Optogenetic activation of dopamine neurons in the ventral tegmental area induces reanimation from general anesthesia

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Dopamine (DA) promotes wakefulness, and DA transporter inhibitors such as dextroamphetamine and methylphenidate are effective for increasing arousal and inducing reanimation, or active emergence from general anesthesia. DA neurons in the ventral tegmental area (VTA) are involved in reward processing, motivation, emotion, reinforcement, and cognition, but their role in regulating wakefulness is less clear. The current study was performed to test the hypothesis that selective optogenetic activation of VTA DA neurons is sufficient to induce arousal from an unconscious, anesthetized state. Floxed-inverse (FLEX)-Channelrhodopsin2 (ChR2) expression was targeted to VTA DA neurons in DA transporter (DAT)-cre mice (ChR2+ group; $n = 6$). Optical VTA stimulation in ChR2+ mice during continuous, steady-state general anesthesia (CSSGA) with isoflurane produced behavioral and EEG evidence of arousal and restored the righting reflex in 6/6 mice. Pretreatment with the D1 receptor antagonist SCH-23390 before optical VTA stimulation inhibited the arousal responses and restoration of righting in 6/6 ChR2+ mice. In control DAT-cre mice, the VTA was targeted with a viral vector lacking the ChR2 gene (ChR2− group; $n = 5$). VTA optical stimulation in ChR2− mice did not restore righting or produce EEG changes during isoflurane CSSGA in 5/5 mice. These results provide compelling evidence that selective stimulation of VTA DA neurons is sufficient to induce the transition from an anesthetized, unconscious state to an awake state, suggesting critical involvement in behavioral arousal.

anesthesia | ventral tegmental area | dopamine | optogenetics | arousal

One of the biggest mysteries of modern medicine is how anesthetic drugs create the state of general anesthesia (1). General anesthesia is a reversible, drug-induced state consisting of unconsciousness, amnesia, analgesia, and immobility with maintenance of physiological stability (2, 3). Hence, general anesthesia is a reversible coma rather than being akin to any stage of sleep (3). Traditionally, studies of the mechanisms of general anesthesia have focused on characterizing the actions of anesthetic drugs at molecular targets (4). Recent studies have begun to characterize how anesthetics act at specific neural circuits to create altered states of arousal (2, 3, 5, 6). For example, the anesthetic propofol impairs intracortical and thalamocortical communication by inducing coherent alpha and incoherent slow oscillations (7, 8). The slow oscillations modulate the alpha oscillations (7) and phase-limited spiking activity (8). Propofol's simultaneous actions in the cortex, thalamus, and in several brainstem nuclei contribute to these dynamics (2, 6, 8, 9). Less research has been done on the neurophysiology of emergence from general anesthesia.

In current clinical practice, emergence from general anesthesia is a passive process whereby the general anesthetic is merely discontinued at the end of surgery, and recovery of consciousness is governed by the pharmacokinetics of anesthetic drug clearance. There are currently no clinically approved pharmacologic or circuit-level interventions that can induce active emergence or reanimation from general anesthesia (10). Dopamine (DA) is a neurotransmitter that is well-known to elicit wakefulness (11). Although stimulants that increase extracellular DA levels such as methylphenidate and amphetamine are commonly used to treat attention deficit hyperactivity disorder (12), these drugs also promote arousal (13–16), and are effective for treating sleep disorders such as narcolepsy (17). Patients with Parkinson's disease have degenerative loss of DA neurons in both the ventral tegmental area (VTA) and substantia nigra (SN) (18), and excessive daytime sleepiness is a common symptom of this disease (19). In mice, selective loss of DA in the brain causes hypoactivity (20). The available evidence strongly suggests that DA plays a critical role in regulating wakefulness, yet the DA circuits in the brain that regulate arousal have not been clearly defined.

It was discovered more than three decades ago that the mean firing rates of DA neurons in the VTA and SN do not change appreciably during sleep or wakefulness (21, 22), suggesting that they do not play a central role in regulating arousal. As a consequence, most studies of DA networks have focused on other functions such as movement (23), reward (24), and cognition (25). However, DA neurons in the VTA and SN send projections to key arousal-promoting brain regions, including the thalamus, dorsal raphe, locus ceruleus, pedunculopontine and laterodorsal tegmental

Significance

Although dopamine is known to promote wakefulness, the specific dopamine circuits in the brain that regulate arousal are not clear. Here we report that selective optogenetic stimulation of ventral tegmental area (VTA) dopamine neurons in mice produces a powerful arousal response sufficient to restore conscious behaviors, including the righting reflex, during continuous, steady-state general anesthesia. Although previous studies found that VTA dopamine neurons do not appear to play a central role in regulating sleep–wake transitions, our findings demonstrate that selective stimulation of these neurons is sufficient to induce the transition from an unconscious, anesthetized state to an awake state. These results suggest that VTA DA neurons play a critical role in promoting wakefulness.

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areas, basal forebrain, and lateral hypothalamus (11). The existence of these projections suggests that DA neurons in the VTA and SN may be intimately involved in maintaining behavioral arousal. VTA lesions in cats have been reported to produce a coma-like state almost entirely devoid of arousal (26), suggesting that DA neurons in the VTA may play a critical role in maintaining wakefulness.

Using continuous, steady-state general anesthesia (CSSGA), we previously demonstrated that DA transporter (DAT) inhibitors (methylphenidate, dextroamphetamine) and a D1 receptor agonist (chloro-APB) reliably restore conscious behaviors, including the righting reflex in rats anesthetized with isoflurane, propofol, and sevoflurane (27–30). The ability to reverse the actions of chemically diverse anesthetics such as propofol (an alkyl phenol) and sevoflurane (a halogenated ether) strongly suggests that DAT inhibitors induce reanimation from general anesthesia not by molecular-level antagonism of the anesthetic but rather by stimulating arousal at the level of neural circuits. In an effort to define the circuit and localize the source of arousal-promoting DA that induces reanimation from general anesthesia, the present study was performed using optogenetics to test the hypothesis that selective stimulation of DA neurons in the VTA induces reanimation from general anesthesia.

Results

Mice expressing Cre recombinase under the transcriptional control of the DAT promoter (DAT-cre mice) were used to target VTA DA neurons. Anesthetized male DAT-Cre mice received bilateral injections of adeno-associated virus carrying floxed-inverse (FLEX)-Channelrhodopsin2 (ChR2) into the VTA (ChR2+ mice), and fiber-optic cannulas were implanted in the injection sites. In addition, extradural EEG and nuchal electromyography (EMG) electrodes were placed. A separate group of control DAT-cre mice underwent the same surgery but received injections of a viral vector encoding YFP but lacking the ChR2 gene (ChR2– mice). To ensure maximal viral expression, the animals had a recovery period of at least 3 wk before experimentation.

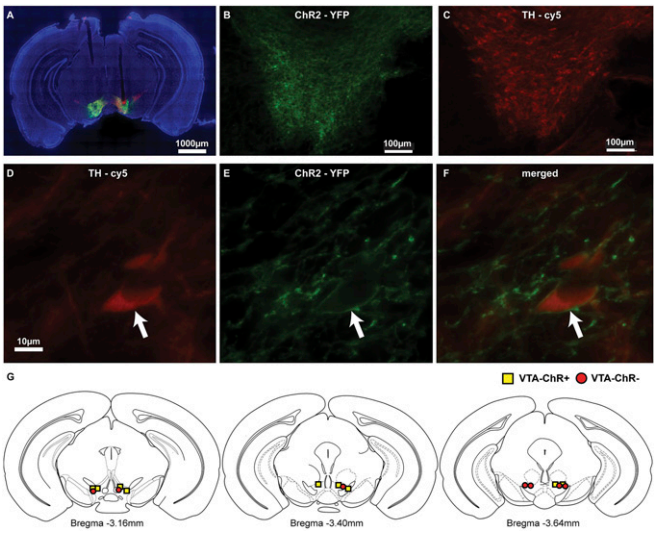

Fig. 1. Targeted expression of ChR2 in VTA DA neurons. (*A*) A representative coronal section shows bilateral fiber-optic cannula tracks. (Only the beginning of the left tract is visualized in this section, as both fiber tips were not in the same plane.) The cannula tips end just above the VTA. The section is stained with DAPI, and the VTA is visualized using immunohistochemistry (composite image of multiple, sequential 10x magnification frames stitched together automatically using Zeiss Blue software). (*B*) The yellow fluorescent protein tag on the ChR2 construct identifies membrane-bound ChR2 expression in cell bodies and axons in the VTA. (*C*) Cytoplasmic tyrosine hydroxylase expression in DA neurons is visualized using cy5 (red). (*D*) Higher magnification showing TH expression in DA neurons (white arrow). (*E*) Higher magnification showing ChR2-YFP expression in the same region as *D*. (*F*) A merged image of *D* and *E* showing colocalized expression of TH and YFP. (*G*) Locations of the fiber-optic probes in areas of viral expression for ChR2+ mice (yellow squares) and ChR2– mice (red circles).

After all experiments were completed, the locations of the fiber-optic cannulas were histologically confirmed, and immunohistochemistry was performed to confirm virus expression and localization (Fig. 1 *A*–*F*). In the ChR2+ group ($n = 6$), five mice had bilateral expression of ChR2, whereas one mouse only had unilateral expression. In the ChR2– group ($n = 5$), three mice had bilateral expression of YFP, whereas two mice only had unilateral expression. The locations of the fiber-optic probes in areas of viral expression are shown in Fig. 1*G*. There was no evidence of thermal injury due to optical stimulation. ChR2 was expressed selectively in dopaminergic neurons in the VTA, although the transfection efficiency was low. Quantification revealed that 40.7% (1,415/3,481) of tyrosine hydroxylase (TH)-positive neurons were positive for ChR2-YFP in the VTA. The ability of ChR2 to excite VTA DA neurons in DAT-cre mice has been previously validated (31, 32).

In all experiments, isoflurane CSSGA was induced and maintained according to the protocol illustrated in Fig. 2*A*, and optical stimulation of DA neurons was initiated using 473-nm light pulses to test for behavioral arousal and return of righting. As shown in Fig. 2*B*, two distinct optical stimulation protocols were tested. Impulse train stimulation (16 pulses, 10-ms duration, 30 Hz, repeated every 2 s) was used to simulate a burst firing pattern, similar to that observed during normal wakefulness (33). In addition, periodic step stimulation (cycles of 5-ms pulses at 50 Hz for 60 s, followed by 30-s pauses with no stimulation) was used, which is similar to the electrical stimulation protocol used in our previous study in rats (34). The two stimulation protocols were tested on separate days, with at least 3 d of rest provided between experiments, in random order.

In the ChR2+ group, both stimulation protocols produced similar behavioral evidence of wakefulness, including leg, head, and whisker movements, righting, and walking, in 6/6 mice during isoflurane CSSGA (Table 1). After initiating impulse train stimulation, the median time to first purposeful movement was 555 s [95% confidence interval (CI) 166 to 865 s] and the median time to righting was 1,097 s (95% CI 662 to 1,343 s), as shown in Fig. 2*C*. Periodic step stimulation produced similar behavioral results (Fig. 2*D*): The median time to first purposeful movement was 437 s (95% CI 50 to 1,091 s) and the median time to righting was 868 s (95% CI 709 to 1,330 s). There was no statistically significant difference between impulse train stimulation and periodic step stimulation for time to first purposeful movement ($P = 0.94$, Mann–Whitney U test) or time to righting ($P = 0.97$, Mann–Whitney U test). No correlation was found between the transfection efficiency and reanimation times. Additionally, because the arousal response of the ChR2+ mouse that only had unilateral ChR2 expression was indistinguishable from the mice that had bilateral expression of ChR2, it was included in the pooled results. These results demonstrate that the two stimulation protocols produced comparable arousal responses during CSSGA, and the reproducibility of the arousal response on separate days indicates that optical stimulation did not cause neuronal damage.

In subsequent experiments, SCH-23390 (0.1 mg/kg, i.p.) was administered during isoflurane CSSGA to the ChR2+ group 40 min before initiating optical stimulation. These additional experiments were performed with both impulse train and periodic step stimulation on separate days (at least 3 d apart in random order) to determine whether D1 receptor blockade would inhibit the arousal response induced by optical stimulation. After the administration of SCH-23390, neither stimulation protocol restored righting despite 30 min of stimulation. As shown in Table 1, SCH-23390 not only inhibited restoration of righting but also greatly attenuated the behavioral arousal response in all six mice.

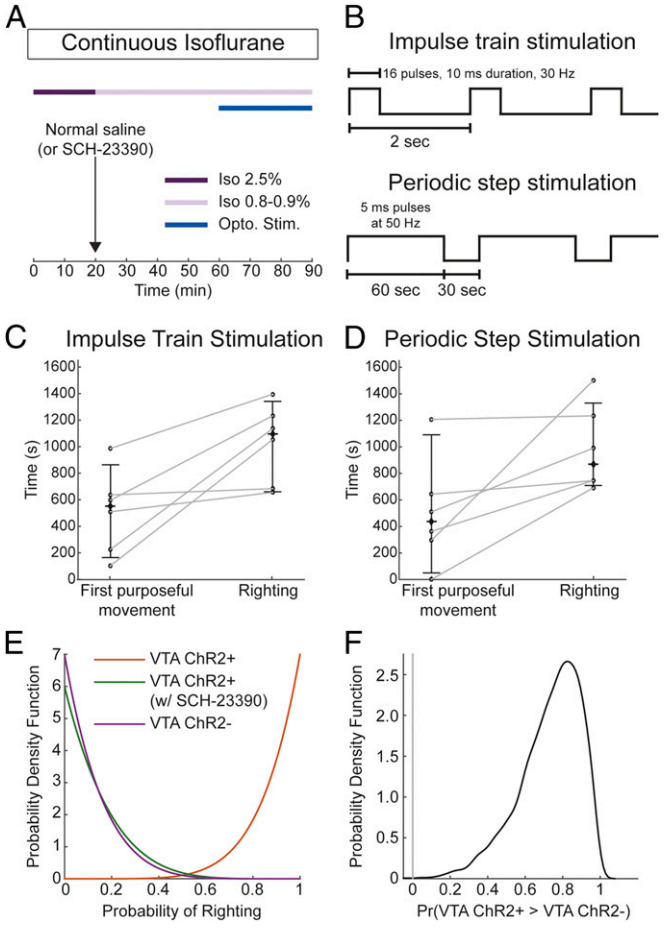

Fig. 2. Optogenetic activation of VTA DA neurons restores righting during CSSGA with 0.8 to 0.9% isoflurane. (*A*) Mice inhaled 2.5% isoflurane for 20 min before receiving vehicle (normal saline, i.p.) or the D1 antagonist SCH-23390 (0.1 mg/kg, i.p.). The mice were then exposed to a dose of isoflurane sufficient to maintain loss of righting (0.8 to 0.9%) for 40 consecutive minutes, and this dose was maintained throughout the remainder of the experiment. Optical stimulation was initiated and continued for 30 min, or until return of righting occurred. (*B*) All mice underwent two different optical stimulation protocols during CSSGA on separate days in random order at least 3 d apart. Impulse train stimulation consisted of bursts of 16 pulses of 10-ms duration at 30 Hz, repeated every 2 s. Periodic step stimulation consisted of cycles of 5-ms pulses at 50 Hz for 60 s, followed by 30 s of no stimulation. (*C*) Time to first purposeful movement and time to righting for each ChR2+ mouse during CSSGA with impulse train stimulation. The horizontal line represents the median, and the bars represent the 95% CI. (*D*) Time to first purposeful movement and time to righting for each ChR2+ mouse during CSSGA with periodic step stimulation. The horizontal line represents the median, and the bars represent the 95% CI. (*E*) Posterior densities for the propensity of righting for ChR2+ (red), ChR2+ after SCH-23390 (green), and ChR2− (purple) groups. Posterior densities are drawn from beta distributions. (*F*) The Bayesian 95% CIs for the difference in the propensity of righting between the ChR2+ group and the ChR2− group were 0.34 to 0.96. The posterior probability of the difference between the ChR2+ group and the ChR2− group being greater than 0 was statistically significant, with a value of 0.9996.

In 5/5 control (ChR2−) mice, neither impulse train stimulation nor periodic step stimulation (on different days, at least 3 d apart, in random order) restored righting during isoflurane CSSGA. As shown in Table 1, impulse train stimulation induced some mild leg and head movements in ChR2− mice but did not restore righting. The beta distribution functions used to model the difference in propensity for righting are shown in Fig. 2*E*. Fig. 2*F* shows the probability distribution of the difference between the two curves shown in Fig. 2*E*. The Bayesian 95% CIs for the difference in propensity for righting between ChR2+ mice and ChR2− mice were 0.34 to 0.96, and the posterior probability that the propensity for righting was higher in the ChR2+ mice than in the ChR2− mice was 0.9996, indicating that optical stimulation in ChR2+ mice caused a statistically significant increase in return of righting compared with optical stimulation of ChR2− mice.

The spectral analysis of EEG data recorded during isoflurane CSSGA was computed for each mouse and correlated with EMG data. Data were then pooled to compute a group analysis. Time windows used to compute grouped power spectral density data comparing a 5-min period of CSSGA before optical stimulation (blue) vs. a 5-min period during periodic step stimulation (red) are shown in Fig. 3*A*. These windows were chosen because (*i*) the EEG changes tended to develop gradually over several minutes, and (*ii*) the data for these two time windows were available for all ChR2+ and ChR2− mice (because the first ChR2+ mouse has restoration of righting at 11 min, EEG data were only available for all animals during the first 10 min of optical stimulation). Constructing 95% CIs around the grouped power spectra revealed that optical activation of VTA DA neurons during isoflurane CSSGA in ChR2+ mice induced statistically significant decreases in low power at frequencies <5 Hz as well as at frequencies between 6 and 17 Hz (Fig. 3*B*). When the ChR2+ group received SCH-23390, the grouped power spectra revealed only a statistically significant decrease in 6- to 17-Hz power with optical stimulation (Fig. 3*C*). In the ChR2− group, there was no statistically significant change in the grouped power spectra with optical stimulation (Fig. 3*D*). Mean power differences with 95% CI are also presented to more clearly highlight these spectral changes.

Discussion

In this study, we found that selective optogenetic activation of VTA DA neurons in ChR2+ mice induced a profound behavioral arousal response during CSSGA with isoflurane. Both impulse train and periodic step stimulation achieved similar behavioral responses that led to restoration of the righting reflex in 6/6 ChR2+ mice. Spectral analysis of extradural EEG recordings revealed that optical stimulation induced statistically significant decreases in δ- and α-power. Systemic administration of the D1 antagonist SCH-23390 in ChR2+ mice before optical stimulation greatly attenuated the arousal response, and 0/6 mice had restoration of righting. This suggests that the arousal response induced by activating VTA DA neurons during CSSGA is mediated primarily by a downstream mechanism involving D1 receptors. In control DAT-cre mice lacking the FLEX-ChR2 gene (ChR2− mice), optical VTA stimulation during CSSGA did not restore the righting reflex, and did not induce statistically significant changes in the EEG power spectrum.

A variety of experimental approaches have been used to study the neural circuits that underlie anesthetic-induced unconsciousness and recovery of consciousness in rodents, including designer receptors exclusively activated by designer drugs (DREADDs) (35), genetic manipulations (36), local and systemic drug administration (10, 37–39), microdialysis (40, 41), targeted brain lesions (42, 43), and electrical stimulation (34). Optogenetics is a novel tool that provides distinct advantages over previous techniques used to study anesthetic-induced unconsciousness and recovery of consciousness in rodents. Unlike electrical stimulation, which recruits multiple pathways, optogenetics provides precise temporal control of specific neuronal populations in vivo (44). Optical stimulation of VTA DA neurons in ChR2+ mice reliably restored behavioral arousal and the righting reflex during CSSGA. However, the arousal response was slower to develop compared with VTA electrical stimulation in rats (34). It is interesting to note that a similar attenuation of effect between optical and electrical stimulation was observed by others in VTA DA-mediated reward behaviors. Optical activation of VTA DA neurons was insufficient

Table 1. Behavioral responses of DAT-cre mice under isoflurane CSSGA during optical stimulation of the VTA

Group	Drug treatment	Leg movement	Head movement	Whisker movement	Righting	Walking	Total score
ChR2+	NS	2	2	2	2	1	9
ChR2+	NS	2	2	2	2	1	9
ChR2+	NS	2	2	2	2	2	10
ChR2+	NS	2	2	2	2	2	10
ChR2+	NS	2	2	2	2	1	9
ChR2+	NS	2	2	2	2	2	10
ChR2+	SCH	1	0	0	0	0	1
ChR2+	SCH	1	0	0	0	0	1
ChR2+	SCH	1	1	0	0	0	2
ChR2+	SCH	1	0	0	0	0	1
ChR2+	SCH	1	0	0	0	0	1
ChR2+	SCH	1	0	0	0	0	1
ChR2−	NS	1	1	0	0	0	2
ChR2−	NS	1	1	0	0	0	2
ChR2−	NS	1	0	0	0	0	1
ChR2−	NS	1	1	0	0	0	2
ChR2−	NS	1	1	0	0	0	2

The arousal scoring system was adapted from Reed et al. (54). Leg, head, and whisker movements were scored as absent, mild, or moderate in intensity (0, 1, or 2, respectively) during 30 min of optical VTA stimulation while mice continuously inhaled 0.8 to 0.9% isoflurane. Righting was scored as 0 if the mouse remained prone, and 2 if all four paws touched the ground. Walking was scored as 0 if the mouse made no further movements after righting, 1 if it crawled but did not raise its abdomen off the floor, and 2 if it walked on four paws with its abdomen off the floor. The total score for each animal is the sum of all categories, with a maximum possible score of 10. Mice were treated with either SCH-23390 (SCH) or normal saline (NS). The total score of ChR2+ mice was significantly higher than ChR2+ mice after SCH-23390 administration ($P = 0.0022$) and ChR2− mice (0.0043), as determined using a Mann–Whitney U test. The total scores of ChR2+ mice after SCH-23390 administration and ChR2− mice were not significantly different ($P = 0.1342$).

to produce self-stimulation reward seen with electrical stimulation (45, 46) but instead required a pairing of a food-seeking behavior to see a significant effect (47).

There are several possible explanations for these observations. First, it is possible that nondopaminergic VTA neurons contributed to the behavioral arousal response elicited by electrical stimulation (48) but were not recruited during selective optogenetic activation of DA neurons. Second, only 40% of DA neurons in the VTA expressed ChR2 and were therefore able to respond to optical stimulation, as opposed to 100% recruitment by electrical stimulation. This low efficiency also likely explains why there was no correlation between the number of neurons expressing ChR2 and time to reanimation. Finally, not all transfected DA neurons are likely to fall under the cone of light emitted by the laser, and there is loss of light intensity as it passes through brain tissue, further limiting recruitment. Although optogenetics allows for precise temporal control of neuronal firing, it is likely that a large increase in extracellular DA is necessary to overcome the powerful hypnotic effects of general anesthesia, which takes several minutes to develop with optical stimulation.

Accelerated time to emergence from an inhaled anesthetic has been reported for a number of pharmacological and circuit-level manipulations, but this end point is greatly affected by physiological variables such as minute ventilation and cardiac output (49). In contrast, this study was performed using CSSGA wherein the concentration of anesthetic in the brain is held constant, so changes in cardiopulmonary physiology cannot alter anesthetic depth (50). This experimental technique controls for interventions that produce isolated increases in respiratory rate that will significantly decrease time to emergence from an inhaled anesthetic without affecting the arousal state of the brain. In this way, arousal from CSSGA is a more robust end point than the more traditional accelerated time to emergence.

Selective activation of VTA DA neurons in this study induced a profound behavioral arousal response yet relatively modest EEG changes (small but statistically significant decreases in low power at frequencies <5 Hz as well as at frequencies between 6 and 17 Hz that correlated with movement and EMG activity). These findings support previous observations that profound arousal-state transitions do not necessarily correlate with large changes in surface EEG activity. For example, atomoxetine, a selective norepinephrine transport inhibitor, elicited large, statistically significant EEG changes, including a shift in peak spectral power from δ (0 to

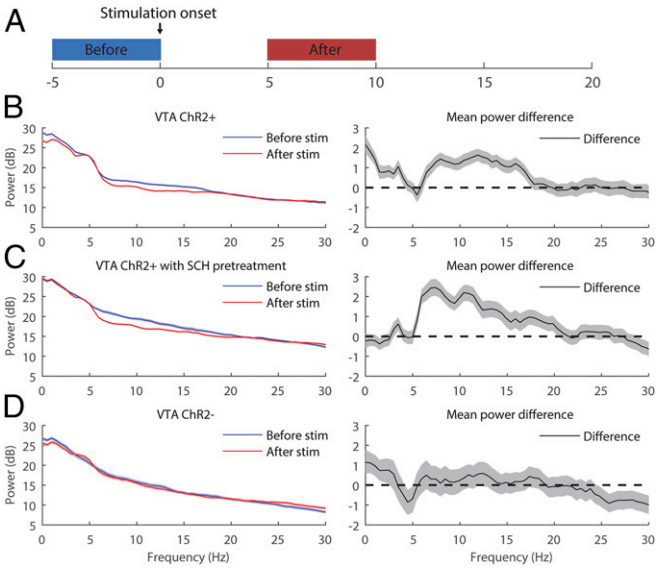

Fig. 3. Spectral analysis of EEG recordings during isoflurane CSSGA and optical stimulation. (*A*) Five-minute time windows were used to compute the grouped power spectral densities with 95% CI as shown (*B–D*, *Left*). Mean power differences of the grouped power spectral densities with 95% CI are shown (*B–D*, *Right*). (*B*) Optical stimulation during CSSGA produced statistically significant decreases in low power at frequencies <2 Hz as well as at frequencies between 6 and 17 Hz in ChR2+ mice. (*C*) After pretreatment with SCH-23390, optical stimulation only produced statistically significant decreased frequencies between 6 and 20 Hz. (*D*) No statistically significant EEG changes occurred with optical stimulation in the ChR2− group.

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4 Hz) to θ (4 to 8 Hz) during CSSGA, but did not induce behavioral arousal (30). Conversely, chloro-APB, a specific D1 receptor agonist, induced a decrease in δ-power but failed to induce an increase in θ-power, and yet produced profound behavioral arousal during CSSGA (28). Taken together, these results suggest that surface EEG changes may not adequately reflect arousal states, and that efforts should be made to investigate changes in local field potentials and their correlation with surface EEG.

Many areas of the brain that are known to promote wakefulness are quiescent during natural sleep (2), but VTA DA neurons have similar mean firing rates over sleep–wake cycles (21, 22), suggesting they are not involved in sleep–wake transitions. However, general anesthetics produce a state of profound unresponsiveness that is distinct from natural sleep (for example, rodents do not lose the righting reflex when they sleep). Therefore, the results of the present study are consistent with the hypothesis that the neural mechanisms underlying emergence from general anesthesia are distinct from those that govern awakening from natural sleep. Although VTA DA neurons may not play a key role in regulating sleep–wake transitions, they may be critically involved in governing the transition from the unconscious, anesthetized state to the awake state.

There are two main DA pathways that project from the VTA: the mesolimbic and mesocortical pathways. The mesolimbic pathway projects to the nucleus accumbens, amygdala, and hippocampus and plays a key role in processing reward, motivation, emotion, and reinforcement (51). It may also play a role in behavioral arousal, as local injection of DA into the nucleus accumbens of unanesthetized, freely moving rats is known to stimulate locomotor activity (52). The mesocortical pathway, which has a major projection to the prefrontal cortex, complements the function of the mesolimbic pathway and also appears to influence arousal (53). The present study encourages further work to define the specific dopaminergic projections from the VTA that promote wakefulness.

In summary, selective optogenetic activation of VTA DA neurons is sufficient to restore behavioral arousal and the righting reflex during isoflurane CSSGA, through a D1 receptor-mediated process. The results of this study provide compelling evidence that VTA DA neurons play an important role in behavioral arousal and recovery of consciousness from general anesthesia. The ability to activate specific subpopulations of neurons in the brain with optogenetics provides a promising approach to elucidate the complex neural circuit mechanisms that underlie awakening from general anesthesia and sleep.

Materials and Methods

Animal Care. All animal procedures were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and the Massachusetts Institute of Technology Committee on Animal Care. Adult DAT-cre mice (Jackson Laboratory; stock no. 006660) were used for all experiments. Mice were kept on a 12:12-h light–dark cycle (lights on at 7:00 AM, lights off at 7:00 PM) with ad libitum access to food and water. Mice had a minimum of 3 wk to recover after surgery, and at least 3 d of rest was provided after each experiment.

Surgery. All mice were anesthetized with 1–3% isoflurane and placed in a stereotaxic frame (David Kopf Instruments). An incision was made in the skin, and craniotomies were made above the target region. In ChR2+ mice ($n = 6$), 280 nL adeno-associated virus carrying FLEX-ChR2 (AAV5-EF1a-DIO-ChR2-EYFP; UNC Vector Core, University of North Carolina at Chapel Hill) was injected bilaterally, targeting the VTA (−3.1 mm anterior/posterior, ±0.8 mm lateral, and −4.25 mm dorsal/ventral to bregma) using a motorized stereotaxic injector (Stoelting). In ChR2− (control) mice ($n = 5$), virus lacking the FLEX-ChR2 gene (AAV5-EF1a-DIO-EYFP) was targeted to the VTA using the same stereotaxic coordinates. In all mice, optical fibers (200 μm inner diameter, N.A. 0.22; Doric Lenses) were lowered into the brain 4.1 mm so that the tip of the fiber was near the dorsal aspect of the VTA. Craniotomies were made for extradural EEG electrodes over the prefrontal cortex, and EMG electrodes were placed in the nuchal muscle. Anchor screws, electrodes, and fibers were adhered to the skull with dental acrylic.

Continuous, Steady-State General Anesthesia with Isoflurane. Mice were placed in an acrylic chamber and anesthetized with isoflurane (2 to 3%) in oxygen. A heating pad was placed under the chamber to maintain normothermia. Gas was continuously sampled from the distal portion of the chamber, and isoflurane, oxygen, and carbon dioxide concentrations in the chamber were monitored using a calibrated Ohmeda 5250 anesthetic agent analyzer (GE Healthcare). After 20 min at an inhaled isoflurane concentration of 2.5%, 1.0 mL vehicle (normal saline, i.p.) or the D1 receptor antagonist SCH-23390 (0.1 mg/kg, i.p.; Sigma-Aldrich) was administered. The isoflurane concentration was then held at a dose that produced loss of righting with no spontaneous movement for 40 consecutive minutes (0.8 to 0.9% isoflurane) as described previously (27). After the 40-min equilibration period, optical stimulation was initiated while each animal continued to inhale the same dose of isoflurane. The experiment was concluded when restoration of righting occurred or after 30 min had elapsed.

Arousal Scoring. Upon completion of the studies, video recordings were used to score the arousal responses of each of the animals. The person who conducted the scoring was blinded to the experimental intervention (e.g., ChR2+ vs. ChR2−; saline vs. SCH-23390) to prevent biasing. After optical stimulation was initiated, the animals were observed for signs of arousal and scored using an adaptation of the method of Reed et al. (54). Spontaneous movements of the limbs, head, and whiskers were recorded on an itemized scoring sheet as absent, mild, or moderate in intensity (0, 1, or 2, respectively). In addition, orienting was scored as 0 if the mouse remained prone, and 2 if all four paws touched the ground. Walking was scored as 0 if the mouse made no further movements after righting, 1 if it crawled but was unable to raise its abdomen off the floor, and 2 if it walked on four paws with its abdomen off the floor. Overall arousal was based on the sum of all categories.

Optical Stimulation and Electrophysiological Recordings. EEG and EMG were recorded using a 64-channel digital recording system (Neuralynx). A patch cord (Doric Lenses) was connected to the implant to deliver blue light from a 473-nm laser (Opto Engine). Stimulation parameters were controlled using Multi Channel Systems stimulus generator STG4000 (ALA Scientific Instruments). The laser intensity was adjusted so that with continuous light the power measured at the tip of the patch cord using a power meter (Thorlabs) was 30 mW. The optical fibers caused loss of ∼50% of the light intensity, so the power at the tip of the fiber was ∼15 mW.

For all mice, two distinct optical stimulation protocols were tested. Impulse train stimulation (16 pulses, 10-ms duration, 30 Hz, repeated every 2 s) was used to simulate a burst firing pattern (33), and periodic step stimulation (cycles of 5-ms pulses at 50 Hz for 60 s, followed by 30-s pauses with no stimulation) was used to simulate the electrical stimulation protocol used in our previous rat study (34). The two stimulation protocols were tested on separate days, with at least 3 d of rest provided between experiments, in random order. In the ChR2+ group, at least 3 d after the two different stimulation protocols had been tested, SCH-23390 (0.1 mg/kg, i.p.) was administered during isoflurane CSSGA 40 min before initiating optical stimulation. These additional experiments were performed with both impulse train and periodic step stimulation on separate days (at least 3 d apart in random order) to determine whether D1 receptor blockade would inhibit the arousal response induced by optical stimulation.

Histology. After all experiments were completed, the optical fiber positions were verified by postmortem histological analysis. Animals were perfused with PBS followed by neutral-buffered formalin. The brains were postfixed in formalin overnight and sliced at 50 μm using a VT1000 S vibratome (Leica Microsystems). Specific expression of ChR2 in DA neurons was confirmed with immunohistochemical staining for tyrosine hydroxylase, a marker of DA neurons (mouse anti-TH; 1:1,000 dilution) (Millipore; catalog no. MAB318) and ChR2 as marked by YFP colocalization (chicken anti-GFP; 1:100 dilution) (Invitrogen; catalog no. A10262). The secondary antibodies were goat anti-mouse conjugated to Alexa 555 (1:200 dilution) (Invitrogen; catalog no. A-21424) and goat anti-chicken conjugated to Alexa 488 (GFP) (1:200 dilution) (Invitrogen; catalog no. A11039). Images were taken with an Imager M2 microscope (Zeiss). Confirmation of optical fiber placement in the correct brain region was performed by comparing images with a mouse brain atlas (55).

Spectral Analysis of EEG Data and Statistical Analysis. Spectral analysis was carried out with MATLAB 8.4 (MathWorks) and the Chronux software package (Cold Spring Harbor Laboratory). Spectrograms were computed from EEG recordings using a 2-s window stepped by 0.05 s and constructed from 0 to 40 Hz using three tapers with a bandwidth of 1 Hz. For each of the three animal conditions (ChR2+, ChR2+ after SCH-23390, and ChR2−), group power spectral densities were calculated from pooled EEG data. "Before" and "after" group power spectral densities reflect the 5-min period preceding stimulation onset and the 5-min period beginning 5 min after periodic step stimulation onset,

respectively. Ninety-five percent CIs around the resulting means were computed using the percentile bootstrap. Ninety-five percent CIs were also computed using the bootstrap analysis of the difference between the median power spectra for the before and after time periods for each of the treatment groups.

A Bayesian Monte Carlo procedure was used to determine the efficacy of optical stimulation for restoring righting during CSSGA, as previously described (27). The Bayesian Monte Carlo gives the posterior probability of the difference in the propensity to right between the two groups (ChR2+ and ChR2–). Statistical significance was determined as a posterior probability greater than 0.95 and Bayesian CIs that did not include zero. Behavioral scoring was tested by performing a two-sided Mann–Whitney *U* test on the total score of the three groups (ChR2+, ChR2+ after SCH-23390, and ChR2–).

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