Glutamatergic Ventral Pallidal Neurons Modulate Activity of the Habenula–Tegmental Circuitry and Constrain Reward Seeking

Supplemental Information

Supplemental Methods and Materials

Animals. Adult *VGlut2-ires-cre, VGAT-ires-cre, ChAT-Cre, PV-Cre* and wild-type litter mates on a C57BL/6J background were used [1-3]. We used both male and female mice for all experiments, while we did not detect differences between males and females in our electrophysiological or behavioral effects, our groups were not adequately powered to include sex as a between-subject variable for experimental analysis. Mice were group housed (2 – 4 / cage, single sex) in the temperature- and humidity-controlled vivarium at the University of Maryland School of Medicine, and were maintained on a reverse 12 h light cycle (lights off at 07:00) with *ad libitum* access to food and water. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, as adopted by the NIH, and with approval of the Institutional Animal Care and Use Committee at the University of Maryland.

Stereotaxic Surgery. Anesthesia was induced and maintained with isoflurane at 5% and 1.5%, respectively. Mice were placed in a stereotaxic frame and craniotomies were performed. For optogenetic, electrophysiology and behavioral experiments, purified and concentrated adeno-associated viruses coding for Cre-inducible ChR2-eYFP (AAV5-EF1a-DIO-hChR2(H134R)-eYFP, 350 nL), mCherry (AAV5-EF1a-DIO-mCherry, 500 nL), rabies virus (AAV8-CAG-DIO-RG, AAV5-EF1a-DIO-TVA-mCherry, 250 nL in a 1:1 mixture), or taCasp (AAV5-flex-taCasp3-TEVp; 350 nL) was bilaterally injected in the VP (coordinates relative to Bregma, AP: +0.2 mm, ML: ±1.5 mm, DV: 4.6 mm). All viruses were obtained from the University of North Carolina Vector Core Facility. Injections were carried out using graduated pipettes broken back to a tip diameter of 10-15 µm, at a rate of 0.05 µl / min, pipettes were left in situ for an additional 8 minutes to allow for

the diffusion of virus. Minimum viral expression time was 28 days. For behavioral experiments, optic fibers (constructed in house as described previously [4]) were implanted bilaterally over the VP and secured with 3 skull screws and dental cement. Animals were allowed to recover for at least 1 week before behavioral training, and only mice with viral infection sites and fiber placements in the VP were included in the analyses.

Immunohistochemistry. Mice were first perfused transcardially with 1X phosphate buffered saline (PBS) then with 4% paraformaldehyde dissolved in PBS. Brains were removed and placed in 4% paraformaldehyde unit sectioning. The brains were sectioned using a vibratome (Leica VT1000S) at 40 µM and used for immunohistochemical staining. For parvalbumin (PV) and choline acetyltransferase (ChAT) staining, slices were permeabilized with 0.3% Triton-X-100 in PBS for one hour at room temperature; blocked in 5% normal goat serum with 0.3% Triton-X-100 in PBS for 2 hours; slices were incubated with mouse polyclonal anti-PV primary antibody (1:1000 in PBS, Swant, PV-235) or rabbit unconjugated polyclonal anti-ChAT primary antibody (1:100 in PBS; ThermoFisher Scientific, PA5-29653) overnight at 4°C; rinsed in PBS three times for thirty minutes each; blocked with 0.3% Triton-X-100 and 2% bovine serum albumin in PBS for ninety minutes; incubated in goat anti-mouse IgG (PV) or donkey anti-goat (ChAT) secondary antibody conjugated to AlexaFluor 488 (1:500 in 0.25% Tween 20 in PBS) for 2 hours at room temperature; rinsed in PBS three times for 30 min each and mounted with SlowFade Diamond Antifade Mountant with DAPI (Invitrogen).

Fluorescent In Situ Hybridization. Mice were euthanized and fresh tissue was frozen on dry ice, stored at -80°C and sectioned with a cryostat (Leica, CM3050) at 15 µM. Tissue was processed using RNAscope® Technology in situ hybridization assay according to provided protocols (ACDBio). Briefly, slides were transferred from -80°C and fixed with formalin, dehydrated with increasing concentrations of ethanol and pretreated with provided protease solution before hybridization with probes targeted against Slc32A1 and Slc17A6 mRNA.

Following 2 h of hybridization at 40°C, sections were washed and fluorescence was amplified with provided buffer solutions. Sections were incubated with DAPI before cover-slipping for visualization.

Microscopy and Imaging. Slides were initially scanned with an Olympus Flowview slidescanning microscope (Olympus BX61VS, Japan) at 10X and visualized with Olivia software to identify areas of interest. For co-localization immunohistochemistry and fluorescent in situ hybridization experiments, sections of the VP through the rostro-caudal extent were imaged with a confocal laser-scanning microscope (LSM2010; Zeiss). For each mouse, 6 images were acquired at 40X oil objective. The brightness and contrast of images were adjusted to optimize visualization, and adjustments were applied uniformly across each image. Cell counting was performed with ImageJ (National Institutes of Health; Bethesda MD). For in situ hybridization experiments, a 40X objective on a slidescanning microscope (Olympus BX61VS, Japan) was used to capture fluorescent images. For rabies tracing experiments, 10x images acquired from Olympus Flowview slide scanning microscope were regions of interest were manually delineated based on the atlas of Paxinos and Watson, and subsequently imported to ImageJ for cell counting.

Patch Clamp Electrophysiology. Whole-cell patch-clamp recordings were made from coronal slices (220 µm) of the VP, LHb, VTA and RMTg were prepared using a vibratome (Leica VT 1000S) in ice cold cutting solution (in mM: 667 $CaCl_2$, 91 $C_5H_{14}ClNO$, 27.5 $C_6H_{12}O_6$, 25 $NaHCO_3$, 7 $MgCl_2$, 11.6 $C_6H_8O_6$, 3.1 $C_3H_3NaO_3$, 2.5 KCl, and 961.5 NaH_2PO_4) continuously bubbled with 95% O_2 and 5% CO_2. Slices were incubated at 32ºC for 30 min in carbogen bubbled artificial cerebrospinal fluid (aCSF; in mM: 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.0 Na_2HPO_4, 26.2 $NaHCO_3$, and 11 glucose) followed by storage at room temperature until electrophysiological recordings were performed. Slices were hemisected and superfused with aCSF at 30 ± 2ºC. Neurons were recorded using borosilicate glass pipettes (5-7 MΩ resistance) pulled on a micropipette puller (Narishige PC-100) filled with cesium-based (in mM: 110 CsMeS, 15 KMeS,

0.02 EGTA, 10 Hepes, 0.5 $MgCl_2$, 1.5 $MgSO_4$, 4 ATP-Na_2, 0.3 GTP-Na_3, 10 $C_6H_{12}O_6$, and 10 Phosphocreatine-Na_2 with 7.3 pH and 289 Osm) or cesium-free physiological internal solution (in mM: 130 $C_6H_{11}KO_7$, 10 $C_4H_{10}N_3O_5P$, 4 $MgCl_2$, 3.4 ATP-Na_2, 0.1 GTP-Na_3, 1.1 EGTA, and 5 Hepes with 7.3 pH and about 289 Osm). Currents were amplified, filtered at 2 kHz, and digitized at 10 kHz using a MultiClamp 700B amplifier and Digidata 1440A (Molecular Devices). Clampex 10.7.0.3 software (Molecular Devices) was used for data acquisition. Series resistance was monitored by a hyperpolarizing step of -15 mV for 5 msec every 10 sec; the cell was discarded if the series resistance changed by more than 15%. Neurons expressing ChR2/eYFP or mCherry were visualized through epifluorescent light using a mercury bulb lamp (Nikon C-SHG1) and stimulated by flashing 470 nm light (4 msec) through the light path of the microscope powered by a shutter driver (Uniblitz VMM-D1). Paired pulse ratios (PPRs) were evoked at an interval of 50 msec. All agents were purchased from Sigma Biosciences.

In Vivo Electrophysiology. After 6 weeks of viral ChR2 expression, *VGlut2-ires-cre* mice were lightly anesthetized with isofluorane. Optic fibers were implanted over the VP (coordinates relative to Bregma, AP: +0.2 mm, ML: ±1.5 mm, DV: -4.6 mm) and craniotomies were made over the LHb (coordinates relative to Bregma, AP: -1.5 mm, ML: ±0.3 mm, DV: -2.35 mm), VTA (coordinates relative to Bregma, AP: -3.8 mm, ML: ±0.8 mm, DV: -4.4 mm) and RMTg (coordinates relative to Bregma, AP: -4.2 mm, ML: ±0.6 mm, DV: -4.2 mm). Silicone electrodes with 16 recording sites (silicon optrode (A1x16-Poly2-5mm-50s- 177, Neuronexus, Ann Arbor, MI) was inserted and lowered from the surface of the brain at approximately 100 μm/sec. After reaching stereotaxic coordinates of the recording site, the electrode was left for 15 minutes for tissue to stabilize before beginning recordings. During recordings, anesthesia was maintained at 0.5%, and blue light was delivered to the VP through the optic probe in 1 sec pulses, presented with a 10 sec inter-trial interval (ISI). Following 60 minutes of recording, the electrode was removed and the procedure was repeated in the additional two recording sites. During the second

day of recording, the same procedure was repeated and the animals were then sacrificed in order to verify the viral expression and the location of the recording electrode. Recordings were performed on a 16 channel PlexControl electrophysiology system and Plexon offline sorter (Plexon, Dallas, TX, USA) was used for spike sorting. NeuroExplorer was used to analyze neural firing rates and changes in neuronal responses to light stimulation. PSTHs were computed with 100 msec bins around onset of 1 sec of blue light stimulation. To test whether a neural response of an isolated unit was significantly modulated by activation of glutamatergic VP neurons, paired t-tests were performed on the average firing activity of each isolated unit in the 1 sec period during light onset relative to activity during the 1 sec baseline period immediately preceding light onset A unit was determined to be light-modulated if the difference between the average activity between baseline and light-onset was statistically significant (paired t-test, $p = 0.05$). We used electrophysiological criteria, histology and modulation of the LFP by blue light to confirm our recording sites; given that recording sites spanned 0.5 mm along the electrode shaft, if there was no significant modulation of LFP by blue light stimulation, units recorded on the same channel were excluded from further analysis. Autocorrelelagrams were performed to exclude duplicate units.

Behavioral Studies.

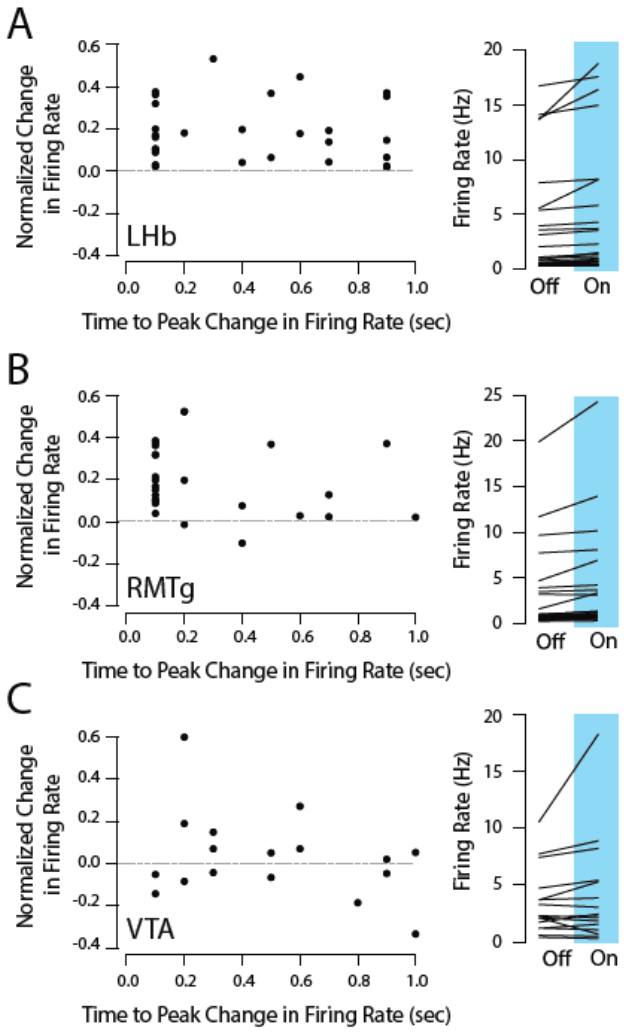
Real time place preference (RTPP): *VGlut2-ires-cre* (n = 4 males, 4 females) and wildtype littermates (n = 3 males, 5 females) were injected with channelrhodopsin in the VP as described above. After 6 weeks of expression, optic fibers were implanted over the VP; following one week of mice were run in a real-time place preference task (RTPP). The mice were individually run in a behavioral arena (black Perspex, 30 cm x 30 cm x 40 cm) that contained three chambers: two experimental zones and a connecting neutral zone. Two experimental zones were distinguished by floor texture and wall patterns. During a pre-test session, mice were placed in the neutral zone of the arena and allowed to move freely for 20 min. During the test session, 4 msec blue light (473

nm) pulses were delivered at 1, 10 or 20Hz to the optic fibers when the mouse entered the "active" experimental zone, there were no consequences while the mouse was in the "inactive" experimental zone. Active and inactive zones were counterbalanced across subjects. The amount of time the mouse spent in each of the zones was detected and recorded by a CCD camera and Ethovision software (Noldus Information Technologies).

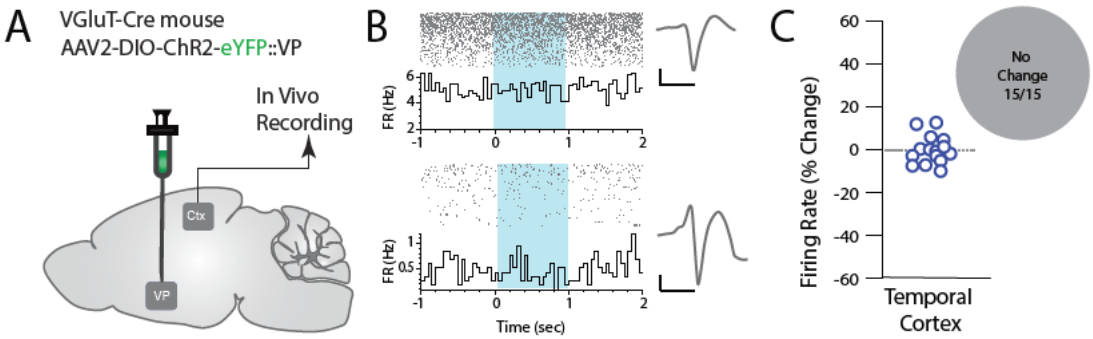
Operant task: Starting at 8 weeks of age, mice (WT n = 4 males, 4 females; *VGluT2-ires-cre* n = 4 males, 3 females) were lightly food restricted (no loss of body weight) before training on a fixed-ratio (FR) operant task. Mice were placed in operant chambers equipped with a house light, active and inactive lever, and a sucrose pellet dispenser (Coulbourn Instruments). During a 60 min session, mice were allowed to lever press for sucrose pellets (Test Diet), an active lever press resulted in a pellet being dispensed. Each press of the active or inactive lever was followed by a 20 sec time-out where further lever presses had no consequence. Following stable lever press responding (less than 20% variation over 5 consecutive test days), mice were moved to an FR2 schedule for 5 days and FR3 schedule for a final five days of training. Following this acquisition, mice were tested for 2 days on an FR5 task, followed by a progressive ratio (PR) session (according to the schedule: 1, 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328). This sequence was repeated three times to achieve baseline measurements. Mice were then injected with AAV5-flex-taCasp-TEV to the VP and allowed three days of recovery following surgery. Testing continued identical to baseline sessions for a total of 10 days.

Conditioned taste aversion: During a 4 day habituation phase, mice (WT n = 4 males, 5 females; *VGluT2-ires-cre* n = 3 males, 3 females) were water deprived and given 30 min/day to consume tap water in the experimental arena. The water was presented in liquid feeding tubes (Bioserv) in a corner of the arena. After 4 days of habituation, the water was replaced with 5% sucrose solution and the mice had 7 days of training. The amount of time the mice spent drinking was recorded using infrared break-beam sensors connected to a microcontroller (Arduino), and sessions were

recorded using a camera. After 4 days of consistent sucrose drinking (defined as < 20% variation in amount of time spent drinking per session), the mice were given an injection of 0.15 LiCl M solution (2% body weight, i.p.) following their 30 min allotted drinking time. The mice were tested during the following day to assess drinking time.



Supplemental Figure S1. Latency to peak change in firing rate of LHb, RMTg and VTA neurons induced by optogenetic activation of glutamatergic VP neurons. Recordings were made in the LHb **(A)**, RMTg **(B)** and VTA **(C)**. For each significantly modulated, isolated unit, the normalized change in firing rate is plotted against the latency from light onset in the VP to the bin with the maximum change in firing rate from baseline (Left). For each significantly modulated, isolated unit, the absolute firing rate during baseline (off) and during light on-set is plotted (on). There was no significant difference in the latency to peak change in firing rate or in variance to peak firing rate between brain areas (F_2 = 2.701, p = 0.075, RMTg latency to peak: 0.28, var: 0.082 sec, LHb latency to peak: 0.43, var: 0.107 sec, VTA latency to peak: 0.50, var: 0.097 sec), nor was there a significant relationship between latency to peak change and magnitude of peak change in firing rate (RMTg R^2 = 0.049, p = 0.405, LHb R^2 = 0.003, p = 0.494, VTA R^2 = 0.105, p = 0.343).
Corresponds to Figure 5.



Supplemental Figure S2. Optogenetic activation of glutamatergic VP neurons has no effect on firing rates of temporal cortex neurons in vivo. **(A)** Experimental design, floxed ChR2-eYFP was injected into the VP of VGluT2-Cre mice, and *in vivo* recordings were performed in the temporal cortex. **(B)** Representative raster plots, perievent histograms aligned to onset of a 1 second optogenetic stimulation of VGluT2-positive VP neurons with waveform insets of two representative cortical units. **(C)** Of the 15 identified units, there was no significant change in firing rate in response to activation of VGluT2-positive VP neurons. Scale bars: 20 µsec, 20 mV. *Corresponds to Figure 5.*

Brain region		Percentage of total inputs
Striatal subregions	NAcC – accumbens core	9.7 (3.7)
	NAcS – accumbens shell	20.5 (6.2)
	DLS – dorsolateral striatum	7.2 (3.9)
	DMS – dorsomedial striatum	9.1 (2.0)
Cortical regions	OFC – orbitofrontal cortex	3.9 (0.8)
	mPFC IL – medial prefrontal cortex, infralimbic	4.5 (1.2)
	mPFC PL – medial prefrontal cortex, prelimbic	1.9 (1.4)
Subcortical nuclei: basal ganglia, septal and habenular nuclei	LSd – lateral septum, dorsal division	0.5 (0.0)
	LSv – lateral septum, ventral division	1.2 (0.3)
	GP – globus pallidus	6.4 (1.7)
	LHb – lateral habenula	0.5 (0.2)
	STN – subthalamic nucleus	2.8 (2.2)
Thalamic nuclei	CM – central medial nucleus	0.05 (0.04)
	PVN – paraventricular nucleus	2.5 (0.1)
Amygdala subregions	BLA – basolateral amygdala	2.6 (1.1)
	MedA – medial amygdala	9.1 (4.3)
	CeA – central amygdala lateral	2.3 (0.5)
	CeA – central amygdala central	3.5 (0.8)
	LA – lateral amygdala	0.3 (0.1)
Midbrain	RMTg – rostromedial tegmental nucleus	0.3 (0.0)
	SN – substantia nigra	4.7 (0.1)
	VTA – ventral tegmental area	4.9 (2.0)
	PAG – periaqueductal gray	0.5 (0.2)

Supplemental Table S1. Pseudo Rabies tracting reveals monosynaptic inputs onto glutamatergic VP neurons. List of individual brain regions examined along with percentage of total inputs arising from each brain region.

Supplemental References

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