and 3.1 mM pyruvic acid, gassed with 95% O_2 and 5% CO_2). Coronal slices of 300 µm containing the amygdaloid complex were cut in dissection buffer using a HM650 Vibrating Microtome (Thermo scientific), and were subsequently transferred to a storage chamber containing artificial cerebrospinal fluid (ACSF; 118 mM NaCl, 2.5 mM KCl, 26.2 mM NaH2PO4, 20 mM glucose, 2 mM MgCl2 and 2 mM CaCl2, at 34 ℃, pH 7.4, gassed with 95% O2 and 5% CO2). After 30 minutes recovery time, slices were transferred to room temperature (20-24 ℃) and were continuously perfused with ACSF. In acute slices, the major subdivisions of the amygdala can be easily identified under trans-illumination 17. Whole-cell, patch-clamp recordings of CeL neurons were obtained with Multiclamp 700B amplifiers (Molecular Devices). Recordings were under visual guidance using an Olympus BX51 microscope equipped with both transmitted light illumination and epifluorescence illumination. Som^+ and Som^- cells were identified based on red fluorescence in Som;Ai14 animals, PKC-δ^+ cells were identified based on their red fluorescence in PKC-δ-FlpO;tdTomato animals. Synaptic responses were low-pass filtered at 1kHz and recorded at holding potentials of -70 mV (for AMPAR-mediated responses) or +40 mV (for NMDAR-mediated responses), depending on the experiment. Recordings were performed in ACSF. The internal solution for voltage-clamp experiments contained 115 mM cesium methanesulphonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl2, 4 mM Na2-ATP, 0.4 mM Na3GTP, 10 mM sodium phosphocreatine and 0.6 mM EGTA (pH 7.2). Miniature excitatory post-synaptic currents (mEPSCs) were recorded in the presence of tetrodotoxin (1 µM) and picrotoxin (100 µM). Spontaneous EPSCs (sEPSCs) and light-evoked recordings were done in the presence of picrotoxin (100 µM). Electrophysiological data were acquired and analyzed using pCLAMP 10 software (Molecular Devices). mEPSCs were analyzed using Mini Analysis Program (Synaptosoft). To evoke synaptic transmission using channelrhodopsin, a single-wavelength LED system (λ= 470 nm, CoolLED.com) was connected to the epifluorescence port of the Olympus BS51 microscope. Two protocols for light stimulation were used, single pulse (0.5 ms duration, 5s sweep, 30 sweeps) or train stimulation (10 Hz, 2s train, 10s sweep duration, 10 sweeps). The light pulses were triggered by a TTL (transistor-transistor logic) signal from the Clampex software (Molecular Devices).

9.6 *In-vivo* calcium imaging

We imaged GCaMP6 signals in behaving mice using a custom-built wide-filed imaging system. The system consisted of four major components: excitation light source, imaging optics, CCD camera and acquisition software, and mechanical parts. An LED (470 nm; PE-100, CoolLED) was used as the excitation light source. During imaging, the light power was adjusted to 5%-10% of the maximum power based on the intensity of GCaMP6 signals. A fluorescence illuminator (BX51, Olympus) was used to transmit light. A filter cube (U-MF2, Olympus), which contained the appropriate optical filters, was included inside the illuminator to ensure that only fluorescence signals with the desired wavelengths are transmitted. The filters used were: excitation (FF02-482/18-25, Semrock), dichroic (FF409/493/573/652-Di01, Semrock) and emission (FF01-520/35-25, Semrock). An objective lens (10x, NA 0.3, WD 11 mm; MPLFLN10X, Olympus) was used to focus the excitation light onto and collect fluorescence signals from the GRIN lens. A tube lens (180 mm; TTL180-A, Thorlabs) was paired with the objective for magnification and forming images onto a CCD camera (Retiga R3, Qimaging), which was used to collect fluorescence signals. During imaging, pixels were binned at 2 by 2 to increase frame rate and signal-to-

noise ratio, and exposure time was set to 50 to 100 ms according to the intensity of GCaMP6 signals. An acquisition software (Micro-manager, University of California San Francisco) was used to control the camera for continuous image acquisition. During the acquisition, the camera was set to external trigger mode by the software, such that an image was captured each time the camera received a TTL signal.

Imaging consisted of three days with 2 sessions on each day. Animals were injected with either saline or methamphetamine (1 mg/kg, i.p.), 20 min before every imaging session. All three days started with a baseline saline session, followed by another saline session on day one and day three, and a methamphetamine session on day 2. Every session consisted of 30 minutes, with three minutes of imaging at the start, middle and end of the session, resulting in 9 minutes of imaging per session.

9.6.1 *In-vivo* calcium imaging data analysis

Imaging data was saved as an imaging stack in tiff format for every imaging session. The imaging stack was spatially down-sampled by a factor of 2. Margin areas in the images that didn't have any signal were cropped from the imaging stack using ImageJ (National Institutes of Health, USA). Motion artifacts were corrected using an algorithm for fast non-rigid motion correction (NoRMCorre) method. After the correction, we applied the extended constrained non-negative matrix factorization optimized for one-photon imaging analysis (CNMF-E) to demix neural signals and get their denoised and deconvolved temporal activity, termed ΔF. We used ΔF for further analysis. The analysis with CNMF-E method was carried out using a custom MATLAB algorithm.

The STD (standard deviation) was calculated across the whole trace for every neuron that was imaged. A threshold of 2 times the STD was set, and the time a trace spent above this threshold was recorded to assess bouts of high neuronal activity [76]. This analysis results in a single value for every neuron per session that gives an indication of the baseline activity of this neuron. When neuronal activity is compared between sessions, only neurons that can be tracked across all relevant sessions were included.

9.7 Perfusion and immunohistochemistry

Immunohistochemistry experiments were performed following standard procedures on 50 µm brain sections fixed with 4% paraformaldehyde. Brain sections were blocked in PBS-T (4% goat serum, 0.4% Triton-X) for 1 hour at room temperature, followed by incubation with primary antibodies overnight at 4 ℃. The antibodies used were anti-PKC-δ antibody (mouse, 1:1000, BD Biosciences), anti-GFP antibody (chicken, 1:1000, Aveslab). Sections were then washed with PBS (3x10 min) and incubated with fluorescent secondary antibodies at room temperature for 2 hours. After washing with PBS (3x10 min), sections were mounted onto slices. Images were taken using an LSM 710 confocal microscope (Zeiss).

9.8 Data analysis and statistics

All statistics are indicated where used. Statistical analyses were performed with GraphPad Prism Software (GraphPad Software, Inc.). All behavioral experiments were controlled by computer systems, and data were collected and analyzed in an automated and unbiased way.