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Supplemental Information

Opposite Consequences of Tonic and Phasic Increases in Accumbal Dopamine on Alcohol-Seeking Behavior

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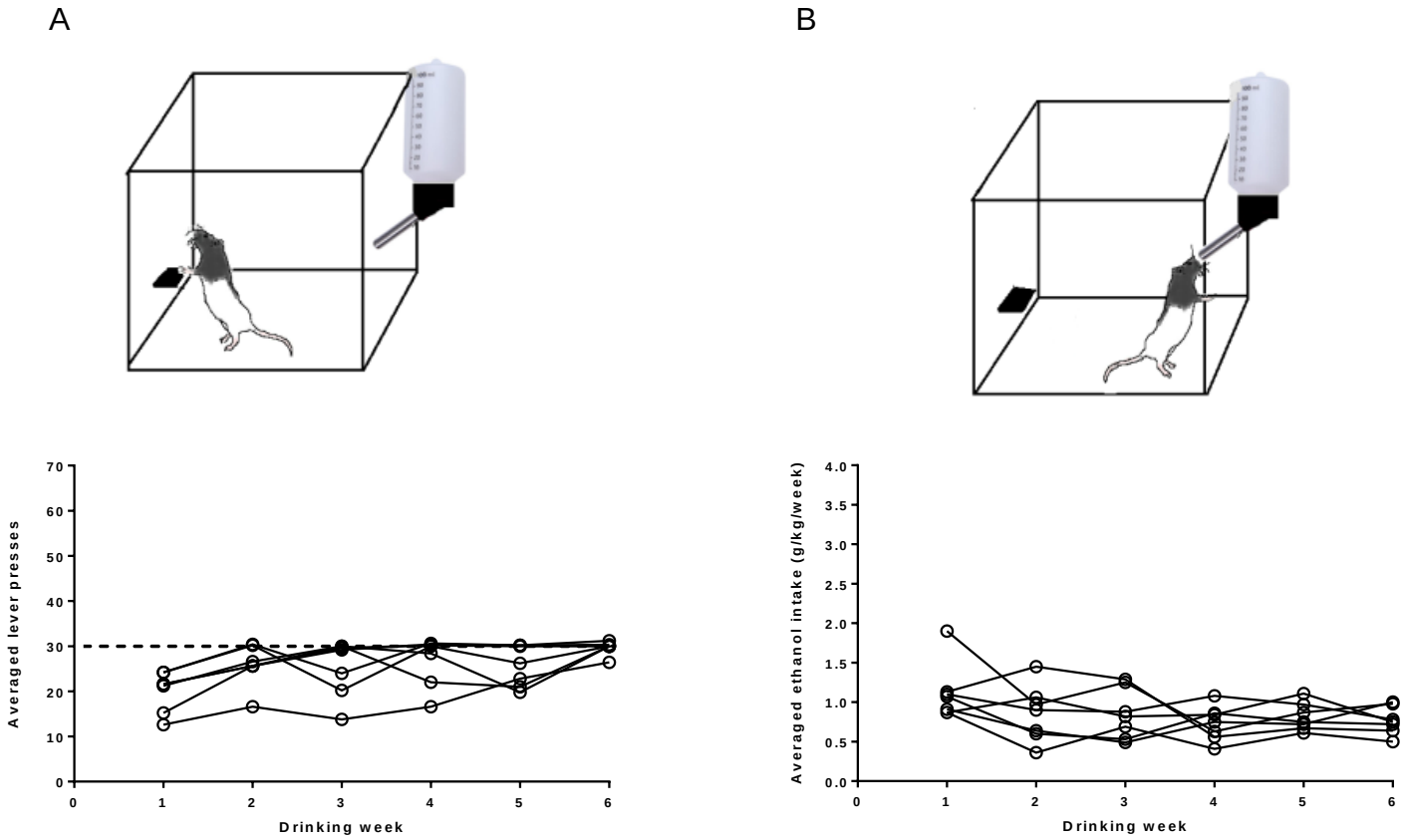

Figure S1. Alcohol seeking and taking behaviors for the 6-week training procedure, related to Figure 1. Following an abbreviated sucrose-substitution protocol, rats (n=7) were provided with 10% ethanol solution that was available after the completion of a response requirement. The requirement was individualized for each rat depending on previous behavior (from 10 to 30 presses) in order to obtain alcohol regularly. By the end of the training period, all rats met the final requirement criterion of 30 lever presses to receive 10% ethanol. The number of lever presses (A) and alcohol intake (g/kg) (B) were averaged per animal per week.

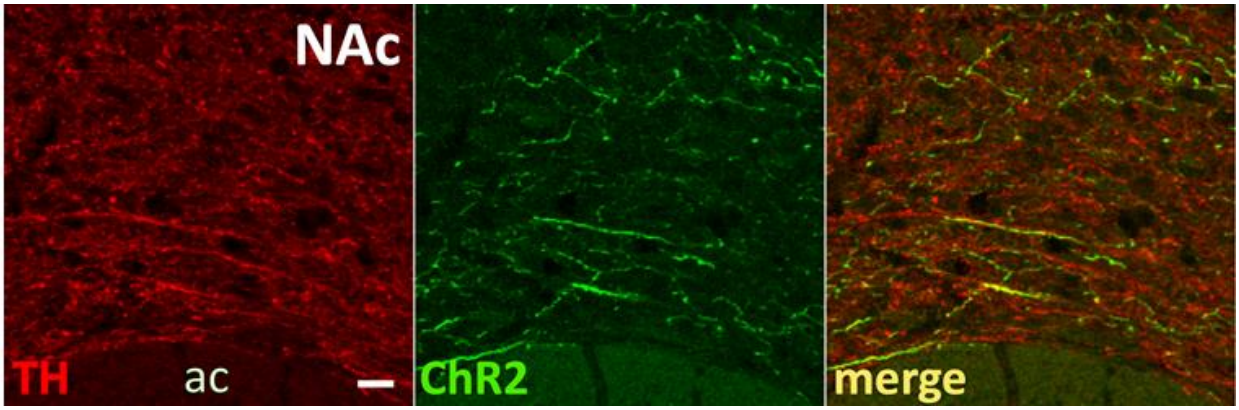

Figure S2. Optogenetically targeting DA terminals within the nucleus accumbens of rats consuming ethanol in an operant behavior test, related to Figure 1. Rats, previously trained to press a lever to obtain access to a 10% ethanol solution for 20 minutes, were anesthetized and injected in the VTA with a combination of DIO-ChR2-EYFP-AAV2/10 and TH-iCRE-AA2/10 via a Hamilton syringe. Immunohistochemical analysis confirmed that ChR2-EYFP was restricted to TH-positive cells in the targeted area. The scale bar is 20 microns.

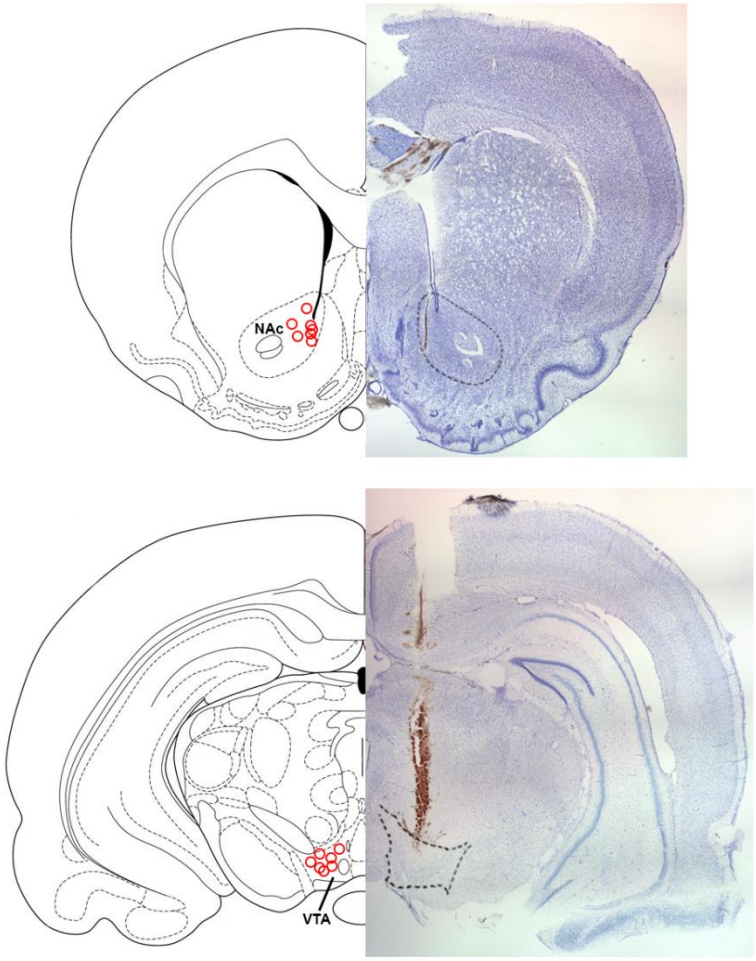

Figure S3. Schematic depictions (left) and representative images (right) of recording electrode (top) and optical fiber (bottom) placements (n=7), related to Figures 2 and 3. Note that all electrochemical recordings were performed on anesthetized animals. Red circles indicate placements of ventral tip of electrodes and fibers.

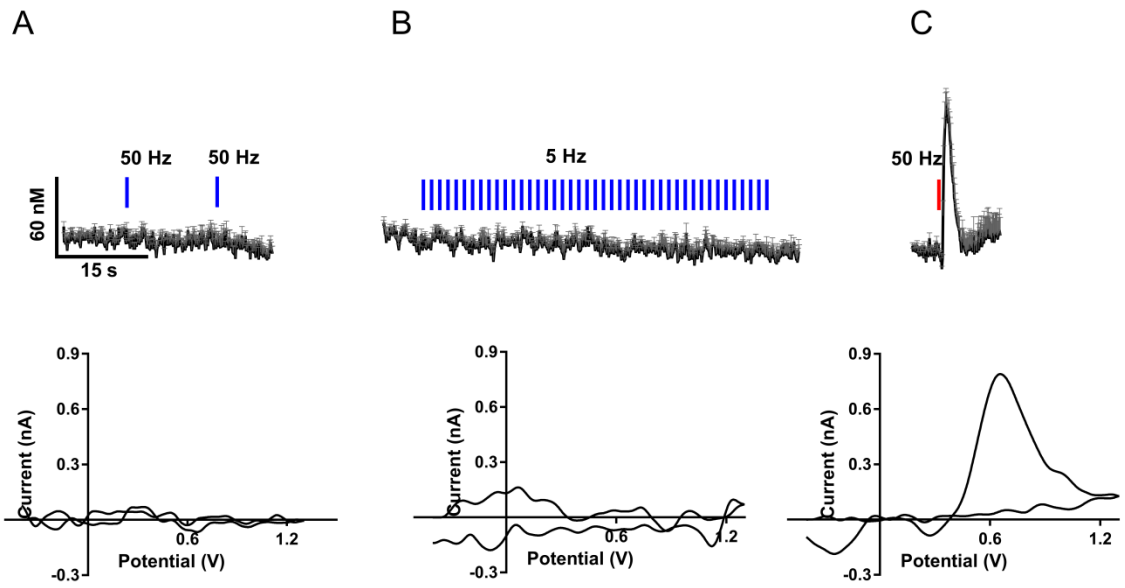

Figure S4. Optogenetic and electrical activation of the VTA in anesthetized rats without light sensitive opsin, related to Figures 2 and 3. Rats were injected in the VTA with viral construct referred in Figures 1 and S2 but with no ChR2. Four weeks later, animals were anesthetized and FSCV recordings in the NAc were performed. Optoactivation of the VTA at 50 Hz frequency for 1 s did not did not alter baseline recordings in the NAc (A). Identically, no effect of the stimulation at 5 Hz for 60 s was found (B). However, when an electrical stimulation (50 Hz, 1 s) was applied, a patent DA release was detected in the same rats (C). These data are presented as a mean ± s.e.m. from 5 rats. Blue bars indicate light stimulation, while the red bar shows an electrical stimulation. The lower panel demonstrates background-subtracted cyclic voltammograms obtained during stimulations. The electrical stimulation only (C, lower panel) induced the signal that was identified as DA based on its electrochemical signature.

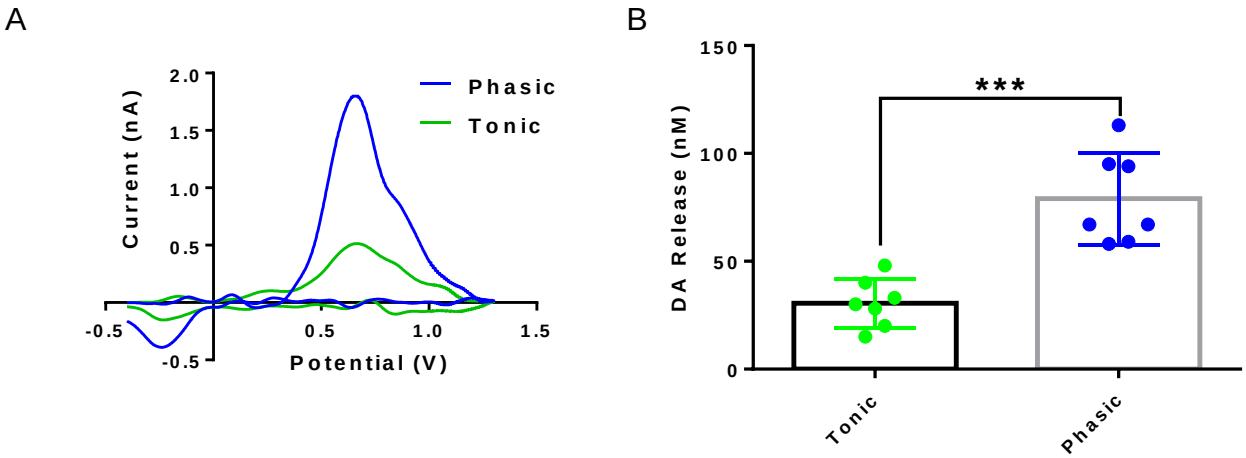

Figure S5. Effect of optogenetic stimulations on DA release *in vivo*, related to Figure 2. DA concentration changes were recorded in the nucleus accumbens of anesthetized rats (n=7) using FSCV. DA was identified by its oxidation (≈0.6 V) and reduction (≈-0.2 V on the negative going scan) features. (A) Representative background-subtracted cyclic voltammograms from tonic (green) and phasic (blue) DA responses are presented. (B) The amplitude of phasic DA release was significantly higher than tonic DA elevation. ***P<0.001 (unpaired t test). These data are presented as a mean ± s.e.m.

Supplemental Information

Transparent methods

Animals. Adult (90-150 days old) male Long-Evans rats, weighing 300-380g, were individually housed in a temperature-controlled vivarium in acrylic cages on a 12/12 h light/dark cycle (lights out at 6:00 PM). Food and water were available ad libitum, except during experimental sessions (30 min), conducted during the light cycle (8:30 AM – 12:30 PM) from Monday to Friday. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

Viral constructs and packaging. Virus packaging and titering were previously described (Bass et al., 2013; Fox et al., 2016; Mikhailova et al., 2016). A standard triple transfection packaging protocol was used to package viruses to generate pseudotyped AAV2/10 (Xiao et al., 1998). All viruses were titered by quantitative PCR and were 1012 vector genome copies per ml. The three plasmids were an AAV2 plasmid, which contained the transgene to be packaged, pHelper (Stratagene, La Jolla, CA) provided adenoviral helper functions, and an AAV2/10 rep/cap plasmid containing the AAV2 replicase and AAV10 capsid genes (Gao et al., 2002; De et al., 2006). The EF1α-DIO-ChR2-EYFP-pAAV and TH-iCRE-pACP plasmids were previously described (Gompf et al., 2015). Briefly, Cre recombinase expression is driven by a rat tyrosine hydroxylase (TH) promoter, which restricts expression to TH+ neurons (Oh et al., 2008; Gompf et al., 2015). In the second construct, ChR2-EYFP is driven by a strong, generalized promoter (EF1α), but this transgene is oriented in a DIO configuration that requires Cre recombinase to reorient it to an active, drivable position in relation to the EF1α promoter. When co-infused into the VTA, the Cre is expressed only in TH+ (dopaminergic) neurons that are then the only cells that express ChR2-EYFP.

Stereotaxic virus injection. Subjects were anesthetized using ketamine hydrochloride (80 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.). Once placed in a stereotaxic frame, the scalp was shaved and wiped with iodine. The skull was uncovered by making an incision centrally along the scalp. Two small drill holes were fashioned for two skull screws to stabilize a cement cap. Two final holes were drilled on the right side above the VTA (from bregma: anterior 5.8 mm; lateral, 0.7 mm) for the virus injection and for the NAc (from bregma: anterior 1.3 mm; lateral, 1.3 mm), into which an optic-fluid cannula (OFC) (Doric Lenses, Canada) was implanted (DV, 7.0 mm). Next, a combination of DIO-ChR2-EYFP-AAV2/10 and TH-iCRE-AAV2/10 were coinjected (1.2 µl total) gradually into the VTA (DV, 7.3mm) over 13 min through the OFC via a Hamilton syringe. Previously we have shown that this combinatorial system restricts ChR2 expression to dopaminergic neurons in the VTA (Gompf et al., 2015; Mikhailova et al., 2016). Dental cement stabilized by skull screws was used to cover the exposed skull. Subjects were returned to their home cages for recovery once the cement was dry.

Operant Self-Administration. *Training.* Daily sessions were performed in commercially available, sound-attenuated operant chambers (Med Associates, East Fairfield, VT) as previously described (Samson et al., 1999). Each chamber contained a house light to signal the beginning of a session, a retractable lever and a sipper tube that extended into the chamber. The system was computer controlled and appetitive and consummatory responses were collected at 2 Hz and analyzed using MedPC software (Med Associates, St. Albans, VT).

To train rats to self-administer alcohol (ethanol), we used an abbreviated sucrose substitution protocol based on the method of Samson (Samson, 1986). Briefly, on the first day of shaping, animals were acclimated to chambers for two hours using a 10% sucrose solution on a fixed ratio (FR1) schedule that resulted in 45-second access to the sipper. Over the next consecutive days, session time, fixed ratio, and sipper access time were modified and the sucrose concentration was decreased by 1% each day and replaced with increasing concentrations of ethanol. The fixed ratio schedule was increased from a FR1 to a FR4 over an 8-day period. On day 9, the schedule was changed such that completion of a response requirement of eight lever presses (RR8) resulted in a twenty minute presentation of the reinforcer. Over the next consecutive sessions at 10% ethanol, the response requirement was gradually increased from RR10 to RR15, 20, 25 and finally 30 for at least 2 days at each response requirement (individualized for each rat's capability). This type of individual training allowed for all animals to reach the criterion of 30 presses per session by the end of the six week period. Appetitive and consummatory behaviors were monitored daily and data were collected using Med Associates software. Animals had 20 minutes to complete the RR30.

Extinction Probe Trials. To obtain an appetitive measure devoid of any consummatory behaviors, extinction probe trials were conducted. On the days when extinction probe trials were performed, rats were placed into the operant

chamber and allowed to respond on the lever for the entire 30 minutes, regardless of the number of lever presses completed. Extinction probe trials were conducted on each animal, at least two weeks apart.

*Optical Stimulation***.** Optical stimulations were performed during extinction tests at least 4 weeks following viral transfection. This interval was necessary to obtain the level of ChR2 expression that is adequate for effective triggering of DA release in terminals (Bass et al., 2010, 2013; Mikhailova et al., 2016). It is important to note that when optical stimulation experiments were performed they were randomized across animals so that some received phasic and some received tonic stimulation. Days of stimulation during the extinction trial were staggered across days of the week and no single animal was stimulated on more than one day per two week period. The optical setup had a laser at wavelength 473 nm (Beijing Viasho Technology Co., Ltd, Beijing, China) with a 100 mW maximum power output. A programmable function generator (Hewlett-Packard model 8116A) provided control signals to modulate the laser via the TTL input control port on the laser power supply. Parameters of the light presentation for tonic stimulation were 3000 pulses at 5 Hz (total light exposure of 10 minutes) and 50 pulses at 50 Hz with 30 s intervals for phasic stimulation (Bass et al., 2013). These light paradigms were applied for the first 10 minutes of the session. The optical pulse procedure began manually by firing a pulse generator (Systron Donner Model 100C), which activated a digital delay generator (SRS Model DG535). The digital delay generator was used to ensure the function generator was appropriately gated to select a finite number of pulses from the continuous waveforms typically produced by the function generator. The function generator produced a series of 5 Hz and 50 Hz square pulses. The total number of pulses in one data stream was gated by the digital delay generator because the temporal length of a gate pulse received by the function generator dictated the number of square pulses produced for each trigger by the function generator. Individual pulses had a temporal width of 4 ms within each series of pulses. A commercial power meter (Thorlabs Model S121C, Newton, New Jersey) was used to measure the laser power output.

Fast-scan cyclic voltammetry recordings. Extracellular DA concentrations, before and after optical stimulation of the VTA, were measured using FSCV. The experiments were performed at least 4 weeks following viral infusion. All subjects were secured in a stereotaxic frame following urethane (1.5 g/kg, i.p.) anesthesia. After the scalp was shaved and cleaned, the skull was uncovered by making a central incision along the scalp. One hole was fashioned above the NAc (from bregma: anterior, 1.3 mm; lateral, 1.3 mm) and another hole above the ipsilateral VTA (from bregma: posterior, 5.8 mm; lateral, 0.7 mm). A final hole was placed on the contralateral hemisphere for the implantation of an Ag/AgCl reference electrode connected to a voltammetric amplifier (UNC Electronics Design Facility, Chapel Hill, NC). A carbon fiber microelectrode (exposed fiber length: 100 µm; diameter: 6 µm) connected to the voltage amplifier and secured to the stereotaxic frame arm was lowered into the hole drilled above the NAc (ventral, 7.4 mm). Photostimulation was achieved via an optical fiber (200 µm diameter) inserted above the VTA and connected to a laser (Viasho, China). To generate tonic and phasic patterns of DA increases, we applied the protocol used in previous studies (Bass et al., 2013; Mikhailova et al., 2016). In the experiment with tonic-phasic interplay (Fig.3), two optical fibers connected to different lasers were placed above the VTA with 750-800 µm distance between tips. A calculation based on the cones of light emanating from the two sources indicated that 93-95 % of the light exposed volume was not overlapped by these two sources of photostimulation. In addition it is the edges of the two separated light cones that overlap, and the intensities are weaker in each of the beams near their peripheries than in their central regions, making the effects of the small overlap even weaker than the 7% volumetric overlap would suggest. This overlap was calculated using the following formula for a cone cut by a plane parallel to the cone's symmetry axis

$$V = \frac{H}{3R}\left[R^3 \cos^{-1}\left(\frac{x}{R}\right) - 2Rx\sqrt{R^2 - x^2} + x^3 \ln\left(\frac{R + \sqrt{R^2 - x^2}}{x}\right)\right],$$

where *H* is the cone height, *R* is the radius at the base of the cone, and x is the distance from the center of one of the cones to the bisecting plane. We have two of these zones contributing to our overlap region, so the overlap volume is $V_{overlap} = 2V$. In our case, *H* = 1.13 mm, which is estimated based on stereotaxic coordinates for the VTA (Paxinos and Watson, 2009), *R* = 0.478 mm, based on the numerical aperture of each fiber (NA = 0.39) and its diameter (200 µm), and *x* = 0.275 mm, is the distance to a point midway between the nearest edges of the two fibers.

Therefore, given the small overlap volume and the weaker intensities in the overlap regions, we can conclude that the majority of DA cell bodies were separately activated with the tonic (5 Hz) or phasic (50 Hz) protocol without any overlap. The light paradigm consisted of 50 light pulses at 50 Hz with 10 s intervals for phasic stimulation and 100 light pulses at 5 Hz for tonic stimulation. It is important to highlight that tonic stimulation was applied 5 s before the first phasic stimulation and ended 5 s after the second phasic stimulation, when these two patterns were combined. Under this circumstance, the increase in tonic DA release should result in an activation of DA autoreceptors that triggers a negative feedback mechanism of DA regulation. Therefore, when the high frequency stimulation was delivered, the presynaptic state was tuned to diminish phasic DA response. The averaged amplitude from two phasic transients, triggered with a 10-second interval, was used for the evaluation of changes in DA release.

Voltammetric recordings occurred at the carbon fiber electrode every 100 ms by applying a triangular waveform (-0.4 to +1.3V, 400 V/s). Oxidation and reduction peaks were observed at +0.6 V and -0.2 V respectively (vs. Ag/AgCl reference) identifying DA as the released chemical. Data were digitized (National Instruments, Austin, TX) and stored on a computer. Calibration of the carbon fiber electrodes was performed with known concentrations of DA (5, 10 µM) *in vitro* (Mateo et al., 2004; Oleson et al., 2009).

Nissl staining. Following the end of the experiment, rats were transcardially perfused with 4% paraformaldehyde and their brains were removed. The brains were then sliced and underwent Nissl staining to confirm proper placement of the recording and stimulating electrodes. Briefly, brains were removed and submerged in sucrose solutions of increasing concentrations (10%, 20%, and 30%) for at least 24h each, allowing the brain to sink before moving to the next concentration and kept at 4°C until further processing. Slices containing the NAc and VTA were taken using a vibratome (50 µm thickness; Vibratome 1000 Plus, The Vibratome Company, St. Louis, MO, USA) and mounted onto slides. After allowing the slides to dry at room temperature overnight, they were then processed for Nissl staining. Slides were submerged in deionized water (DI H_2O) and a series of ethanol solutions (50%, 70%, 90%, and 100%) for 3 min each rinse. They were then placed into 2 separate baths of xylene for 10 and 15 min each, respectively. Following this, the sections were rehydrated through a series of descending ethanol concentrations (100%, 95%, 70%, and 50%) followed by DI H_2O for 3 min each rinse. Next, the slices were submerged and stained in the cresyl violet solution (cresyl violet acetate, 0.1% aqueous, Electron Microscopy Sciences #26089-01) until the desired staining intensity had been achieved (typically 5-10 submersions) and then moved to a differentiation solution (0.5% glacial acetic acid) for 1-2 quick rinses. Then following 3 rapid DI H_2O rinses, the slices were dehydrated through a series of increasing ethanol concentrations (50%, 70%, 95%, and 100%) and 2 additional rinses of 100% ethanol for 3 min each concentration. Finally, the slides were submerged in xylene for 10 min before being placed in a final bath of xylene from which they were taken and coverslipped using DePeX mounting medium (Serva #18243.01). After allowing to dry overnight, slides were visualized and the locations of the recording and stimulating electrode tips were noted (see Fig. S3).

Immunohistochemistry. Rats were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then transcardially perfused with 10% normal buffered formalin. After removal, brains were soaked overnight in fixative at 4º C and then incubated in a 25% sucrose solution overnight until the brains sank. Fifty µm thick sections were obtained on an American Optical 860 sliding microtome. Free-floating coronal sections, which contained the midbrain, were processed for immunohistochemistry. Briefly, sections were washed in PBS for 5 min followed by 3 x 10 min rinses in PBS + 0.5% triton X-100. Primary antibody diluted in PBS + 0.3% triton X-100 was applied overnight at 4ºC while shaking. Primary antibodies used were mouse anti-tyrosine hydroxylase (ImmunoStar #22941) at a 1:4000 dilution and a rabbit anti-GFP (Invitrogen #A6455, also cross reacts with EYFP) at a 1:2000 dilution. The following day, sections underwent 3 x 10 min PBS rinses and then were incubated with secondary antibodies of Alexa 555 donkey anti-mouse (Invitrogen, #A31570, 1:4000) and Alexa 488 goat anti-rabbit (Invitrogen #A11034, 1:2000) at room temperature for 2 hours while shaking. A last set of 3 x 10 min PBS rinses were applied to the sections that were then mounted onto slides and coverslipped with Prolong Gold media. Slides were visualized via a Zeiss LSM 710 confocal microscope.

Statistical analysis. Data were analyzed in GraphPad Prism (GraphPad Software, San Diego, CA). The sample size (n=7 rats per each experimental group) was based on power analyses and was similar to those reported in previous studies (Bass et al., 2013, Fox et al., 2016; Mikhailova et al., 2016). The Shapiro-Wilk normality test was used to attest to the normality of the data sets. Based on results of this evaluation, the data were further analyzed with parametric (one-way ANOVA with multiple comparison test) or nonparametric approaches (Kruskal-Wallis,

Wilcoxon or Mann-Whitney test) to determine statistical significance. Spearman correlation analysis was used to evaluate the relationship between lever pressing behavior (alcohol seeking) and alcohol consumption. The data were presented as mean ± s.e.m. and the criterion of significance was set at $P<0.05$.

Supplemental References

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