Ablation of Ventral Midbrain/Pons GABA Neurons Induces Mania-like Behaviors with Altered Sleep Homeostasis and Dopamine D₂R-mediated Sleep Reduction

**HIGHLIGHTS**

- Hyperactivity and anti-depressive behaviors are induced by loss of VMP GABA neurons
- Homeostatic sleep rebound is lost together with largely shorten daily sleep
- Dopamine D₂ receptors mediate the daytime sleep loss

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Ablation of Ventral Midbrain/Pons GABA Neurons Induces Mania-like Behaviors with Altered Sleep Homeostasis and Dopamine D2R-mediated Sleep Reduction

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SUMMARY

Individuals with the neuropsychiatric disorder mania exhibit hyperactivity, elevated mood, and a decreased need for sleep. The brain areas and neuronal populations involved in mania-like behaviors, however, have not been elucidated. In this study, we found that ablating the ventral medial midbrain/pons (VMP) GABAergic neurons induced mania-like behaviors in mice, including hyperactivity, anti-depressive behaviors, reduced anxiety, increased risk-taking behaviors, distractibility, and an extremely shortened sleep time. Strikingly, these mice also showed no rebound sleep after sleep deprivation, suggesting abnormal sleep homeostatic regulation. Dopamine D2 receptor deficiency largely abolished the sleep reduction induced by ablating the VMP GABAergic neurons without affecting the hyperactivity and anti-depressive behaviors. Our data demonstrate that VMP GABAergic neurons are involved in the expression of mania-like behaviors, which can be segregated to the short-sleep and other phenotypes on the basis of the dopamine D2 receptors.

INTRODUCTION

Sleep problems are the core components of mood disorders, such as bipolar disorder characterized by periods of depression and mania (Benca et al., 1997). The clinical criteria for mania include persistently elevated mood or energy and decreased need for sleep (American Psychiatric Association, 2013). In humans, infarctions of the midbrain or pontine areas induces secondary mania (Antelmi et al., 2014; Caplan, 2010; Drake et al., 1990; Satzer and Bond, 2016; Shen et al., 2005). In the present study, we focused on the ventral medial midbrain/pons (VMP) area, a region that contains sleep-regulating GABAergic neurons (Chowdhury et al., 2019; Takata et al., 2018; Yu et al., 2019). To elucidate the physiologic functions of VMP GABAergic neurons, we selectively ablated these neurons in mice with Cre-dependent viral expression of the diphtheria toxin subunit A (DTA) in the VMP of vesicular GABA transporter (VGAT; a marker of GABAergic neurons) Cre mice (VGAT-CreDTA/VMP mice: Figures 1A and 1B). For controls, we prepared mice expressing humanized Renilla reniformis-derived green fluorescent protein (hrGFP) in the VMP GABAergic neurons (VGAT-Cre hrGFP/VMP). These mice were subjected to a comprehensive behavioral test battery including various tasks that are reliably used to assess locomotor activity, depression-like behavior, sociability, anxiety, species-typical behavior, and learning and memory (Crawley, 2007; Crawley and Paylor, 1997).

RESULTS

GABAergic VMP Ablation Induces Hyperactivity, Anti-depressive Behaviors, and Reduced Anxiety

Our behavioral test battery revealed that VGAT-CreDTA/VMP mice exhibited increased locomotor activity with a longer distance traveled in the open field test compared with control VGAT-CrehrGFP/VMP mice (p < 0.001, Figure 1C). VGAT-CreDTA/VMP mice also showed consistently decreased immobility in both the tail suspension test (p < 0.01, Figure 1D) and the forced swim test (p < 0.0001, Figure 1E), suggesting anti-depressive behaviors. In addition, VGAT-CreDTA/VMP mice exhibited increased preference for the zone
Figure 1. Selective Ablation of the VMP GABAergic Neurons Induces Hyperactivity, Anti-depressive Behaviors, and Reduced Anxiety

(A) VGAT-Cre mice were injected with AAV-FLEX-hrGFP (VGAT-CrehrGFP/VMP mice; control) or AAV-FLEX-DTA (VGAT-CreDTA/VMP mice) into the VMP region.

(B) Brain sections stained against VGAT mRNA. VMP GABAergic neurons were selectively ablated in VGAT-CreDTA/VMP mice but not in VGAT-CrehrGFP/VMP mice. RN, red nucleus; VTA, ventral tegmental area; SN, substantia nigra; IP, interpeduncular nucleus. Scale bar, 500 μm.
with mice compared with control VGAT-Cre<sup>hGFP/VMP</sup> mice ($p < 0.05$, Figure 1F). In the elevated plus maze, VGAT-Cre<sup>DTAVMP</sup> mice spent more time in the open arms ($p < 0.01$, Figure 1G), indicating less anxiety and increased risk-taking behavior. In addition, VGAT-Cre<sup>DTAVMP</sup> mice had lower scores in the nest building test ($p < 0.001$, Figure 1H). We also assessed learning and memory. In the novel object recognition task, control VGAT-Cre<sup>hGFP/VMP</sup> mice showed increased exploratory preference for novel objects ($p < 0.001$, Figure 1I). On the other hand, VGAT-Cre<sup>DTAVMP</sup> mice demonstrated no preference for novel objects (not significant, n.s., $p = 0.131$, Figure 1I), suggesting impaired novel object recognition. Conversely, in the Y maze as a test for working memory, VGAT-Cre<sup>DTAVMP</sup> mice exhibited no difference from control mice in spontaneous alternation rate (n.s., $p = 0.260$, Figure 1J), but the number of entries into each arm was increased, consistent with their hyperactivity ($p < 0.01$, Figure 1J). Overall, the series of behavioral tests provided us landmark information to support the involvement of VMP GABAergic neurons in the various aspects of manic episodes described in both humans and rodent models (American Psychiatric Association, 2013; Logan and McClung, 2016; Perry et al., 2009; Young et al., 2007), including hyperactivity, increased risk-taking behavior, e.g., attention-deficit/hyperactivity disorder (Marangoni et al., 2015). Therefore, we next performed a detailed analysis of the sleep/wake behaviors in VGAT-Cre<sup>DTAVMP</sup> mice.

GABAergic VMP Ablation Induces Reduced Daily Sleep Amounts and Loss of Rebound Sleep

Sleep consists of rapid eye movement sleep (REMS) and slow-wave sleep (SWS), also known as non-REM sleep, which are distinguished on the basis of electroencephalography (EEG) and electromyography (EMG) characteristics. It is commonly considered that sleep need is represented during SWS as EEG power in delta frequency (0.5–4 Hz), which increases in animals after prolonged wakefulness (Daan et al., 1984; Lazarus et al., 2019) as well as in mutant mice with prolonged daily sleep amount (Funato et al., 2016; Honda et al., 2018). We first measured the daily sleep amount of VGAT-Cre<sup>DTAVMP</sup> mice. The VGAT-Cre<sup>DTAVMP</sup> mice displayed reduced sleep amounts and increased wake amounts compared with control VGAT-Cre<sup>hGFP/VMP</sup> mice (SWS 24 h: $p < 0.0001$, SWS light: $p < 0.0001$, SWS dark: $p < 0.0001$, wake 24 h: $p < 0.0001$, wake light: $p < 0.0001$, wake dark: $p < 0.0001$, REMS 24 h: $p < 0.0001$, REMS light: $p < 0.0001$, REMS dark: $p < 0.05$, Figures 2A and 2B), consistent with our previous study (Takata et al., 2018). Next, we calculated the delta power during daily SWS to evaluate the mean level of sleep need. In contrast to the severe sleep reduction in VGAT-Cre<sup>DTAVMP</sup> mice, there was no difference in the EEG power spectra ($p = 0.650$, Figure S1) and the delta power ($p = 0.378$, Figure 2C) during SWS over 24 h compared with control VGAT-Cre<sup>hGFP/VMP</sup> mice. This finding suggests that VGAT-Cre<sup>DTAVMP</sup> mice are able to maintain the same level of sleep need compared with control mice, despite lower daily sleep amounts. Alternatively, although the daily sleep amount is constantly reduced, it would be an adequate amount for VGAT-Cre<sup>DTAVMP</sup> mice such that it does not evoke changes in the mean level of the SWS delta power. Thus, VGAT-Cre<sup>DTAVMP</sup> mice may not be under higher sleep pressure or suffering from greater “sleepiness” despite the reduced sleep amounts. To further investigate sleep/wake phenotypes, we performed sleep deprivation (SD) experiments.
Involvement of D2 receptors (D2R) in notable behaviors in VGAT-CreDTA/VMP mice, we generated D2R knockout (KO) mice using fertilized egg donors of VGAT-Cre mice by the CRISPR-Cas9 technique (Knockout [KO] mice using fertilized egg donors of VGAT-Cre mice by the CRISPR-Cas9 technique). Only detected in VGAT-Cre;D2R+/+ mice and not in VGAT-Cre;D2R−/− mice. Functional D2R deficit, we administered a moderate dose (45 mg/kg) of modafinil, a dopamine transporter blocker that has an arousal effect and is largely ineffective in D2R-deficient mice (Qu et al., 2008). Consistent with the previous study, our newly generated VGAT-Cre;D2R−/− mice did not exhibit arousal after modafinil administration (p = 0.788, Figure 3C), in contrast to the significant arousal effect observed in VGAT-Cre;D2R+/+ mice (p < 0.001, Figure 3C).

Although sleep latency after SD was extended in VGAT-CreDTA/VMP mice (p < 0.05, Figure 2E), we found that EEG delta power during SWS was increased after SD and dissipated along with SWS time (not total time) in VGAT-CreDTA/VMP mice (0–10 min versus 30–40 min: p < 0.05, 0–10 min versus 40–50 min: p < 0.01, Figure 2F), despite lacking sleep rebound (Figure 2D). These data suggest that the mechanisms regulating delta power during SWS and sleep amounts are dissociable, consistent with findings from a previous study (Suzuki et al., 2013). Remarkably, SWS delta power dissipation along with SWS time after SD was noticeably similar between VGAT-CreDTA/VMP and the control VGAT-CreEGFP/VMP mice (n.s., p = 0.999, the main effect of viral transduction, ANOVA; Figure 2F). In contrast, the gradual decay of delta power was not observed during wakefulness (n.s., p = 0.074, the main effect of time, ANOVA; Figure 2G). These observations suggest that the neural mechanisms generate delta power after SD function mainly during SWS and pause during wakefulness. Overall, we revealed that VMP GABAergic neurons have critical roles in the regulation of both daily sleep amounts and homeostatic sleep as a response to SD.

**Dopamine D2 Receptors Mediate Sleep Reduction Induced by GABAergic VMP Ablation**

Our previous pharmacologic study suggested that D2 and/or D3 receptors mediate the wake-promoting effect induced by ablating or inhibiting VMP GABAergic neurons (Takata et al., 2018). To evaluate the involvement of D2 receptors (D2R) in notable behaviors in VGAT-CreDTA/VMP mice, we generated D2R knockout (KO) mice using fertilized egg donors of VGAT-Cre mice by the CRISPR-Cas9 technique (VGAT-Cre;D2R−/− mice; Figure 3A). We first histologically confirmed that immunostaining for D2R was only detected in VGAT-Cre;D2R+/+ mice and not in VGAT-Cre;D2R−/− mice (Figure 3B). To verify an in vivo functional D2R deficit, we administered a moderate dose (45 mg/kg) of modafinil, a dopamine transporter blocker that has an arousal effect and is largely ineffective in D2R-deficient mice (Qu et al., 2008). Consistent with the previous study, our newly generated VGAT-Cre;D2R−/− mice did not exhibit arousal after modafinil administration (p = 0.788, Figure 3C), in contrast to the significant arousal effect observed in VGAT-Cre;D2R+/+ mice (p < 0.001, Figure 3C).
To assess the D2R involvement in sleep behaviors in VGAT-CreDTA/VMP mice, we next injected AAV-FLEX-DTA or control AAV-FLEX-hrGFP virus into the VMP of VGAT-Cre;D2R+/+ mice or VGAT-Cre;D2R−/− mice. We first confirmed that VGAT-CreDTA/VMP;D2R+/+ mice exhibited reduced amounts of SWS over 24 h (p < 0.0001, the main effect of viral transduction, ANOVA; Figure 4A) in both light and dark periods (light: p < 0.001, dark: p < 0.01, Figure 4B) compared with control VGAT-CrehrGFP/VMP;D2R+/+ mice. Although VGAT-CreDTA/VMP;D2R−/− mice also showed reduced SWS amounts over 24 h (p < 0.001, the main effect of viral transduction, ANOVA; Figure 4C), strikingly, VGAT-CreDTA/VMP;D2R−/− mice showed no difference in SWS amounts during the light period compared with control VGAT-CrehrGFP/VMP;D2R−/− mice (light: n.s., p = 0.182, dark: p < 0.001, Figure 4D).

In other words, the reduced sleep amounts observed in VGAT-CreDTA/VMP mice during the light period (Figure 2B) were functionally rescued by the loss of D2R. This finding indicates that the sleep reduction induced by ablating VMP GABAergic neurons is mainly mediated by D2R.

We next examined the effect of SD, which resulted in the loss of rebound sleep by ablation of VMP GABAergic neurons regardless of D2R+/+ or D2R−/− conditions (p = 0.111, VGAT-CreDTA/VMP;D2R+/+ basal versus SD; p = 0.406, VGAT-CreDTA/VMP;D2R−/− basal versus SD; Figure 4B) compared with control VGAT-CrehrGFP/VMP;D2R+/+ mice. Although VGAT-CreDTA/VMP;D2R−/− mice also showed reduced SWS amounts over 24 h (p < 0.001, the main effect of viral transduction, ANOVA; Figure 4C), strikingly, VGAT-CreDTA/VMP;D2R−/− mice showed no difference in SWS amounts during the light period compared with control VGAT-CrehrGFP/VMP;D2R−/− mice (light: n.s., p = 0.182, dark: p < 0.001, Figure 4D).

In other words, the reduced sleep amounts observed in VGAT-CreDTA/VMP mice during the light period (Figure 2B) were functionally rescued by the loss of D2R. This finding indicates that the sleep reduction induced by ablating VMP GABAergic neurons is mainly mediated by D2R.

We then assessed VGAT-CreDTA/VMP mice in a set of behavioral tasks to examine the D2R dependency of hyperactivity and anti-depressive behaviors. In the open field test, both VGAT-CreDTA/VMP;D2R+/+ and VGAT-CreDTA/VMP;D2R−/− mice exhibited increased travel distance (p < 0.01, VGAT-CrehrGFP/VMP;D2R+/+ versus
In the present study, we found that ablating GABAergic neurons in the VMP induced various behavioral phenotypes, including hyperactivity, reduced depression and anxiety, loss of sleep rebound, and reduced sleep. The phenotypes in VGAT-CreDTA/VMP mice with hyperactivity in the open field (Figure 1C), reduced immobility in tail suspension (Figure 1D) and forced swim tests (Figure 1E), and increased social interaction (Figure 1F) may be consistent with the facets of mania symptoms in humans, such as increased goal-directed activity and psychomotor agitation, which is described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013; Johnson, 2005). Hyperactivity and reduced depression are common features of manic patients quantitively bears a resemblance to the hyperactivity in hyperdopaminergic rodent models on the basis of cross-species translational studies (Perry et al., 2009; Young et al., 2007, 2011a). Further, activation of dopaminergic neurons in the ventral tegmental area (VTA) induces hyperactivity in rodents (Boekhoudt et al., 2016). Because the VTA region is anatomically included in the VMP (Figure 1B), ablation of VMP GABAergic neurons may unlock inhibitory outputs on VTA dopaminergic neurons, which could lead to locomotor hyperactivity. To further dissect the detailed mechanisms underlying hyperactivity phenotypes in future studies, dopamine levels should be measured around the nucleus accumbens, a major target area of the VTA, in VGAT-CreDTA/VMP mice. The reduced anxiety and increased risk-taking behavior of VGAT-CreDTA/VMP mice in the elevated plus maze (Figure 1G) are presumably related to excessive involvement in activities with a high potential for a painful consequence in humans (American Psychiatric Association, 2013; Leathy, 1999). Increased risk-taking behaviors are also reported in both manic patients and dopamine transporter knockdown (DAT-KD) hyperdopaminergic mice using comparable tasks (van Enkhuizen et al., 2014; Young et al., 2011b). In addition, the low nest-building performance (Figure 1H) supports the aspect of distractibility described in the...
dependent dopaminergic system. Further research using VGAT-CreDTA/VMP mice may help to elucidate the difference with female mice provides further information about our findings.

All the experiments were performed using male mice in the present study, investigating the phenotypical contribution to the development of safer and more effective drugs.

The mechanisms related to hyperactivity, anti-depressive behaviors, and reduced sleep amount during the dark period in VGAT-CreDTA/VMP mice remain unclear. Recent studies revealed GABAergic projections from the VTA to the lateral hypothalamus (Taylor et al., 2014; Yu et al., 2019) and especially onto orexin neurons (Chowdhury et al., 2019), which are critical for maintaining wakefulness (Chenelli et al., 1999). Optogenetic activation of GABAergic VTA terminals in the lateral hypothalamus suppresses wakefulness (Chowdhury et al., 2019; Yu et al., 2019). Moreover, lateral hypothalamic kindling induces mania-like behaviors (Abulseoud et al., 2014). Therefore, the lateral hypothalamic might play a role in the mania-like behaviors induced by GABAergic VMP ablation.

In summary, as illustrated in Figure 4J, our findings revealed that VMP GABAergic neurons are involved in various aspects of mania-like behaviors, and the reduced sleep amounts are mediated through the D2R-dependent dopaminergic system. Further research using VGAT-CreDTA/VMP mice may help to elucidate the different aspects of mania as well as the detailed neural mechanisms of sleep homeostasis and contribute to the development of safer and more effective drugs.

Limitations of the Study
Pharmacological studies in VGAT-CreDTA/VMP mice, such as of lithium and valproic acid, which are the commonly used treatments for bipolar disorder (Chiu et al., 2013; Logan and McClung, 2016), are important for further investigation of the effects on individual behavioral phenotypes and D2R-dependency. Because all the experiments were performed using male mice in the present study, investigating the phenotypical difference with female mice provides further information about our findings.
Resource Availability

Lead Contact

Further information and requests for reagents and resources should be directed to and will be fulfilled by the Lead Contact, Yo Oishi (oishi.yo.fu@u.tsukuba.ac.jp).

Materials Availability

The mouse line generated in this study (D_{2R} KO strain) will be made available with a completed Material Transfer Agreement.

Data and Code Availability

The data that support the findings of this study are available from the Lead Contact on reasonable request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101240.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.H. and Y.O.; Investigation, T.H., Y.T., and Y.O.; Resources, Y.C., S.M., F.S., and S.T.; Writing—Original Draft, T.H. and Y.O.; Writing—Review & Editing, Y.T., Y.C., S.M., F.S., S.T., H.F., M.Y., and M.L.; Supervision, T.H., M.L., and Y.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Ablation of Ventral Midbrain/Pons GABA Neurons
Induces Mania-like Behaviors with Altered Sleep
Homeostasis and Dopamine D2R-mediated Sleep Reduction

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Figure S1. EEG power spectra during 24 h SWS, Related to Figure 2. Two-way repeated measures ANOVA. $F (1, 12) = 0.2163, p = 0.6502$ (not significant), the main effect of viral transduction. $n = 6$–8 mice for each group. Data are presented as the mean ± SEM.
## TRANSPARENT METHODS

### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-D2R | Millipore | AB5084P  |
| Sheep polyclonal anti-Digoxigenin | Roche | 11093274910 |
| **Bacterial and Virus Strains** |        |            |
| AAV-FLEX-DTA       | Takata et al., 2018 | N/A      |
| AAV-FLEX-hrGFP     | Takata et al., 2018 | N/A      |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) | Millipore-Sigma | B1911 |
| Avidin–biotin complex (Vectastain ABC Elite Kit) | Vector Laboratories | PK-6100 |
| Modafinil         | Sigma-Aldrich | M6940 |
| **Experimental Models: Organisms/Strains** |        |            |
| Mouse: VGAT-Cre   | Vong et al., 2011 | N/A      |
| Mouse: VGAT-Cre; D$_2$R KO | This paper | N/A      |
| Mouse: C57BL/6J   | The Jackson Laboratory | JAX: 006494 |
| **Oligonucleotides** |        |            |
| Primers for VGAT riboprobe | Takata et al., 2018 | N/A      |
| Forward: 5'-GCATGTTCGTGGCGCTACC-3'  |
| Reverse: 5'-CAGCGCAGCGTCAGCCCCCAG-3' conjugated with the T7 promoter |
| Genotyping Primers for VGAT-Cre; D$_2$R KO mice | This paper | N/A      |
| Forward: 5'-GAGCCAAAATAATCCCGTCA-3'  |
| Reverse: 5'-GCACTGAGCAAGAATGACCA-3'  |
| **Software and Algorithms** |        |            |
| SMART              | Panlab | [https://www.panlab.com/en/products/smart-video-tracking-software-panlab](https://www.panlab.com/en/products/smart-video-tracking-software-panlab) |
| SLEEPSIGN          | Kissei Comtec | [http://www.sleepsign.com/](http://www.sleepsign.com/) |
| GraphPad Prism 7.0 | GraphPad Software | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/) |

### Animals

The VGAT-Cre (Vong et al., 2011) (kindly provided by Dr. Bradford Lowell, Harvard Medical School, C57BL/6 and 129 mixed background), VGAT-Cre;D$_2$R KO (newly generated in this study), and C57BL/6J wild-type (WT; The Jackson Laboratory, used in social interaction test)
mouse strains were used in this study. Male mice (9–27 weeks) used in the behavioral experiments were single-housed in insulated sound-proof chambers maintained at an ambient temperature of 23 ± 0.5°C with 55 ± 3% humidity on a 12 h light/dark cycle (lights on at 8:00). Food and water were provided ad libitum. All experiments were performed in accordance with the Animal Care Committee of the University of Tsukuba, and every effort was made to minimize the number of animals used as well as any pain or discomfort.

**Surgery**

The mice used in the behavioral experiments were anesthetized with isoflurane (4% for induction, 2% for maintenance) for brain microinjection. To selectively ablate GABAergic neurons in the VMP, VGAT-Cre, VGAT-Cre;D2R−/−, or VGAT-Cre;D2R+/− mice were injected bilaterally into the VMP (3.4 mm posterior and 0.2 mm lateral to bregma, 4.4 mm below the dura) with AAV-FLEX-DTA (Takata et al., 2018) (120 nl, 8.6 × 10¹⁰ particles ml⁻¹) or AAV-FLEX-hrGFP (Takata et al., 2018) (120 nl, 1.5 × 10¹¹ particles ml⁻¹) using a glass micropipette and an air pressure injector system. For monitoring sleep/wake behavior, the mice were chronically implanted with electroencephalography (EEG) and electromyography (EMG) electrodes for polysomnography (Oishi et al., 2016). As EEG electrodes, two stainless steel screws were implanted into the skull. As EMG electrodes, two insulated Teflon-coated silver wires were placed bilaterally into the trapezius muscles. The electrodes were fixed to the skull using dental acrylic.

**Histology**

For histologic analyses, the mice were deeply anesthetized with an overdose of chloral hydrate (500 mg/kg, i.p.) and perfused through the left ventricle of the heart with saline, followed by neutral buffered 10% formalin. The brains were removed and placed in 20% sucrose in phosphate-buffered saline overnight at 4°C to reduce freezing artifacts. The brains were then
sectioned at 40 μm on a freezing microtome. For in situ hybridization, a 918-bp digoxigenin-labeled riboprobe for VGAT mRNA was generated by PCR amplification with the forward (5’-GCATGTTCGTGCTGGCCTACC-3’) and reverse (5’-CAGCGCAGCGTCAGCCCCCAG-3’) primers conjugated with the T7 promoter using mouse tail genomic DNA (gDNA) as a template, followed by in vitro transcription. The brain sections were then incubated with a 1 μg ml⁻¹ of the VGAT probe in 5× sodium citrate buffer containing 50% formamide at 50°C overnight, washed in 1× saline sodium citrate buffer at 50°C, incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Roche) overnight, and visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Millipore). Immunohistochemistry for D₂R was performed on free-floating sections, the brain sections were incubated in 0.3% hydrogen peroxide for 30 min and then for two nights at 4°C and one night at room temperature with a rabbit anti-D₂R antibody (1:500; Millipore). Sections were then treated with avidin–biotin complex (1:1000; Vectastain ABC Elite kit; Vector Laboratories) for 1 h, and immunoreactive cells were visualized by reaction with 3,3’-diaminobenzidine, 0.01% nickel ammonium sulfate, 0.005% cobalt chloride, and 0.1% hydrogen peroxide.

**Behavioral experiments**

During the entire behavioral testing period, the mice were single-housed in insulated sound-proof chambers, and each cage was visually isolated with partitions. The cages were changed weekly. Only male mice were used in the behavioral experiments. The mice were handled for 2 min twice a day for 3 days total prior to the behavioral experiments. All behavioral assays were performed during the light period (8:00-20:00) except the EEG/EMG recordings, which required 24 h data sampling. The behavioral room was maintained at 23.5 ± 2.0°C with 55 ± 10% humidity. During the behavioral tests, white noise was constantly generated in the room. Before and after each individual test, all the apparatuses were sterilized with weak acidic water.
The behavioral test battery (Figures 1 and 4) was performed in the following order with at least a 6-day interval between tasks: nest building, open field, social interaction, elevated plus maze, novel object recognition, Y-maze, tail suspension, and forced swim. Different groups of mice were used for the EEG/EMG recording (Figures 2 and 4) to avoid the effect of EEG/EMG wire implantation on other behavioral tests.

**Open field test**

The open field test was performed according to the previously described procedures (Crawley, 2007; Seibenhener and Wooten, 2015) with minor modifications. The mice were individually placed in a white plastic box (40 cm wide × 40 cm high × 40 cm deep). The light level at the center of the box was adjusted to 100–130 lux. Spontaneous locomotor activity was recorded using a video camera. Total distance traveled (m) during the 10-min period was analyzed automatically using video tracking software (SMART, Panlab).

**Tail suspension test**

The tail suspension test was performed according to the previously described procedures (Can et al., 2012; Crawley, 2007) with minor modifications. A transparent plastic hollow cylinder (4 cm long, 4 mm diameter) was placed around the mouse tail to prevent climbing behavior. The mice were placed individually in a lane of a plastic tail suspension apparatus comprising four lanes (each lane: 15 cm wide, 60 cm high), and each lane was visually isolated from the others by opaque walls. The mice were suspended 30 cm above the floor, using a 15-cm piece of adhesive tape attached 1 cm from the tip of the tail. Immobility (s) during the 6-min period was manually analyzed. Immobility was defined as the absence of active movements, including shaking of the body and running-like motions using all four legs.
**Forced swim test**

A forced swim test was performed according to the previously described procedures (Crawley, 2007; Yankelevitch-Yahav et al., 2015) with minor modifications. The mice were placed individually in a plastic chamber (23 cm high × 20 cm diameter) filled with water (23.5 ± 2.0°C, 15 cm deep), and spontaneous activity was recorded using a video camera. Immobility (s) during the last 4 min of the recorded 6-min period was manually analyzed. Immobility was defined as the absence of movements except minimal motions required to keep the head above water.

**Sociability and social interaction test**

The social interaction test was performed according to the previously described procedures (Crawley, 2007; Moy et al., 2004) with some modifications. An unfamiliar mouse (C57BL/6J, same age and same sex as the subject mouse) that had no prior contact with the subject mouse, was placed in a transparent plastic chamber (15 cm high × 7 cm diameter containing a band of small holes up to 4 cm from the bottom, which allows the mice to make nose contact) and set in one corner of a plastic white box (40 cm wide × 40 cm high × 40 cm deep). An empty identical chamber was set in the diagonally opposite corner. The subject mouse was then individually placed in one corner of the box where no chambers were set. The light level at the center of the box was adjusted to 100–130 lux. Spontaneous activity was recorded using a video camera. The ratio (%) of time spent in the zone surrounding the unfamiliar mouse chamber to the time spent in both zones surrounding empty and unfamiliar mouse chambers during the 10-min period was analyzed automatically using video tracking software (SMART, Panlab).

**Elevated plus maze**

The elevated plus maze task was performed based on the previous studies (Crawley, 2007; Komada et al., 2008) with minor modifications. A gray plastic plus-maze comprising two open
arms (5 cm wide × 30 cm long) and two closed arms (5 cm wide × 30 cm long with 20 cm high walls) was elevated 50 cm above the floor. The light level was adjusted to 150–170 lux for the open arms and 10–20 lux for the closed arms. The mice were set on the center of plus-maze, and their spontaneous activity was recorded using a video camera. The time spent in the center was also counted as the time in the open arms. The ratio (%) of time spent in the open arms to the time spent in the entire area during the 5-min period was manually analyzed.

**Nest building**

Mice were placed in new individual cages, each containing a single square piece of pressed cotton (5 × 5 cm, 2.5 g, Nestlets, Ancare). Nest building score was evaluated 1 h later based on the previously described five-point scale criteria (Deacon, 2006; Eban-Rothschild et al., 2016).

**Novel object recognition**

The novel object recognition test was performed as previously described (Leger et al., 2013) with minor modifications. Two different objects (object A: Falcon tissue culture flask filled with sand: 10 cm high × 4 cm wide × 2.5 cm deep, object B: Lego brick tower: 10 cm high × 3 cm wide × 3 cm deep) were prepared. On Day 1 as a familiarization session, two identical copies of object A were each set in the center of opposite quadrants of a white plastic box (40 cm wide × 40 cm high × 40 cm deep). The mice were placed individually in one of the corners where no objects were placed and exposed to object A for a 10-min period as familiarization. The light level at the center of the box was adjusted to 100–130 lux. Spontaneous activity was recorded using a video camera. After the 24-h interval, on Day 2 as a test session, one of the copies of object A was replaced by object B as a novel object for the mice. The ratio (%) of time spent in the familiar/novel object zone to the time spent in both zones during the 10-min period was analyzed automatically using video tracking software (SMART, Panlab).
**Y maze**

The Y-maze task was performed according to the procedures described previously (Miedel et al., 2017; Sarnyai et al., 2000) with minor modifications. The mice were individually placed in one arm of the gray plastic Y-shaped maze comprising three arms (3 cm wide × 38 cm long, 12 cm high angled walls), facing the center, and spontaneous activity was recorded using a video camera. The number of entries into each arm and the spontaneous alternation rate during the 10-min period was manually analyzed. When all four legs of the mouse entered an arm, it was counted as an arm entrance. The spontaneous alternation rate (%) was calculated using the following formula.

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\text{Spontaneous alternation (\%)} = \frac{\text{Number of 3 consecutive entries to different arms}}{\text{Total number of arm entries} - 2} \times 100
\]

**EEG/EMG recordings**

EEG/EMG recordings were performed based on the previous study (Oishi et al., 2016). Briefly, after allowing 1–2 weeks for postoperative recovery and transgene expression, the mice were connected with EEG/EMG recording cables. The EEG/EMG signals were amplified and filtered by an amplifier (Biotex; EEG: 0.5–64 Hz, EMG: 16–64 Hz), digitized at a sampling rate of 128 Hz, and recorded using SLEEPSIGN software (Kissei Comtec). Vigilance states were scored offline by characterizing 10-s epochs into three stages: awake, SWS, and REMS according to standard criteria (Oishi et al., 2016). Sleep latency after SD was defined as the time from the end of SD to the appearance of the first SWS episode lasting longer than 30 s. Delta power (%) was calculated based on the ratio of delta (0.5–4.0 Hz) frequency EEG power to the total (0–25.0 Hz) EEG power during SWS (Figure 2C). Although VGAT-Cre\textsuperscript{DTA/VMP} mice took time to exhibit the first SWS episode after SD (Figure 2E), once they were into SWS episodes, the delta power was calculated for every 10-min SWS (not necessary to be a single continuous SWS episode) and the averaged values (across animals) for every 10-min SWS
period after SD were used in Figure 2F. For pharmacologic experiments, modafinil (MilliporeSigma) was dissolved in saline containing 10% DMSO and 2% (w/v) cremophor immediately before use. After baseline recordings for 24 h, VGAT-Cre;D_{2}R^{-/-} or VGAT-Cre;D_{2}R^{+/+} mice were intraperitoneally injected with vehicle or modafinil (45 mg/kg).

**Generation of VGAT-Cre;D_{2}R KO mice by the CRISPR/Cas9 system**

For generation of the CRISPR-engineered VGAT-Cre;D_{2}R KO mice with the electroporation method, guide RNA (gRNA), donor single-stranded oligodeoxynucleotide (ssODN), Cas9 protein (Thermo Fisher Scientific), and fertilized eggs of homozygous VGAT-Cre mice were used. The gRNA (5’-GACATTGTATTATCTCCAGG-3’) containing the splicing acceptor (SA) site located across intron 2 and exon 3 of the D_{2}R gene was synthesized using a GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific). The donor ssODN was designed to induce a deletion of the SA site to knockout the D_{2}R gene with the production of a stop codon at exon 4. The 120-bp ssODN was synthesized (Integrated DNA Technologies) with deletion of the SA site (encoded as GG) in the center and two 60-nucleotide arms at the 5’ and 3’ ends. Female homozygous VGAT-Cre mice were injected with pregnant mare serum gonadotropin and human chorionic gonadotropin at a 48-h interval. Fertilized one-cell embryos were collected from the oviducts. gRNA, ssODN, and Cas9 protein were introduced into the pronuclei of these one-cell embryos using the electroporator (NEPA21, Nepa Gene). These one-cell embryos were then transferred into pseudopregnant ICR mice for F0 mouse production. Direct sequencing from the gDNA of F0 mice was performed to check SA site deletion. For later genotyping, the gDNA was amplified the forward (5’-GAGCCAAAAATAATCCCGTCA-3’), and the reverse (5’-GCACTGAGCAAGAATGACCA-3’) primers, and the PCR products (D_{2}R KO: 361 bp, WT: 363 bp) were digested with NcoI enzyme. Because SA site deletion resulted in the production of a NcoI site, the PCR products of the D_{2}R KO allele were digested into 201-bp and 160-bp fragments whereas the WT allele was detected as a 363-bp product.
The obtained F0 VGAT-Cre;D_{2}R KO mice were mated with homozygous VGAT-Cre mice for the F1 production of heterozygous VGAT-Cre;D_{2}R KO mice. These heterozygous VGAT-Cre;D_{2}R KO mice were maintained in colonies and mated to produce homozygous VGAT-Cre;D_{2}R KO mice and littermate WT mice for behavioral experiments.

**Quantification and statistical analysis**

Statistical analysis was performed using Graph Pad Prism 7.0 (GraphPad Software). All data were subjected to D’Agostino–Pearson (Omnibus K2) and Shapiro-Wilk normality tests for Gaussian distribution and variance. For the dataset showing a Gaussian distribution ($p > 0.05$ in normality test), we performed parametric tests such as two-tailed unpaired/paired t test and ANOVA followed by Sidak’s multiple comparisons. For datasets that failed to show a Gaussian distribution, we performed a nonparametric test, such as the Mann-Whitney U test. Significance levels in the figures are represented as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Error bars in the graphs represent mean ± SEM.

**Supplemental References**

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