Cell Reports, Volume *42*

Supplemental information

Lateral preoptic area glutamate neurons relay nociceptive information to the ventral tegmental area

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Supplementary Figures and Legends

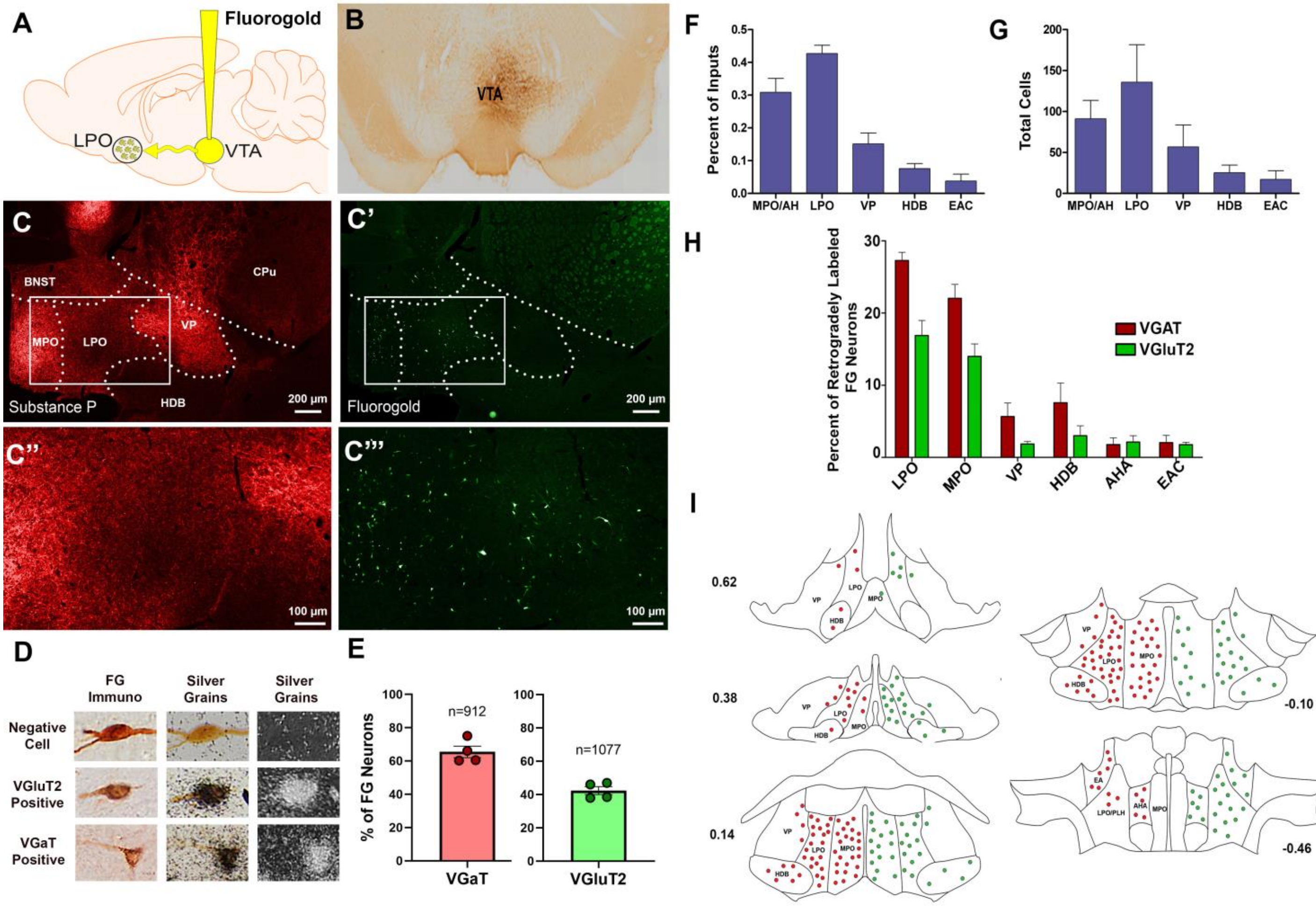


Figure S1. The preoptic area provides GABAergic and glutamatergic inputs to the ventral tegmental area. A, Iontophoretic delivery of the retrograde tract tracer Fluorogold (FG) into the VTA**. B,** VTA FG injection site**. (C-C'''),** Basal forebrain subdivisions based on substance P immunodetection (C and high magnification in C'') showing higher concentration of FG neurons in the lateral preoptic area (LPO) (C' and high magnification in C'''). **D,** FG-positive LPO neurons (brown) innervating VTA. In sequential tissue series, we quantified the number of FG neurons lacking VGluT2 or VGaT mRNA (negative cell; top), as well as those co-expressing FG (brown) and VGluT2 mRNA (middle) or VGaT mRNA (bottom). **E,** Frequency of LPO-VGaT (65.4 ± 3.5%), or LPO-VGluT2 (42.3 ± 2.4%) neurons innervating VTA. mean ± SEM from 4 rats. MPO, medial area; LPO, lateral preoptic area; BNST, Ventral Bed Nucleus of the Stria Terminalis; CPu, caudate putamen/striatum; VP, ventral pallidum and HDB, horizontal nucleus of the diagonal band. **F,** Percentage of neurons innervating the VTA (fluorogold cells, FG) in subregions of the basal forebrain. **G,** Total cell count of neurons innervating the VTA in each basal forebrain subregion. **H,** Percentage of VGaT (n = 4 mice) and VGluT2 (n = 4 mice) neurons innervating the VTA in each subregion of the basal forebrain: LPO (27.3 ± 1.2% VGaT, 16.9 ± 2.1% VGluT2); MPO (22.0 ± 1.9% VGaT, 14.0 ± 1.7% VGluT2); VP (5.7 ± 1.9% VGaT, 1.9 ± 0.4% VGluT2), HDB (7.6% ± 2.7% VGaT, 3.0% ± 1.4% VGluT2), AHA (1.8 ± 0.9% VGaT/ 2.1 ± 0.9% VGluT2), and EAC (2.1 ± 1.0% VGaT, 1.8± 0.3% VGluT2) **I,** Summary of LPO-VGluT2 neurons innervating the VTA (Green; Right) or LPO-VGaT (Red; Left) neurons at different anteroposterior coordinates. LPO, the lateral preoptic area; MPO, medial preoptic area; VP, ventral pallidum; HDB, horizontal diagonal band; AHA, anterior hypothalamic area anterior part; EAC, extended amygdala central part.

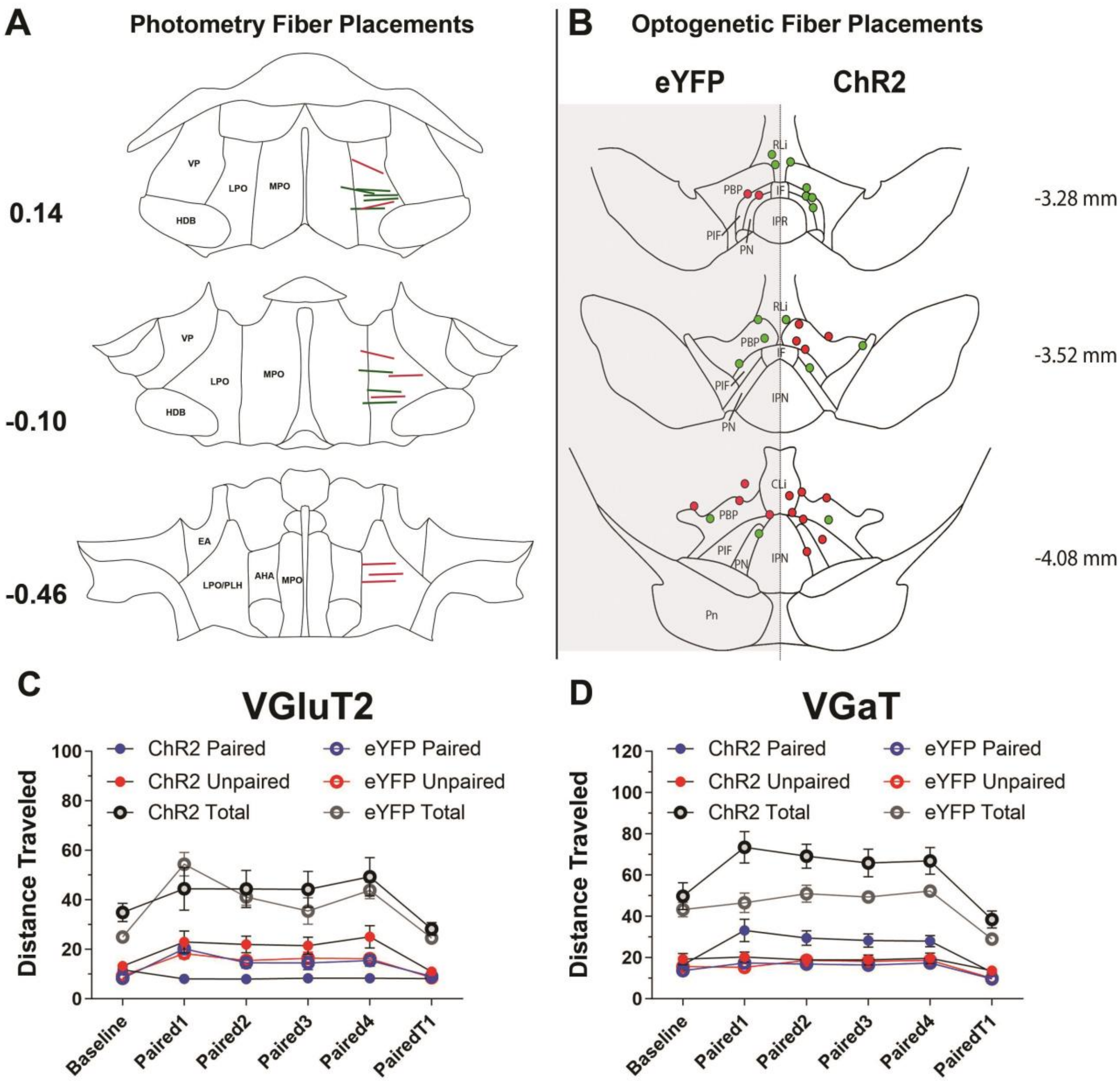


Figure S2. Fiber optic placements over the LPO for fiber photometry and VTA for optogentic stimulation. A, Lines represent the placement of fiber optics for recording LPO signals in response to optogenetic stimulation of nociceptors in the hindpaw. Red lines indicate placements in *vgat:Cre* mice, while green lines indicate placements in *vglut2:Cre* mice. **B,** Dots representing fiber placements for optogenetic stimulation of LPO terminals in the VTA. Dots on the left side represent fiber placements for eYFP controls, while dots on the right represent the placements for ChR2-eYFP mice. Red dots indicate placements in *vgat:Cre* mice, while green dots indicate placements in *vglut2:Cre* mice. **C-D,** Distance traveled by *vglut2:Cre* and *vgat:Cre* mice in the real time place conditoning task (**Figure 4**) during the first week of testing for the photostimulation paired chamber, unpaired chamber, and the total distance traveled (paired+unpaired+connecting chambers) **C,** *vglut2:Cre* mice with ChR2 traveled similar distances to eYFP controls, but spent less time in the paired chamber. **D,** *vgat:Cre* mice showed increased locomotion in the photostimulation-paired chamber, which is reflected in their overall greater distance traveled. Note: The baseline and test (T1) session are 15 minutes in duration, while pairing sessions 1-4 are 30 minutes in duration.

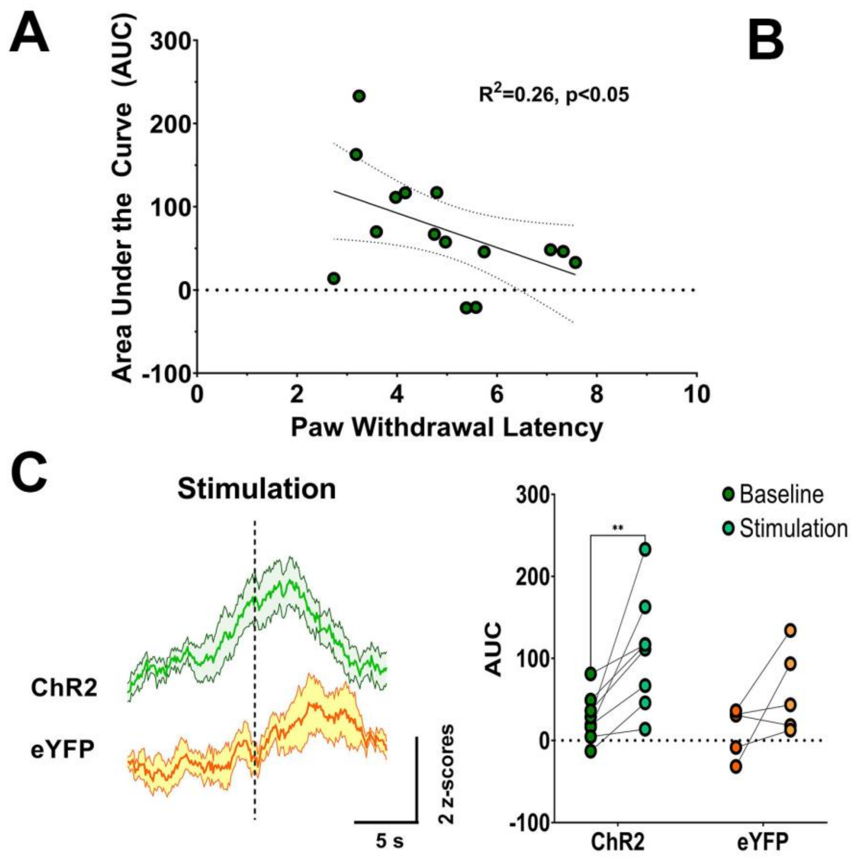


Figure S3. The activity in LPO VGluT2 neurons targeting the VTA is correlated with behavioral responses and specific to optogenetic stimulation. A, Paw withdrawal latency in response to optogenetic stimulation of the hindpaw correlates with the magnitude of activity in LPO-VGluT2 neurons innervating the VTA ($R^2 = 0.26$, $p = 0.045$). **B,** *vglut2::cre* mice injected whose hindpaw was injected with an AAV6-Syn-eYFP control vector show significantly longer paw withdrawal latencies ($t_{(12)} = 5.592$, $p = 0.0001$), with several mice hitting the 10 second cutoff time. **C,** Mice with ChR2 in the hindpaw showed increased activity in LPO-VGluT2 neurons innervating the VTA when compared to their pre-stimulation baseline ($p = 0.0074$), while mice expressing eYFP did not ($p = 0.204$). **D,** Responses for LPO-VGluT2 neurons innervating the VTA in response to the first (bottom) and last (top) tone presentation during the first conditioning session. Tone responses are absent in the first trial, but develop by the end of the first conditioning session.

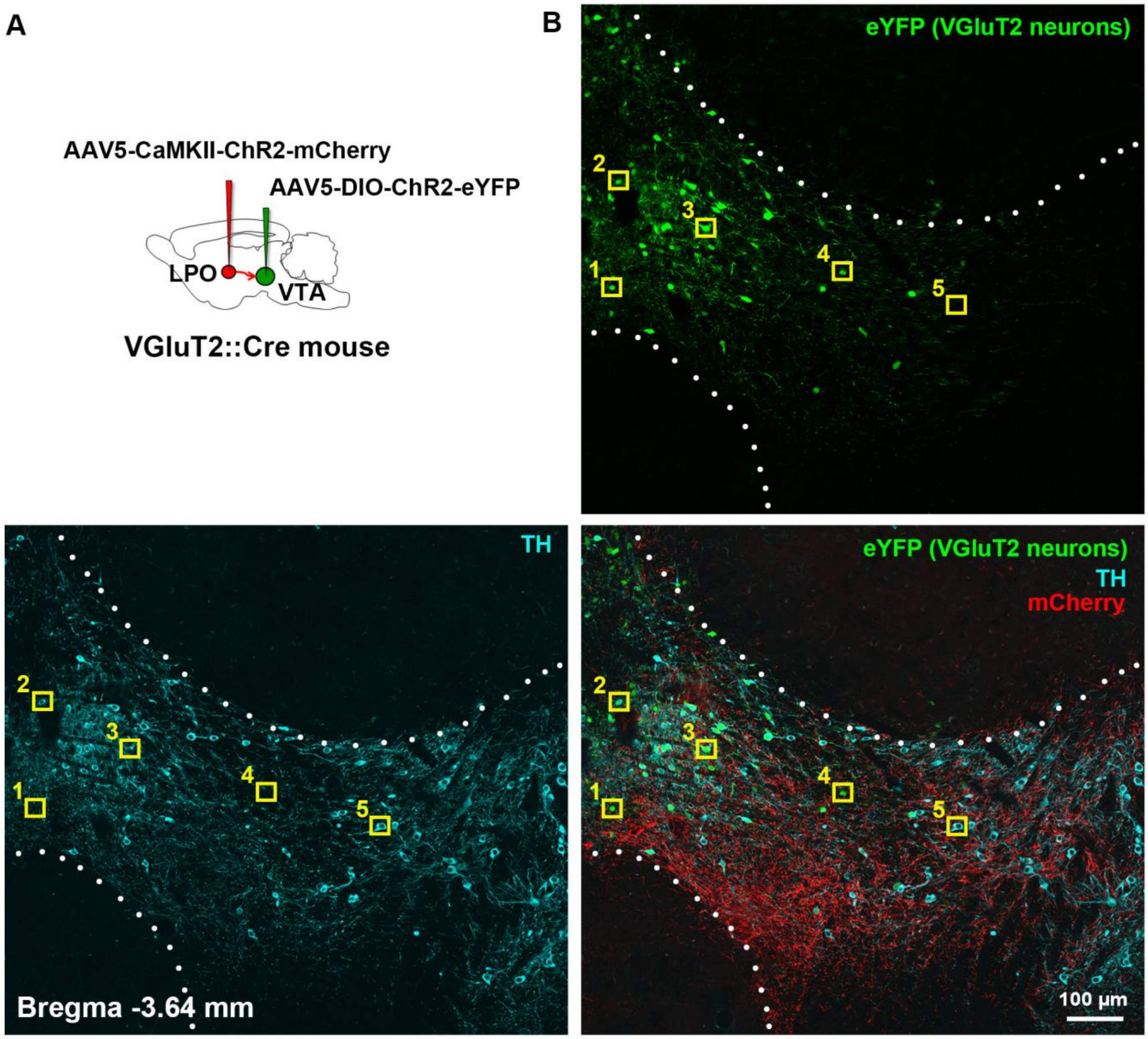


Figure S4. LPO neurons established synapses on VTA TH and VGluT2 neurons A, Diagram of viral injection of AAV5-CaMKII-ChR2-mCherry into the LPO and AAV5-DIO-ChR2-eYFP into the VTA of *vglut2:cre* mice. **B,** Confocal micrograph of VTA at low magnification; VGluT2-eYFP neurons (green), TH (cyan) and mCherry fibers from LPO (red). Neuron 1 in the medial VTA and neuron 4 in the mediolateral VTA represent eYFP-only neurons. Neuron 2 in the medial VTA and neuron 3 in the mediolateral VTA represent dual TH-eYFP neurons. Neuron 5 in the lateral VTA represent TH-only neurons.

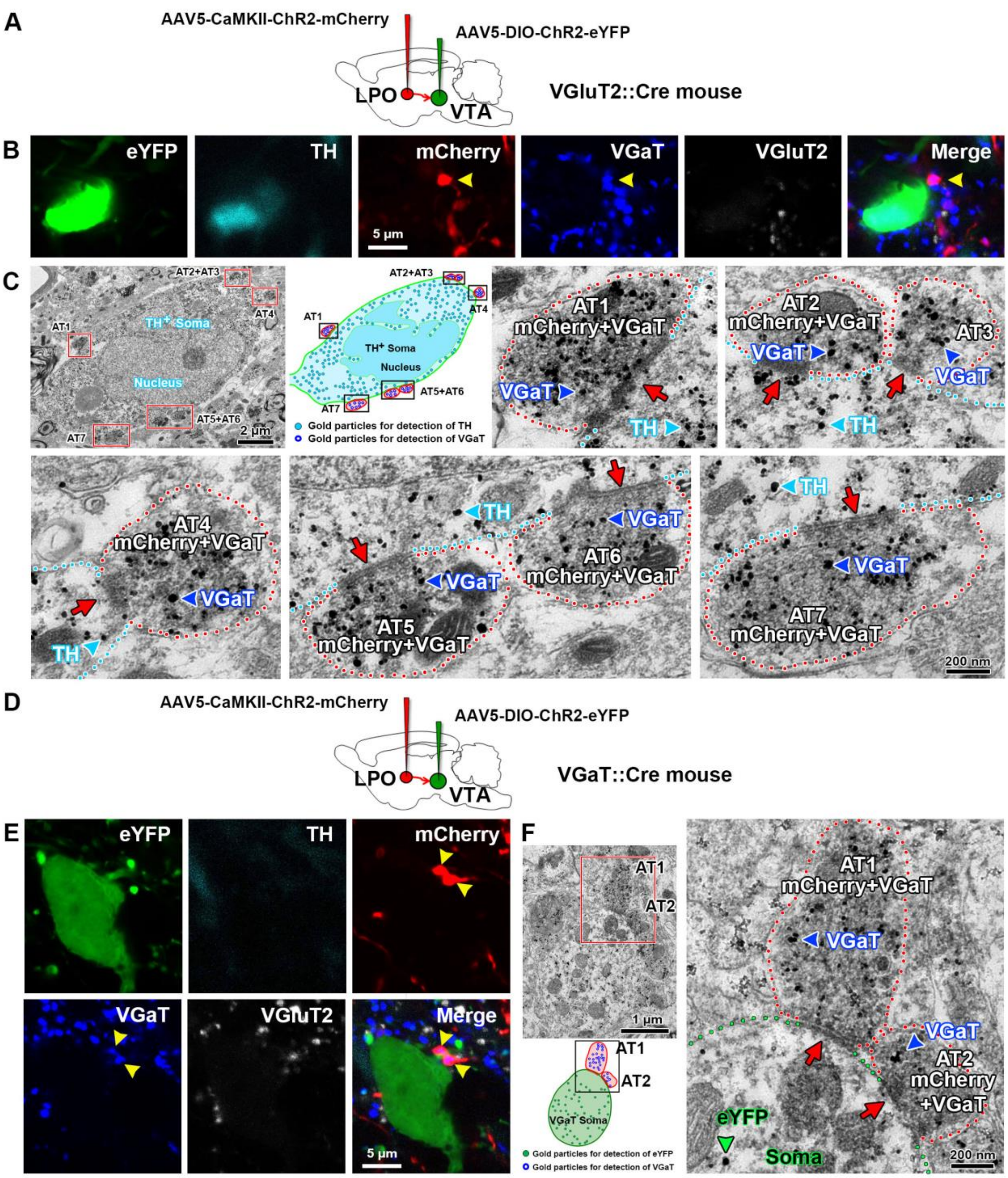


Figure S5. Axon terminals from the LPO VGaT neurons establish symmetric synapses on VTA TH, VGluT2, or VGaT neurons. A, Diagram of viral injection of AAV5-CaMKII-ChR2-mCherry into the LPO and AAV5-DIO-ChR2-eYFP into the VTA of *vglut2:cre* mice. **B,** Confocal micrographs at higher magnification showing the soma of a VTA neuron co-expressing TH and eYFP in contact with an LPO-terminal co-expressing mCherry and VGaT protein (arrowheads). **C,** Electron micrographs and corresponding diagram showing seven VGaT ATs (AT1-7, red outlines) from LPO neurons co-expressing mCherry (scattered dark material) and VGaT, (blue arrowheads, gold particles) making symmetric synapses (red arrows) on a common soma (cyan outline) of a neuron expressing TH (detected by gold particles, cyan arrowheads). **D,** Diagram of viral injection of AAV5-CaMKII-ChR2-mCherry into the LPO and AAV5-DIO-ChR2-eYFP into the VTA of *vgat:cre* mice. **E,** Confocal micrographs at higher magnification showing two LPO-VGaT terminals co-expressing mCherry and VGaT protein (arrowheads) in contact with the soma of a VTA VGaT-eYFP. **F,** Electron micrographs and corresponding diagram showing two axon terminals (AT1-2, red outlines) from LPO neurons co-expressing mCherry (scattered dark material) and VGaT (blue arrowheads, gold particles) making symmetric synapses (red arrows) on a common soma (green outline) of a VTA-VGaT-eYFP neuron (eYFP detection by gold particles, green arrowhead).

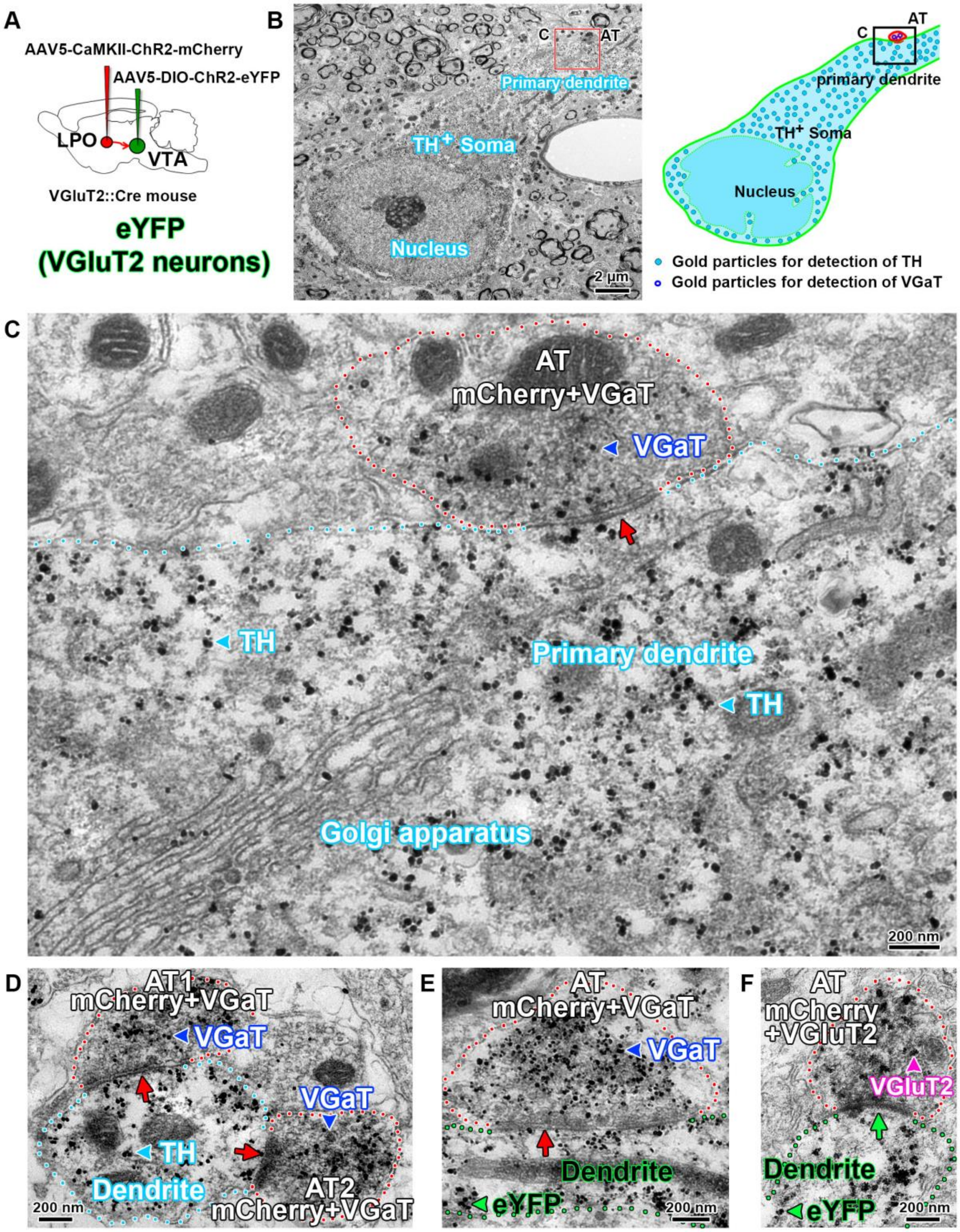


Figure S6. Axon terminals from the LPO VGaT neurons establish symmetric synapses with TH and VGluT2 dendrites. A, Diagram of injections of the AAV5-CaMKII-ChR2-mCherry viral vector into the LPO and AAV5-DIO-eYFP viral vector into the VTA of *vglut2:cre* mice. **B-C,** Electron micrograph at low magnification (**B**) higher magnification (**C**) and corresponding diagram showing a showing a VGaT axon terminal (AT, red outlines) from a LPO neuron co-expressing mCherry (scattered dark material) and VGaT (blue arrowhead, gold particles) establishing a symmetric synapse (red arrow) with the primary dendrite of a TH neuron (cyan outline and arrowheads, gold particles). **D,** Two VGaT axon terminal (AT1-2, red outlines) from LPO neuro co-expressing mCherry (scattered dark material) and VGaT (blue arrowheads, gold particles) establishing symmetric synapses (red arrows) with common TH dendrite (cyan outline and arrowhead, gold particles). **E,** A VGaT axon terminal (AT, red outlines) from a LPO neuron co-expressing mCherry (scattered dark material) and VGaT (blue arrowhead, gold particles) establishing a symmetric synapse (red arrow) with an VTA-eYFP-VGluT2 dendrite (green outline and arrowhead, gold particles). **F,** A VGluT2 axon terminal (AT, red outlines) from a LPO neurons co-expressing mCherry (scattered dark material) and VGluT2 (pink arrowhead, gold particles) establishing an asymmetric synapse (green arrow) with an eYFP-VGluT2 dendrite (green outline and arrowhead, gold particles).

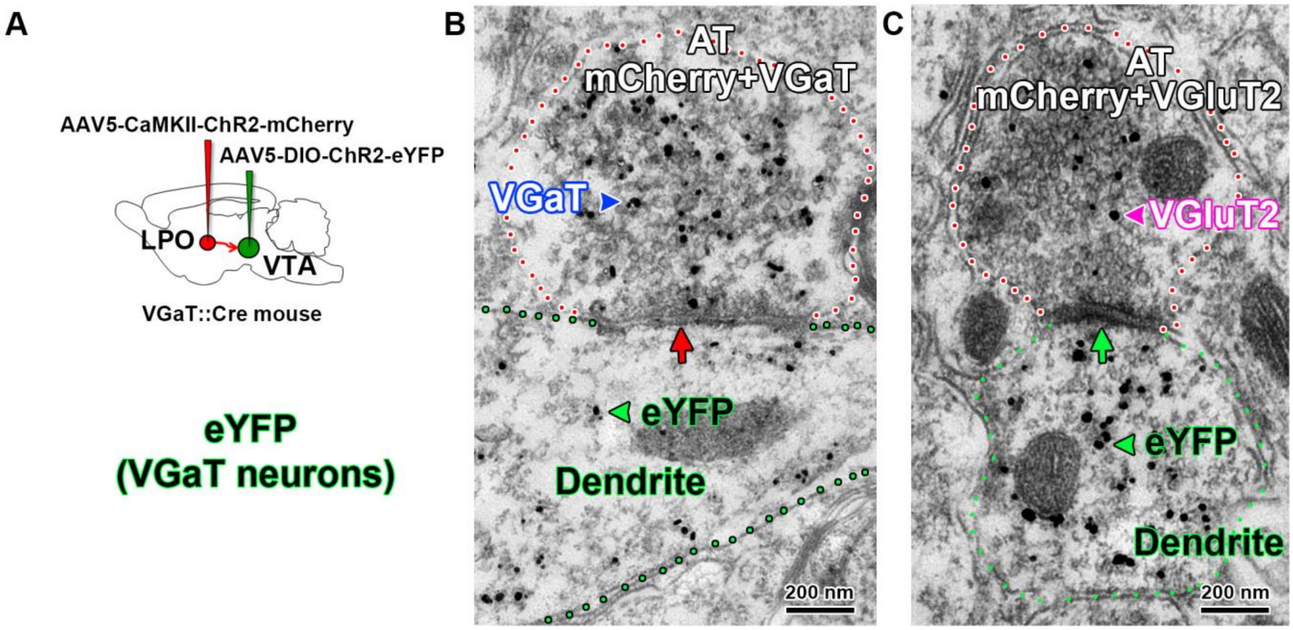
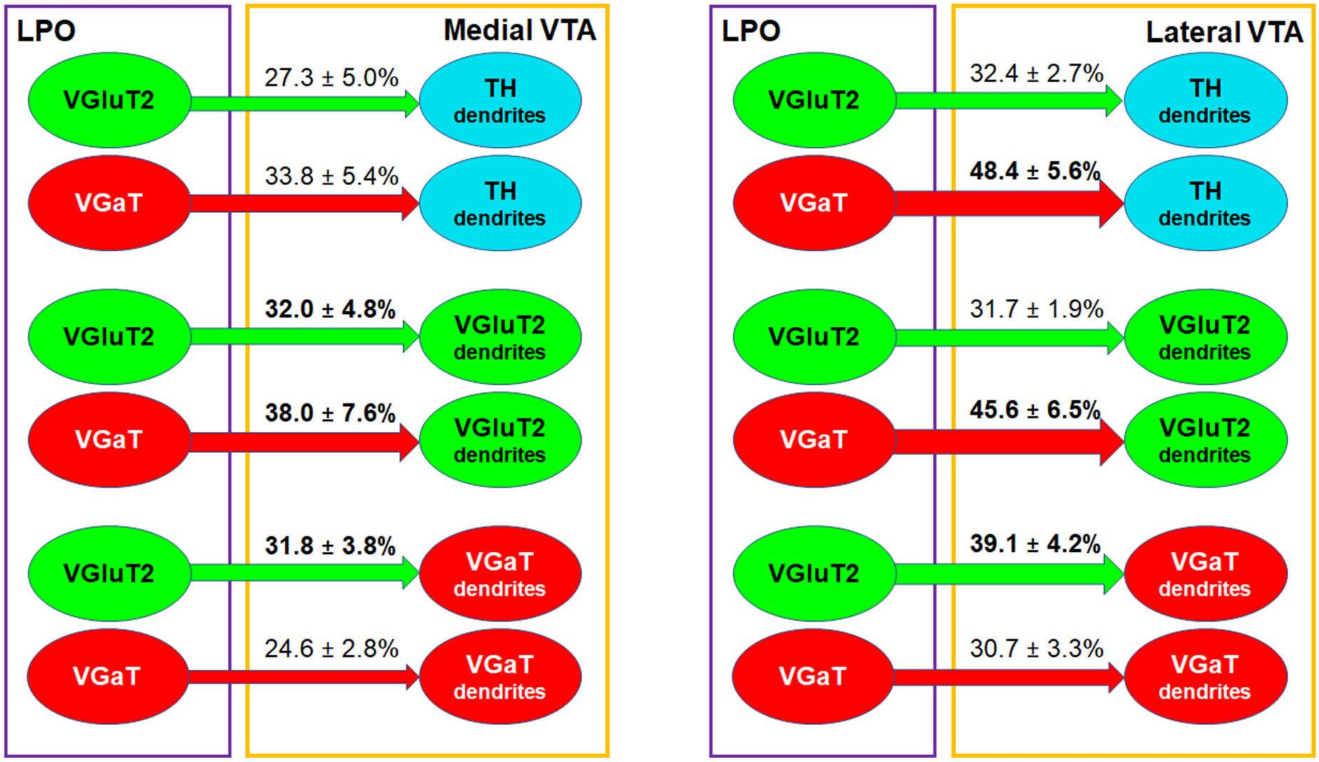


Figure S7. Axon terminals from the LPO VGaT- or VGluT2-neurons establish synapses on VTA eYFP-VGaT neurons. A, Diagram of injections of the AAV5-CaMKII-ChR2-mCherry viral vector into the LPO and the AAV5-DIO-eYFP viral vector into the VTA of *vgat:cre* mice. **B,** A VGaT axon terminal (AT, red outlines) from LPO neuron co-expressing mCherry (scattered dark material) and VGaT (blue arrowhead, gold particles) establishing a symmetric synapse (red arrow) with an eYFP-VGaT dendrite (green outline and arrowhead, gold particles). **C,** A VGluT2 axon terminal (AT, red outlines) from LPO co-expressing mCherry (scattered dark material) and VGluT2 (pink arrowhead, gold particles) establishing an asymmetric synapse (green arrow) with an eYFP-VGaT dendrite (green outline and arrowhead, gold particles).

A. Dendritic Synapses



B. Somatic Synapses

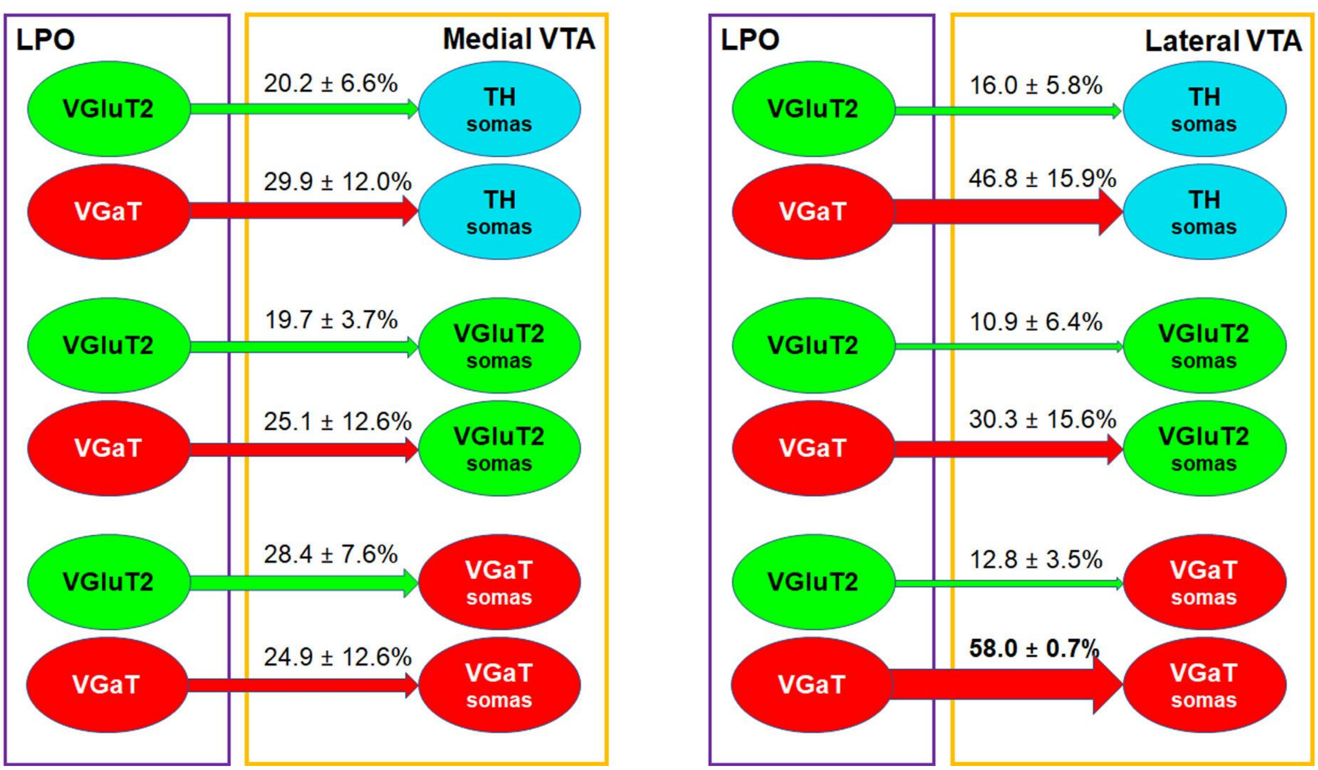


Figure S8. Axon terminals from LPO-VGluT2 or LPO-VGaT neurons differentially established synapse on dendrites and somas of VTA-TH, VTA-VGluT2 or VTA-VGaT neurons distributed within the medial or lateral aspects of the VTA. **A)** Within the medial VTA, LPO-VGluT2 neurons synapsed on VGluT2 or VGaT dendrites with higher frequency than on TH dendrites (32.0 ± 4.8% ,61/190 synapses on VGluT2 dendrites; 31.8 ± 3.8% ,68/220 synapses on VGaT dendrites; and 27.3 ± 5.0%, 31/114 synapses on TH dendrites). In contrast, within the lateral VTA, LPO-VGluT2 neurons established synapse with higher frequency on VGaT dendrites and with comparable frequency on TH or VGluT2 dendrites (39.1 ± 4.2%, 65/153 synapses on VGaT dendrites; 32.4 ± 2.7%, 52/162 synapses on TH dendrites; and 31.7 ± 1.9%, 66/207 synapses on VGluT2 dendrites). In common with synapses from LPO-VGluT2 neurons, LPO-VGaT neurons differentially established synapses on dendrites of the distinct classes of VTA neurons. Within the medial VTA, LPO-VGaT neurons more frequently synapsed on VGluT2 dendrites followed by synapses on TH or VGaT dendrites (38.0 ± 7.6%, 102/263 synapses on VGluT2 dendrites; 33.8 ± 5.4%, 69/ 208 synapses on TH dendrites and 24.6 ± 2.8%, 50/189 synapses on VGaT dendrites). In contrast, within the lateral VTA, LPO-VGaT neurons synapse with comparable frequency on TH and VGluT2 dendrites and with lowest frequency on VGaT dendrites (48.4 ± 5.6%, 103/205 synapses on TH dendrites; 45.6 ± 6.5%, 68/154 synapses on VGluT2 dendrites and 30.7 ± 3.3%, 46/152 synapses on VGaT dendrites). **B)** Within the medial VTA, LPO-VGluT2 neurons synapsed on VGaT somas with higher frequency and with comparable frequency on TH or VGluT2 somas (28.4 ± 7.6%, 23/83 synapses on VGaT somas; 20.2 ± 6.6%, 13/70 synapses on TH somas; and 19.7 ± 3.7%, 21/113 synapses on VGluT2 somas). In contrast, within the lateral VTA, LPO-VGluT2 neurons established synapses with more frequently synapsed on TH somas followed by synapses on VGaT or VGluT2 somas (16.0 ± 5.8%, 13/78 synapses on TH somas; 12.8 ± 3.5%, 7/54 synapses on VGaT somas; and 10.9 ± 6.4%, 6/53 synapses on VGluT2 somas. In common with synapses from LPO-VGluT2 neurons, LPO-VGaT neurons differentially established synapses on somas of the distinct classes of VTA neurons. Within the medial VTA, LPO-VGaT neurons synapsed on TH somas with higher frequency and with comparable frequency on VGluT2 or VGaT somas (29.9 ± 12.0%, 24/70 synapses on TH somas; 25.1 ± 12.6%, 18/66 synapses on VGluT2 somas; and 24.9 ± 12.6%, 15/62 synapses on VGaT somas). In contrast, within the lateral VTA, LPO-VGaT neurons established synapses with more frequently synapsed on VGaT somas followed by synapses on TH or VGluT2 somas (58.0 ± 0.7%, 51/88 synapses on VGaT somas; 46.8 ± 15.9%, 32/68 synapses on TH somas; and 30.3 ± 15.6%, 30/68 synapses on VGluT2 somas).

Table S1. Frequency of neurons co-expressing c-Fos-immunoreactivity, TH-immunoreactivity, VGluT2 mRNA or VGaT mRNA in the VTA of VGluT2-ChR2-eYFP or VGluT2-eYFP mice[a]

VTA neuronal phenotypes[b]	VGluT2-ChR2-eYFP mice			VGluT2-eYFP mice		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
c-Fos/VGluT2	*n* = 233	*n* = 313	*n* = 189	*n* = 93	*n* = 65	*n* = 93
c-Fos/VGluT2/TH	*n* = 66	*n* = 84	*n* = 106	*n* = 16	*n* = 39	*n* = 64
c-Fos/VGaT	*n* = 132	*n* = 100	*n* = 91	*n* = 36	*n* = 19	*n* = 33
c-Fos/VGluT2/VGaT	*n* = 45	*n* = 32	*n* = 59	*n* = 16	*n* = 21	*n* = 7
c-Fos/TH	*n* = 7	*n* = 2	*n* = 16	*n* = 5	*n* = 5	*n* = 6
c-Fos only	*n* = 59	*n* = 27	*n* = 58	*n* = 42	*n* = 27	*n* = 31
Total*	*n* = 542	*n* = 558	*n* = 519	*n* = 208	*n* = 176	*n* = 234

[a] Phenotypes of neurons expressing c-Fos in VTA of both VGluT2-ChR2-eYFP and VGluT2-eYFP mice induced by VTA optical activation of LPO-VGluT2 fibers.

[b] VTA c-Fos immunoreactive neurons co-expressing VGluT2 mRNA (c-Fos/VGluT2), VGaT mRNA (c-Fos/VGaT), TH immunoreactivity (c-Fos/TH), both VGluT2 mRNA and VGaT mRNA (c-Fos/VGluT2/VGaT), both VGluT2 mRNA and TH immunoreactivity (c-Fos/VGluT2/TH) or lacking VGluT2 mRNA, VGaT mRNA and TH immunoreactivity (c-Fos only) were counted in the entire VTA from three mice (fifteen sections at bregma -2.92 to -3.88 mm). * means $P < 0.0001$ in comparison to the total number of c-Fos counted neurons in VGluT2-ChR2-eYFP mice to those in VGluT2-eYFP mice. T-test $t_{(4)} = 16.49$.

Table S2. Number of axon terminals from LPO-VGluT2 or LPO-VGaT making synapses on VTA dendrites

Axon terminals from LPO-VGluT2 neurons synapsing on VTA dendrites			Axon terminals from LPO-VGaT neurons synapsing on VTA dendrites		
Synapses on medial VTA	**Synapses on lateral VTA**	**Total number of synapses**	**Synapses on medial VTA**	**Synapses on lateral VTA**	**Total number of synapses**
Synapses on TH dendrites (n = 31) (31/114 = 27.3 ± 5.0%)	Synapses on TH dendrites (n = 52) (52/162 = 32.4 ± 2.7%)	Synapses on TH dendrites (n = 83) (83/276 = 29.8 ± 2.8%)	Synapses on TH dendrites (n = 69) (69/ 208 = 33.8 ± 5.4%)	Synapses on TH dendrites (n = 103) **(103/205 = 48.4 ± 5.6%)**	Synapses on TH dendrites (n = 172) (172/413 = 41.1 ± 4.8%)
Synapses on VGluT2 dendrites (n = 61) **(61/190 = 32.0 ± 4.8%)**	Synapses on VGluT2 dendrites (n = 66) (66/207 = 31.7 ± 1.9%)	Synapses on VGluT2 dendrites (n = 127) (127/397 = 31.9 ± 2.3%)	Synapses on VGluT2 dendrites (n = 102) **(102/263 = 38.0 ± 7.6%)**	Synapses on VGluT2 dendrites (n = 68) **(68/154 = 45.6 ± 6.5%)**	Synapses on VGluT2 dendrites (n = 170) (170/417 = 41.8 ± 4.8%)
Synapses on VGaT dendrites (n = 68) **(68/220 = 31.8 ± 3.8%)**	Synapses on VGaT dendrites (n = 65) **(65/153 = 39.1 ± 4.2%)**	Synapses on VGaT dendrites (n = 133) (133/373 = 35.5 ± 3.0%)	Synapses on VGaT dendrites (n = 50) (50/189 = 24.6 ± 2.8%)	Synapses on VGaT dendrites (n = 46) (46/152 = 30.7 ± 3.3%)	Synapses on VGaT dendrites (n = 96) (96/341 = 27.7 ± 2.4%)
Total terminals in medial VTA (n= 160) (160/524 = 30.4 ± 1.6%)	Total terminals in lateral VTA (n = 183) (183/522 = 34.4 ± 2.4%)	Total terminals in VTA (n = 343) (343/1046 = 32.4 ± 1.6%)	Total terminals in medial VTA (n= 221) (221/660 = 32.1 ± 3.9%)	Total terminals in lateral VTA (n = 217) (217/511 = 41.6 ± 5.5%)	Total terminals in VTA (n = 438) (438/1171 = 36.8 ± 4.6%)

Table S3. Number of axon terminals from LPO-VGluT2 or LPO-VGaT making synapses on somas of VTA neurons

Axon terminals from LPO-VGluT2 neurons synapsing on somas of VTA neurons			Axon terminals from LPO-VGaT neurons synapsing on somas of VTA neurons		
Synapses on medial VTA	**Synapses on lateral VTA**	**Total number of synapses**	**Synapses on medial VTA**	**Synapses on lateral VTA**	**Total number of synapses**
Synapses on TH somas (n = 13) (13/70 = 20.2 ± 6.6%)	Synapses on TH somas (n = 13) (13/78 = 16.0 ± 5.8%)	Synapses on TH somas (n = 26) (26/148 = 18.1 ± 4.1%)	Synapses on TH somas (n = 24) (24/ 70 = 29.9 ± 12.0%)	Synapses on TH somas (n = 32) (32/68 = 46.8 ± 15.9%)	Synapses on TH somas (n = 56) (56/138 = 38.4 ± 9.7%)
Synapses on VGluT2 somas (n = 21) (21/113 = 19.7 ± 3.7%)	Synapses on VGluT2 somas (n = 6) (6/53 = 10.9 ± 6.4%)	Synapses on VGluT2 somas (n = 27) (27/166 = 15.3 ± 3.8%)	Synapses on VGluT2 somas (n = 18) (18/66 = 25.1 ± 12.6%)	Synapses on VGluT2 somas (n = 30) (30/68 = 30.3 ± 15.6%)	Synapses on VGluT2 somas (n = 48) (48/134 = 27.7 ± 9.0%)
Synapses on VGaT somas (n = 23) **(23/83 = 28.4 ± 7.6%)**	Synapses on VGaT somas (n = 7) (7/54 = 12.8 ± 3.5%)	Synapses on VGaT somas (n = 30) (30/137 = 20.6 ± 5.1%)	Synapses on VGaT somas (n = 15) (15/ 62 = 24.9 ± 12.6%)	Synapses on VGaT somas (n = 51) **(51/88 = 58.0 ± 0.7%)**	Synapses on VGaT somas (n = 66) (66/150 = 41.5 ± 9.3%)
Total terminals synapsing on somas in medial VTA (n = 57) (57/266 = 22.8 ± 2.8%)	Total terminals synapsing on somas in lateral VTA (n = 26) (26/185 = 13.2 ± 1.5%)	Total terminals synapsing on somas in VTA (n = 83) (83/451 = 18.0 ± 1.5%)	Total terminals synapsing on somas in medial VTA (n = 57) (57/198 = 26.6 ± 1.6%)	Total terminals synapsing on somas in lateral VTA (n = 113) (113/224 = 45.0 ± 8.0%)	Total terminals synapsing on somas in VTA (n = 170) (170/422 = 35.8 ± 4.2%)