Supplemental Information

Aversion or Salience Signaling by Ventral Tegmental Area Glutamate Neurons

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Transparent methods

All animal procedures were performed in accordance with the National Institutes of Health Guidelines, and approved by the National Institute on Drug Abuse Animal Care and Use Committee.

**Subjects.** Male VGluT2-IRES::Cre mice were used (n = 14, 25-30g body weight; *Slc17a6tm2(cre)Lowl* in C57BL/6J background from The Jackson Laboratories, Bar Harbor, ME).

**Surgical procedures.** Mice were anesthetized with 1–5% isoflurane. AAV5-EF1α-DIO-hChR2(H134R)-eYFP (UNC Vector Core, Chapel Hill, North Carolina USA) was injected (350 nl) in the VTA [-3.3 mm anteroposterior (AP), 0.0 mm mediolateral (ML), -4.3 mm dorsoventral (DV)] at a flow rate of 100 nl/min. Injections were made using the UltraMicroPump, Nanofil syringes, and 35 g needles (World Precision Instruments, Sarasota, FL USA). Syringes were left in place for 10 min following injections to minimize diffusion. At least 3 weeks after virus injection, mice were implanted with microwire electrodes in the midbrain (-2.9 to -3.8 AP, -0.2 to 1.5 ML at 10º angle, -4.5 DV; Microprobes, Gaithersberg MD USA) (Barker et al., 2014). The microwire array consisted of a 4 x 4 array of polyimide-coated stainless steel microelectrodes (25 µm diameter) surrounding a 200 µm multimode optical fiber (Figure 1). Electrodes were separated by 250 µm anterior to posterior and medial to lateral, except for the electrodes closest to the optical fiber, which were separated by 500-600 µm. The optical fiber was approximately 500 µm dorsal to the electrode tips. An additional stainless-steel electrode was implanted 250 µm posterior to the optical fiber and used in conjunction with a stainless-steel electrode wrapped around a stainless-steel screw implanted in the skull as a ground.

**Apparatus.** Mice were trained in a 7”W x 7”D x 12”H conditioning chamber (Mouse Test Cage, Coulbourn, Holliston, MA USA) within a sound attenuating cubicle (ENV-018V, Med-Associates, Fairfax, VT USA). A syringe pump (PHM-100, Med-Associates) delivered 8% sucrose to a custom-made plastic reward port with infrared head entry detector (ENV-302HD, Med-Associates). Auditory cues were delivered by a programmable audio generator (ANL-926, Med-Associates).

**Behavioral procedures.** One week following electrode implantation, mice were restricted to 85% free-feeding body weight for the duration of all behavioral experiments. Two days prior to training, mice were pre-exposed to 8% sucrose in their home cage for 1 hr. One day prior to training, mice were placed in the conditioning chamber and delivered 8% sucrose (40 µl) every 30 sec for 30 min. On day 1 of training, mice were placed in the conditioning chamber for 2 h, and a conditioned stimulus, CS+ (7000 Hz, 80 dB, 10 sec duration) predicting 8% sucrose was delivered at 0 and 5 sec after CS+ onset (20 µl at each delivery). A different conditioned stimulus, CS– (white noise, 80 dB, 10 sec duration), did not result in sucrose delivery. Cues were presented with a variable 25-45 sec inter-trial interval. The same training procedure was used for electrophysiological recordings, except that the CS+ tone resulted in sucrose delivery on 90% of CS+ trials. On 10% of randomly distributed CS+ presentations, the CS+ tone was played without sucrose delivery. These trials were termed CS+ error trials (CS±). Following the reward task recordings, access to the port was blocked, and the same mice were exposed to 20-30 airpuffs of nitrogen delivered to the face with a distance of approximately 4 cm from the snout (25 PSI, 200 ms duration, Picospritzer III, Parker, Pine Brook NJ USA). Following airpuff delivery, fiber optic ferrules were connected to a rotary joint (Doric Lenses, Quebec, Quebec Canada) and 473 nm light pulses (1 Hz, 5 ms, 10-20 mW) were delivered.

**Electrophysiological and video recordings.** Neural discharges were amplified and digitized within a headstage (ZD32, TDT, Alachua FL USA), routed through a motorized commutator (ACO32, TDT), differentially amplified against another microwire that did not exhibit a single unit (PZ4, TDT), bandpass filtered 300-5000 Hz, and acquired in software at 25 kHz/channel (OpenWorkbench and OpenController, TDT). Video recordings synchronized with neuronal acquisition clocks were acquired at 30 Hz (RV2, TDT).

**Behavioral video analysis.** Frame by frame timestamps were overlayed onto video files by Avidemux v2.6 and recorded for approaches toward the reward port in MPC-HC v1.7.10. Approach onset was determined by the start of a change in direction toward the reward port and approach offset was the breaking of the reward port photocell (Root et al., 2013). Approach onset was typically a leftward or rightward head turn, or the start of
alternating limb movements toward the reward port. Approaches greater than 3 seconds in duration were excluded from analysis.

**Optogenetic classification.** We used organized arrays of microwires with optical fibers similar to those used by Kravitz and colleagues (Kravitz et al., 2013). Optical Microwire Arrays allow for verification of individual recording sites within brain tissue (Barker et al., 2014). However, this organization causes light to be delivered more strongly to microwires proximal to the fiber and less to microwires distal to the fiber (Kravitz et al., 2013). To account for differences in light distribution, individual wires were recorded at increasing light intensities (mW). For each neuron, the light intensity that generated the shortest latency responses and highest fidelity was designated as the optimal light intensity (Kravitz et al., 2013). After identification of optimal light intensity, we classified single-units as channelrhodopsin (ChR2)-responsive by the following criterion: (1) median latency to fire less than 15 ms following light onset (Kravitz et al., 2013) and (2) greater than 60% fidelity to fire an action potential following light onset.

**Electrophysiological analyses.** Isolation and separation of individual neural waveforms from background noise and waveforms of other neurons recorded from the same microwire were conducted offline using spike sorting and separation software (SciWorks, DataWave, Loveland CO USA) as previously described (Root et al., 2013). Neural discharges were sorted by waveform parameters, including principal components 1 through 3, peak voltage, spike height, peak voltage time, and voltages at user-defined time cursors. Interspike interval histograms were constructed for each neuron to confirm that no discharges occurred during refractory period (<1.8ms). Cross-correlations were used to confirm that populations of neurons from single microwires were distinct neurons. Waveforms exhibiting signal-to-noise ratios less than 2:1 were discarded. Each mouse was recorded one or two times, typically separated by 5 or 6 days if recorded twice. When similar mean waveform and interspike interval histograms patterns were recorded from the same microwire on both days (Coffey et al., 2015), only one recording was included in the dataset of optogenetically-identified neurons.

Changes in firing rate were computed using a standardized change ratio, \((E - B) / (E + B)\), where \(B\) is the mean baseline firing rate across trials and \(E\) is the mean event firing rate across trials. Using this standardized change ratio a 50% change from baseline firing rate during an event equals \(0.20\) (increase) or \(-0.20\) (decrease), and no change in firing from baseline equals 0. To examine changes in firing rate in response to the \(CS^+\) or \(CS^-\), \(B\) equaled the firing rate -150 to 0 ms prior to all cue presentations (cue baseline), and \(E\) equaled the firing rate between 0-150 ms following \(CS^+\) or \(CS^-\). The 150 ms duration for cue baseline was chosen to assess firing based on cue presentation without influence of cue-induced movements (Ghitza et al., 2003). To examine changes in firing rate prior to and following reward-seeking behaviors, \(B\) equaled the firing rate from -6 to -3 sec prior to reward port entries (movement baseline), determined by infrared photobeam breaks, and \(E\) equaled the firing rate -2 to 0 sec prior to reward port entry (pre-reward) or 0 to 2 sec following reward port entry (post-reward). If reward port entries occurred within 3 sec of each other they were discarded from neural examination. Reward port entries were divided into all reward port entries, trials in which the \(CS^+\) was played and sucrose was delivered, trials in which the \(CS^+\) was played and sucrose was not delivered, and trials in which the \(CS^-\) was played and sucrose delivered (Coffey et al., 2015). To examine changes in firing rate following reward port entry on \(CS^+\) trials, \((E - B) / (E + B)\), \(B\) = the firing rate 0-2 sec following reward port entry on \(CS^+\) trials, and \(E\) = the firing rate 0 to 2 sec following reward port entry on \(CS^-\) trials. To examine changes in firing rate response to the airpuff, \(A\) equaled the firing rate 200 to 0 ms prior to airpuffs, and \(B\) equaled the firing rate 0 to 200 ms during airpuffs.

**Histology.** Mice were anesthetized with 1-5% isoflurane and anodal current (50 µA, 2 sec) was passed through each microwire to mark the tip location. Mice were then perfused with phosphate buffer (PB, 0.1M, pH 7.4) followed by 4% paraformaldehyde. The brain was extracted and stored in 4% paraformaldehyde for 2h prior to storing overnight in 18% sucrose at 4°C. Brains were coronally sectioned (40 µm) through the VTA and processed for tyrosine hydroxylase (TH) immunohistochemistry to identify the borders of the VTA. VTA sections were washed in PB, treated with 0.01% \(H_2O_2\) for 15 min before washing again in PB, pretreated for 1 h in blocking buffer (PB supplemented with 0.1% Triton X-100 and 4% bovine serum albumin), and incubated overnight at 4°C with blocking buffer supplemented with mouse anti-TH (1:1000, MAB318, Millipore, Burlington, MA USA, RRID:AB_2201528). Brains were then washed in PB, incubated for 1h at room
temperature with blocking buffer supplemented with goat anti-mouse biotinylated secondary (BA1000, 1:200, Vector Labs, Burlingame, CA USA), washed in PB, incubated for 1h at room temperature with PB supplemented with avidin-biotinylated horseradish peroxidase (1:200, ABC kit, Vector Labs). Sections were washed in PB and peroxidase reaction was developed with 0.05% 3,3’-diaminobenzidine tetrahydrochloride and 0.003% H₂O₂. To identify recording sites, sections were mounted on coated slides and incubated in 5% potassium ferrocyanide and 10% HCl. Sections were imaged by a VS120 (Olympus, Center Valley, PA USA) microscope at 20X. The positions of the microwires were identified by their tracks in the brain and the blue potassium ferrocyanide iron deposits. Recordings from microwires localized outside of the tyrosine hydroxylase-labeled VTA were not included. Subdivisions of the VTA were identified based on prior research of tyrosine hydroxylase immunoreactions (Root et al., 2014) and mouse brain atlas (Paxinos and Franklin, 2001).

**Statistics.** A 2 (CS) x 2 (Day) repeated measures ANOVA was used to examine acquisition of the reward task. Sidak-adjusted pairwise comparisons evaluated specific changes between days and cues. Paired t-tests were used to evaluate changes in standardized changes in firing rate between two variables. Correlation coefficients of changes in firing rate between two variables were compared by Fisher’s r-to-z transformation followed by asymptotic covariance tests (Steiger, 1980, Lee and Preacher, 2013). A hierarchical clustering analysis and K-means principle component analysis was used to determine if neurons clustered into subpopulations based on their changes in firing rate following reward port entries during CS⁺ trials and their changes in firing rate in response to the airpuff. Inspection of the scree plot “elbow” was used to determine number of clusters. Heat plots were constructed in Matlab (Mathworks) using the standardized change in firing rate formula (E – B) / (E + B), where B was baseline and E was each bin of the perievent time histogram for an event. Movement or airpuff bins were 50 ms and cue bins were 10 ms. All tests were performed in SPSS (SPSS, IBM, Armonk NY USA) except cluster analyses, which were performed in R.

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