Supporting Information

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SI Materials and Methods

Animals. Ninety adult male Sprague–Dawley rats (275–300 g; Elevage Janvier) were used. They were housed three or four per cage under controlled conditions (22–23 °C, 40% relative humidity, a 12-h light/dark cycle with light on at 7:00 AM, and food and water ad libitum) and were acclimatized to laboratory conditions 1 wk before use.

Drugs. ***Systemic drug injection.*** The jugular vein was cannulated for i.v. administration of morphine hydrochloride (1 mg/kg) prepared in isotonic saline.

Local drug microinfusion. Double-barrel pipettes were used to infuse drugs while simultaneously recording ventral tegmental area (VTA) or the tail of the VTA (tVTA)/rostromedial tegmental nucleus (RMTg) firing activity (1). Microinjections were performed using brief pulses of pneumatic pressure allowing an ejection rate of 10 nL in 5 s (Picrospritzer; Intracel). The microejected drugs in this study were morphine hydrochloride (1 mg/mL, 60 nL), naltrexone (100 μM), picrotoxin (1 mM, 60 nL), and a mixture of 100 μM AP5 and 50 μM CNQX (60 nL) (Sigma-Aldrich) and artificial cerebrospinal fluid (aCSF; 60 nL). All drugs were dissolved in aCSF.

Surgery. Stereotaxic surgeries for electrophysiology were performed under halothane anesthesia as previously described (1). Microelectrodes (recording and stimulating) and ejection pipettes were lowered into the VTA, the tVTA/RMTg, or the lateral habenula (LHb) at the following coordinates: VTA: −5.3 mm from bregma, 0.7 mm from midline, 7.5 mm from brain surface; tVTA/RMTg: −6.8 mm from bregma, 0.4 mm from midline, 7.4 mm from brain surface; LHb: −3.2 mm from bregma, 0.8 mm from midline, 4.5 mm from brain surface. Stereotaxic surgeries for tract-tracing experiments were performed under ketamine (87 mg/kg)/xylazine (13 mg/kg) anesthesia as previously described (2).

VTA and tVTA/RMTg Recordings. ***VTA-dopamine neuron recordings.*** A glass micropipette (tip diameter, 2–3 μm; 4–6 MΩ) filled with 2% pontamine sky blue solution in 0.5 M sodium acetate was lowered into the VTA. VTA-DA (dopamine) neurons were identified according to well-established electrophysiological features (3, 4): (*i*) an action potential width ≥1.1 ms (measured from the start of action potential to the negative trough); (*ii*) slow spontaneous firing rate (<10 Hz); (*iii*) single and burst spontaneous firing patterns (characterized by spike–amplitude decrement); and (*iv*) inhibition of spontaneous activity by DA receptor agonists and subsequent reversal by DA receptor antagonists (data not shown). Characteristic waveforms of VTA-DA neuron spikes are shown in the *Insets* of Figs. 3*C* and 4*B*. Through these electrodes, the extracellular potential was recorded with an Axoclamp2B amplifier (Axon Instrument) in the bridge mode versus a reference electrode maintained in contact with the skull. The extracellular potential amplified 10 times by the Axoclamp2B amplifier was further amplified 100 times and filtered (low-pass filter at 300 Hz and high-pass filter at 0.5 kHz) via a differential AC amplifier (model 1700; A-M Systems). Single-neuron spikes were discriminated and digital pulses were collected online using a laboratory interface and software (CED 1401, Spike2; Cambridge Electronic Design).

VTA-DA neuron juxtacellular labeling. A glass micropipette (tip diameter, 2–3 μm; 4–6 MΩ) filled with 2% neurobiotin (Vector Labs; solution in 0.5 M sodium acetate) was lowered into the VTA. Recordings were similar to those described above for VTA-DA neurons. Once the activity of a putative VTA-DA neuron was recorded, juxtacellular injection of neurobiotin was achieved, as previously described (5), by applying positive current pulses (1–4 nA, 500 ms, 50% duty cycle for 1–4 min) through the bridge circuitry of the amplifier.

tVTA/RMTg neuron recordings. A glass micropipette (tip diameter, 2 μm; 10–12 MΩ) was lowered into the tVTA/RMTg. Recordings were similar to those described above for VTA-DA neurons.

tVTA/RMTg Inactivation. An injection pipette (tip diameter, 30 μm) was filled with the $GABA_A$ agonist muscimol covalently attached to a fluorescent tag (bodipy; Molecular Probes). For inactivation of tVTA/RMTg, 1 mg of muscimol-bodipy was dissolved in 2 mL of 0.01 M phosphate-buffered 0.9% saline (PBS) (0.8 mM; 500 nL). VTA-DA neuron recordings were performed during a 60-min period following the muscimol-bodipy infusion. One hour after tVTA/RMTg inactivation, rats were perfused and their brain collected to control the location and the extent of muscimol-bodipy infusion. Brain slices containing the tVTA/RMTg were imaged at 5× magnification in transmission light (black field) and at 40× magnification using a Leica DMR epifluorescence microscope.

Electrical Stimulation of the LHb. As previously shown (6), excitatory response at a short latency (<8 ms), evoked by LHb stimulation, was used to identify tVTA/RMTg neurons. Bipolar electrical stimulation of the LHb was conducted with a concentric electrode (250-μm diameter overall, 100-μm diameter inner electrode which extended 100 μm beyond the outer electrode; Phymep). This electrode was inserted into the LHb. Electrical stimulation (0.2–1.0 mA, 0.5 Hz, 0.5-ms duration pulses) was administered using a square pulse stimulator (CED 1401, Spike2; Cambridge Electronic Design) and stimulus isolator (DS3; Digitimer). Responses to electrical stimulation of the LHb were evaluated and a peristimulus time histogram (PSTH) was generated online (Spike2 software; Cambridge Electronic Design) for each neuron.

Histology. At the end of each recording experiment, the electrode placement was marked with an iontophoretic deposit of pontamine sky blue dye (−20 μA, continuous current for 12–15 min). To mark electrical stimulation sites, +20 μA of positive current was passed through the stimulation electrode for 2 min. After dye ejection, rats were deeply anesthetized with halothane (5%) and decapitated. Brains were removed, snap-frozen in isopentane at −80 °C, sectioned (30-μm coronal sections), mounted, and stained with neutral red to enable histological determination of recording and stimulation electrode sites.

Tract Tracing. The anterograde tracer biotinylated dextran amine (BDA) (molecular weight 10,000; 5% in 0.25 M potassium acetate; Molecular Probes) or the retrograde tracer cholera toxin B subunit (CTb) (0.25% in 0.1 M Tris, 0.1% NaCl; Sigma) were iontophoretically delivered (1–6 μA, 7-s on/off cycles for 15 min) with glass micropipettes (tip diameter, 10–40 μm) as previously described (2).

Histochemistry. The rat perfusion was done under anesthesia 1–2 wk after surgery. Coronal sections of the brain (40-μm) were obtained on a vibratome (VT1000S; Leica). BDA and neurobiotin histochemistry was done as described (2, 5, 7) with streptavidin Alexa Fluor 488 (Invitrogen; S32354; 1/400). Immunohistochemistry was done as previously described (2, 7). Primary antibodies were specific for CTb (Sigma; C3062; 1/20,000), μ-opioid receptor (Chemicon; AB1774; 1/5,000), tyrosine hydroxylase (TH) (Chemicon; AB1542; 1/500), or glutamic acid decarboxylase 67

kDa (GAD67) (Chemicon; MAB5406; 1/10,000). Fluorescent revelation was carried out with fluorophore-labeled secondary antibodies (Jackson ImmunoResearch; Cy3- or FITC-labeled antibodies from donkey; 1/400). Colorimetric revelation was carried out with a biotinylated secondary antibody (Vector Labs; anti-guinea pig; 1/200), avidin biotin peroxidase complex (ABC Elite; Vector Labs), and hydrogen peroxide with diaminobenzidine as chromogene.

Data Analysis. For in vivo electrophysiological experiments, three parameters of VTA-DA neuron impulse activity were computed before and after drug administration: firing rate, bursting rate (number of burst events over time), and mean number of spikes per burst (mean spikes per burst). The onset of a burst was defined as the occurrence of two spikes with an interspike interval <80 ms (3). Cumulative PSTHs (5-ms bin width) of VTA-DA neuron activity were generated during electrical stimulation of the LHb for each neuron recorded. Results are expressed throughout as mean ± SEM. When two means were compared, the statistical significance of their difference was assessed using Student's *t* tests. For multiple comparisons, values were subjected to a one- or two-way ANOVA followed by post hoc Newman–Keuls tests. For tract-tracing experiments, microphotographs for fluorescence double labeling were taken using a Leica SP5 II confocal microscope. ImageJ (National Institutes of Health) was used to adjust contrast, brightness, and sharpness. For merged pictures, the plugin colocalization finder was used to point out the colocalized pixels in white. Counting analysis and juxtacellular identification were done using a Leica DMRD epifluorescence microscope. Microphotographs in direct light were taken using a Leica microscope with a digital camera. For these pictures, Adobe Photoshop 7.0 was used to adjust contrast, brightness, and sharpness. Abbreviations and structure limits are based on frontal diagrams from rat brain atlas (8).

Statistical Analysis. Fig. 1*G*: Analysis of the firing rate (% of basal firing rate) and bursting activity (% of basal bursting rate; % of the mean spikes per burst) of VTA-DA neurons after control conditions (saline i.v. condition pooled with intra-VTA aCSF condition because of the lack of significant difference), i.v. injection of morphine, and intra-VTA morphine ejection. Firing-rate values (%): control, $94.3 \pm 2.6, n = 29$; i.v. morphine, $122.1 \pm 6.1, n = 11$; intra-VTA morphine, 141.4 ± 9.5, $n = 14$; one-way ANOVA, $F_{2,53} = 21.91$, $P < 0.0001$; post hoc Newman–Keuls tests, $^{**}P < 0.01$, $^{***}P < 0.005$. Bursting-rate values (%): control, 98.6 ± 5.3, $n = 29$; i.v. morphine, $182.3 \pm 32.36, n = 11$; intra-VTA morphine, 209.5 ± 49.1, $n = 14$; one-way ANOVA, $F_{2,53} = 6.02$, $P < 0.005$; post hoc Newman–Keuls tests, $^{*}P < 0.05$, $^{**}P < 0.01$. Mean spikes per burst values (%): control, 97.0 ± 4.4, $n = 29$; i.v. morphine, $134.8 \pm 9.9, n = 11$; intra-VTA morphine, $151.2 \pm 13, n = 14$; one-way ANOVA, $F_{2,53} = 13.96$, $P < 0.0001$; post hoc Newman–Keuls tests, $^{**}P < 0.01$, $^{***}P < 0.005$.

Fig. 3*D*: Analysis of the firing rate (% of basal firing rate) and bursting activity (% of basal bursting rate; % of the mean spikes per burst) of VTA-DA neurons after intra-tVTA/RMTg infusion of PBS or muscimol-bodipy followed by intra-VTA infusion of morphine. Firing-rate values (%): PBS intra-tVTA/RMTg, before/after morphine, $98.3 \pm 2.4/143.0 \pm 17.7$, $n = 5$; muscimol-bodipy intra-tVTA/RMTg, before/after morphine, $98.4 \pm 2.0/104.0 \pm 7.05$, $n = 7$; two-way ANOVA, $F_{1,20} = 8.6$ for interaction, $P < 0.05$; post hoc Newman–Keuls tests, $^{**}P < 0.01$. Bursting-rate values (%): PBS intra-tVTA/RMTg, before/after morphine, $96.7 \pm 4.0/137.0 \pm 30.7$, $n = 5$; muscimol-bodipy intra-tVTA/RMTg, before/after morphine, $96.3 \pm 4.9/87.2 \pm 13.4, n = 7$; two-way ANOVA, $F_{1,20} = 1.03$, not significant. Mean spikes per burst values (%): PBS intra-tVTA/RMTg, before/after morphine, $100.0 \pm 15.4/169.2 \pm 15.4$, $n = 5$; muscimol-bodipy intra-tVTA/RMTg, before/after morphine, $93.7 \pm 10.9/108.9 \pm 15.3, n = 7$; two-way ANOVA, $F_{1,20} = 8.51$, $P < 0.01$; post hoc Newman–Keuls tests, $^{*}P < 0.05$.

Fig. 4*D*: Analysis of the firing rate (% of basal firing rate), the bursting activity (% of basal bursting rate; % of the mean spikes per burst) of VTA-DA neurons, after intra-VTA infusion of aCSF or CNQX+AP5 followed by systemic injection of morphine (1 mg/kg i.v.) or an equal amount of saline 0.9%. Firing-rate values (%): aCSF-VTA/saline i.v., 97.7 ± 4.0, $n = 13$; aCSF-VTA/morphine i.v., $122.1 \pm 6.1, n = 11$; CNQX+AP5-VTA/morphine i.v., 105.1 ± 7.9, $n = 6$; one-way ANOVA, $F_{2,29} = 5.75$, $P < 0.01$; post hoc Newman–Keuls tests, $^{**}P < 0.01$. Bursting-rate values (%): aCSF-VTA/saline i.v., 109.7 ± 8.2, $n = 13$; aCSF-VTA/morphine i.v., 182.3 ± 32.4, $n = 11$; CNQX+AP5-VTA/morphine i.v., 76.1 ± 12.6, $n = 6$; one-way ANOVA, $F_{2,29} = 5.47$, $P < 0.05$; post hoc Newman–Keuls tests, $^{*}P < 0.05$. Mean spikes per burst values (%): aCSF-VTA/saline i.v., 100.0 ± 0.0, $n = 13$; aCSF-VTA/morphine i.v., $134.8 \pm 9.9, n = 11$; CNQX+AP5-VTA/morphine i.v., 105.6 ± 5.6, $n = 6$; one-way ANOVA, $F_{2,29} = 8.92$, $P < 0.01$; post hoc Newman–Keuls tests, $^{**}P < 0.01$.

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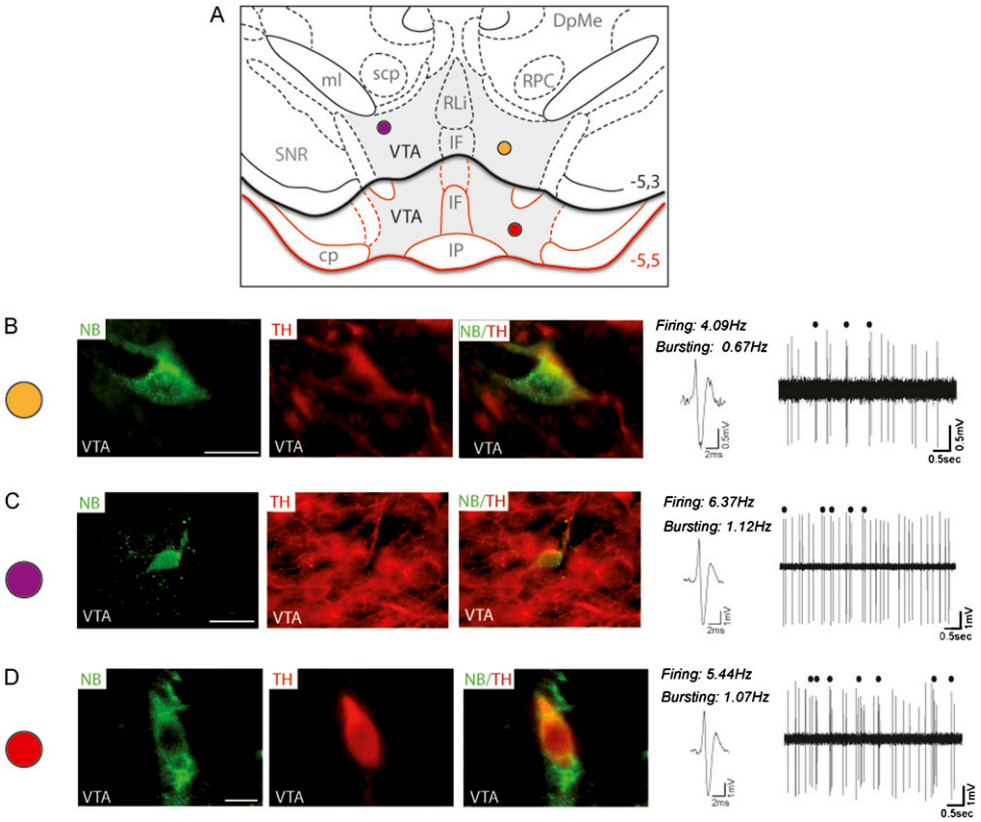

Fig. S1. Characterization of VTA-DA neurons. (*A*) Schematic presentation of neuronal juxtacellular recording sites in the VTA at 5.3 and 5.5 mm posterior to the bregma. Each dot correspond to a VTA TH-positive neuron marked with neurobiotin (NB). (*B–D*) Microphotographs of VTA neurons filled with neurobiotin (*Left*) and immunopositive for tyrosine hydroxylase (TH) (*Center*). The merge of both stainings (*Right*). For each neuron are represented a spike trace and the neuronal activity during 5 s. Firing- and bursting-activity values are also indicated. [Scale bars, 20 μm (*B* and *D*), 100 μm (*C*).] cp, cerebral peduncle; DpMe, deep mesencephalic nucleus; IF, interfascicular nucleus; IP, interpeduncular nucleus; ml, medial lemniscus; RPC; red nucleus, parvicellular part; RLi, rostral linear nucleus of the raphe; scp, superior cerebellar peduncle; SNR, substantia nigra pars reticulata.

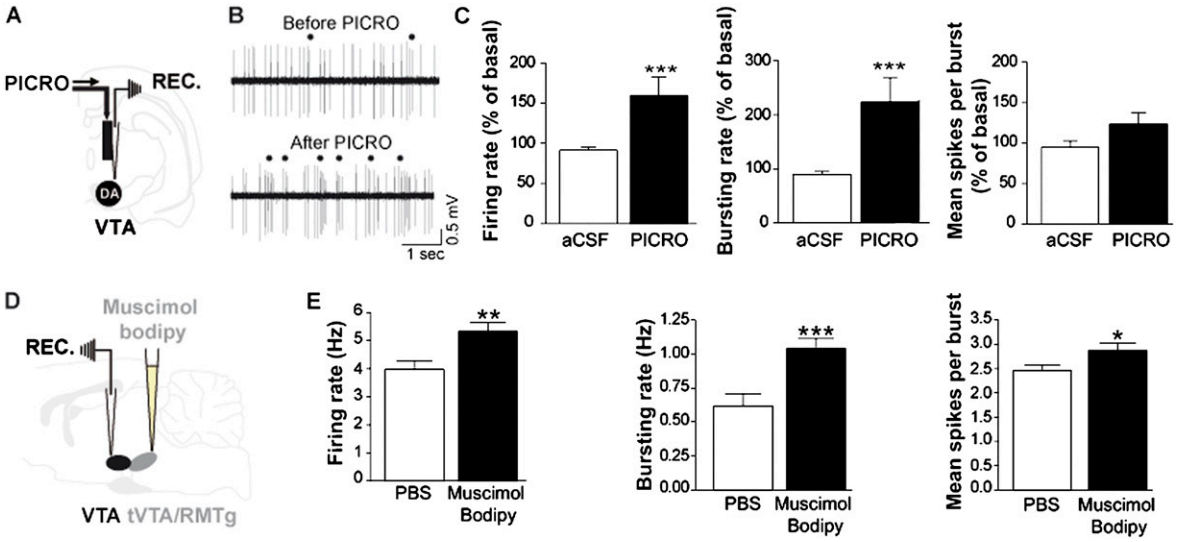

Fig. S2. tVTA/RMTg induced an inhibitory tone onto VTA-DA neurons. (*A*) DA neurons were recorded while microinjecting the $GABA_A$ receptor antagonist picrotoxin (PICRO; 1 mM) or aCSF in their vicinity. Picrotoxin (60 nL) was microinfused through a pipette adjacent to the recording electrode. (*B*) Representative traces of a VTA-DA neuron before and after intra-VTA infusion of picrotoxin. Circles above traces represent burst occurrences. (*C*) Analysis of firing rate (% of basal), bursting activity (% of basal), and mean spikes per burst (% of basal) after intra-VTA ejection of aCSF ($n = 16$) or picrotoxin ($n = 5$). Injection of picrotoxin within the VTA increased the VTA-DA neuron firing rate (aCSF: 91.62 ± 3.4; picrotoxin: 159.3 ± 23.00; Student's t test, $^{**}P < 0.01$) and bursting activity (aCSF: 91.86 ± 6.114; picrotoxin: 223.4 ± 45.15; Student's t test, $^{***}P < 0.005$). Note that mean spikes per burst does not change after picrotoxin ejection. (*D*) tVTA/RMTg was inactivated by muscimol, a $GABA_A$ agonist, covalently attached to a fluorescent tag (bodipy, 0.8 mM, 500 nL). The control experiment consisted of ejecting PBS within the tVTA/RMTg. VTA-DA neurons were recorded within the first hour following muscimol-bodipy ($n = 54$) or PBS ($n = 44$) infusion into tVTA/RMTg. (*E*) Inactivation of tVTA/RMTg increased VTA-DA neuron firing rate (PBS: 3.9 ± 0.3 Hz; muscimol-bodipy: 5.3 ± 0.3 Hz; Student's t test, $^{**}P < 0.01$), bursting activity (PBS: 0.6 ± 0.1 Hz; muscimol-bodipy: 1.0 ± 0.08 Hz; Student's t test, $^{***}P < 0.005$), and mean spikes per burst (PBS: 2.5 ± 0.1; muscimol-bodipy: 2.9 ± 0.2; Student's t test, $^{*}P < 0.05$).

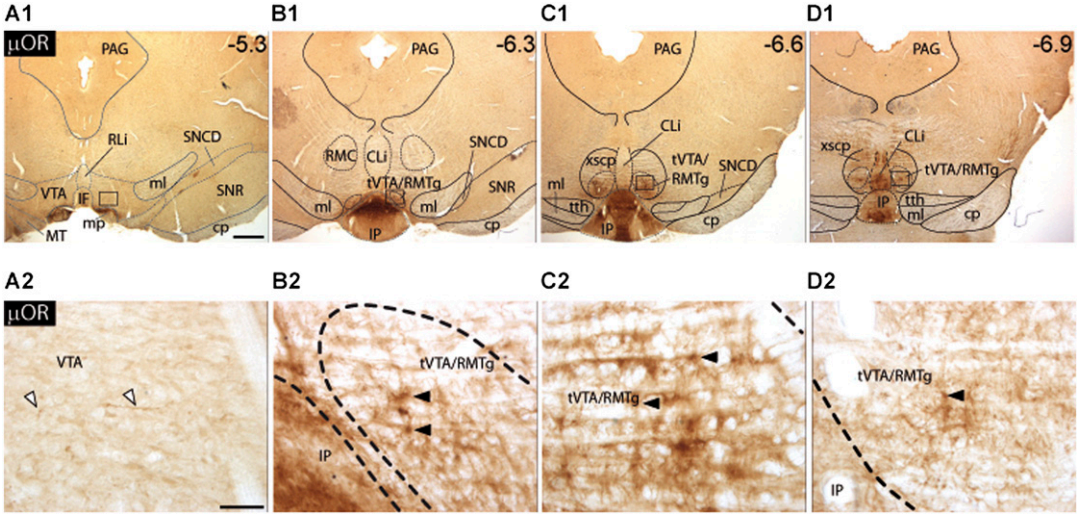

Fig. S3. μ opioid receptor (μOR) staining is detected throughout the rostrocaudal extent of the tVTA/RMTg. (*A1–D1*) Microphotographs illustrating μOR staining on coronal sections at the VTA (*A1*) and tVTA/RMTg (*B1–D1*) levels. (Scale bar, 500 μm.) (*A2–D2*) Magnifications of boxed areas in *A1–D1* showing μOR-positive fibers in the VTA (white arrowheads) and μOR-positive cell bodies in the tVTA/RMTg region (black arrowheads). (Scale bar, 100 μm.) Numbers refer to the antero-posterior distance from the bregma in millimeters. Dashed lines indicate structures boundaries. CLi, caudal linear nucleus of the raphe; cp, cerebral peduncle; IF, interfascicular nucleus; IP, interpeduncular nucleus; ml, medial lemniscus; mp, mammillary peduncle; MT, medial terminal nucleus of accessory optic tract; PAG, periaqueductal gray; RLi, rostral linear nucleus of the raphe; RMC, red nucleus, magnocellular part; SNCD, substantia nigra pars compacta dorsal part; SNR, substantia nigra pars reticulata; tth, trigeminothalamic tract; xscp, superior cerebellar peduncle decussation.

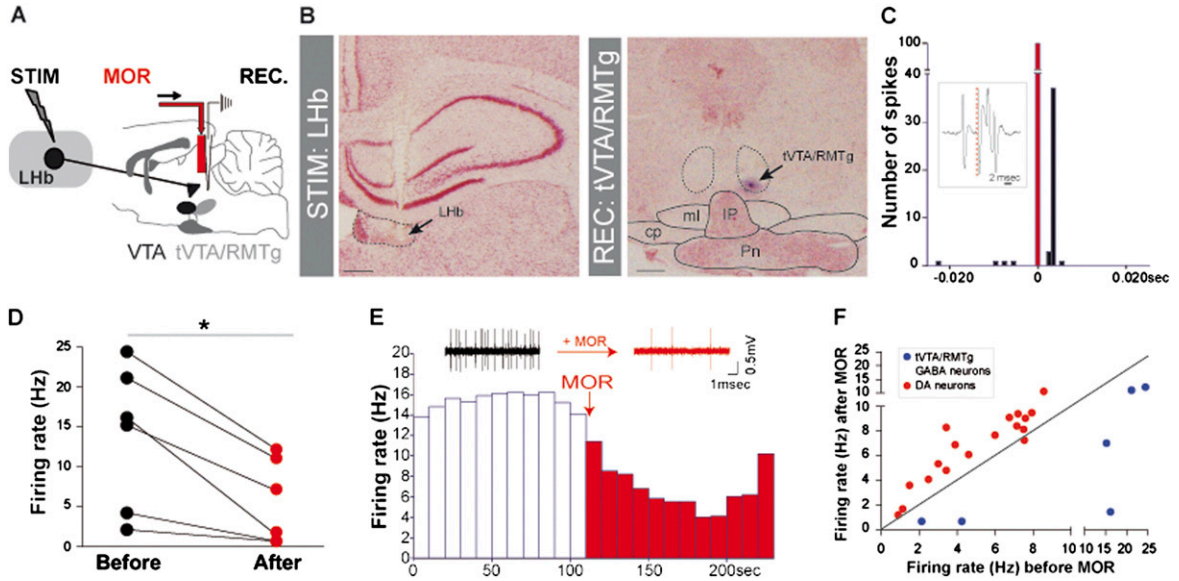

Fig. S4. Local infusion of morphine decreases tVTA/RMTg GABA neuron activity. (*A*) Experimental protocol. As previously shown (6), only tVTA/RMTg neurons with a robust excitatory response to LHb stimulation were selected in this experiment. (*B*) (*Left*) Electrical stimulation site in the LHb (lesioned area, black arrow). (*Right*) Recording location for a tVTA/RMTg neuron (blue spot, black arrow). (Scale bars, 500 μm.) (*C*) Typical PSTH showing LHb-evoked response in a VTA-DA neuron. Each bin represents 1 ms. In red is represented the bar corresponding to the stimulus artifact. (*Inset*) Orthodromic spikes evoked by stimulation of LHb (red bar). (*D*) Effect of local infusion of morphine (1 mg/mL, 60 nL) on tVTA/RMTg neuron firing rate. Morphine infused locally induced a 61% decrease in tVTA/RMTg neuron firing rate. Firing-rate values (Hz): before morphine, 13.9 ± 3.7, $n = 6$; after morphine, 5.5 ± 2.2, $n = 6$; Student's t test, $^*P < 0.05$. (*E*) Example of tVTA/RMTg neuron activity following an intra-tVTA/RMTg infusion of morphine (1 mg/mL, 60 nL). Above, two traces of the same tVTA/RMTg neuron before and after morphine ejection are represented. (*F*) Scatter plot depicting activity changes for VTA-DA (red dots) and tVTA/RMTg (blue dots) neurons following local morphine infusion. Pn, pontine nuclei.

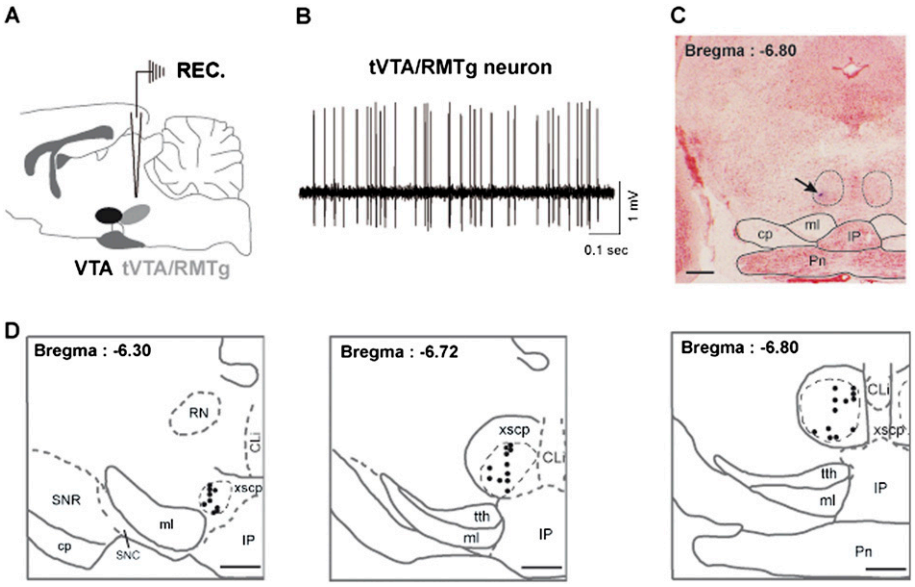

Fig. S5. Electrophysiological parameters for tVTA/RMTg neuron recordings. (*A*) Experimental protocol. (*B*) Trace of a representative tVTA/RMTg neuron. (*C*) Microphotograph of a coronal section through the tVTA/RMTg (dotted black line). Recording location for a tVTA/RMTg neuron (blue spot) at black arrow. (Scale bar, 500 μm.) (*D*) Schematic presentation of neuronal recording sites in the tVTA/RMTg. tVTA/RMTg neurons are represented by black dots. Numbers refer to stereotaxic coordinates. RN, red nucleus. (Scale bars, 200 μm.)

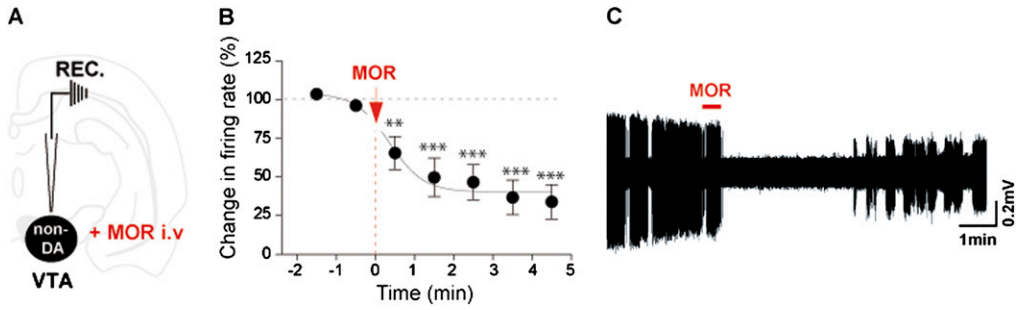

Fig. S6. Local morphine injection decreases non-DA VTA neuron activity. (*A*) Experimental protocol. (*B*) Effect of local morphine injection (1 mg/mL) on non-DA VTA neuron firing rate in function of time (min). A one-way ANOVA was performed on repeated measures followed by a Dunnet post hoc test. $P < 0.0001$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*C*) Traces of a non-DA VTA neuron before, during, and after morphine injection within the VTA. Note that morphine blocks its activity within minutes.