Dysfunction of ventral tegmental area GABA neurons causes mania-like behavior

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Abstract

The ventral tegmental area (VTA), an important source of dopamine, regulates goal- and reward-directed and social behaviors, wakefulness and sleep. Hyperactivation of dopamine neurons generates behavioral pathologies. But any roles of non-dopamine VTA neurons in psychiatric illness have been little explored. Lesioning or chemogenetically inhibiting VTA GABAergic (VTA Vgat) neurons generated persistent wakefulness with mania-like qualities: locomotor activity was increased; sensitivity to D-amphetamine was heightened; immobility times decreased on the tail suspension and forced swim tests; and sucrose preference increased. Furthermore, after sleep deprivation, mice with lesioned VTA Vgat neurons did not catch up on lost sleep, even though they were starting from a sleep-deprived baseline, suggesting that sleep homeostasis was bypassed. The mania-like behaviors, including the sleep loss, were reversed by valproate, and re-emerged when treatment was stopped. Lithium salts and lamotrigine, however, had no effect. Low doses of diazepam partially reduced the hyperlocomotion and fully recovered the immobility time during tail suspension. The mania-like behaviors mostly depended on dopamine, because giving D1/D2/D3 receptor antagonists reduced these behaviors, but also partially on VTA Vgat projections to the lateral hypothalamus (LH). Optically or chemogenetically inhibiting VTA Vgat terminals in the LH elevated locomotion and decreased immobility time during the tail suspension and forced swimming tests. VTA Vgat neurons help set an animal’s (and perhaps human’s) mental and physical activity levels. Inputs inhibiting VTA Vgat neurons intensify wakefulness (increased activity, enhanced alertness and motivation), qualities useful for acute survival. In the extreme,
however, decreased or failed inhibition from VTA\textsuperscript{Vgat} neurons produces mania-like qualities (hyperactivity, hedonia, decreased sleep).

\section*{Introduction}

During the mania phase of bipolar disorder, patients sleep little and have elevated mood (\textit{e.g.} increased energy and hyperactivity, impulsivity, decreased depression)\textsuperscript{1–5}. In mice, pathological hyperactivity and elevated mood can be generated by various gene mutations and deletions: \textit{e.g.} Clock \textDelta 19\textsuperscript{6}, REV-erb\textalpha\textsuperscript{7}, ErbB4 tyrosine kinase deletion in noradrenergic locus ceruleus cells\textsuperscript{8}, GSK-3\textbeta overexpression\textsuperscript{9}, dopamine transporter knockdown\textsuperscript{10, 11}, SHANK2 knockout\textsuperscript{12}, SHANK3 overexpression\textsuperscript{13}, ANK3 disruptions\textsuperscript{14}, ionotropic glutamate/AMPA receptor GluA1 knockout\textsuperscript{15}, ionotropic glutamate/kainate GluK2 knockout\textsuperscript{16}, phospholipase c\textgamma 1 knockout\textsuperscript{17}, histidine triad nucleotide-binding protein 1 knockout\textsuperscript{18}, glutamate-cysteine ligase modifier unit knockout\textsuperscript{19}, and the Na/K-ATPase\textalpha 3 Muskhin (Myk/+) mutation\textsuperscript{20–22}. Some of these gene manipulations alter excitation-inhibition (E-I) balance\textsuperscript{8, 13, 15, 16, 23}, and/or elevate catecholamines\textsuperscript{6–8, 10, 24}, suggesting common themes that could underlie the emergence of some types of mania.

Both these themes come together in the ventral tegmental area (VTA). The VTA an important source of dopamine, regulates goal- and reward-directed and social behaviors\textsuperscript{25, 26}, as well as wakefulness and sleep\textsuperscript{27–29}. Exciting VTA dopamine neurons with well-chosen rhythms can produce mania-like behaviors in the day and euthymia at night\textsuperscript{30}. In addition to dopamine neurons, there is a rich heterogeneity of glutamate and GABA neurons in the different anatomical subdomains of the VTA, and neurotransmitter co-release (\textit{e.g.} dopamine-glutamate, GABA-glutamate, GABA-dopamine) from VTA neurons is common\textsuperscript{25, 31–33}.

For this paper, we focus on the midline VTA, which contains GABA (VTA\textsuperscript{Vgat}) and glutamate/nitric oxide synthase (VTA\textsuperscript{Vglut2}) neurons\textsuperscript{25, 28, 34–37}. These VTA\textsuperscript{Vgat} and VTA\textsuperscript{Vglut2/NOS1} neurons inhibit and excite, respectively, the dopamine cells, but also, by projecting out of the VTA, exert effects independent of dopamine\textsuperscript{25, 28, 36}. The VTA\textsuperscript{Vgat} neurons co-release GABA and glutamate\textsuperscript{33}, but the majority of these VTA neurons’ actions locally are GABAergic\textsuperscript{28}. Locally they mostly inhibit VTA\textsuperscript{Vglut2}, VTA\textsuperscript{DA} and VTA\textsuperscript{Vglut2/DA} cells, but also elicit a small number of pure excitatory responses\textsuperscript{28}. Chemogenetic inhibition and chronic lesion of midline VTA\textsuperscript{Vgat} neurons causes sustained wakefulness\textsuperscript{28, 29}, and excitation of VTA\textsuperscript{Vglut2} cells and VTA dopamine cells also causes wakefulness\textsuperscript{28, 29}, VTA\textsuperscript{Vgat} neurons limit arousal by inhibiting dopamine neurons and via projections to the lateral hypothalamus (LH)\textsuperscript{28, 36, 38}; VTA\textsuperscript{Vglut2} neurons produce wakefulness, also independently of dopamine, by projecting to the LH and nucleus accumbens\textsuperscript{28}.

Here, we characterize the type of wakefulness produced by inhibiting or lesioning the VTA\textsuperscript{Vgat} neurons or exciting VTA\textsuperscript{Vglut2} neurons. We find that the wakefulness induced by diminishing or removing VTA\textsuperscript{Vgat} neuron inhibition contains behavioral endophenotypes that are mania-like, and are treatable with valproate and diazepam, although not with lithium or lamotrigine. On the other hand, the extended but quiet wakefulness produced by activating midline VTA\textsuperscript{Vglut2} neurons has no endophenotypes characteristic of mania.
We suggest that the mania-like symptoms resulting from diminished VTA $V_ga t$ function are generated by changing the E-I balance in both the VTA and the LH.

**Material and Methods**

**Mice and housing**

All experiments were performed in accordance with the UK Home Office Animal Procedures Act (1986); all procedures were approved by the Imperial College Ethical Review Committee and the Ethics Committee for Animal Experimentation of Xijing Hospital, Xi’an, and were conducted according to the Guidelines for Animal Experimentation of the Chinese Council institutes. The following strains of mice were used: $V_gat$-ires-Cre: $S l c 3 2 a ^ { t m 2 ( c r e ) L o w l } J$ kindly provided by B.B. Lowell, JAX stock 016962; $V g l u t 2$-ires-Cre: $S k l 7 a ^ { t m 2 ( c r e ) L o w l } J$, kindly provided by B.B. Lowell, JAX stock 016963. Mice were maintained on a 12 hr:12 hr light:dark cycle at constant temperature and humidity with ad libitum food and water. The “lights-on” period started at 6:00 pm (18:00); the “lights-off” period started at 6:00 am (06:00). Lighting intensity was around 5 Lux during the red light “lights off period”; and the white light level around 150 Lux during the “lights on” period.

**AAV**

$p A A V . h S y n - D I O - h M 4 D i - m C h e r r y$, $p A A V . h S y n - D I O - h M 3 D q - m C h e r r y$ and $p A A V . h S y n - D I O - m C h e r r y$ were gifts from Bryan L. Roth (Addgene plasmid 44362, 44361 and 50459); $p A A V . E F 1 a - D I O - t a C A S P 3 - T E V$ was a gift from Nirao Shah (Addgene plasmid 45580); we packaged the transgenes into AAV capsids (capsid serotype 1/2) in house as described previously. $r A A V 2 / 9 - E F 1 a - D I O - e N p H R 3 . 0 - m C h e r r y$ and $r A A V 2 / 9 - E F 1 a - D I O - C h R 2 - m C h e r r y$ were packaged by BrainVTA (Wuhan, China).

**Surgery**

10-12-week-old male mice were anesthetized with 2% isoflurane in oxygen by inhalation and received buprenorphine (0.1 mg/kg) and carprofen (5 mg/kg) injections, and then placed on a stereotaxic frame (Angle Two, Leica Microsystems, Milton Keynes, Buckinghamshire, UK). The AAV was injected through a stainless steel 33-gauge/15mm/PST3 internal cannula (Hamilton) attached to a 10 μl Hamilton syringe, at a rate of 0.1 μl min$^{-1}$. For the AAV injections, virus was bilaterally injected into the VTA, 50 nl for each side of the VTA. The injection co-ordinates were VTA: (ML = ±0.35 mm, AP = -3.52 mm, DV = -4.25 mm). After injection, the cannula was left at the injection site for 5 min and then slowly pulled out. After injections, mice that were to undergo the sleep experiments were implanted with 3 gold-plated miniature screw electrodes (−1.5 mm Bregma, +1.5 mm midline; +1.5 mm Bregma, −1.5 mm midline; −1 mm Lambda, 0 mm midline – reference electrode) with two EMG wire (AS634, Cooner Wire, CA). The EMG electrodes were inserted between the neck musculature. The Neurologger 2A EEG-EMG device was affixed to the skull with Orthodontic Resin power and Orthodontic Resin liquid (TOCental, UK). For the fiber optogenetic and chemogenetic experiments, after virus injection above the VTA (ML = ±0.35 mm; AP = −3.52 mm; DV = −4.25 mm), mice received surgical bilateral implantations above the LH of a monofiberoptic cannula (ML = ±0.36 mm; AP = −3.54 mm; DV = −4.25 mm).
DV = −4.0 mm) (200 μm; Doric Lenses, Inc., Quebec, Canada) or guide cannula (ML = ±0.36 mm; AP = −3.54 mm; DV = −3.5 mm) (World precision instruments, USA) for CNO delivery.

**Drug treatments**

For all drug treatments, mice first received vehicle injections, and behavioral tests were performed; 2 weeks later, the same group of mice were given drug injections, and behavioral tests were performed. For the long-term valproate treatments, we further tested mice behaviors 2 weeks after valproate treatment was withdrawn. Baseline behaviors of the mice were tested during this period without any treatment.

**Chemogenetics**

Clozapine-N-oxide (CNO) (C0832, Sigma-Aldrich, dissolved in saline, 1 mg/kg) or saline was injected i.p. 30 minutes before the start of the behavioral tests. For VTA Vglut2-hM3Dq mice, CNO or saline was injected during the “lights off” active phase. 1 μl of 1 mM CNO was infused into the LH through the guide cannula at a rate of 0.1 μl min⁻¹ with an injector needle (33-gauge, Hamilton).

**D-amphetamine**

D-Amphetamine (2813/100, Tocris Bioscience, dissolved in saline, 2 mg/kg) or saline was injected i.p. into the mice 13, 21, and the mice were assessed directly after D-amphetamine injection.

**Valproate, lithium, diazepam and lamotrigine**

Sodium valproate (2815, TOCRIS, dissolved in saline, 200 mg/kg), LiCl (Sigma-Aldrich, dissolved in saline, 100 mg/kg) or saline (vehicle) was injected i.p. into the mice. For acute treatments (repeated injections) with lithium or valproate, mice received vehicle injections, and then behaviors (locomotion and tail-suspension) were tested; after 2 weeks, the same group of mice were given lithium or valproate injections. For the Li-H₂O treatment, mice were treated with LiCl in drinking water (300 mg/L) for 2 weeks. Mice were first given normal water, and behaviors were tested, and the same group of mice were placed on Li-H₂O for 2 weeks. Valproate or LiCl treatments were as previously reported13, 17: valproate or LiCl were injected 3 times (10:00, 14:00 and 17:00) one day before the behavioral assays; and during the day of the behavioral assay, valproate or LiCl was injected 2 times (10:00, 14:00). Locomotion or tail-suspension tests were performed 30 min after injection (14:30). After the locomotion or tail-suspension tests (see below), mice received a final valproate injection (17:00), and then the 24-hour sleep-wake recordings were then performed (see section “EEG analysis, sleep-wake behavior and sleep deprivation”).

Diazepam (2805, TOCRIS, dissolved in 0.9% saline containing 0.1% Tween 80, 1 mg/kg) and lamotrigine (1611, TOCRIS, diluted in 0.9% saline, 10 mg/kg) or their control vehicles were injected i.p. into the mice. For single treatments with diazepam (1 mg/kg) or lamotrigine (10 mg/kg), mice received vehicle injections, and then behaviors (locomotion and tail-suspension) were tested; after 2 weeks, the same group of mice were given diazepam or lamotrigine injections (14:00). For repeated treatments, diazepam (1 mg/kg)
or lamotrigine (10 mg/kg) were injected 3 times (10:00, 14:00 and 17:00) one day before the behavioral assays; and during the day of the behavioral assay, diazepam or lamotrigine were injected 2 times (10:00, 14:00). The locomotion or tail-suspension tests were performed 30 min after injection (14:30).

Serum lithium

Blood samples were collected before and after lithium injections via transcutaneous cardiac puncture and the serum was separated for determination of lithium levels. Mice were maintained on anesthesia and blood samples were collected directly from the left ventricle before killing them. Serum lithium was analyzed using a Roche Cobas c311 analyzer.

Dopamine receptor antagonists experiment

Dopamine antagonists SCH-23390 (0.03 mg/kg, dissolved in saline) and raclopride (1 mg/kg, dissolved in saline), for D1 and D2/D3 receptors respectively, were injected serially i.p. into the VTA/Vgat-mCherry mice or VTA/Vgat-CASP3 mice 20 minutes before the locomotion, TST or sleep experiment. For the experiments with chemogenetic inhibition combined with dopamine receptor antagonists, SCH-23390 and raclopride were injected serially i.p. into the VTA/Vgat-hM4Di mice, and 20 minutes later, saline or CNO was injected into the antagonists-injected mice, and 30 minutes later, the locomotion test and TST was performed.

Locomotor activity

The locomotor activity and time spent in stereotypy was detected in an activity test chamber (Med Associates, Inc) with an ANY-maze video tracking system (FUJIFILM co.) and measured by ANY-maze software (Stoelting Co. US.). VTA/Vgat-mCherry or VTA/Vgat-CASP3 mice were directly put into the activity test chamber; for VTA/Vgat-hM4Di mice, the mice were put into the test chamber 30 minutes after saline or CNO injection; for D-Amphetamine experiments, mice were first put into chamber for 30 min, and the mice received vehicle or amphetamine injections. The locomotor activity was detected straight after injection for 1-h.

Home cage activity

Mice were habituated in the cage for 24 h with mock Neurologgers before recordings. The home cage activities were then recorded using the accelerometer built into the Neurologger 2A devices for a 24-h period and analyzed using Spike2 software.

Tail-suspension test (TST)

The TST was performed as described. After a habituation period in the test room, mice were suspended 60 cm above the floor by their tails by taping the tail tip. Behaviors were video recorded and blindly scored manually and measured by ANY-maze software (Stoelting Co. US.).
**Forced swimming test (FST)**

Mice were placed in a borosilicate glass cylinder (5L, 18 cm diameter, 27 cm high) filled with water (25 °C, water depth 14 cm) for 6 min. The immobility time during the last 4 min was manually measured. Immobility time was defined as the time spent without any movements except for a single limb paddling to maintain flotation.

**Sucrose preference test (SPT)**

This was a 2-choice test between 1% sucrose and water. Mice were habituated to a dual delivery system (one bottle with water and one bottle with 1% sucrose) for 3 days. Sucrose preference was then assessed over 3 consecutive days. For the chemogenetic experiments, animals were water-restricted overnight before saline or CNO injection. 30 min after saline or CNO injection, the mice were given free access to the 2-water delivery system for 4 hours. Sucrose preference (%) was calculated as (weight of sucrose consumed) / (weight of water consumed + weight of sucrose consumed) × 100%.

**Elevated plus maze test**

The elevated plus maze apparatus (Global Biotech Inc. Shanghai) was opaque and consisted of a central platform (10 cm x 10 cm), two open arms (50 cm x 10 cm), and two closed arms (50 cm x 10 cm) with protective walls 40 cm high which was 70 cm above the ground. Animals were placed in the central platform facing one open arm of the apparatus and were free to explore the arms for 5 min. The apparatus was cleaned with 75% ethanol before and after each session. The traces were recorded by an overhead camera and shown by average heatmap of each group. The ratio of exploring time in the open arm was calculated by Video Tracking Software (ANY-maze, Stoelting Co., Ltd.).

**Timing of behavioral tests**

10-12 weeks male mice were given virus injection (see Surgery section), and 4-6 weeks after this, behavioral tests were performed (the mice were approximately 14-18 weeks old by this stage), or drug treatments were started. During the drug treatment experiments, after vehicle injections, behavioral tests were performed (the mice were approximately 14-18 weeks old); and 2 weeks later, mice received drug injection, and behavioral tests were performed (by this stage, the mice were approximately 16-20 weeks old). All behavioral tests (locomotion, TST, FST or SPT) took place during the “lights-off” phase of the light-dark cycle, when the mice were most active, particularly between 14:00 pm-17:00 (2 PM-5 PM), except for the sleep deprivation experiments (see section “EEG analysis, sleep-wake behavior and sleep deprivation ”).

**Mouse groupings for behavioral tests**

For chemogenetic experiments, VTA\textsubscript{Vgat}-hM4Di mice were randomly split into two groups that received saline or CNO injection, and the locomotion test, TST, FST or SPT were then performed. After 1-2 weeks, the same mice were given CNO or saline injection, and the locomotion test, TST, FST or SPT were again performed. For the saline control experiments, VTA\textsubscript{Vgat}-hM4Di mice received saline injections, and then the locomotion test, TST, FST or
SPT were performed, and after 1-2 weeks, the same mice were given saline injection, and the locomotion test, TST, FST or SPT were performed.

**EEG analysis, sleep-wake behavior and sleep deprivation**

EEG and EMG signals were recorded using Neurologger 2A devices\(^{28,44}\). NREM sleep and wake states were automatically classified using a sleep analysis software Spike2 and then manually scored. The sleep deprivation protocol was as described previously\(^{45}\). At the start of ‘lights on’ period, when sleep pressure is the highest, mice fitted with Neurologgers were put into novel cages, and at one-hour intervals, novel objects were introduced. After 5 hours sleep deprivation, mice were then put back into their home cages for 19 hours. In total, a 24-hour sleep-wake state was recorded. For the sleep experiments with dopamine receptor antagonists, mice were given SCH-23390 (0.03 mg/kg) and raclopride (2 mg/kg) injections in the middle of the ‘lights off’ active period.

**Optogenetic stimulation**

For the optogenetic behavioral experiments, a fiber patch cord was connected to the laser generator, and dual optic fibers were connected to the fiber patch through a rotary joint (ThinkerTech Nanjing BioScience Inc. China.). Before the experiments, a monofiberoptic cannula was connected to the fiber patch cord. \(\text{VTA}^{\text{Vgat}}\text{-eNpHR-mCherry}\rightarrow\text{LH}\) mice or \(\text{VTA}^{\text{Vgat}}\text{-ChR2-mCherry}\rightarrow\text{LH}\) mice were bilaterally opto-stimulated (20 Hz, 0.5 s duration with 0.5 s interval, 593 nm, 20 \(\mu\)W) in the LH during the “lights off” phase. The stimulation was given for the duration of the behavioral tests.

**Immunohistochemistry**

These procedures were carried as described previously\(^{28}\). Primary antibodies used were rat monoclonal mCherry (1:2000, Thermo Fisher, M11217); mouse monoclonal TH (1:2000, Sigma, T2928); secondary antibodies were Alexa Fluor 488 goat anti-mouse (1:1000, Invitrogen Molecular Probes, A11001), Alexa Fluor 594 goat anti-rat (1:1000, Invitrogen Molecular Probes, A11007). Slices were mounted on slides, embedded in Mowiol (with 4,6-diamidino-2-phenylindole), cover-slipped, and analyzed using an upright fluorescent microscope (Nikon Eclipse 80i, Nikon).

**Slice electrophysiology**

Three weeks after injection of \(\text{AAV2/9-hSyn-DIO-hM3Dq-mCherry}\) into the VTA of \(\text{VGlut2-Cre}\) mice, brain slices containing the VTA were prepared for electrophysiological recordings. Mice were anesthetized with isoflurane and then killed. Brains were rapidly removed and placed in ice-cold oxygenated cutting ACSF (containing in mM: 252 sucrose, 2.5 KCl, 6 MgSO\(_4\), 0.5 CaCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 2.5 NaHCO\(_3\), 10 glucose). Horizontal VTA slices (300 \(\mu\)m) were cut using a vibratome (VT1200S, Leica). Brain slices containing the VTA were transferred to recording ACSF with 124 mM NaCl, 2.5 mM KCl, 1.2 mM NaH\(_2\)PO\(_4\), 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 24 mM NaHCO\(_3\), 5mM HEPES and 12.5 mM glucose. Brain slices were incubated at 34\(^\circ\)C for 30 min and were then kept at room temperature under the same conditions for 45 min before transfer to the recording chamber at room temperature (22-25\(^\circ\)C). The ACSF was perfused at 2 mL/min. The brain slices...
were visualized with a fixed upright microscope (BX51WI, Olympus) equipped with a water immersion lens (40X/0.8 W) and a digital camera (C13440, Hamamatsu). Patch pipettes were pulled from borosilicate glass capillary tubes using a pipette puller (P97, Sutter). The resistance of pipettes varied between 3 and 5 MΩ. For current clamp recording of action potentials, pipettes were filled with solution (130 mM potassium gluconate, 4 mM KCl, 1 mM MgCl2, 4 mM ATP-Mg, 10 mM phosphocreatine, 0.3 mM EGTA, 0.3 mM GTP-Na, 10 mM HEPES, pH 7.3). The hM3Dq-mCherry-positive neurons in the VTA were patched in current-clamp mode. We injected a constant positive current to bring the membrane potential to around −45 mV to induce stable and persistent AP firing. CNO (100μM) was bath applied. The signals were recorded with a MultiClamp 700B amplifier (Molecular Devices), Digidata 1550 interface, and Clampex 10.6 software (Molecular Devices).

Quantification and statistics
Statistical tests were run in “Origin 2019b” (Origin Lab). The individual tests, and the number (n) of mice for each experimental group/condition, are given in the figure legends. All data are given as mean ± SEM and ‘center values’ are the means. We tested for normality and equal variances. When the data were non-normal, we used non-parametric tests (stated in relevant figure legends). Mice were assigned randomly to the experimental and control groups. For chemogenetic experiments, saline or CNO injection was blinded. For analyzing chronic lesioning experiments, VTA\textsuperscript{Vgat}\textsuperscript{-}mCherry and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice, experimenters were blinded. For drug treatments, vehicle or drug injections were not done blinded. Experimental data analysis, including animal behavior that was scored from videos and the analysis of EEG data, was done blinded.

Results
Chemogenetic inhibition or lesioning of VTA\textsuperscript{Vgat} neurons produces increased locomotor activity and sensitivity to amphetamine
We delivered AAV-DIO-hM4Di-mCherry into the VTA of Vgat-ires-cre mice to express hM4Di-mCherry (an inhibitory receptor activated by the CNO ligand\textsuperscript{40}) specifically in VTA\textsuperscript{Vgat} neurons to generate VTA\textsuperscript{Vgat-hM4Di} mice (Figure 1a)\textsuperscript{28}. As previously\textsuperscript{28}, we also ablated VTA\textsuperscript{Vgat} neurons by injecting AAV-DIO-CASP3 and/or AAV-DIO-mCherry (as a control) into the VTA of Vgat-ires-cre mice to generate VTA\textsuperscript{Vgat-CASP3} mice and VTA\textsuperscript{Vgat-mCherry} mice respectively. After 5-6 weeks post-injection, mCherry-positive VTA\textsuperscript{Vgat} neurons were detected in control mice that had only received AAV-DIO-mCherry injections, whereas mCherry-positive VTA\textsuperscript{Vgat} neurons were mostly absent in caspase-injected mice (Figure 1b), but tyrosine hydroxylase-positive (dopamine) neurons were still present (Figure 1b).

We next assessed locomotion behaviors of these animals. After CNO injection, VTA\textsuperscript{Vgat-hM4Di} mice had higher locomotor activity in an open-field arena with more distance travelled (Figure 1c) and had elevated average and maximum speeds over 30 min compared with saline-injected VTA\textsuperscript{Vgat-hM4Di} mice (Supplementary Figure 1a). As a control, the locomotor baseline activity of VTA\textsuperscript{Vgat-hM4Di} mice did not differ over the extended experimental period: mice that received an initial saline injection, and then another saline
injection two weeks later, had the same locomotor activity (Supplementary Figure 1b). Consistent with these chemogenetic inhibition results, VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice also produced hyperlocomotion in the open field – more distance travelled (Figure 1d), and higher average and maximum speeds over 30 min compared with the distance travelled by VTA\textsuperscript{Vgat}\textsuperscript{-}mCherry control mice (Figure 1d; Supplementary Figure 1c). VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice were also hyperactive in their home cages (Supplementary Figure 1d). Notably, the hyperlocomotion of the VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice was still seen when the mice were retested in the open field four months post-lesion (Supplementary Figure 1e). By the end of this period, the body weight of VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice was reduced compared with that of VTA\textsuperscript{Vgat}\textsuperscript{-}mCherry control mice (Supplementary Figure 1f).

Mice suggested to have mania-like characteristics, or patients with bipolar-related mania, are hypersensitive to D-amphetamine\textsuperscript{13, 21}, whereas mice and patients posited to have an attention deficit hyperactivity (ADHD)-like disorder become less active with D-amphetamine\textsuperscript{46, 47}. Treating CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice with D-amphetamine significantly increased their locomotion speed above their high baseline speed, and increased the distance travelled (Figure 1e, f) and the time spent in stereotypy (Supplementary Figure 1g, h), suggesting that both groups of mice (VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 and CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice) were not ADHD-like.

**Acute inhibition or chronic lesioning of VTA\textsuperscript{Vgat} neurons elevates mood**

We assessed mood-related behaviors using the tail-suspension test (TST), the forced swimming test (FST), the sucrose preference test (SPT) and the elevated plus maze. During the TST, the immobility times of CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice were greatly decreased compared with saline-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di control mice and VTA\textsuperscript{Vgat}\textsuperscript{-}mCherry (Figure 2a, b). Both CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice also had decreased immobility times during the FST compared with saline-injected or VTA\textsuperscript{Vgat}\textsuperscript{-}mCherry controls (Figure 2c, d). This could suggest that reducing VTA\textsuperscript{Vgat} neuronal output, by either chemogenetic inhibition or lesioning, produces less depressive-like behaviors. In addition, during the SPT, CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice consumed more sucrose than their control littermates (Figure 2e, f), possibly indicating a raised hedonic state. As a control, the baseline behavior of VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice did not differ over the extended experimental period: mice that received an initial saline injection, and then another saline injection two weeks later, had the same performance during the TST, FST or SPT (Supplementary Figure 1i). We characterized the anxiety-like behavior of our mouse models using the elevated plus maze. The time spent in the open arms of the maze by both CNO-injected VTA\textsuperscript{Vgat}\textsuperscript{-}hM4Di mice and VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice increased (Fig 2g, h), suggesting their anxiety was reduced.

**Mice with lesioned VTA\textsuperscript{Vgat} neurons have less sleep need after sleep deprivation**

VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice have a long-term sleep deficit\textsuperscript{28}. To further characterize the need of VTA\textsuperscript{Vgat}\textsuperscript{-}CASP3 mice for sleep, we performed a sleep deprivation experiment. After sleep deprivation, there is usually a rebound in lost NREM sleep, a process termed sleep homeostasis\textsuperscript{48}. Mice were kept awake for 5 hours with novel objects presented each hour and were then tested to see if they caught up on lost sleep in their home cage. Control
VTA \textit{Vgat-}mCherry mice had rebound NREM sleep after 5 hours sleep deprivation, and thereby after 19 hours they had regained 90% of their sleep loss (Figure 2i). Surprisingly, the VTA \textit{Vgat-CASP3} mice, despite already starting from a chronic sleep-deprived baseline, did not catch up on lost NREM sleep after 5 hours continuous sleep deprivation (Figure 2i).

The mania-like behavior produced by lesioning VTA \textit{Vgat} neurons can be pharmacologically rescued by valproate and diazepam but not lithium or lamotrigine

We assessed if lithium could treat the manic-like state of VTA \textit{Vgat-CASP3} mice. We confirmed that serum lithium was elevated to therapeutic levels during the acute treatment (Supplementary Figure 2a), and that the behavioral baselines of VTA \textit{Vgat-mCherry} or VTA \textit{Vgat-CASP3} mice did not change during the treatment period (Supplementary Figure 2b-f). However, neither acute (100 mg/kg) (Supplementary Figure 3a-e) nor chronic (300 mg/L) treatments (Supplementary Figure 3f-j) had any effects (Of note, chronic Li-H\textsubscript{2}O treatment decreased the immobility time of control mice during the TST (Supplementary Figure 3i)).

We next examined valproate (200 mg/kg) treatments (Figure 3a). Acute injection of valproate did not affect the locomotor activity of VTA \textit{Vgat-mCherry} control mice (Figure 3b). By contrast, the hyperlocomotor activity of VTA \textit{Vgat-CASP3} mice in the open field was restored down to control levels by valproate treatment (Figure 3c). However, 2 weeks after the acute valproate treatment was withdrawal from VTA \textit{Vgat-CASP3} mice, their hyperactivity in the open field had returned (Figure 3c). Similarly, during the tail suspension test, valproate treatment did not affect VTA \textit{Vgat-mCherry} control mice (Figure 3d), but significantly increased the immobility time of VTA \textit{Vgat-CASP3} mice back up to control levels (Figure 3e). Two weeks after valproate had been removed, however, the abnormally high agitation of VTA \textit{Vgat-CASP3} mice had re-emerged (Figure 3e).

We assessed if valproate could reduce the sustained wakefulness of VTA \textit{Vgat-CASP3} mice. The wake time of VTA \textit{Vgat-CASP3} mice significantly decreased after treatment with valproate, whereas NREM sleep time substantially increased (Figure 3f). VTA \textit{Vgat-CASP3} mice also have a pathological sleep architecture with fewer episodes of wake and NREM sleep, prolonged duration of each wake episode, and substantially decreased numbers of wake-NREM transitions\textsuperscript{28}. Valproate treatment normalized the sleep-wake architecture of the VTA \textit{Vgat-CASP3} mice: the episode number (Supplementary Figure 4a), episode duration of wake was restored to control levels (Supplementary Figure 4b), as were the number of transitions between wake and NREM sleep (Supplementary Figure 4c).

We next tested if a low dose of the GABA\textsubscript{A} receptor positive allosteric modulator diazepam, an anxiolytic, influenced the behavior of VTA \textit{Vgat-CASP3} mice (Supplementary Figure 5a-j). Treatment of VTA \textit{Vgat-CASP3} mice with either single or repeated doses of 1 mg/kg diazepam partially reduced the hyperlocomotion (Supplementary Figure 5c, h) and fully recovered the immobility time during the TST (Supplementary Figure 5e, j). These results suggest that diazepam has a potential treatment effect on VTA \textit{Vgat-CASP3} mice.

We also treated VTA \textit{Vgat-CASP3} mice with another anti-epilepsy and bipolar disorder drug – lamotrigine (Supplementary Figure 6a-j). Lamotrigine (10 mg/kg) decreased
the locomotion (Supplementary Figure 6b, g) and immobility time during the TST (Supplementary Figure 6d, i) of VTA\textsubscript{Vgat}-mCherry mice. However, lamotrigine had no effect on treatment of VTA\textsubscript{Vgat-CASP3} mice. Neither locomotion (Supplementary Figure 6c, h) nor immobility time during TST (Supplementary Figure 6e, j) were affected.

Note: with the exception of chronic lithium treatment, none of these drug treatments affected the overall body weight of the mice during and after treatments (Supplementary Figure 7a-d); body weight was reduced during chronic lithium treatment (Supplementary Figure 7c).

The extended wakefulness produced by activating VTA\textsubscript{Vglut2} neurons is not mania-like

Activating VTA\textsubscript{Vglut2} neurons also promotes wakefulness\textsuperscript{28}. To investigate if this type of artificially-induced wake is also mania-like, we expressed the excitatory chemogenetic hM3Dq receptor in VTA\textsubscript{Vglut2} neurons (VTA\textsubscript{Vglut2-hM3Dq} mice; Figure 4a)\textsuperscript{8}. We prepared acute brain slices containing the VTA from VTA\textsubscript{Vglut2-hM3Dq} mice, and first confirmed that CNO application triggered action potentials in hM3Dq-mCherry-expressing cells in VTA glutamatergic neurons (Figure 4b). Chemogenetic activation of VTA\textsubscript{Vglut2} neurons by CNO injection did not alter the locomotor activity or distance travelled of VTA\textsubscript{Vglut2-hM3Dq} mice compared with saline injected mice (Figure 4c) (see also data in ref.\textsuperscript{28}). Moreover, the immobility time during the TST and FST did not differ between CNO injected- and saline injected VTA\textsubscript{Vglut2-hM3Dq} mice (Figure 4d, e). This data serves as an internal negative control, showing that not all artificially induced wakefulness in mice will be mania-like: the activation of VTA\textsubscript{Vglut2} neurons did not produce hyperactivity and mood-related deficits, suggesting a different kind of wakefulness from that generated by inhibition of VTA\textsubscript{Vgat} neurons.

VTA GABAergic neurons contribute to mania-like behaviors via dopamine signaling and projections to the LH

To examine whether the hyperactive behaviors in the CNO-injected VTA\textsubscript{Vgat-hM4Di} mice or VTA\textsubscript{Vgat-CASP3} mice were produced by increased dopamine signaling, we gave a dopamine receptor antagonist mixture i.p. (containing SCH-23390 and raclopride for D1 and D2/D3 receptors, respectively) (these antagonists were given prior to saline or CNO injection to VTA\textsubscript{Vgat-hM4Di} mice). In the open-field test, the dopamine antagonists reduced the hyperlocomotion and distance travelled of saline-injected VTA\textsubscript{Vgat-hM4Di} and VTA\textsubscript{Vgat-mCherry} control mice (Figure 5a, b), and also largely reduced the hyperlocomotion of CNO-injected VTA\textsubscript{Vgat-hM4Di} mice (Figure 5a), but only had a partial effect on VTA\textsubscript{Vgat-CASP3} mice (Figure 5b). In the TST, dopamine receptor antagonists increased the immobility time of both saline- or CNO-injected VTA\textsubscript{Vgat-hM4Di} mice and VTA\textsubscript{Vgat-mCherry} control or VTA\textsubscript{Vgat-CASP3} mice (Figure 5c, d). However, the immobility time of CNO-injected VTA\textsubscript{Vgat-hM4Di} mice or VTA\textsubscript{Vgat-CASP3} mice that received dopamine receptor antagonist injection was still significantly lower than saline-injected VTA\textsubscript{Vgat-hM4Di} mice or VTA\textsubscript{Vgat-mCherry} mice injected with dopamine receptor antagonists (Figure 5c, d). We also found that the extended wakefulness of VTA\textsubscript{Vgat-CASP3} mice was substantially attenuated by the dopamine receptor antagonists (Supplementary Figure 8a-b). The above results suggest that

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blocking dopamine signaling substantially reduces the mania-like behaviors of VTA\textsuperscript{Vgat} mice.

VTA\textsuperscript{Vgat} neurons project prominently to the LH\textsuperscript{28}. We conducted optogenetic and chemogenetic experiments to examine if the VTA\textsuperscript{Vgat} to LH projection contributes to hyperactivity and mood-related behaviors. An AAV carrying a Cre-dependent eNpHR3.0-mCherry transgene was injected into the VTA of Vgat-ires-cre mice to express inhibitory halorhodopsin in VTA\textsuperscript{Vgat} neurons to generate VTA\textsuperscript{Vgat-NpHR} mice (Figure 5e). Optic fibers were placed above the LH of VTA\textsuperscript{Vgat-NpHR} mice, where dense NpHR-mCherry projections arising from the VTA\textsuperscript{Vgat} cell bodies can be seen (Figure 5e). Optogenetic inhibition of the VTA\textsuperscript{Vgat} to LH projection, by activating NpHR-mCherry in the LH, elevated the locomotion of mice in the open field (Figure 5f), whereas it decreased the immobility times during the TST (Figure 5g) and FST (Figure 5h). We confirmed this using specific chemogenetic inhibition of the VTA\textsuperscript{Vgat} to LH projection. CNO was infused directly into the LH of VTA\textsuperscript{Vgat-hM4Di} mice, which expressed the inhibitory CNO receptor in VTA\textsuperscript{Vgat} neurons (Supplementary Figure 9a). CNO infusion increased locomotion speed (Supplementary Figure 9b), and decreased immobility time during the TST (Supplementary Figure 9c). These results indicate that inhibiting GABAergic tone from VTA\textsuperscript{Vgat} neurons to the LH produces hyperlocomotion and less depressive-like behaviors. We next injected AAV-DIO-ChR2-mCherry into the VTA of Vgat-cre mice to express excitatory channel rhodopsins in VTA GABAergic cells. Although opto-activation of the VTA\textsuperscript{Vgat} to LH projection did not affect the locomotion of mice in the open field (Figure 5i), it did increase the immobility times during the TST (Figure 5j) and FST (Figure 5k). The above results suggest that the VTA\textsuperscript{Vgat} to LH projection, together with VTA\textsuperscript{Vgat} local inhibition of the dopamine system, contributes to generating mania-like behaviors.

Discussion

We found that endophenotypes (hyperactivity, elevated mood and reduced sleep) resembling aspects of mania could be produced by lesion or acute inhibition of GABAergic VTA\textsuperscript{Vgat} neurons. In mice with lesioned or inhibited VTA\textsuperscript{Vgat} neurons, D-amphetamine further increased the hyperactivity, suggesting the mania-like behavior of these mice could be the type associated with bipolar disorder in humans. The mania effects were partially blocked by D1, D2 and D3 receptor antagonists. Thus, hyperdopaminergia could originate from VTA\textsuperscript{Vgat} cells failing to inhibit dopamine neurons (Figure 5l). VTA\textsuperscript{Vgat} cells, however, are not simply VTA inhibitory interneurons that reduce dopamine’s actions. Part of their actions in promoting increased movement in the FST and TST depends on their inhibitory projections to the LH. Our results on hyperactivity produced by disinhibiting LH circuits are consistent with previous findings. In rats, repeated delivery of subthreshold stimuli (kindling) to the LH induces mania-like behavior\textsuperscript{49}. Indeed, the LH promotes physical activity and motivated behavior\textsuperscript{50}; LH lesions in rodents and people produce a state of wakefulness with no motion\textsuperscript{51}. The LH contains orexin/glutamate neurons that promote arousal, motivation and energy expenditure\textsuperscript{52}, and GABAergic neurons whose activation induces wakefulness and locomotion\textsuperscript{50, 53, 54}. It is likely that the VTA\textsuperscript{Vgat} neurons inhibit both the orexin neurons and/or the arousal/locomotor-promoting GABA neurons in the LH\textsuperscript{28, 36}. 

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Given the hub-like nature of the GABAergic VTA
Vgat
neurons, the mania phenotype produced by lesioning or inhibiting these neurons is likely a consequence of the disinhibition of multiple targets, e.g. dopamine cells in the VTA, but also to some extent other cell types in the LH. On the other hand, VTA
Vgat
neurons also locally inhibit VTA glutamate neurons28, but disinhibition of glutamate neurons in the VTA by lesioning or inhibiting the VTA
Vgat
neurons probably does not contribute to the mania-phenotype. Direct pharmacogenetic excitation of these glutamate cells produces calm wakefulness (Figure 4)28.

Inability to sleep is one of the diagnostic criteria for the mania phase of bipolar disorder2, 3, 55, and certainly VTA
Vgat
mice sleep consistently less than control mice, such that they have 100% wakefulness during the “lights off” phase compared with control mice that take about 4 hours NREM sleep during this time28. Usually, the longer that wakefulness persists, the stronger the urge to sleep becomes, until sleep is inescapable. A remarkable finding is that VTA
Vgat-CASP3 mice bypassed the homeostatic process of having NREM recovery sleep after sleep deprivation – they did not catch up on lost sleep in spite of already starting from a strongly sleep-deprived background. The mechanisms underlying sleep homeostasis are not well understood48, 56, 57. Sleep homeostasis is thought to reflect the function of sleep, (otherwise why catch up on lost sleep?), and it will be interesting to investigate if the chronic lack of sleep of VTA
Vgat-CASP mice will be detrimental metabolically.

Sleep deprivation can sometimes trigger mania episodes in humans3, 55, 58. Consequently, an interesting question is whether the chronic sleep deprivation phenotype of VTA
Vgat-CASP3 mice actually causes their mania-like symptoms. Mice chronically sleep derived with the flower pot method - the animals stay on top of a raised platform surrounded by water, and when they fall asleep, they fall into water and wake up - also develop mania-like behavior59. This type of sleep deprivation is, however, likely to be stressful. In the VTA
Vgat-CASP3 mice, the sleep-deprivation phenotype is internally generated within the brain, and could be less stressful per se.

The mania-like behaviors of VTA
Vgat-CASP3 mice, including the strong sleep loss and abnormal sleep architecture, were reversed by valproate, and re-emerged when valproate treatment was stopped. Valproate has diverse actions: for example, it enhances GABAergic transmission and reduces action potential firing (reviewed in ref 13), but by inhibiting histone deacetylases60, 61, valproate treatments also change the expression of many genes. We found that low doses of diazepam were also efficacious in reducing the hyperarousal of VTA
Vgat-CASP3 mice. Diazepam a GABA_A receptor positive allosteric modulator, enhances GABA transmission62. At the low doses tested here in mice, diazepam is an anxiolytic, but at higher doses (which we did not test) it induces sleep62. Two other important drugs used for treating bipolar disorder, lithium and lamotrigine, had no effect in treating VTA
Vgat-CASP3 mice. Although its mechanism of action is unclear, lithium treatment is the first choice to treat mania episodes, although a subset of bipolar patients with rapidly cycling mania and depression phases are resistant to lithium (reviewed in ref 13). In most mouse models of mania, both valproate and lithium are usually effective treatments (reviewed in ref. 1). But similar to our results, mice with SHANK3
overexpression in the neocortex, hippocampus and basal ganglia, have mania-like symptoms treatable with valproate but not lithium. Similar arguments could apply to lamotrigine. It could be that the VTA\textsuperscript{Vgat}-CASP3 mice and SHANK3-overexpressing mice models reflect a specific mania type.

In summary, the model based on reducing VTA\textsuperscript{Vgat} neuronal function could provide further insight into the genesis of some types of mania-like behaviors. One hypothesis is that VTA\textsuperscript{Vgat} neurons help set the level of mental and physical activity. Physiologically, inputs that inhibit VTA\textsuperscript{Vgat} neurons will transiently intensify aspects of wakefulness useful for acute success or survival: increased activity, enhanced alertness and motivation, reduced sleep (Figure 5I). Taken to the extreme, however, pathology could emerge and decreased or failed inhibition from VTA\textsuperscript{Vgat} neurons will produce mania-like qualities (Figure 5I).

\textbf{Supplementary Material}

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Selective inhibition or lesioning of VTA<sub>Vgat</sub> neurons increased locomotor activity and sensitivity to D-amphetamine-induced hyperlocomotion.

(a) Generation of VTA<sub>Vgat</sub>-hM4Di mice. Staining by immunohistochemistry (mCherry, red) for hM4Di-mCherry expression in the VTA. Scale bar: 150 μm.

(b) Generation of VTA<sub>Vgat</sub>-CASP3 and VTA<sub>Vgat</sub>-mCherry control mice. Immunohistochemical staining for mCherry (red) and dopamine neurons (TH, tyrosine hydroxylase, green) in the VTA of control mice that expressed mCherry in VTA<sub>Vgat</sub> neurons (left-hand images), or in mice where VTA<sub>Vgat</sub> neurons were lesioned with caspase (right-hand images). Scale bar: 150 μm.

(c) Video-tracked paths, locomotion speed and distance travelled of VTA<sub>Vgat</sub>-hM4Di mice (n=8 mice) following CNO (1 mg/kg) injection compared with saline injection in the open field over a 30-minute period. Paired t-test, t(7)=-3.68, **p=0.007.
(d) Video-tracked paths, locomotion speed and distance travelled of VTA $\text{Vgat}^-$mCherry (n=8 mice) and VTA $\text{Vgat}^-$CASP3 mice (n=9 mice) in the open field over a 30-minute period. Unpaired t-test, $t(15)=-5.43$, ****$p=0.00006$.

(e) Locomotion speed and distance traveled of VTA $\text{Vgat}^-$hM4Di mice (n=6 mice) in the open field after saline or D-amphetamine injection (subsequent to saline or CNO injection). Repeated measures two-way ANOVA and Bonferroni-Holm post hoc test. $F(1,5)=66$. Saline vs. saline + amphetamine $t(5)=3.69$, *$p=0.01$; CNO vs. CNO + amphetamine $t(5)=13.6$, ****$p=0.00038$.

(f) Locomotion speed and distance traveled in the open field test of VTA $\text{Vgat}^-$mCherry (n=7 mice) or VTA $\text{Vgat}^-$CASP3 mice (n=6 mice) after vehicle or D-amphetamine injection. Two-way ANOVA and Bonferroni-Holm post hoc test. $F_{(mCherry \ or \ CASP3)}=93$; $F_{(saline \ or \ amphetamine)}=26$; $F_{(interaction)}=5.64$; mCherry + vehicle vs. mCherry + amphetamine $t(6)=-3.39$, *$p=0.01$; CASP3 + vehicle vs. CASP3 + amphetamine $t(5)=-4.97$, **$p=0.004$.

All error bars represent the SEM.
Figure 2. Dysfunction of VTA\textsuperscript{Vgat} neurons elevates mood and impairs sleep homeostasis

(a) Immobility time during the tail suspension test (TST) of VTA\textsuperscript{Vgat-hM4Di} mice (n=8 mice) after saline or CNO injection. Paired t-test, t(7)=53.8, ****p=1.98e^{-10}.

(b) Immobility time during the tail suspension test (TST) of VTA\textsuperscript{Vgat-CASP3} (n=9 mice) or VTA\textsuperscript{Vgat-mCherry} mice (n=8 mice). Unpaired t-test, t(15)=14.3, ****p=3.7e^{-10}.

(c) Immobility time during the forced swimming test (FST) of VTA\textsuperscript{Vgat-hM4Di} mice (n=6 mice) after saline or CNO injection. Paired t-test, t(5)=5, **p=0.003.
(d) Immobility time during the FST of VTA\textsuperscript{Vgat\,-CASP3} (n=6 mice) or VTA\textsuperscript{Vgat\,-mCherry} mice (n=6 mice). Unpaired t-test, \(t(10)=6, ***p=0.0001\).

(e) Sucrose preference of VTA\textsuperscript{Vgat\,-hM4Di} mice (n=6 mice) after saline or CNO injection. Paired t-test, \(t(5)=-3.5, *p=0.01\).

(f) Sucrose preference of VTA\textsuperscript{Vgat\,-CASP3} (n=6 mice) or VTA\textsuperscript{Vgat\,-mCherry} mice (n=8 mice). Unpaired t-test, \(t(12)=-4.8, ***p=0.0004\).

(g) Elevated plus maze: VTA\textsuperscript{Vgat\,-hM4Di} mice received saline injection and two weeks later a second saline injection (n=8 mice), paired t-test, \(t(7)=-1.06, p=0.32\); or saline and two weeks later a CNO injection (n=8 mice), paired t-test, \(t(7)=-5.28, **p=0.001\).

(h) Elevated plus maze of VTA\textsuperscript{Vgat\,-CASP3} (n=6 mice) or VTA\textsuperscript{Vgat\,-mCherry} mice, paired t-test, \(t(5)=-3.45, *p=0.01\).

(i) The accumulative recovery sleep (time in NREM sleep) after 5 hours of sleep deprivation (SD) of VTA\textsuperscript{Vgat\,-CASP3} (n=4 mice) or VTA\textsuperscript{Vgat\,-mCherry} mice (n=4 mice). Mann-Whitney test, *\(p=0.03\). The light and dark shading represents the “lights on” and “lights off” phases.
Figure 3. Valproic acid can successfully treat the mania-like behavior of VTA\textsuperscript{Vgat}\textsuperscript{+}\textsuperscript{-}\textsuperscript{CASP3} mice

(a) Pharmacological treatment protocol for valproic acid. The top arrows indicate vehicle or valproic acid injection (depending on mouse group) and the stars indicate when the behavioral experiments were undertaken.

(b) Locomotion speed and distance travelled for VTA\textsuperscript{Vgat}\textsuperscript{+}\textsuperscript{-}\textsuperscript{mCherry} mice (n=6 mice) that received either vehicle or valproic acid treatment. Paired t-test, t(5)=-0.36, p=0.73.

(c) Locomotion speed and distance travelled for VTA\textsuperscript{Vgat}\textsuperscript{+}\textsuperscript{-}\textsuperscript{CASP3} mice (n=6 mice) received either vehicle or valproic acid treatment, or where the valproic acid treatment had been removed for 2 weeks. Repeated measures one-way ANOVA and Bonferroni-Holm post hoc test. F(2,10)=47; vehicle vs. valproate t(10)=8.68, ****p=0.000005; valproate vs. 2 weeks after valproate withdrawal t(10)=8.19, ****p=0.000009; vehicle vs. after valproate withdrawal t(10)=0.48, p=0.63.

(d) Time spent immobile on the TST of VTA\textsuperscript{Vgat}\textsuperscript{+}\textsuperscript{-}\textsuperscript{mCherry} mice (n=6 mice) received vehicle or valproate injection. Paired t-test, t(5)=0.71, p=0.5.

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(e) Time spent immobile on the TST of VTA<sup>V<sub>GAT</sub></sup>-CASP3 mice (n=6 mice) received vehicle, valproate injection, or 2 weeks after valproate withdrawal. Repeated measures one-way ANOVA and Bonferroni-Holm post hoc test. F(2,10)=32; vehicle vs. valproate t(10)=7.41, ****p=0.00002; 2 weeks after valproate withdrawal; t(10)=6.32, ****p=0.00008; vehicle vs. remove t(10)=1.08, p=0.3.

(f) Percentage and time of wake and NREM for VTA<sup>V<sub>GAT</sub></sup>-mCherry mice and VTA<sup>V<sub>GAT</sub></sup>-CASP3 mice over the 12 hours “lights off” period that received saline or valproate injection. mCherry + vehicle n=6 mice; mCherry + valproate n=5 mice; CASP3 + vehicle n=6 mice; CASP3 + valproate n=7 mice. Two-way ANOVA and Bonferroni-Holm post hoc test. Wake: F<sub>(mCherry or CASP3)</sub>=66; F<sub>(Vehicle or valproate)</sub>=17; F<sub>(interaction)</sub>=21; mCherry + vehicle vs. mCherry + valproate t(9)=0.38, p=0.38; CASP3 + vehicle vs. CASP3 + valproate t(11)=6.89, ****p=0.00002. NREM: F<sub>(mCherry or CASP3)</sub>=65; F<sub>(Vehicle or valproate)</sub>=32; F<sub>(interaction)</sub>=20; mCherry + vehicle vs. mCherry + valproate t(9)=-0.92, p=0.37; CASP3 + vehicle vs. CASP3 + valproate t(11)=-6.85, ****p=0.00002. All error bars represent the SEM.
Figure 4. Activation of VTA$^{Vglut2}$ neurons does not produce mania-like behaviors
(a) Generation of VTA$^{Vglut2}$-hM3Dq mice. Staining by immunohistochemistry (mCherry) for hM3Dq-mCherry expression in the VTA. Scale bar: 150 μm.
(b) Example of a spontaneously active VTA glutamatergic neuron with action potential firing before, during and after CNO application in an acute brain slice prepared from a VTA$^{Vglut2}$-hM3Dq mouse. The bar graph shows the average normalized frequency of action potentials of all cells recorded. One-way repeated ANOVA and Bonferroni-Holm post hoc test.
(c) Video-tracked paths, locomotion speed and distance travelled of VTA\textsuperscript{$V_{glut2}$}-hM3Dq mice (n=8 mice) following CNO (1 mg/kg) injection compared with saline injection in the open field arena over a 30-minute period. Paired t-test, t(7)=-0.72, p=0.49.

(d) Immobility time during the tail suspension test (TST) of VTA\textsuperscript{$V_{glut2}$}-hM3Dq mice (n=8 mice) after saline or CNO injection. Paired t-test, t(7)=-0.73, p=0.88

(e) Immobility time during the forced swimming test (FST) of VTA\textsuperscript{$V_{glut2}$}-hM3Dq mice (n=6 mice) after saline or CNO injection. Paired t-test, t(4)=-0.01, p=0.98.
Figure 5. Circuit mechanisms underlying the mania-like behaviors mediated by VTA\textsuperscript{Vgat} neurons
(a) Locomotion speed and distance travelled of VTA\textsuperscript{Vgat}-hM4Di mice that received injections with vehicle or a dopamine receptor antagonist mixture (SCH-23390 and raclopride for D1 and D2/D3 receptors, respectively) and either saline (n= 7 mice) or CNO (n=7 mice). Two-way ANOVA and Bonferroni-Holm post hoc test. F\textsubscript{(saline or CNO)}=13.6; F\textsubscript{(vehicle or antagonists)}=91.2; F\textsubscript{(interaction)}=7.4; Vehicle + saline vs. antagonists + saline t(12)=5, ***p=0.0002; Vehicle + CNO vs. antagonists + CNO t(12)=8.34, ****p=2E-6. All error bars represent the SEM.

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(b) Locomotion speed and distance travelled of VTA\textsuperscript{V\textsubscript{gat}}-mCherry and VTA\textsuperscript{V\textsubscript{gat}}-CASP3 mice that received injections with either vehicle (n=6 mice for mCherry and n=10 mice for CASP3) or a dopamine receptor antagonist mixture (n=6 mice for mCherry and n=13 mice for CASP3). Two-way ANOVA and Bonferroni-Holm post hoc test. 
\[ F_{(\text{mCherry or CASP3})}=10.8; \ F_{(\text{saline or antagonists})}=4.5; \ F_{(\text{interaction})}=0.03; \text{mCherry + vehicle vs. mCherry + antagonists} \ t(10)=4.14, **p=0.002; \text{CASP3 + vehicle vs. CASP3 + antagonists} \ t(21)=1.64, p=0.11. \] All error bars represent the SEM.

(c) Time spent immobile for the tail suspension assay of VTA\textsuperscript{V\textsubscript{gat}}-hM4Di mice that received injections of either vehicle or the dopamine receptor antagonist mixture and saline or CNO. Two-way ANOVA and Bonferroni-Holm post hoc test. 
\[ F_{(\text{saline or CNO})}=36; \ F_{(\text{Vehicle or antagonists})}=27; \ F_{(\text{interaction})}=0.35; \text{vehicle + saline vs. antagonists + saline} \ t(12)=-7.59, ****p=6E-6; \text{vehicle + CNO vs. antagonists + CNO} \ t(12)=-2.54, *p=0.02; \text{antagonists + saline vs. antagonists + CNO} \ t(12)=3.8, **p=0.002. \] All error bars represent the SEM.

(d) Time spent immobile for the tail suspension assay of VTA\textsuperscript{V\textsubscript{gat}}-mCherry and VTA\textsuperscript{V\textsubscript{gat}}-CASP3 mice that received injections of either vehicle (n=8 mice for mCherry and n=9 mice for CASP3) or the dopamine receptor antagonist mixture (n=8 mice for mCherry and n=9 mice for CASP3). Two-way ANOVA and Bonferroni-Holm post hoc test. 
\[ F_{(\text{mCherry or CASP3})}=209; \ F_{(\text{saline or antagonists})}=25; \ F_{(\text{interaction})}=4.38; \text{mCherry + vehicle vs. mCherry + antagonists} \ t(14)=-4.87, ***p=0.0002; \text{CASP3 + vehicle vs. CASP3 + antagonists} \ t(16)=-2.16, *p=0.04; \text{mCherry + antagonists vs. CASP3 + antagonists} \ t(15)=13, ****p=7e^{-10}. \] All error bars represent the SEM.

(e) AAV-DIO-NpHR-mCherry was injected the VTA of \textit{V\textsubscript{gat}-ires-cre} mice, and optic fibers were bilaterally implanted above the LH. The NpHR-mCherry fibers projecting from the VTA\textit{V\textsubscript{gat}} neurons into the LH were stained by immunohistochemistry (mCherry, red). Scale bar: 200 μm.

(f) Video-tracked paths and distance travelled in the open field by VTA\textsuperscript{V\textsubscript{gat}}-NpHR mice (n=7 mice) with and without opto-inhibition of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH over 5 minute period. Paired t-test, \( t(6)=-4.08, **p=0.006. \)

(g) Time spent immobile on the tail suspension test (TST) of VTA\textsuperscript{V\textsubscript{gat}}-NpHR mouse (n=7 mice) with and without opto-inhibition of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH. Paired t-test, \( t(6)=5.3, **p=0.001. \)

(h) Time spent immobile on the forced swimming test (FST) of VTA\textsuperscript{V\textsubscript{gat}}-NpHR mouse (n=6 mice) with and without opto-inhibition of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH. Paired t-test, \( t(5)=6.77, **p=0.001. \)

(i) Video-tracked paths and distance travelled in the open field by VTA\textsuperscript{V\textsubscript{gat}}-ChR2 mice (n=8 mice) with and without opto-activation of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH over 5-minute period. Paired t-test, \( t(7)=0.18, p=0.85. \)

(j) Time spent immobile on the tail suspension test (TST) of VTA\textsuperscript{V\textsubscript{gat}}-ChR2 mice (n=8 mice) with and without opto-activation of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH. Paired t-test, \( t(7)=-3.11, *p=0.01. \)

(k) Time spent immobile on the forced swimming test (FST) of VTA\textsuperscript{V\textsubscript{gat}}-ChR2 mice (n=6 mice) with and without opto-activation of VTA\textsuperscript{V\textsubscript{gat}} terminals in the LH. Paired t-test, \( t(5)=-4. *p=0.01. \)
(l) Conceptual circuit diagram illustrating VTA $V_{gat}$ neurons inhibiting VTA dopamine (DA) neurons and circuitry in the LH. When the VTA $V_{gat}$ cells have acutely diminished or absent function, activity of VTA dopamine neurons and arousal-promoting neurons in the LH increase. Valproate can reverse the effects of these changes.