

Original Article

Involvement of substantia nigra dopaminergic neurons in propofol anesthesia

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Abstract: Objective: Multiple lines of evidence indicated that the central dopaminergic system modulates the response to general anesthesia, and dopaminergic neurotransmission is a potential mediator of arousal maintenance. Here we assessed if sensitivity to propofol anesthesia is affected by selective lesioning of dopaminergic neurons in the substantia nigra (SN). Methods: We bilaterally infused 6-hydroxydopamine (6-OHDA) into the SN of male Sprague Dawley rats and measured dopaminergic cell loss by investigating tyrosine hydroxylase (TH) expression with immunohistochemistry and immunofluorescence. Two weeks after 6-OHDA infusion, we assessed the loss of righting reflex (LORR) to examine the hypnotic properties of propofol, and electroencephalogram (EEG) activity was recorded under propofol anesthesia. Results: Compared with vehicle-treated rats, 6-OHDA-treated rats showed 43% loss of dopaminergic cells. Lesioned rats took significantly longer time to recover from propofol anesthesia, but there was no difference in the onset time and 50% effective dose (ED₅₀). Visual cortex EEG delta power in the lesioned rats was greater than in vehicle rats after a propofol bolus (9 mg/kg). Conclusion: Collectively, our results suggest that SN dopaminergic neurons may have important implications for propofol-induced consciousness.

Keywords: Propofol, substantia nigra, dopamine, loss of righting reflex, electroencephalogram

Introduction

General anesthetics have been used in clinical application for more than 170 years [1], and yet their mechanisms of loss of consciousness are still obscure. A considerable body of evidence suggests that general anesthetics induce loss of consciousness by suppressing arousal pathways or enhancing sleep pathways. Arousal from general anesthesia may rely on activating cholinergic [2], histaminergic [3, 4], noradrenergic [5], or orexinergic [6] arousal pathways. In addition, the dopaminergic system is implicated in various physiological processes, such as movement [7], reward [8], emergence and sleep [9].

In the mammalian brain, dopaminergic neurons are mainly located in the midbrain, covering the ventral tegmental area (VTA), substantia nigra (SN) and ventral periaqueductal gray matter (vPAG) [10]. It is unclear which population is responsible for modulating wakefulness. Recent studies indicated that dopaminergic pathways

play a critical role in promoting and maintaining wakefulness. Co-application of D1 receptor agonist [11] and methylphenidate [12] can drive rat emergence from isoflurane anesthesia. Similarly, VTA activation via local electrical stimulation hastened the return of righting reflex (RORR) [13]. It has been reported that specific damage to tyrosine hydroxylase-immunoreactive (TH-ir) neurons of the VTA delayed emergence from general anesthesia [14]. Interestingly, the total daily sleep increased by 20% after damage of dopaminergic cells in the vPAG, and none of the TH-ir cells in the vPAG expressed c-fos during sleep [10].

Despite this knowledge, the effect of dopaminergic SN lesions on general anesthesia has not been reported. Although SN stimulation is insufficient to induce arousal, due to current technical limitations (the small region of stimulation and the existence of nondopaminergic neurons), the role of dopaminergic SN neurons in mediating the sleep-awake cycle should not be ignored.

In the present study, 6-hydroxydopamine (6-OHDA) was used to specifically damage the SN dopaminergic neurons in rats. We hypothesized that the behavioral effects of propofol would be enhanced by SN dopaminergic lesion, and that induction and emergence time might be altered in the rat with lesion. An intravenous (IV) general anesthetic (propofol) was used, and we examined the loss of righting response (LORR) together with neocortical EEG changes.

Materials and methods

Animals

All experimental surgical procedures were performed according to the guidelines of the committees on the Care and Use of Laboratory Animals in Zunyi Medical College. We used 36 adult male Sprague Dawley rats (8-12 weeks old, 250-320 g), which were supplied by the animal center of third military medical university (Chongqing, China). The rats lived in comfortable, standard animal cages under a 12 h light-dark cycle at 23°C with free access to food and water.

SN lesion

Dopaminergic SN neurons were bilaterally lesioned with infusion of 6-OHDA (Sigma-Aldrich, St. Louis, MO) as previously described [15]. Briefly, the rats were placed in a stereotaxic frame after intraperitoneal anesthesia (sodium pentobarbital, 50 mg/kg). For 6-OHDA infusion, a pair of stainless steel cannula (outer diameter = 0.41 mm) was implanted into the SN (from bregma: P 5.4 mm, L ± 2.2 mm, and 7.5 mm ventral to the skull surface) [16]. 6-OHDA hydrochloride was dissolved in 0.9% saline containing 0.2% ascorbic acid to prevent its decomposition. The rats were bilaterally infused with 2 µl 6-OHDA (3.5 µg/µl) through the inner needle that was 0.5 mm longer than the cannula tip. The control group rats were received equal volume of vehicle into the SN. Recording electrodes were placed bilaterally in the primary motor cortex (M1; at anterior 1.5, L2, V1.5), visual cortex (VC; P7, L3, V1.5). Two weeks after agent administration, we began to test their responses to propofol anesthesia.

Behavioral test and EEG recording

We used LORR as the behavioral indicator of general anesthesia in rats because it is rele-

vant to loss of consciousness (LOC) in humans [17]. The rats were placed on their back to test the righting reflex. We determined the anesthetic response to propofol based on the time of LORR and recovery from propofol anesthesia. We first determined the 50% effective dose (ED50) of propofol through IV injection. A rat was repeatedly injected with propofol at an incremental dose, first at 4 mg/kg and then increased by 1 mg/kg at 30 s intervals. We recorded the dose that caused LORR on the successive trials. The dose-response curve was matched using the formula of $Y = Y_{\min} + (Y_{\max} - Y_{\min}) / [1 + 10^{\log(ED50-X)^{-m}}]$. We determined the time of LORR from administration of propofol (48 mg/kg/h, IV) and RORR starting from the termination of propofol (9 mg/kg, IV) corresponding to the ED95 dose (95% effective dose). After anesthetic administration, each rat was left on a warming pad in a supine position in room air.

Cortex EEG recordings were made from 2 to 5 min after propofol administration (9 mg/kg, IV), because this was the period when the maximal effect was observed. EEG signals from the M1 and VC recording electrodes were amplified by a Model 3000 High-Gain AC/DC Differential Amplifier (0.1 Hz-3 kHz, gain: 1000 ×; A-Msystems, Inc., Sequim, WA, USA) and analyzed with a Spike 2 system and software (Cambridge Electronic Design, Cambridge, UK).

Immunohistochemistry and histology

Rats were deeply anesthetized with pentobarbital (60 mg/kg) at the end of all experiments. After perfusion of 400 ml 0.9% saline followed by 500 ml 4% paraformaldehyde solution via the heart, the brains were harvested and fixed in 4% paraformaldehyde overnight. Afterwards, the brains were incubated in 30% sucrose for dehydration until the tissue sank to the bottom.

Blocks containing the SN were cut into 40 µm-thick sections using a freezing microtome. We selected two sections from each rat depending on the anatomic characteristics of the structures, in particular the shape of the aqueduct and the third ventricle. Half of tissue was used for immunohistochemistry and the other was used for immunofluorescence. The immunohistochemistry [14] and immunofluorescence [15] experiments were performed using a detection kit as described previously. Briefly, the sections

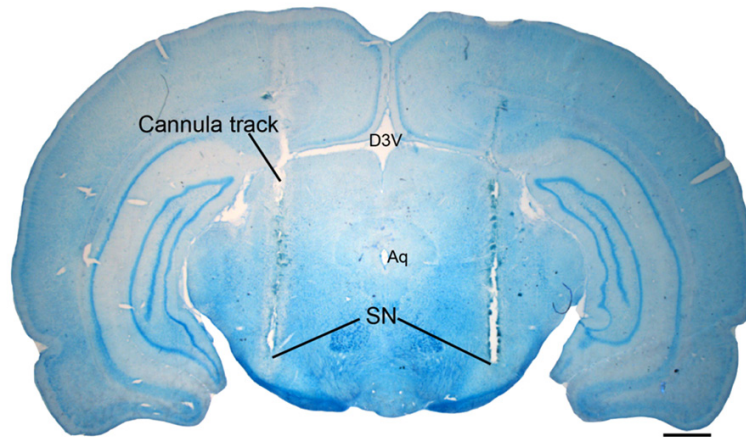


Figure 1. A representative coronal brain section showing the location of bilateral microinjection sites in the SN. Aq, aqueduct; D3V, dorsal 3rd ventricle; SN, substantia nigra. Scale bar =1.0 mm.

were incubated with the TH primary antibody raised in rabbit (at 1:400, Abcam, Cambridge, UK) for 24 hours at 4°C, followed by incubation with biotinylated goat anti-polyvalent (1:100, Abcam) for immunohistochemistry and followed by incubation with fluorescent secondary antibodies (Cy3 Donkey anti-rabbit IgG at 1:1000, BioLegend, San Diego, CA) for immunofluorescence at 37°C for 2 h. The number of TH-positive neurons and TH-staining intensity were calculated using Image-Pro plus 6.0. The data were averaged to mean values. Microinjection placements in the SN were verified in 100-μm frozen brain sections stained with thionin (**Figure 1**).

Statistical analysis

Data are expressed as the mean ± SD. The numbers and fluorescence intensities of TH-immunoreactive cells in the SN were averaged to obtain a mean value for each brain. GraphPad Prism software version 5.0 (GraphPad Prism, Inc., San Diego, CA) was used for drawing LO-RR dose-response curves by nonlinear regression. The Prism equation $Y = Y_{min} + (Y_{max} - Y_{min}) / [1 + 10^{\log(ED50 - X) * m}]$; where Y is the percentage of the rats anesthetized, X is the logarithmic drug dose, m describes the steepness of the family of curves; and ED50 is the propofol dose that produces a half-maximal effect.

Spike 2 software was used to online analysis power spectrum. After administration of propofol, 3 min of M1 and VC EEG data was sampled

in each rat. Delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-25 Hz), and gamma (25-60 Hz) frequencies were accepted from the M1 and VC EEG power spectrum. Each integrated power of the EEG was normalized to the maximal values for comparison.

One-sample Kolmogorov-Smirnov test were used to determine whether the data were normally distributed. Unpaired Student's *t* tests were performed to compare cell count, ED50 values, LORR and RORR time, and EEG power be-

tween the control and lesion groups. Differences were considered significant at *P*<0.05. All statistical analyses were performed using SPSS version 16 software (SPSS Inc., Chicago, IL).

Results

SN 6-OHDA lesion induced dopaminergic neuron loss

Dopaminergic neurons were lesioned by bilateral infusions of 6-OHDA in the SN, and TH was served as adopamine cell marker (**Figure 2**). Immunohistochemistry showed that compared with the vehicle group (100±5%, n=5), the number of TH-immunoreactive neurons in the SN of the lesion group was significantly reduced (57±4%, n=5, **P*<0.05). No differences were observed in the VTA. Quantification of TH intensity showed that immunoreactivity was reduced by ~46% in 6-OHDA-lesion rats compared with vehicle-lesioned rats (n=5 for vehicle and n=5 for 6-OHDA; **P*<0.05, compared with vehicle). TH-ir in the VTA was not significantly different between the vehicle and lesion group.

Anesthetic response to propofol

Figure 3A, 3B depicts a schematic for testing LORR and RORR time. Continuous infusion of propofol (48 mg/kg/h, IV) was used for measuring LORR and a bolus dose of propofol (9 mg/kg, IV) equivalent to ED95 was injected to test RORR. We first determined whether lesion of dopaminergic neurons in the SN affected the

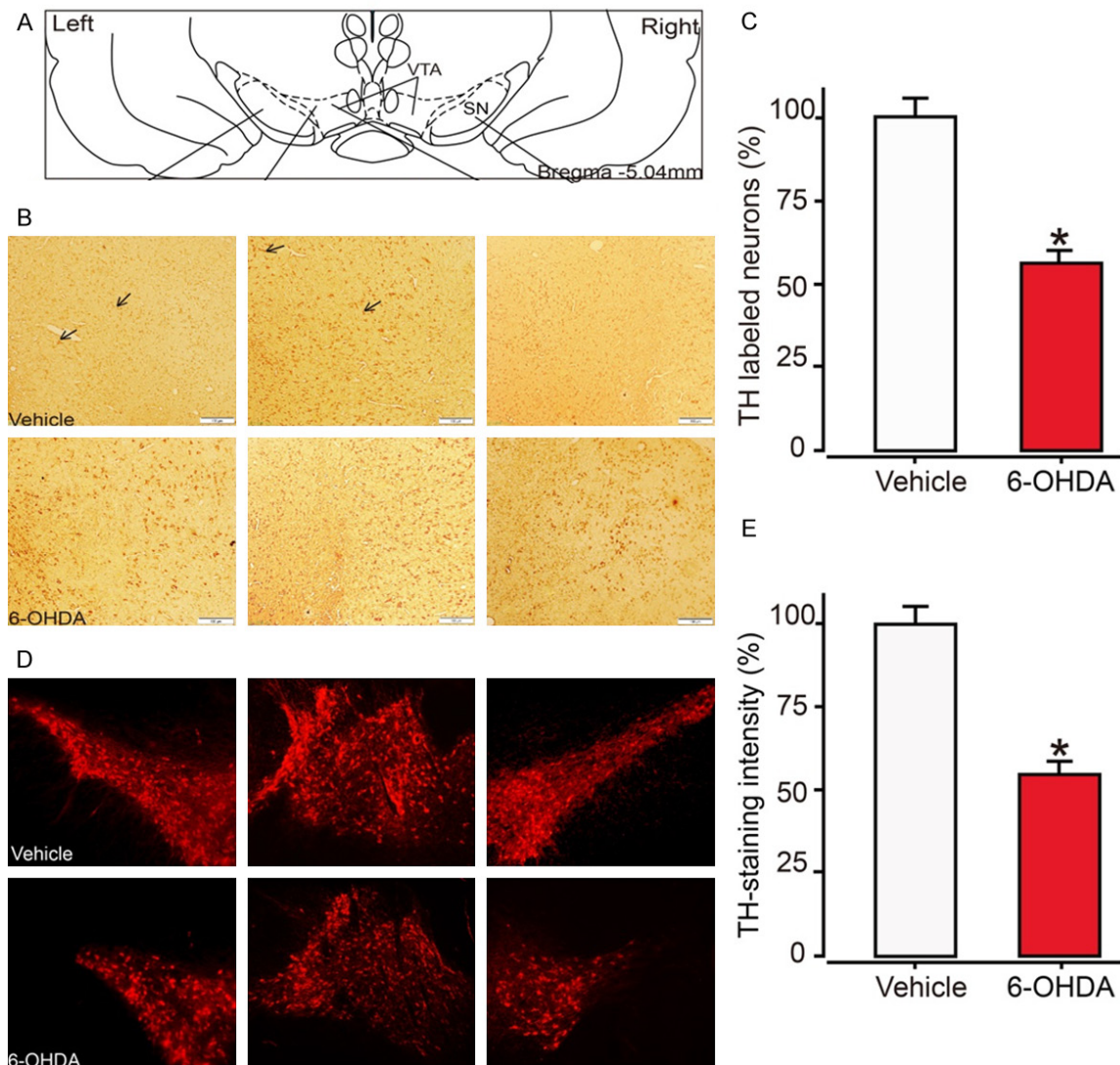


Figure 2. Bilateral infusion of 6-hydroxydopamine (6-OHDA) into the substantia nigra (SN) induced loss of tyrosine hydroxylase (TH) immunoreactive neurons. Schematic of the coronal section through SN and VTA, modified from the rat brain atlas (A). Immunohistochemistry (B) shows TH-positive neurons (quantified in C) and immunofluorescence (D) shows TH-staining intensity (quantified in E). The ratio of TH-positive neurons and TH-staining intensity between vehicle group and lesion group were averaged to obtain mean values. Values represent mean \pm SD. 6-OHDA induced a significant degeneration of SN dopaminergic neurons (both $n=10$, $P<0.05$). Scale bar = 100 μ m.

sedative response to propofol. SN-lesioned and vehicle rats had similar ED50 values (lesion: 6.8 ± 0.7 mg/kg, vehicle: 7.1 ± 0.8 mg/kg, $n=10$ for both, $P>0.05$, **Figure 3C**). As shown in **Figure 3D**, the onset time of propofol in bilateral SN lesioned rats was similar to that of the sham-lesioned rats (lesion: 781 ± 36 s, $n=10$; vehicle: 811 ± 33 s, $n=10$ for both, $P>0.05$). However, the time to recovery from propofol anesthesia was significantly longer in the SN-lesioned group (lesion: 706 ± 24 s, $n=10$; vehicle: 483 ± 17 s, $n=10$ for both, $P<0.05$).

Lesion of SN dopaminergic neurons increased visual cortex delta power

We examined the effects of SN dopaminergic neurons damage on the primary motor cortex (M1) and visual cortex (VC) EEG power spectrum. After 14 days, the EEG at the M1 and VC were recorded after IV 9 mg/kg propofol injection during 2 to 5 min. We compared the powers of the delta, theta, alpha, and beta band after propofol and the frequency of EEG power was from 0 to 60 Hz (**Figure 4**). The integrated

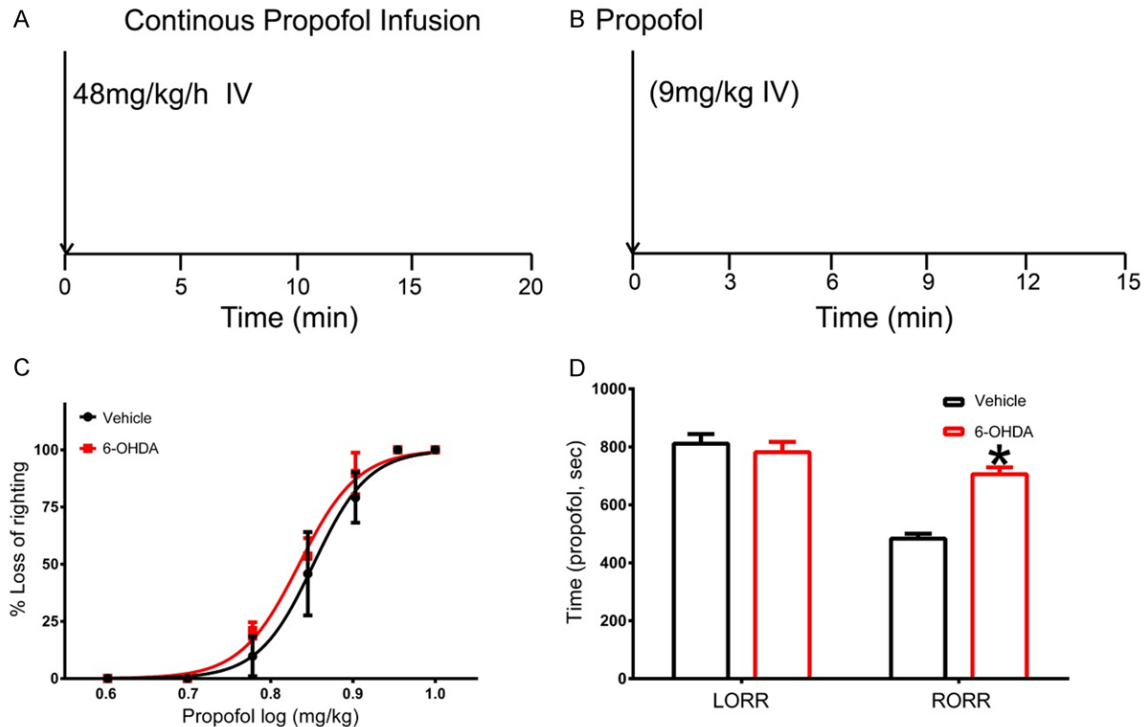


Figure 3. SN lesioned rats exhibited delayed emergence from propofol anesthesia, but there were no differences in propofol ED50 or LORR. A: Rats received continuous propofol infusion (48 mg/kg/h IV) to assess the time of onset. B: A propofol bolus (9 mg/kg IV) was given to determine the emergence time from anesthesia. C: Dose-response curves showing the LORR percentages with cumulative propofol. The ED50 of lesion rats (red) was smaller than vehicle rats (black) (both $n=10$, $P>0.05$). D: 6-OHDA-lesioning failed to change time of propofol anesthesia onset (both $n=10$, $P>0.05$) but significantly prolonged emergence time (both $n=10$, $*P<0.05$). ED50, 50% effective dose; IV, intravenous; LORR, loss of righting reflex; 6-OHDA: 6-hydroxydopamine; RORR, return of righting reflex; Vehicle, 0.9% saline containing 0.2% ascorbic acid.

EEG power at M1 was not significantly different between the vehicle and lesion groups after propofol administration. The delta EEG power (0-4 Hz) recorded from M1 of the lesion group was only slightly higher than the vehicle group ($P>0.05$; **Figure 4A, 4C**). In contrast, when we compared the lesion and vehicle groups for power at the visual cortex electrode, the VC EEG waveforms in lesioned rats showed a high-voltage slow activity pattern (**Figure 4B**) and the integrated delta EEG power was significantly larger in lesioned rats (lesion: 0.7348 ± 0.0726 , $n=8$; vehicle: 0.4474 ± 0.0417 , $n=8$, $P<0.05$, **Figure 4D**). However, the differences in alpha (8-12 Hz), beta (12-25 Hz) and gamma (25-60 Hz) in VC were not significant between the two groups.

Discussion

We found that 6-OHDA lesioning SN dopaminergic neurons significantly delayed emergen-

ce from propofol anesthesia and increased visual cortex delta power. The cortex electroencephalogram (EEG) activity has been used to indicate the depth of general anesthesia; as the depth of anesthesia increases, the EEG shows slow wave sleep accompanied with high-amplitude slow activity (delta frequency, 0-4 Hz) [18]. Lesioning cholinergic neurons in the basal forebrain increases delta EEG power and prolongs the LORR response [19]. Thus, the low delta power can indicate the decrease of cerebral cortex activation. **Figure 4** shown the representative visual cortex electroencephalogram waveforms of vehicle group (alpha and beta oscillations) and lesion group (slow and alpha oscillations). Then, our results revealed that lesion of SN neurons increased VC delta power according with previous studies. Unfortunately, no significant differences were found in the primary motor cortex EEG power. Moreover, we did not measure the effects of lesioning on M1 EEG power prior to propofol

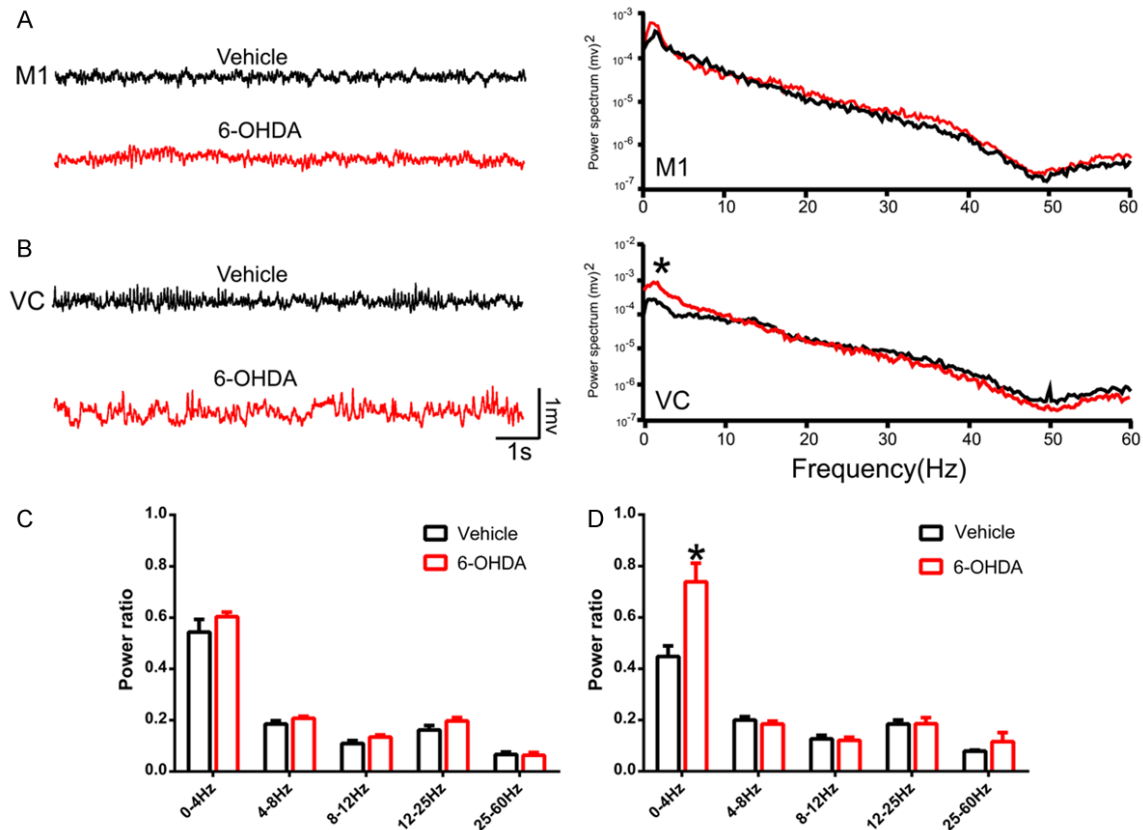


Figure 4. Representative examples of electroencephalographic waveforms and power spectra recorded in the primary motor cortex (A) and visual cortex (B) after a bolus of propofol (9 mg/kg IV). The 3-min periods from 2 min to 5 min after propofol were used to compute power spectra between the vehicle (black) and lesion (red) groups. Lesion group have no significant influence on EEG power in M1 compared with vehicle group (C). The EEG power spectra in VC (D) showed a significant increase in delta power in the lesion group as compared with the vehicle group. Data are mean \pm SD, $n=8$ per group. $*P<0.05$, between the vehicle and lesion groups, unpaired t test. The scale plate represents 1 mV and 1 s. EEG, electroencephalogram; 6-OHDA: 6-hydroxydopamine; Vehicle, 0.9% saline containing 0.2% ascorbic acid.

administration. However, we did eliminate the concerns that LORR depends on motor abilities that may be affected by SN neurons under propofol anesthesia.

In the late 1950s, dopamine was deemed an independent neurotransmitter [20], and previous research mainly focused on locomotor activity and motivation. Several studies have elucidated the role of dopamine in the regulation of sleep and behavioral arousal. The firing rates of dopaminergic neurons in the SN and VTA didn't seem to change substantially across sleep-wake states or during anesthesia [21, 22]. One study reported that the rate of extracellular DA release in the striatum and prefrontal cortex was higher during the wake period than the sleep period [23]. Furthermore, the dorsal striatum mainly receives projections

from dopaminergic neurons in the SN. Recently, the effects of dopaminergic agents such as methylphenidate [12] and chloro-APB (aD1 agonist) [11] on behavioral arousal were reported, restoring righting during continuous isoflurane anesthesia ($1.0\pm0.1\%$) or decreasing emergence time from 1.5% isoflurane anesthesia.

No studies have measured the effect of SN dopaminergic neurons inactivation on general anesthesia. Here we examined the effects of 6-OHDA dopaminergic neuron depletion in the SN on propofol anesthesia, although SN stimulation wasn't sufficient to induce recovery from anesthesia. We found that bilateral SN lesions significantly prolonged emergence time of propofol anesthesia. Previous studies demonstrated that stimulation and lesion of certain nerve nuclei were not equal and generated variable

anesthetic efficacy. Electrical stimulation of the VTA changed the anesthesia responses to isoflurane and propofol, inducing recovery during exposure to general anesthetic [13]. VTA dopaminergic neuron lesioning [14] delayed emergence from propofol, but not isoflurane anesthesia. Differences in the experimental measures and the limitation of stimulated regions may account for the discrepancy. In addition, SN stimulation was not restricted to dopaminergic neurons, it affected other neuron subtypes. On the other hand, the VTA and SN are the major dopaminergic nuclei and receive projections from the brainstem (locus coeruleus-norepinephrine, the pedunculopontine and laterodorsal tegmental nuclei-acetylcholine), the hypothalamus (tuberomammillary-histamine, the lateral hypothalamus-orexin, the preopticaire-GABA), and the basal forebrain (BF-acetylcholine) [9]. These innervations are reciprocal and involved in modulation sleep and waking. Lesions of SN dopaminergic neurons inhibit the ascending arousal pathway, disturbing the intrinsic nerve projection, which may explain the difference in SN electrical stimulation.

In this study, we found that dopaminergic SN neurons play a critical role in the regulation of arousal from propofol general anesthesia, but they did not affect induction. The ED50 value and onset time of propofol anesthesia were not affected by bilateral SN lesioning. Micro-injection of orexin-A into the basal forebrain was previously shown to induce similar effects [24]. Genetic ablation of orexinergic neurons [25] in the lateral hypothalamus/perifornical region prolongs emergence from inhalation anesthesia (isoflurane and sevoflurane) without changing anesthetic induction. However, rats with cholinergic medial septum lesions [26] have been reported to modulate the sensitivity to isoflurane anesthesia, which affected both the induction and emergence. Based on these reports, it suggests that emergence from anesthesia is not simply the inverse process of induction and we propose that dopaminergic SN neurons don't modulate propofol anesthesia induction.

These findings improve our understanding the mechanisms underlying the effect of dopaminergic SN neurons on propofol anesthesia. Although previous investigation [27, 28] studied the pharmacologic properties of intraperitoneal propofol administration, the influence of com-

plex pharmacokinetics after systemic administration can't be ignored. The strength of our study is that we first determined the ED95 value of propofol through IV injection using a cumulative-dose protocol. The acquired propofol dose corresponding to the ED95 guaranteed the safety of rats during RORR and cerebral cortex EEG assessment. At the same time, the dopaminergic neurons in bilateral SN were only reduced by 43%. Considering that symptoms of akinesia and tremor could be induced by dopaminergic SN neuron lesioning, the infusion dose of 6-OHDA was just 7 μ g as determined in our pilot study.

Conclusion

In summary, our results show that lesioning SN dopaminergic neurons markedly increases visual cortex delta power and delays recovery from propofol anesthesia. These findings build on existing evidence that dopaminergic SN neurons are involved in modulating the emergence from propofol anesthesia, and may contribute to exploring the roles and mechanisms of SN dopamine neurons underlying propofol general anesthesia.

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Disclosure of conflict of interest

None.

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