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Supplemental Information

A Midbrain Circuit that Mediates Headache Aversiveness in Rats

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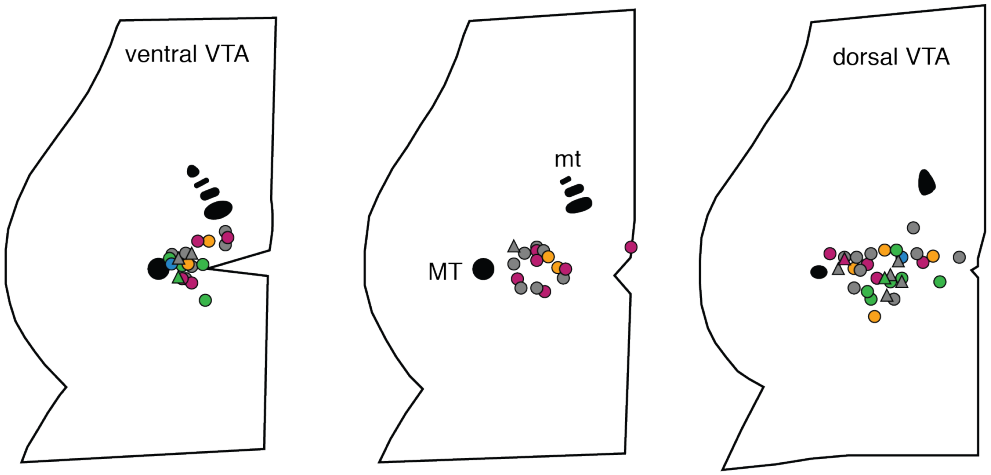
A



mt = mamillothalamic tract
MT = medial terminal nucleus of the accessory optic tract

- Neuron with no input from the PAG
- Neuron with undetermined input from the PAG
- Neuron with excitatory input from the PAG
- Neuron with inhibitory input from the PAG
- Neuron with both excitatory and inhibitory input from the PAG
- △ Confirmed TH-positive neuron

B

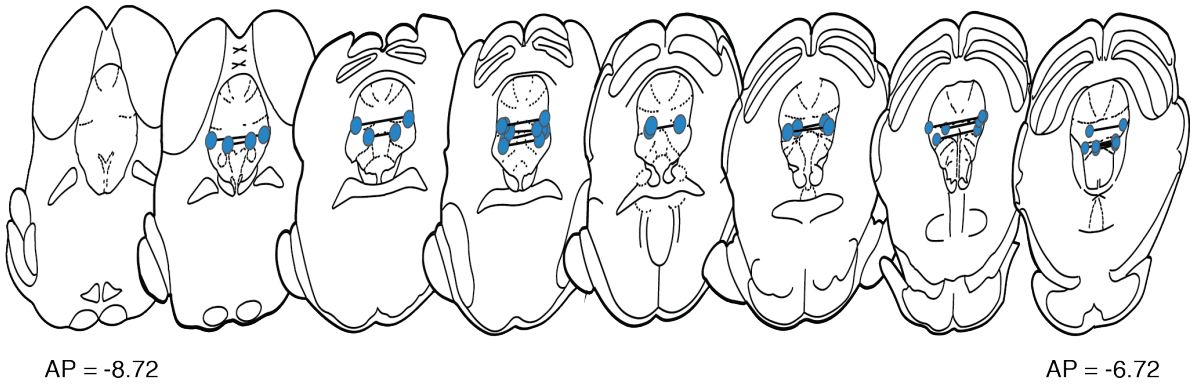


Figure S1. VTA neuron locations and PAG injection sites. Related to Figure 2. (A) Location of recorded VTA neurons labeled to indicate response to light stimulation and TH immunocytochemistry from animals previously injected with AAV2-hSyn-ChR2-mCherry into the vlPAG. (B) Localization of vlPAG injections in rats used for recordings, mapped by the presence of dense mCherry(+) cell bodies and fibers. Coronal brain outlines in this figure were adapted for use with permission from the publisher of "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson, 1998.

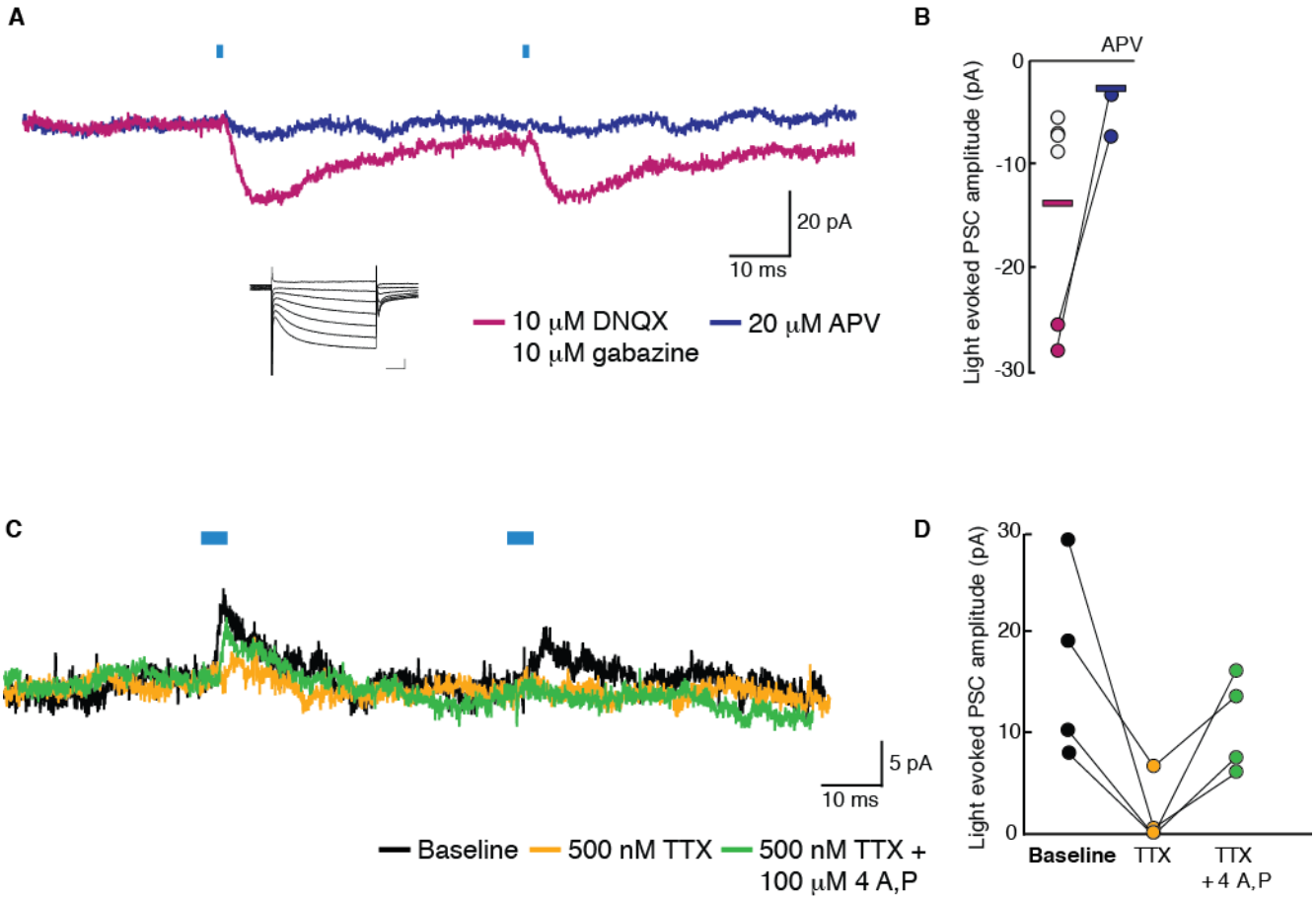


Figure S2. NMDAR EPSCs and monosynaptic GABA IPSCs. Related to Figure 2. (A) Sample light-activated NMDAR current from a VTA neuron in the presence of DNQX and gabazine, abolished by APV. (B) Peak EPSC amplitudes plotted before and after APV application. Each circle represents one neuron: white circles represent observations of EPSCs where APV was not bath applied. Bars represent mean of EPSC amplitudes. (C) Sample trace demonstrating inhibition of optogenetically-induced IPSC by tetrodotoxin (TTX) and subsequent rescue with 4- aminopyridine (4 A,P), indicating a monosynaptic connection. (D) Peak IPSC amplitudes plotted at baseline, after TTX, and after TTX + 4 A,P bath application.

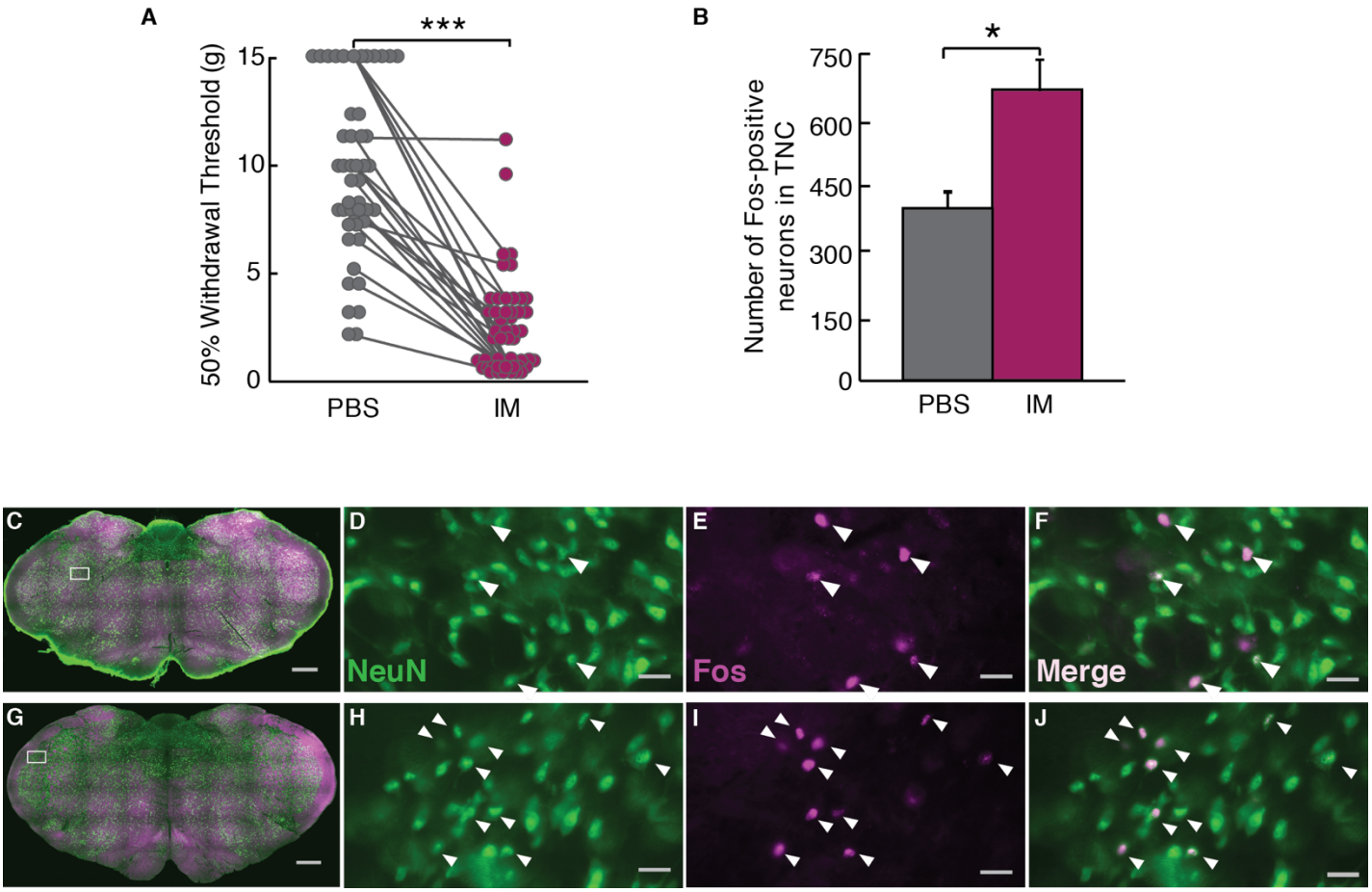


Figure S3. Fos immunostaining in the TNC and withdrawal thresholds after dural IM. Related to Figure 3. (A) Summary of mechanical threshold measurements obtained using the up-down method in rats treated with dural infusions of PBS or IMs. Individual animals are plotted in gray dots and lines. Some animals only underwent infusions of dural PBS or IMs (*** $p < 0.001$). (B) Counts of Fos(+) cells per TNC section ($n = 3$ animals per condition, 8 slices per animal, * $p < 0.05$). Images of example TNC slices treated with dural PBS (C) or IMs (G) were acquired and stitched with 2D slide scan in MBF Stereoinvestigator, *NeuN = green, Fos = magenta* (scale bar = 500 μm). High magnification images of Fos (E,I, *magenta*), NeuN (D,H, *green*) immunostaining in TNC slices treated with dural PBS (D-F) or IMs (H-J). White arrowheads represent Fos(+) cells identified in the plane of focus (scale bar = 50 μm).

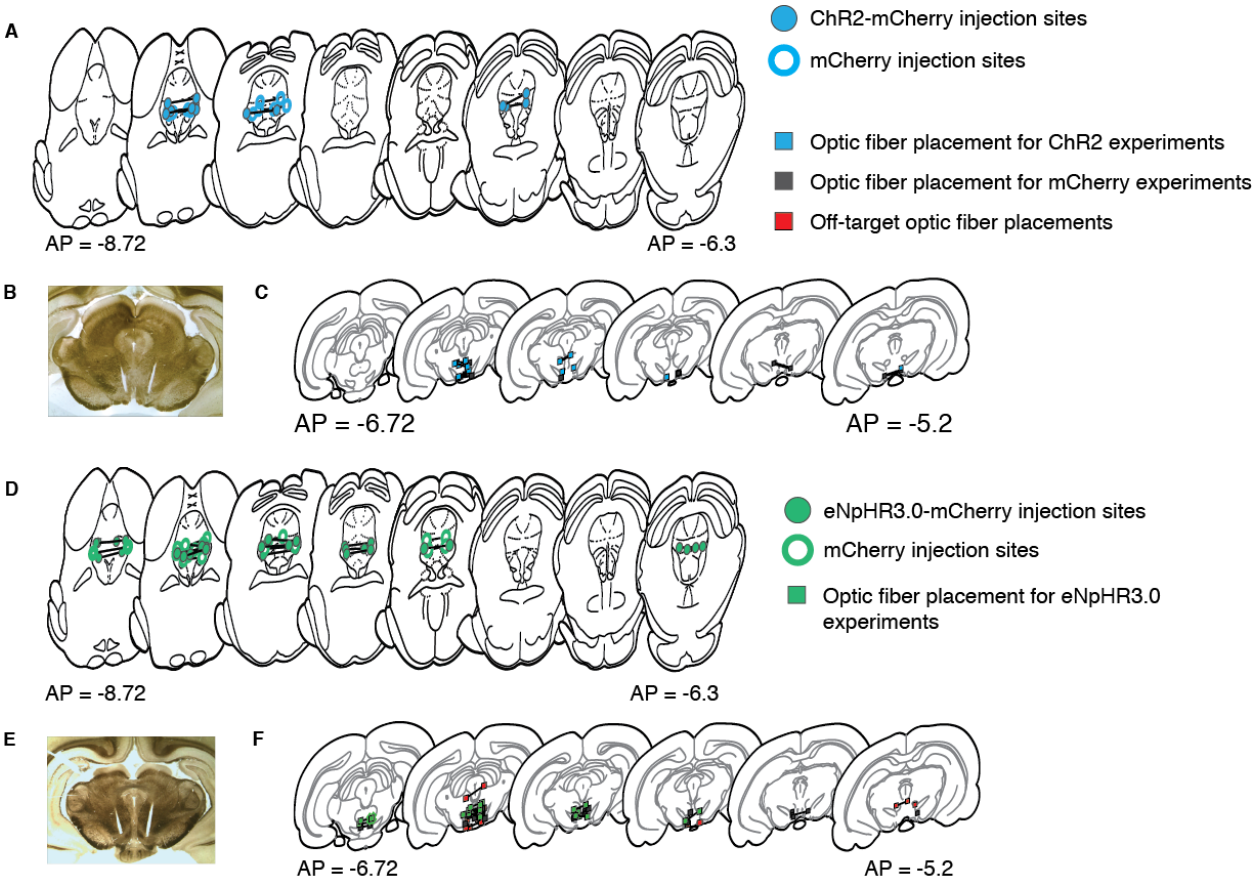


Figure S4. Behavior Timelines with PAG injection sites and VTA optic fiber placements. Related to Figure 4. (A) Location of ChR2-mCherry expression in animals injected with AAV2-hSyn-ChR2-mCherry into the vlPAG (B) Example slice with optic fiber tracts positioned dorsal to the VTA. (C) Optic fiber placements in the bilateral VTA for animals used in vlPAG to VTA terminal fiber activation experiments. Open circles indicate animals injected with sham AAV2-hSyn-mCherry virus. (D) Placements of halorhodopsin-mCherry expression in animals injected with AAV2-hSyn-eNpHR3.0-mCherry into the vlPAG. (E) Example slice with optic fiber tracts positioned dorsal to the VTA (F) Bilateral optic fiber placements in the VTA for animals used in vlPAG to VTA terminal fiber inactivation experiments. Coronal brain outlines in this figure were adapted for use with permission from the publisher of "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson, 1998.