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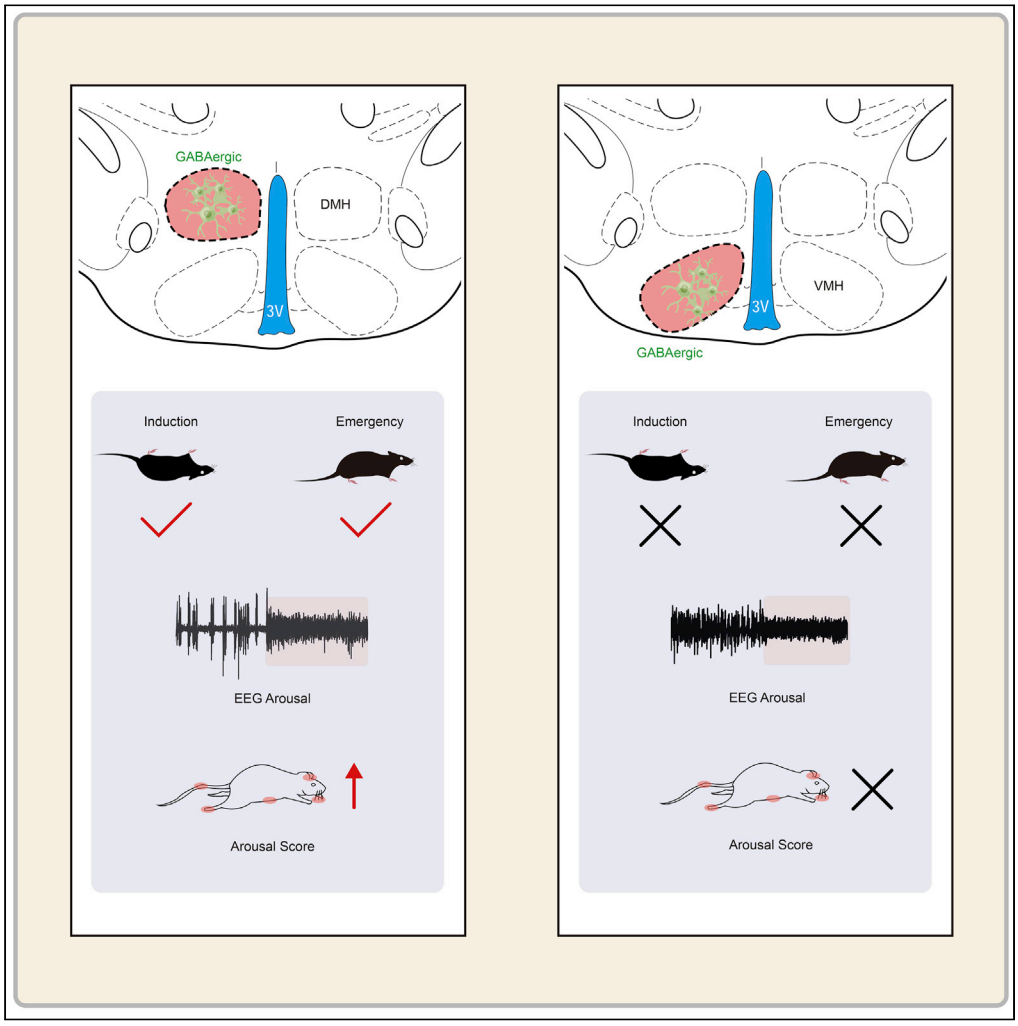

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Highlights

Activation of the DMH^{GABA} not VMH^{GABA} neurons promotes arousal in sevoflurane anesthesia

DMH^{GABA} neurons activity is closely related to arousal during sevoflurane anesthesia

Inhibition of DMH^{GABA} neurons can deepen sevoflurane anesthesia

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GABAergic neurons in the dorsomedial hypothalamus regulate states of consciousness in sevoflurane anesthesia

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SUMMARY

The neural inhibitory gamma-aminobutyric acid (GABA) system in the regulation of anesthetic consciousness is heterogeneous, and the medial hypothalamus (MH), consisting of ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH), plays an important role in sleep and circadian rhythm. However, the role of MH GABAergic neurons (MH^{GABA}) in anesthesia remains unclear. In this study, we used righting reflex, electroencephalogram (EEG), and arousal behavioral score to evaluate the sevoflurane anesthesia. Activation of MH^{GABA} or DMH^{GABA} neurons prolonged the anesthesia induction time, shortened the anesthesia emergence time, and induced EEG arousal and body movement during anesthesia; meanwhile, VMH^{GABA} neurons activated only induced EEG changes during 1.5% sevoflurane anesthesia. Furthermore, inhibition of DMH^{GABA} neurons significantly deepened sevoflurane anesthesia. Therefore, DMH^{GABA} neurons exert a strong emergence-promoting effect on induction, maintenance, and arousal during sevoflurane general anesthesia, which helps to reveal the mechanism of anesthesia.

INTRODUCTION

General anesthetics are a convenient tool for studying consciousness. The mechanisms of anesthesia and change in consciousness during anesthesia induction or recovery have not been fully clarified. Currently, the regulation of neural networks is playing an increasingly prominent role in the study of the anesthesia awakening mechanism, in which different nuclei display different functions.

The hypothalamus plays an important role in regulating human physiological processes, such as homeostasis, sleep, and circadian rhythm.[1] Orexinergic, glutamatergic, and gamma-aminobutyric acid (GABA)-ergic neurons in the lateral hypothalamus exert an obvious effect on the conversion of consciousness during general anesthesia,[2–4] but the effect of the medial hypothalamus (MH) on anesthesia remains unclear.

Dopamine, noradrenaline, glutamate, acetylcholine, and other excitatory neurotransmitters are thought to be involved in the regulation of arousal and anesthesia.[5,6] GABA is the most abundant inhibitory neurotransmitter in the brain, and its mediated inhibitory neural circuits are related to sleep initiation and maintenance.[5,7] Moreover, activation of GABA neurons in the ventral tegmental area (VTA) can significantly promote anesthesia with sevoflurane, whereas inhibition of VTA^{GABA} has the opposite effect.[8] However, researchers have found that GABA in the lateral hypothalamus plays a significant role in promoting arousal under isoflurane anesthesia by projecting to the thalamic reticular nucleus (TRN).[4] Activation of preoptic GABAergic neurons does not alter anesthetic induction or recovery time but alters the sleep-wake architecture.[9] Likewise, GABAergic neurons of the lateral septum participate in the control of wakefulness and promote recovery from isoflurane anesthesia.[10] These investigations indicate that inhibitory GABA neurons in different nuclei can play different roles in inhalation anesthesia. The excitation-inhibition balance of the neural network may affect conscious maintenance.

Two crucial nuclei in the MH, the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH), play essential roles in regulating circadian rhythms, sleep, energy balance, stress response, and aggression, which need heightened arousal.[1,11,12] General anesthesia (GA) can interrupt arousal-based

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behaviors, so there may be a close relationship between the MH and GA-induced changes in consciousness. What's more, the DMH is a substantial relay of circadian regulation of arousal.[13] Adjacent to the DMH, the VMH can affect sleep patterns, and its neuronal activity is significantly correlated with paradoxical sleep.[14,15] Therefore, the MH may play a key role in regulating states of consciousness in GA; the role of GABAergic neurons in these two nuclei during sevoflurane anesthesia remains an important scientific issue and warrants further discussion.

In the present study, we used chemogenetic methods to activate MH^{GABA} neurons to explore their roles in sevoflurane anesthesia. Additionally, we took advantage of optogenetics to further study the roles of DMH^{GABA} and VMH^{GABA} neurons during sevoflurane anesthesia induction and recovery and found that GABA neurons in the DMH, but not in the VMH, were critical for promoting arousal under sevoflurane anesthesia.

RESULTS

Chemogenetic activation of MH^{GABA} neurons has a significant emergence-promoting effect in sevoflurane anesthesia

Glutamate decarboxylase 67 (GAD67) is the rate-limiting enzyme in the modulation of GABA synthesis, and is responsible for over 90% of the basal GABA synthesis.[16,17] In the field of neuroscience, viruses carrying the GAD67 promoter are commonly used to specifically transfect GABAergic neurons.[18,19] To explore the role of MH^{GABA} neurons in sevoflurane anesthesia, we injected a 200 nL virus (DMH and VMH) of *rAAV2/9-GAD67-hM3Dq-mCherry* (hM3Dq group) with the GABA-specific recognition promoter GAD67 into the MH. The control group was injected with *rAAV2/9-GAD67-mCherry* (mCherry group) (Figure 1A).

After four weeks of virus transfection and expression, behavioral tests and fluorescence verification were performed. Immunofluorescence staining showed that the virus was fully expressed in GABA neurons (Figure 1B). One hour after the intraperitoneal injection of clozapine-N-oxide (CNO), the staining results showed that the proportion of neurons co-expressing c-fos/mCherry in the hM3Dq group was significantly increased, suggesting that GABAergic neurons were widely activated (Figures 1E and 1F).

Mice were intraperitoneally injected with CNO, and the righting reflex test was performed 1 h later. The results showed that the loss of right reflex (LORR) time in the hM3Dq group could be significantly delayed, while the recovery of righting reflex (RORR) time was remarkably shorter (Figure 1D). Electroencephalogram (EEG) analysis showed that the burst suppression ratio (BSR) in the hM3Dq group was notably reduced during anesthesia (Figures 1G and 1I). The mice were anesthetized with 1.5% sevoflurane for 30 min at intervals of 3 days after full recovery. Concurrently, data on the total time of body movement and the percentage of each EEG frequency band in the total power during anesthesia were collected. The results showed that the percentage of power in the δ band decreased significantly in the hM3Dq group during anesthesia, whereas that in the γ band increased significantly (Figures 1H and 1J). Additionally, we found that the total movement time of mice during anesthesia in the hM3Dq group was significantly increased (Figure 1K, Videos S1 and S2).

Based on behavioral results and EEG analysis, we found that activation of MH^{GABA} neurons prolonged sevoflurane anesthesia induction time and shortened emergence time, induced more body movement and high-frequency bands related to anesthesia awareness, and reduced BSR, which was positively correlated with the depth of anesthesia, causing an emergence-promoting effect in sevoflurane anesthesia.

Optogenetic activation of the DMH^{GABA} neurons prolongs the induction and promotes the emergence of sevoflurane anesthesia

To assess the function of GABA neurons in different regions of the MH during sevoflurane anesthesia, we used optogenetic methods with higher temporal and spatial resolution to activate GABA neurons in the VMH or DMH. To explore whether MH^{GABA} promotes arousal in anesthesia through DMH, we injected virus of *rAAV2/9-GAD67-hChR2(H134R)-GFP* (ChR2 group) or *rAAV2/9-GAD67-GFP* (GFP group) in DMH with a total volume of 100 nL (Figure 2A). Immunofluorescence of GABA staining showed that the virus was fully expressed in GABA neurons (Figures 2B and S2A). For c-fos staining, the mice were subjected to light stimulation (5 mW, 20 Hz, 10-ms duration, and 1 s on-/9 s off-cycle) for 30 min and were sacrificed and perfused for brain samples after another 30 min. The results showed that the c-fos/GFP co-expression rate significantly increased in the ChR2 group (Figures 2E and 2F).

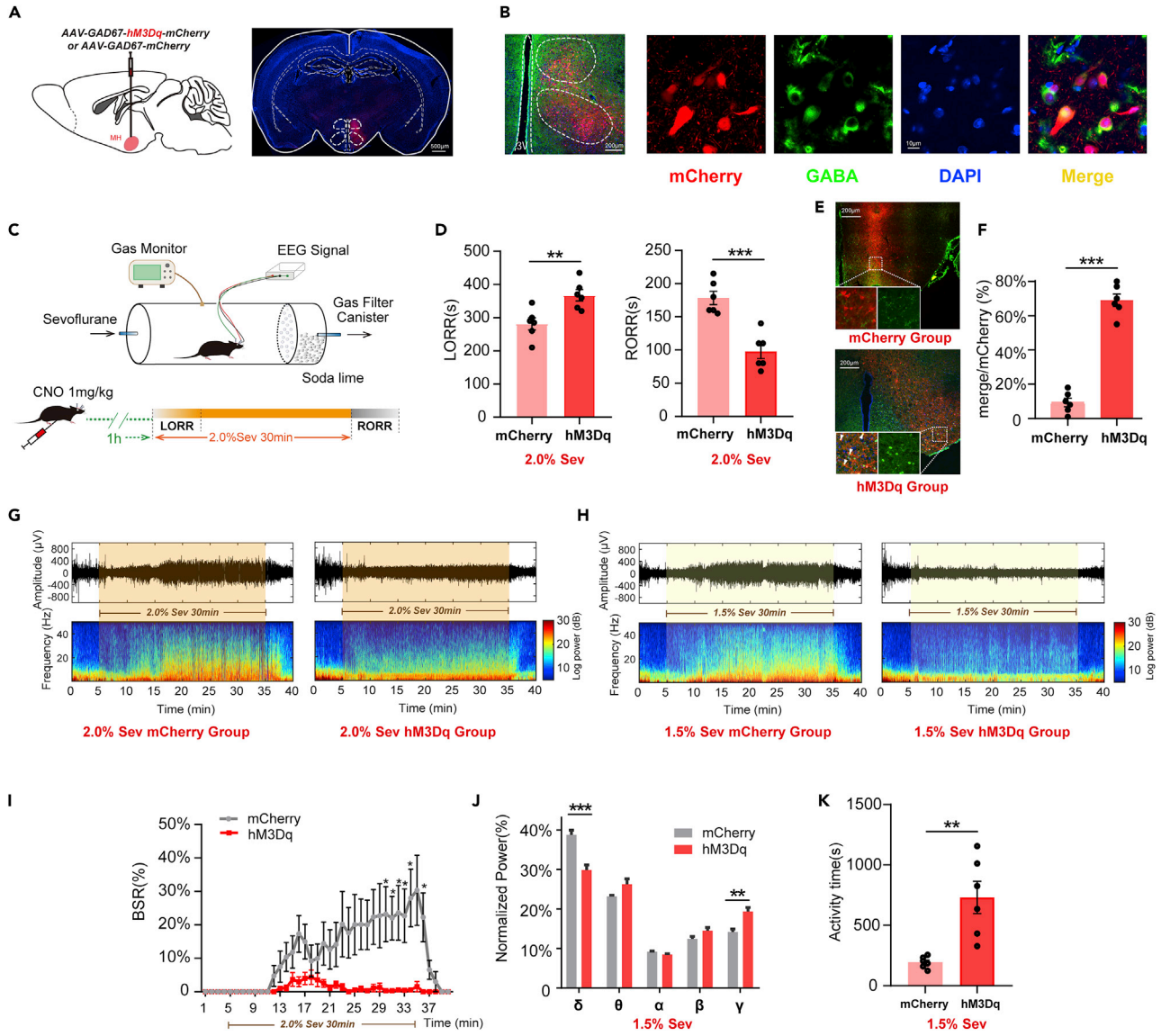

Figure 1. Chemogenetic activation of MH^{GABA} neurons has a significant emergence-promoting effect in sevoflurane anesthesia

(A) Schematic diagram of chemogenetic virus injection into the MH (left) and virus expression in MH (right).

(B) Expression of chemogenetic virus (mCherry, red) in the GABAergic neurons (green).

(C) Top: schematic of the EEG recording configuration and righting reflex detection. Bottom: process of detection of anesthesia induction and emergence times.

(D) Activation of MH^{GABA} significantly prolongs LORR time (mCherry vs. hM3Dq: 281.7 ± 18.3 s vs. 367.5 ± 17.1 s, $p = 0.006$, $n = 6$) (left) and shortens RORR time (178.3 ± 10.1 s vs. 98.0 ± 11.0 s, $p < 0.001$, $n = 6$) (right).

(E) Co-labeled immunofluorescence of virus autofluorescence (mCherry, red) and c-fos (green) (white triangle indicates the merge neuron).

(F) The rate of merge/mChreey expression was higher in the hM3Dq group activated by chemogenetics (9.3 ± 2.5% vs. 69.0 ± 3.7%, $p < 0.001$, $n = 6$).

(G) Representative EEG spectrum in 2.0% sevoflurane anesthesia.

(H) Representative EEG spectrum in 1.5% sevoflurane anesthesia.

(I) Burst suppression ratio (BSR) of EEG in the hM3Dq group is lower than that in the mCherry group under 2.0% sevoflurane anesthesia ($F(9,351) = 29.3$, $p < 0.001$, $n = 5$ vs. 6).

(J) Power spectral analysis of EEG signals showing that in the hM3Dq group the percentage of power in the δ band (38.8 ± 1.2% vs. 29.9 ± 1.3%, $p < 0.001$, $n = 6$) decreased, while that in the γ band (14.2 ± 0.7% vs. 19.4 ± 1.0%, $p = 0.002$, $n = 6$) increased.

(K) In 1.5% sevoflurane anesthesia, the movement time of mice in the hM3Dq group is longer than that in the mCherry group (193.8 ± 19.7 s vs. 731.3 ± 132.8 s, $p = 0.003$, $n = 6$). Data are expressed as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

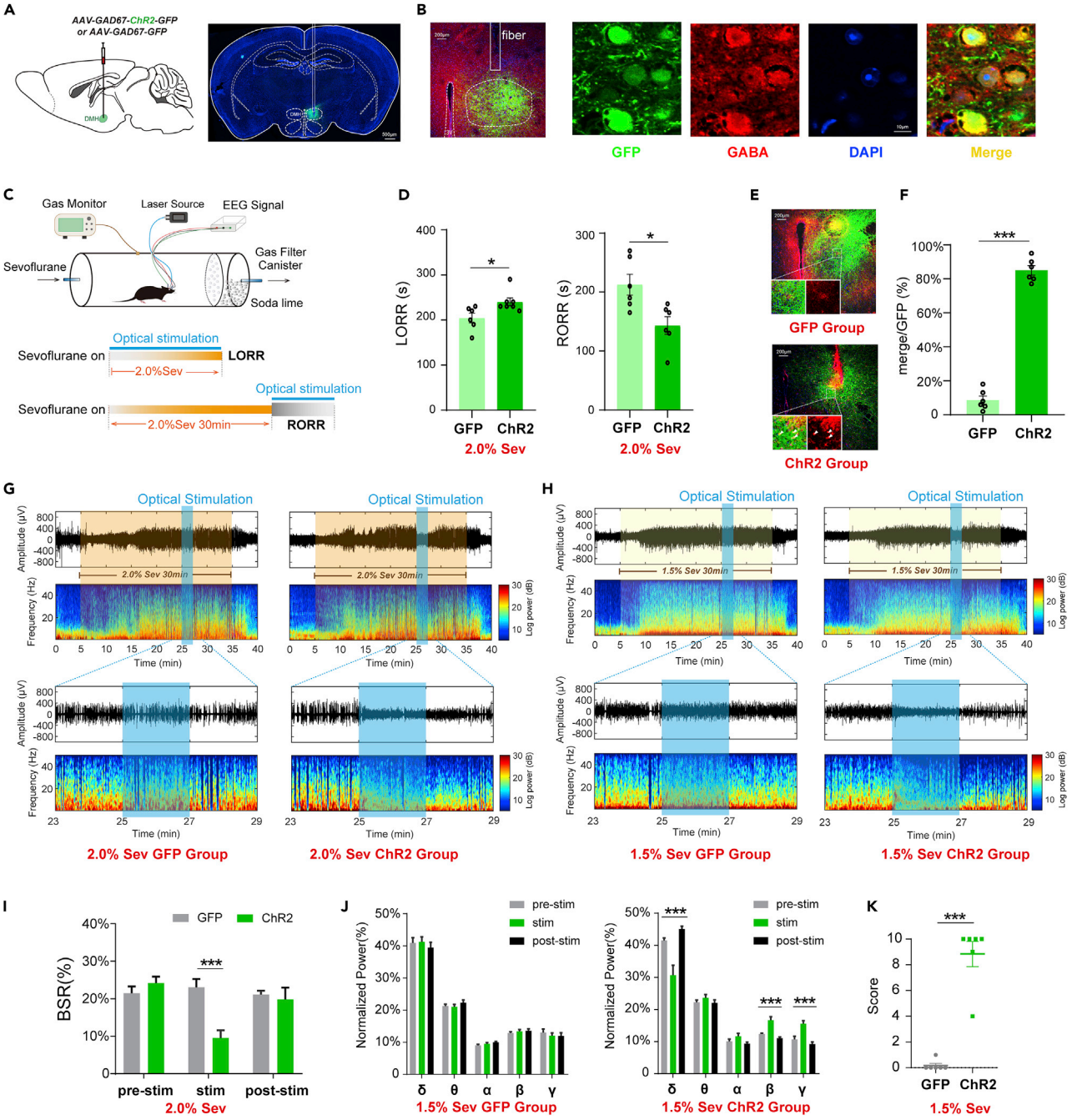

Figure 2. Optogenetic activation of the DMH^{GABA} neurons prolongs the induction time, shortens the emergence time of sevoflurane anesthesia, and induces EEG arousal and body movement during anesthesia

(A) Schematic diagram of optogenetic virus injection into the DMH (left), needle track, and virus expression in DMH (right).

(B) Immunofluorescence image of optogenetic virus (GFP, green) expression in DMH^{GABA} neurons (red).

(C) Schematic of the EEG recording configuration and righting reflex detection.

(D) Optogenetic activation of DMH^{GABA} prolongs LORR time (GFP vs. ChR2: 204.2 ± 10.8 s vs. 240.0 ± 8.7 s, p = 0.024, n = 6 vs.7) (left) and shortens RORR time (212.5 ± 17.7 s vs. 143.3 ± 15.0 s, p = 0.014, n = 6) (right).

(E) Co-labeled immunofluorescence of virus autofluorescence (GFP, green) and c-fos (red) (white triangle indicates the merge neuron).

(F) A significant increase of c-fos expression in GFP-positive neurons activated by optogenetics (8.7 ± 2.4% vs. 85.0 ± 2.8%, p < 0.001, n = 6).

(G) Representative EEG spectrum in 2.0% sevoflurane anesthesia.

(H) Representative EEG spectrum in 1.5% sevoflurane anesthesia.

Figure 2. *Continued*

(I) BSR of EEG in the ChR2 group is lower than that in the GFP group at 20–22 min of light stimulation under 2.0% sevoflurane anesthesia (23.1 ± 2.2% vs. 9.5 ± 2.1%, $p < 0.001$, $n = 5$).

(J) At 20–22 min of light stimulation, the percentages of power in the δ band ($F(2,27) = 15.6$, $p < 0.001$, $n = 5$) decreased, while those in the β ($F(2,27) = 19.4$, $p < 0.001$, $n = 5$) and γ bands ($F(2,27) = 13.4$, $p < 0.001$, $n = 5$) increased in the ChR2 group under 1.5% anesthesia. However, there is no difference in EEG spectrum in the GFP group.

(K) At 20–22 min of light stimulation during 1.5% sevoflurane anesthesia, the arousal scores in the ChR2 group are higher than that in the GFP group (0.2 ± 0.2 vs. 8.8 ± 1.0, $p = 0.0022$, $n = 6$). Data are shown as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

We adjusted the laser parameters (5 mW, 20 Hz, 10-ms duration, and 1 s on-/1 s off-cycle) and connected the ceramic inserts and optical fibers to the mice. Next, the mice were placed in the righting reflex barrel for 5 min before 2.0% sevoflurane anesthesia was administered, and light stimulation was performed at the same time. The LORR time was recorded (Figure 2C). It was found that the LORR time was significantly prolonged in the ChR2 group (Figure 2D). After three days, the RORR time was measured. Anesthesia with 2.0% sevoflurane was maintained for 30 min and light stimulation was initiated as soon as anesthesia was completed. The results showed that RORR was significantly shortened in the ChR2 group (Figure 2D). We also recorded the EEG signals of mice under 2.0% sevoflurane anesthesia and light stimulation at 20–22 min of anesthesia (Figure 2G). We analyzed the BSR data in three time periods (2 min before, during, and after light stimulation) and found that the BSR of the ChR2 group significantly decreased after light stimulation (Figure 2I).

During 20–22 min light stimulation under 1.5% sevoflurane anesthesia (Figure 2H), the percentages of power in the δ band were significantly reduced, whereas those in the β and γ bands were significantly increased in the ChR2 group (Figure 2J). Furthermore, a notable increase in body movement was observed (Figure 2K and Table S1, Videos S3, S4, and S8). What's more, we confirmed the results of our study using *Vgat-ires-Cre* transgenic mice (Figure S2 and Videos S10).

These behavioral results and EEG findings revealed that the activation of DMH^{GABA} neurons prolonged the induction time and shortened the emergence time of anesthesia. Arousal changes in EEG and more body movements related to wakefulness were discovered by activating DMH^{GABA} neurons, which had an emergence-promoting effect on MH in sevoflurane anesthesia.

Optogenetic activation of the VMH^{GABA} neurons has no significant effect on sevoflurane anesthesia

To clarify the role of VMH^{GABA} in sevoflurane anesthesia, we also used the optogenetic method by injecting 100 nL *rAAV2/9-GAD67-hChR2(H134R)-GFP* or *rAAV2/9-GAD67-GFP* into the VMH (AP −1.4 mm, ML +0.45 mm, and DV –5.25 mm) of mice (Figures 3A and S2A). Four weeks later, c-fos staining results showed a significant increase in the c-fos/GFP co-expression rate in the ChR2 group (Figures 3E and 3F).

Comparing the ChR2 group with the GFP group during 2.0% sevoflurane anesthesia, we found that there was no significant difference in the LORR or RORR time (Figure 3D). Moreover, the BSR of the EEG signals showed no statistically significant difference between the two groups during 20–22 min of light stimulation (Figures 3G and 3I). However, under 1.5% sevoflurane anesthesia, when we performed light stimulation at 20–22 min, there was a decrease in the percentage of power in the δ band, whereas that in the β and γ bands significantly increased (Figure 3J). Nevertheless, no obvious body movements were observed in the mice (Figure 3K, Table S2, Videos S5, S6, and S9).

To summarize, there was no significant effect on anesthesia induction and emergence after activation of VMH^{GABA} neurons, and only EEG changes occurred in 1.5% of anesthesia cases. These results indicated that the arousal-promoting effect of MH^{GABA} neurons was not mediated by VMH^{GABA} neurons and that VMH^{GABA} neurons played a very limited role in this process.

Calcium signal indicates that DMH^{GABA} neurons activity is closely related to arousal during sevoflurane anesthesia

To further explore the role of DMH^{GABA} neurons during sevoflurane anesthesia, calcium signal virus of 100 nL *rAAV2/9-GAD67-GCaMP6s* or *rAAV2/9-GAD67-GFP* was injected into the DMH region (Figure 4A). The fiber was connected to a single-channel photometric recording system. After the calcium signal was

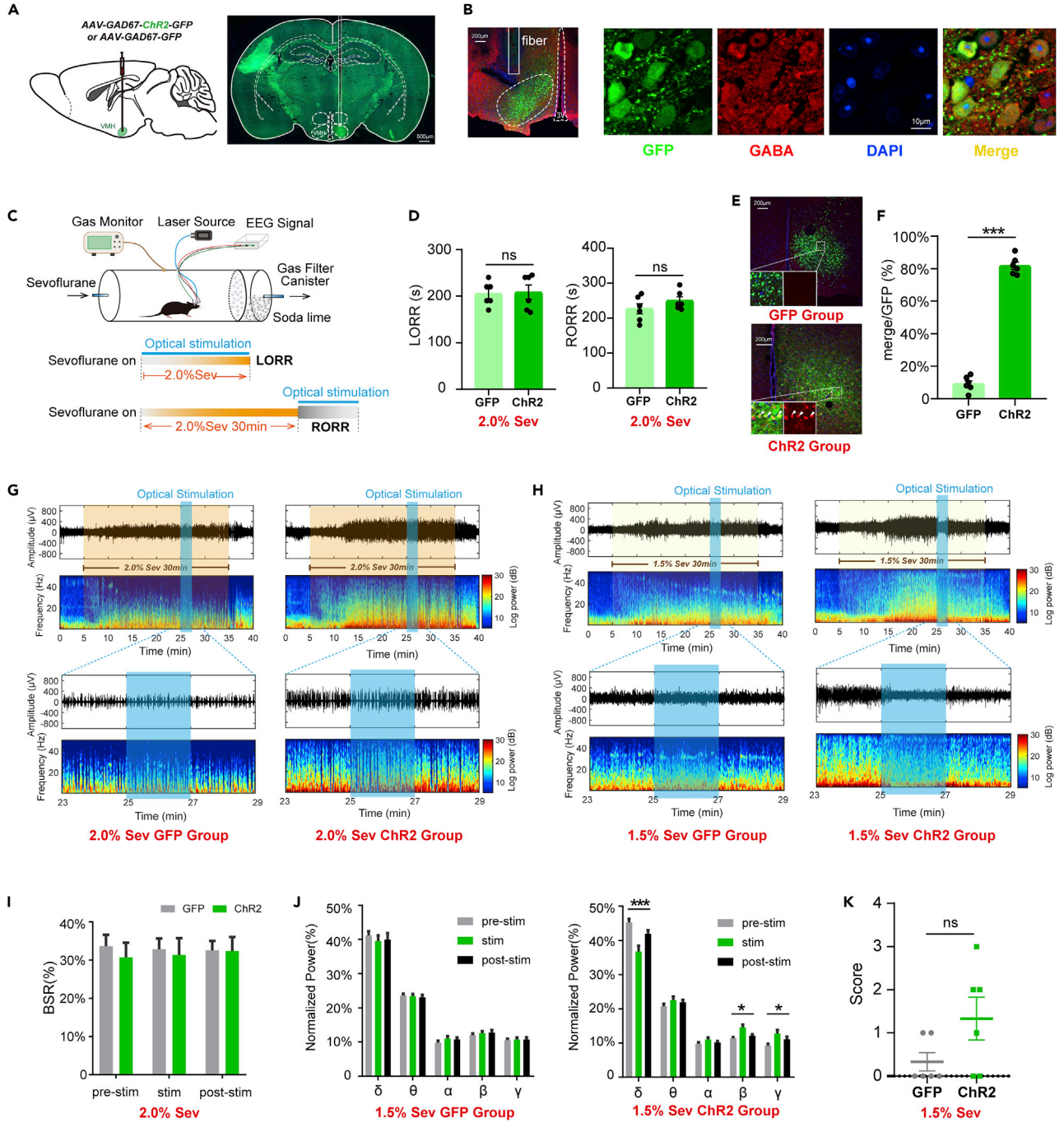

Figure 3. Only EEG changes occur in 1.5% of anesthesia after optogenetic activation of the VMH^{GABA} neurons

(A) Schematic diagram of optogenetic virus injection into the VMH (left) and needle track and virus expression in VMH (right).

(B) Immunofluorescence image of optogenetic virus (GFP, green) expression in VMH^{GABA} neurons (red).

(C) Schematic of the EEG recording configuration and righting reflex detection.

(D) Optogenetic activation of VMH^{GABA} has no significant effect on LORR time (GFP vs. ChR2: 205.0 ± 10.6 s vs. 208.3 ± 15.3 s, p = 0.861, n = 6) (left) and RORR time (226.7 ± 14.5 s vs. 250.5 ± 11.0 s, p = 0.229, n = 6) (right).

(E) Co-labeled immunofluorescence of virus autofluorescence (GFP, green) and c-fos (red) (white triangle indicates the merge neuron).

(F) Significant increase of c-fos expression in GFP-positive neurons activated by optogenetics (9.2 ± 1.9% vs. 81.8 ± 2.3%, p < 0.001, n = 6).

(G) Representative EEG spectrum in 2.0% sevoflurane anesthesia.

(H) Representative EEG spectrum in 1.5% sevoflurane anesthesia.

(I) There is no significant difference in the BSR of EEG between the ChR2 and GFP groups at 20–22 min of light stimulation under 2.0% sevoflurane anesthesia.

Figure 3. *Continued*

(J) At 20–22 min of light stimulation, the percentages of power in the δ band ($F(2,33) = 10.6$, $p < 0.001$, $n = 6$) decreased, while those in the β ($F(2,33) = 8.2$, $p = 0.001$, $n = 6$) and γ bands ($F(2,33) = 4.5$, $p = 0.018$, $n = 6$) increased in the ChR2 group under 1.5% anesthesia. However, there is no difference in the GFP group.

(K) At 20–22 min of light stimulation during 1.5% sevoflurane anesthesia, the arousal scores between the ChR2 and GFP groups showed no obvious difference. Data are displayed as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

recorded for 5 min in the awake state, 2.0% sevoflurane anesthesia was administered for 30 min, and the calcium signal was recorded for 10 min when anesthesia was switched off (Figure 4C). From the beginning of anesthesia, we found that DMH^{GABA} neuronal activity gradually decreased as sevoflurane concentration progressively increased (Figure 4D). Additionally, compared with 2 min before anesthesia, the ΔF/F value was significantly reduced 4–6 min after anesthesia (Figure 4F). In line with the above result, DMH^{GABA} neuronal activity increased gradually with a decrease in anesthesia concentration when anesthesia was stopped (Figure 4E), the ΔF/F values were significantly higher at 4–6 min after anesthesia was stopped than at 2 min before anesthesia was stopped (Figure 4G), and there was no obvious change in the GFP group during sevoflurane anesthesia.

Additionally, compared with the gradual decrease in DMH^{GABA} neuronal activity during sevoflurane anesthesia, the calcium signal of DMH^{GABA} neurons in mice after sevoflurane was stopped showed a sharp increase for approximately 10 s (Figure 4H), while, the mice quickly woke up and their burst suppression on EEG disappeared (Figure 4I). Furthermore, the percentage of power in the δ band gradually decreased, whereas that in the β and γ bands progressively increased (Figure 4J). By analyzing the proportions of bands in the first and 10th s, we found that the percentage of power in the δ band in the 10th s declined significantly, but the γ band increased significantly (Figure 4K and Video S7).

Chemogenetic inhibition of DMH^{GABA} neurons can deepen sevoflurane anesthesia

We used chemogenetic methods to inject 100 nL of *rAAV2/9-GAD67-hM4Di-mCherry/rAAV2/9-GAD67-mCherry* into the DMH to explore the effect of inhibiting DMH^{GABA} neurons under anesthesia (Figures 5A and S2A). After four weeks, the mice were injected with CNO, which specifically inhibits DMH^{GABA} neurons. C-fos staining results showed that the c-fos/mCherry co-staining rate of mice in the hM4Di group was significantly decreased (Figures 5E and 5F).

In the hM4Di group, we found a prominent reduction in LORR time, but a more marked increase in RORR time (Figure 5D), as well as a higher BSR of EEG during 2.0% sevoflurane anesthesia. Likewise, under 1.5% sevoflurane anesthesia, the BSR in the hM4Di group was significantly higher than that in the mCherry group (Figures 5H and 5J). However, in the first 5 min of 1.5% sevoflurane anesthesia without burst suppression, our analysis showed that the percentage of power in the δ band increased significantly in the hM4Di group, whereas that in the γ band declined significantly (Figure 5K).

In conclusion, inhibition of DMH^{GABA} neurons could shorten the induction time, prolong the emergence time, and increase the BSR during anesthesia, thus promoting sevoflurane anesthesia.

Chemogenetic activation of DMH^{GABA} neurons delays induction and accelerates emergence from sevoflurane anesthesia

To investigate the sensitivity of sevoflurane and the righting reflex test after chemogenetic activation of DMH^{GABA} neurons, we injected 100 nL *rAAV2/9-GAD67-hM3Dq-mCherry/rAAV2/9-GAD67-mCherry* into the DMH (Figure 6A). The c-fos staining results showed that c-fos/mCherry in the hM3Dq group was significantly increased (Figures 6H and 6I).

In behavioral experiments, we found that the LORR of chemogenetic activation in the hM3Dq group was significantly prolonged (Figure 6B), whereas the RORR time was significantly shorter (Figure 6C). In addition, after the activation of DMH^{GABA} neurons, the dose-response curve of the LORR was right shifted (Figure 6D), suggesting that hM3Dq group required higher doses of sevoflurane to display LORR than the mCherry group, whereas the 50% effective concentration (EC50) of sevoflurane for LORR was 1.65% (95% confidence interval [CI]: 1.57%–1.73%) for the hM3Dq group and 1.38% (95% CI: 1.29%–1.46%) for the mCherry group (Figure 6E). The dose-response curve of RORR was left-shifted after activation of DMH^{GABA} neurons (Figure 6F), indicating that the RORR of hM3Dq group mice could be recovered under

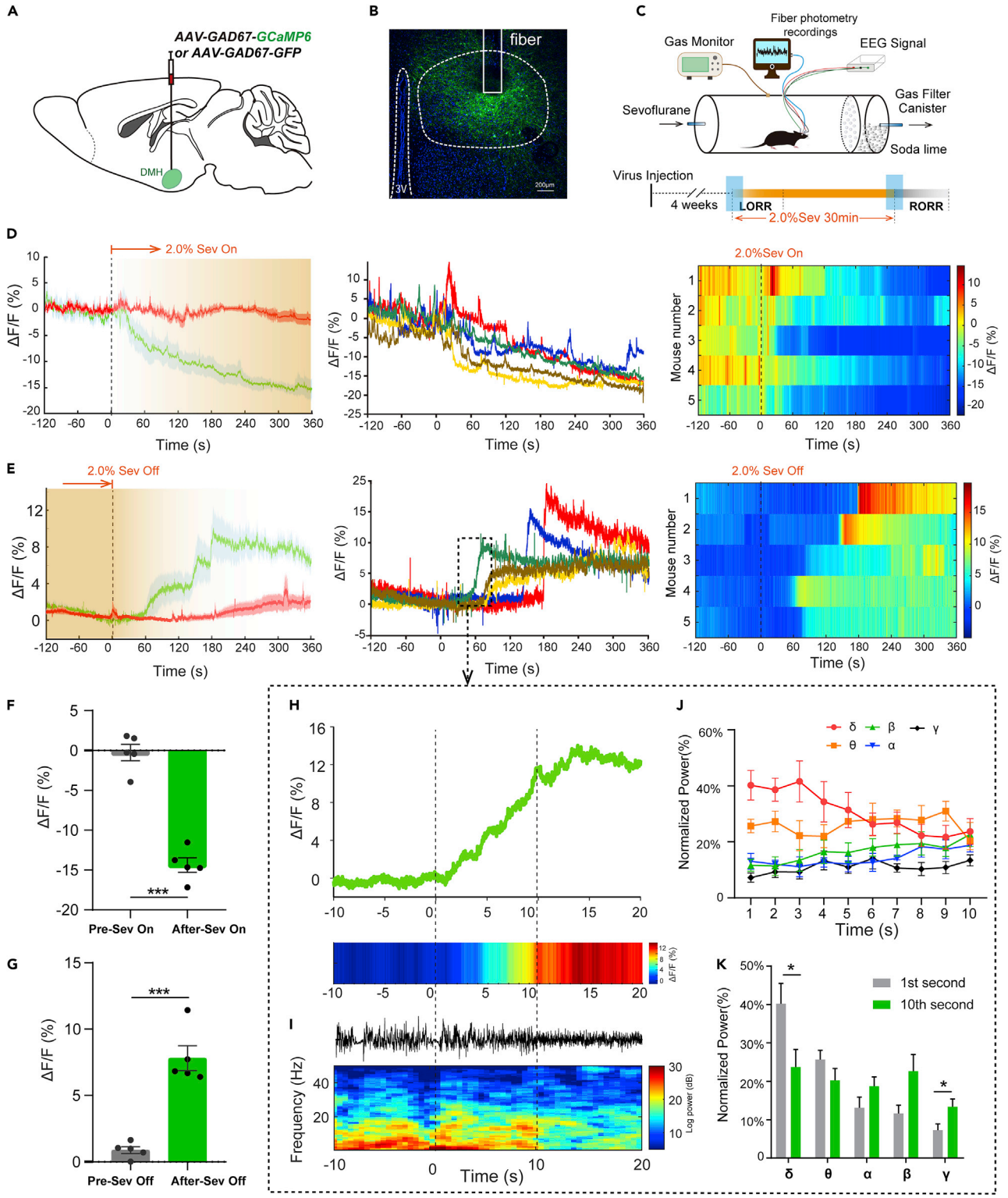

Figure 4. Calcium signal indicates that DMH^{GABA} neurons activity is closely related to arousal during sevoflurane anesthesia

(A) Schematic diagram of calcium signal virus injection into the DMH.

(B) Representative immunofluorescence showing the virus expression into DMH and ceramic ferrule track.

(C) Schematic of the calcium signal recording configuration.

Figure 4. *Continued*

(D) After initiation of 2.0% sevoflurane anesthesia, the activity of DMH^{GABA} neurons gradually decreased, there was no obvious change in the GFP group (left), and changes in DMH^{GABA} neuronal activity of five mice (middle) and their heatmaps (right) are shown.

(E) After the end of 2.0% sevoflurane anesthesia, the activity of DMH^{GABA} neurons gradually increased, there was no obvious change in the GFP group (left), and the changes in DMH^{GABA} neuronal activity of five mice (middle) and their heatmaps (right) are shown.

(F) The ΔF/F value of DMH^{GABA} neurons decreased significantly after initiation of anesthesia (Pre vs. After: $-0.3 \pm 1.0\%$ vs. $-14.4 \pm 0.9\%$, $p < 0.001$, $n = 5$).

(G) The ΔF/F value of DMH^{GABA} neurons increased significantly after the end of anesthesia ($0.9 \pm 0.3\%$ vs. $7.8 \pm 0.9\%$, $p < 0.001$, $n = 5$).

(H) Representative changes of calcium signal in DMH^{GABA} neurons and heatmaps in mice during the rapid awakening period (about 10 s) after anesthesia.

(I) Representative EEG spectrum during the rapid awakening period.

(J) Trend diagram of the percentage of power in the different frequency bands of mice during the rapid awakening period.

(K) Compared with the first second, the percentage of power in the δ band (first vs. 10th: $40.2 \pm 5.3\%$ vs. $23.7 \pm 4.6\%$, $p = 0.046$, $n = 5$) in the 10th s declined, and the γ band ($7.2 \pm 1.7\%$ vs. $13.4 \pm 2.0\%$, $p = 0.044$, $n = 5$) increased significantly. Data are expressed as the mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

high-concentration sevoflurane anesthesia, with an EC50 of 1.65% (95% CI: 1.57%–1.73%) for the hM3Dq group and 1.43% (95% CI: 1.37%–1.48%) for the mCherry group (Figure 6G).

Sevoflurane sensitivity and righting reflex tests further clarified that DMH^{GABA} neurons play a wake-promoting role in sevoflurane induction and emergence.

DISCUSSION

In our study, MH^{GABA} neurons were first activated by chemogenetic manipulations, and we found that it could prolong sevoflurane anesthesia induction time and shorten emergence time. We then revealed that the emergence-promoting effect of MH^{GABA} neurons was mainly modulated by GABA neurons in the DMH rather than in the VMH during sevoflurane anesthesia induction, maintenance, and recovery. The changes in calcium signal and chemogenetic inhibition tests in DMH^{GABA} neurons during anesthesia further confirmed the effect of DMH^{GABA} neurons on emergence from sevoflurane anesthesia. Therefore, our study provides evidence of the important role of the hypothalamic nucleus in general anesthesia and enriches the modulatory aspect of the DMH in sevoflurane anesthesia as a powerful arousal nucleus. The excitation-inhibition balance of the neural network may affect conscious maintenance.

The DMH, as a substantial relay of circadian regulation of arousal, can mediate the suprachiasmatic nucleus to regulate the sleep-wake cycle.[13,20] Researchers have found that DMH galaninergic neurons are a subtype of GABAergic neurons that modulate rapid eye movement (REM) and non-REM by projecting to the preoptic area (POA) and raphe pallidus nuclei.[21] It has also been reported that the cannabinoid system in the prefrontal $cortex^{GLU}$-DMH-ventrolateral preoptic (VLPO) (GLU-DMH-VLPO)/perifornical nucleus tertiary projection promotes awakening during isoflurane anesthesia.[22] Additionally, the VMH can affect sleep patterns, and its neuronal activity is significantly correlated with paradoxical sleep.[14,15] In recent years, studies on VMH have mainly focused on behaviors of defense, attack, sex, and social and predator fear,[23–25] but rarely on anesthesia.

Different GABAergic neurons in the brain nuclei may play different roles during general anesthesia. Research has shown that optogenetic activation of GABAergic neurons in the TRN decreases arousal.[26] Activation of preoptic GABAergic neurons does not alter anesthetic induction or recovery time but alters the sleep-wake architecture.[9] Chemogenetic activation of GABAergic neurons in the rostromedial tegmental nucleus facilitates sevoflurane anesthesia in mice.[27] The lateral septum GABAergic neurons exerted a significant effect in promoting arousal during isoflurane anesthesia, while inhibition of GABAergic neurons during anesthesia did not deepen anesthesia.[10] In contrast, the activation of DMH^{GABA} neurons under sevoflurane anesthesia has a strong stimulatory effect on arousal. When DMH^{GABA} neurons were inhibited, the BSR was significantly increased and deeper anesthesia was observed, indicating that the activity of DMH^{GABA} neurons was closely related to the depth of anesthesia. Furthermore, EEG and calcium signals during the 10 s rapid arousal period showed that burst suppression disappeared; the percentage of power in the δ band gradually decreased, and that in the γ band gradually increased, which also revealed that the neural activity of DMH^{GABA} neurons was closely related to the degree of awakening.

The induction and recovery of anesthesia may be mediated by different cerebral nuclei and pathways, rather than a simple mirror image role.[28] In the three main processes of induction, maintenance, and

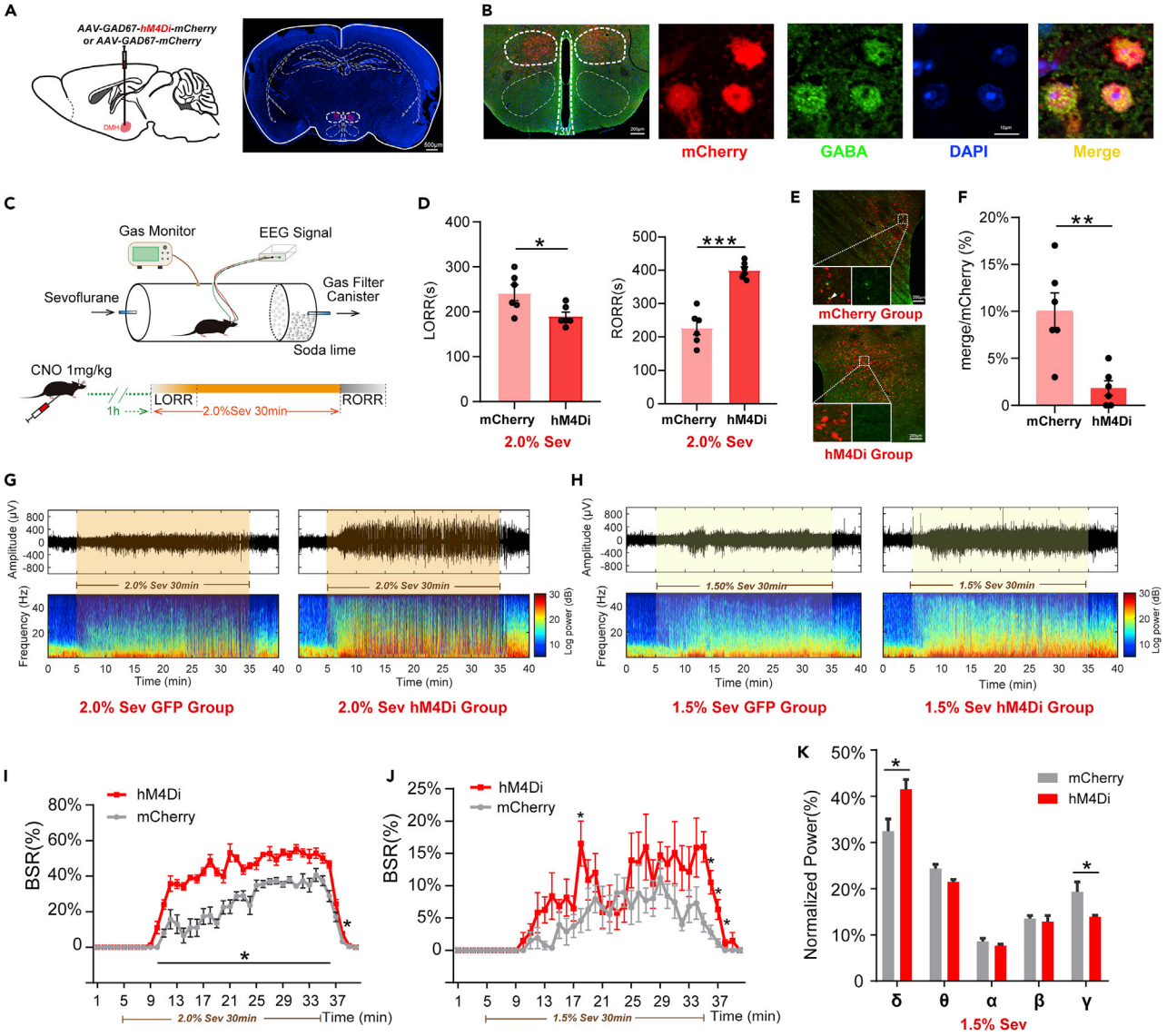

Figure 5. Chemogenetic inhibition of DMH^{GABA} neurons can deepen sevoflurane anesthesia

(A) Schematic diagram of chemogenetic virus injection into the DMH bilaterally (left) and virus expression in DMH (right).

(B) Expression of chemogenetic virus (mCherry, red) in the GABAergic neurons (green).

(C) Schematic of the EEG recording configuration and righting reflex detection.

(D) Inhibition of DMH^{GABA} shortens LORR time (mCherry vs. hM4Di: 242.5 ± 17.6 s vs. 190.0 ± 9.2 s, p = 0.024, n = 6) (left) and prolongs RORR time (227.5 ± 20.7 s vs. 400.0 ± 10.1 s, p < 0.001, n = 6) (right).

(E) Co-labeled immunofluorescence of virus autofluorescence (mCherry, red) and c-fos (green) (white triangle indicates the merge neuron).

(F) The rate of merge/mCheey expression was lower in the hM4Di group inhibited by chemogenetics (10.0 ± 2.0% vs. 1.8 ± 0.8%, p = 0.003, n = 6).

(G) Representative EEG spectrum in 2.0% sevoflurane anesthesia.

(H) Representative EEG spectrum in 1.5% sevoflurane anesthesia.

(I) BSR of EEG in the hM4Di group is higher than that in the mCherry group under 2.0% sevoflurane anesthesia (F (10,390) = 11.16, p < 0.001, n = 6).

(J) In 1.5% sevoflurane anesthesia, BSR in the hM4Di group is also higher than that in the mCherry group (F (8,312) = 14.6, p < 0.001, n = 5).

(K) In the first 5 min of 1.5% sevoflurane anesthesia without burst suppression, the percentage of power in the δ band (32.4 ± 2.6% vs. 41.5 ± 2.1%, p = 0.027, n = 5) increased in the hM4Di group, whereas that of the γ band (19.3 ± 2.1% vs. 13.9 ± 0.3%, p = 0.035, n = 5) declined. Data are shown as the mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

recovery of anesthesia, some neural nuclei participate in the entire process of anesthesia, such as parabrachial nucleus glutamatergic neurons,[29] basal forebrain cholinergic neurons,[30] VTA GABAergic neurons,[8] and lateral hypothalamic glutamatergic neurons.[3] Some other nuclei may participate in only one or two

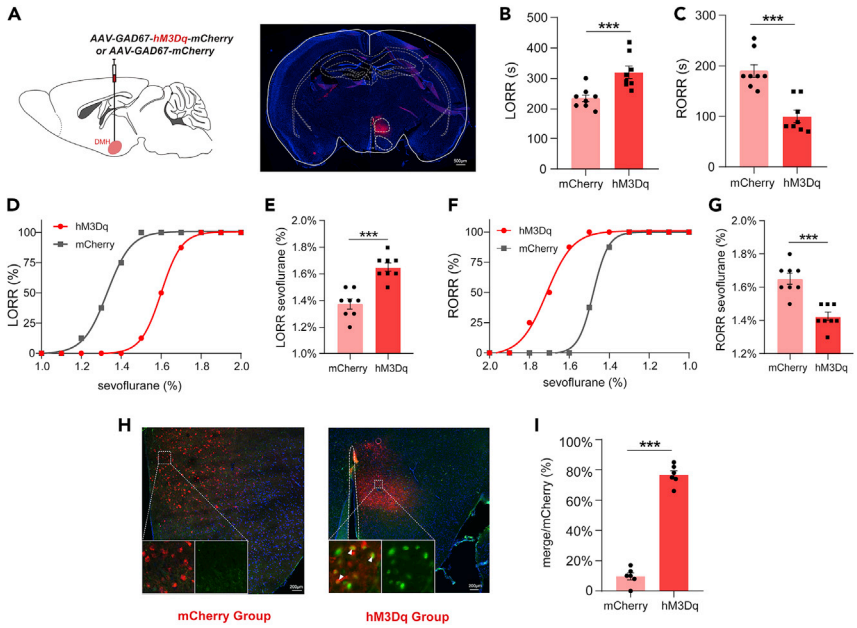

Figure 6. Chemogenetic activation of DMH^{GABA} neurons delays induction and accelerates emergence from sevoflurane anesthesia

(A) Schematic diagram of chemogenetic virus injection into the DMH (left) and virus expression in DMH (right).

(B) Activation of DMH^{GABA} prolongs LORR time (mCherry vs. hM3Dq: 233.8 ± 12.0 s vs. 320.6 ± 20.0 s, p = 0.002, n = 8).

(C) Activation of DMH^{GABA} shortens RORR time (190.6 ± 13.1 s vs. 100.6 ± 11.6 s, p < 0.001, n = 8).

(D) Dose-response curve of LORR was right-shifted after chemogenetic activation of DMH^{GABA} neurons.

(E) Sevoflurane concentrations at which each mouse exhibited LORR (mCherry vs. hM3Dq: 1.38 ± 0.04% vs. 1.65 ± 0.03%, p < 0.001, n = 8).

(F) Dose-response curve of RORR is left-shifted after chemogenetic activation of DMH^{GABA} neurons.

(G) Sevoflurane concentrations at which each mouse exhibited RORR (1.43 ± 0.03% vs. 1.65 ± 0.03%, p < 0.001, n = 8).

(H) Co-labeled immunofluorescence of virus autofluorescence (mCherry, red) and c-fos (green) (white triangle indicates the merge neuron).

(I) The rate of merge/mChreey expression was higher in the hM3Dq group activated by chemogenetics (9.7 ± 2.3% vs. 76.7 ± 2.7%, p < 0.001, n = 6). Data are shown as the mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

processes. For example, orexinergic neurons located in the lateral hypothalamus are involved in both the maintenance and arousal stages of anesthesia but do not affect anesthesia induction.[2,28,31–33] Many studies have focused only on the role of certain nuclei in recovery.[10,34,35] We found that VMH^{GABA} neurons activation only showed EEG changes during maintenance of 1.5% sevoflurane anesthesia. However, activation of DMH^{GABA} neurons not only shortened the emergence time and induced EEG and behavioral arousal during anesthesia but also significantly prolonged the induction time of anesthesia and had an emergence-promoting effect.

Only EEG arousal changes, but no movement behavior, could be induced by microinjection activation of the nucleus basalis magnocellularis and optogenetic activation of the parabrachial nucleus or paraventricular thalamus glutamate neurons during anesthesia.[29,35–37] However, when DMH^{GABA} neurons were activated during anesthesia, mice showed obvious body movements in addition to EEG arousal changes, which was similar to the results of powerful emergence-promoting nuclei of the VTA and nucleus accumbens.[34,36]

Body temperature affects the potency of general anesthetics,[38] and a recent study has identified that chemogenetic activation of DMH^{GABA} neurons leads to elevated body temperature.[39] However, no significant changes in body temperature were observed during anesthesia when optogenetic activation of DMH^{GABA} neurons occurred for shorter light stimuli (Figure S1), indicating that the emergence-promoting effect of DMH^{GABA} neurons in this experiment was mainly a regulatory effect of the nucleus itself. Furthermore, the hypothalamus is the center that regulates autonomic nerve activity, and the anesthesia induction

time may be altered by changes in cardiac output and respiration. Therefore, the relationship between cardiorespiratory function, body temperature, and anesthesia arousal should be considered in future studies.

Different nuclei may play different roles in anesthesia awakening. DMH^{GABA} neurons can project to nuclei related to anesthesia and sleep, such as VLPO, locus coeruleus, and POA; the inhibitory neurons were inhibited in these nuclei and showed a disinhibition effect,[10,11,13,21,22] which probably accounts for the emergence-promoting effect of the DMH. However, the efferent projections of the VMH^{GABA} neurons are fewer;[40] therefore, the differences in anesthesia regulation mechanisms between the DMH and VMH may be the result of variations in the projections. Many general anesthetics are thought to cause a loss of wakefulness, in part, by enhancing GABA neurotransmission.[41] Accordingly, exploring the role of GABA neurons in different nuclei and their projections may be of great significance in revealing the mechanism of anesthesia.

Collectively, DMH^{GABA} but not VMH^{GABA} neurons of the MH exerted a powerful and crucial emergence-promoting effect during the induction, maintenance, and arousal of sevoflurane general anesthesia. DMH GABAergic neurons in the regulation of anesthesia are of great significance for exploring the mechanism of conscious maintenance.

Limitations of the study

In the present study, we did not explore which subtype of GABAergic neurons, such as somatostatin neurons, vasoactive intestinal peptide neurons, and parvalbumin neurons, played a role in regulating anesthesia arousals and we did not explore the projections of DMH or VMH GABAergic neurons. Furthermore, changes in DMH^{GABA} neuronal activity during anesthesia recovery may be related to restlessness, sometimes observed at lower doses of anesthetic drugs, which requires further study.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105913.

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AUTHOR CONTRIBUTIONS

Y.-F.W. conceived, designed, and performed the experiments, analyzed and interpreted the data, prepared the figures, and wrote the manuscript. Y.-P.S. designed and performed the experiments, analyzed and interpreted the data, prepared the figures, and wrote the manuscript. L.T. and L.W. performed the experiments and analyzed data. J.-B.C., G.Q., X.-Y.L., and W.-D.M. analyzed the data and revised the manuscript. E.W. and Y.-X.G. conceived and designed the experiments and wrote and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit GABA antibody	GeneTex	CAT#GTX125988
Mouse anti-c-fos antibody	Abcam	CAT#ab208942
Alexa Fluor® 488-labelled goat anti-mouse lgG	Servicebio	CAT#GB25301
Alexa Fluor® 488-labelled goat anti-rabbit lgG	Servicebio	CAT#GB25303
Cy3-labelled donkey anti-mouse lgG	Servicebio	CAT#GB21401
Cy3-labelled donkey anti-rabbit lgG	Servicebio	CAT#GB21403
Bacterial and virus strains		
rAAV2/9-GAD67-hM3Dq-mCherry	Obio Biotechnologies	N/A
rAAV2/9-GAD67-hM4Di-mCherry	Obio Biotechnologies	N/A
rAAV2/9-GAD67-mCherry	Obio Biotechnologies	N/A
rAAV2/9-GAD67-hChR2(H134R)-GFP	Obio Biotechnologies	N/A
rAAV2/9-GAD67-GFP	Obio Biotechnologies	N/A
rAAV2/9-GAD67-GCaMP6s	Obio Biotechnologies	N/A
rAAV2/9-EF1α-DIO-hChR2(H134R)-GFP	Obio Biotechnologies	N/A
rAAV2/9-EF1α-DIO-GFP	Obio Biotechnologies	N/A
Chemicals, peptides, and recombinant proteins		
DAPI Fluoromount-G	Southern Biotech	CAT#SBA-0100-20
clozapine-N-oxide	MedChemExpress	CAT#HY-17366
sevoflurane	Baxter Healthcare Corp	CAT#CN2L9117
Normal Donkey Serum	Solarbio	CAT#SL050
Experimental models: Organisms/strains		
Male C57BL/6 mice	Experimental Animal Center of Central South University	N/A
Vgat-ires-Cre mice	the Jackson Laboratory	JAX stock 016962
Software and algorithms		
Prism 8.0	GraphPad	https://www.graphpad.com/
Adobe Illustrator CC 2018	Adobe	https://www.adobe.com/products/illustrator.html
SPSS statistics 25	IBM	www.ibm.com/products/spss-statistics
MATLAB	MathWorks	https://www.mathworks.com/products/matlab

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, gyxinbin@163.com (Yongxin Guo).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male C57BL/6 mice (6–8 weeks old, 20–25 g) were obtained from the Experimental Animal Center of Central South University (Changsha, Hunan, China), and *Vgat-ires-Cre* mice were purchased from the Jackson Laboratory (*Vgat-ires-Cre*: *Slc32a1*$^{tm2(cre)Lowl}$/*J*: JAX stock 016962),[42] and were group-housed in clean cages at room temperature of (22 ± 2 °C) with a 12/12-h light/dark cycle. Behavioral tests, such as righting reflex, electroencephalogram (EEG), and calcium signals, were conducted 4–6 weeks after the virus injection, from 8 am to 6 pm (Beijing time). All animal experimental procedures were approved by the Ethics Committee for Animal Experimentation of Central South University and were conducted according to the guidelines of the National Institutes of Health (United States) regarding the care and use of animals.

METHOD DETAILS

Stereotaxic surgery

Mice were anesthetized with 2% pentobarbital sodium (50 mg/kg, intraperitoneally, i.p.) and were placed on a stereotaxic frame (RWD68046, Shenzhen, China), with their body temperature maintained at 35 °C using a heating blanket. The virus was injected through a microinjection pump (Harvard Apparatus Pump 11 Elite, USA) and a 10-μL micro-syringe (Hamilton 1701RN, Switzerland), 100 nL per nucleus for all experiments. After the injection, the syringe was indwelled at the injection site for 10 min before being slowly pulled out. Three EEG electrodes were implanted into the skulls of mice. A ceramic ferrule (Inper, Hangzhou, China) with a diameter of 200 μm was placed into the target brain region with its depth elevated by 50 μm to conduct optogenetics and calcium imaging operations. Finally, EEG electrodes and optical fibers were affixed to the skull with diluted dental cement.

The coordinates of the microinjections were as follows: positive EEG electrode (AP: +1.0 mm, ML: −2.0 mm); negative EEG electrode (AP: −4.0 mm, ML: −2.5 mm); reference electrode (AP: −4.0 mm, ML: +2.5 mm). DMH (AP: −1.4 mm, ML: +0.45 mm, DV: –5.25 mm), VMH (AP: −1.4 mm, ML: +0.45 mm, DV: –5.8 mm), MH (AP: −1.4 mm, ML: +0.45 mm, DV: –5.25 and −5.8 mm).

Examination of induction and emergence times

The righting reflex barrel was connected to a gas monitor (Datex-Engstron, Finland), and the oxygen flow was adjusted to 2 L/min at 40% oxygen concentration. A thermostatic heating pad (37 °C) was placed under an anesthesia barrel (diameter: 15 × 40 cm). First, the mice were placed in the righting reflex barrel for 5 min to adapt to the electrode wire and experimental environment. Subsequently, 2.0% sevoflurane anesthesia was initiated, and the circular barrel was rotated 180° every 15 s from the beginning of anesthesia. When the mice were in an abnormal position (limbs up) and could not voluntarily return to the normal position, this behavior was defined as the loss of righting reflex (LORR), which is considered the time of sevoflurane anesthesia induction. Sevoflurane anesthesia was continued for 30 min after the righting reflex disappeared, and the anesthesia machine was turned off, but the oxygen concentration was maintained. By rotating the barrel, the mouse was kept in a position with its back touching the ground and limbs facing upwards. If the mouse automatically returned to the normal position (all limbs touching the ground) from the position where the righting reflex disappeared, it was considered to have recovered. The time from the end of anesthesia to the recovery of righting reflex (RORR) was defined as the time of emergence from sevoflurane anesthesia. A commutator was used during the LORR and RORR behavioral experiments to ensure that the fiber did not interfere with the experiment and was recorded separately from the EEG.

Chemogenetics

Mice in the experimental and control groups were intraperitoneally administered clozapine-N-oxide CNO (1 mg/kg) or the same amount of sterile saline. One hour after the injection, the mice were connected to EEG electrodes and placed in the righting reflex barrel. After 5 min of adaptation, 2.0% sevoflurane anesthesia was administered, and the loss of righting reflex (LORR), recovery of righting reflex (RORR), and EEG signals were recorded. Three days after recovery, 1.5% sevoflurane anesthesia was administered and the

total movement time and EEG signals during anesthesia were recorded. To determine the dose–response curve for LORR, sevoflurane was initially delivered at 1.0% for 30 min and was increased in increments of 0.1% every 15 min until the mouse reached LORR. To determine the dose–response curve for RORR, sevoflurane was initially delivered at 2.0% for 30 min, and then the sevoflurane concentration was reduced by 0.1% every 15 min until the mouse recovered.[36]

The viruses used for the chemogenetic procedures were as follows: (1) *rAAV2/9-GAD67-hM3Dq-mCherry*, (2) *rAAV2/9-GAD67-mCherry*, (3) *rAAV2/9-GAD67-hM4Di-mCherry* (OBiO, Shanghai, China).

Optogenetics

The power of the blue light (473 nm) from a laser (Thinker Tech, China) was adjusted to approximately 5 mW using an optical power meter (SANWA, Japan). Mice were placed in a righting reflex barrel for 5 min, light stimulation (5 mW, 20 Hz, 10-ms duration, 1 s on-/1 s off-cycle) was performed at the beginning of 2.0% sevoflurane anesthesia, and the LORR time of mice was recorded. Three days later, light stimulation was administered after 30 min of 2.0% sevoflurane anesthesia, and the RORR time was recorded. In addition, EEG and behavioral changes were observed after 2 min of light stimulation under 2.0% and 1.5% anesthesia (20–22 min), respectively. Before the next experiment was conducted, the mice needed three days to recover from each light stimulation.

The viruses used for the optogenetic procedures were as follows: (1) *rAAV2/9-GAD67-hChR2(H134R)-GFP*, and (2) *rAAV2/9-GAD67-GFP* (OBiO, Shanghai, China).

EEG recording and analysis

The EEG monitor PowerLab and LabChart 8.0 software (AD Instruments, USA) were used to record the EEG activity of the mice during anesthesia. EEG (0.3–50 Hz) signals were amplified and filtered, MATLAB (R2020a, MathWorks, USA) software was used for analysis, the interference wave of 50-Hz alternating current signal was filtered out, and data of 0.3–50 Hz were intercepted. EEG power spectra within the frequency range of 0.3–50 Hz were calculated, as were the relative changes in the total power and burst suppression ratio (BSR) of each EEG frequency band (δ [0.3–4 Hz], θ [4–10 Hz], α [10–15 Hz], β [15–25 Hz], and γ [25–50 Hz]).[6]

Arousal scoring

When the mice reached a stable anesthesia state in the optogenetic experiment, 20–22 min after receiving 1.5% sevoflurane anesthesia, they were administered light stimulation and scored according to their body movement, which mainly consisted of five aspects: leg movement, head movement, whisker movement, righting, and walking.[2] No, mild, and moderate body movement was scored as 0, 1, and 2 respectively. If the mice were kept in a supine position, the score was 0. If all four paws of each mouse touched the barrel, the score was 2. If the mice righted or did not move, the score was 0. If the mice crawled but could not lift their abdomen off the bottom of the barrel, the score was 1. If the mice walked and separated their bellies from the barrel, the score was 2. The final awakening behavior score was the sum of all behavioral scores. Each mouse had a maximum score of 10 points. This score was applied equally to chemogenetic manipulations. The entire experimental process was recorded using a video system and scored by two blinded researchers for verification.

Fiber photometry recording

The calcium signaling virus *rAAV2/9-GAD67-GCaMP6s* (OBiO, Shanghai, China) was injected, and a ceramic insert was buried in the DMH of the mice. Four weeks later, the fiber was connected to a single-channel photometry recording system (Thinker Tech, China), the 480 nm excitation optical power was adjusted to 40–50 μW, and the offset calcium signal value F_{offset} was recorded in a black box for 5 min. The ceramic was then connected to an optical fiber, and the calcium signal was recorded during the awake state for 5 min. The 2.0% sevoflurane was maintained for 30 min and then stopped, and the calcium signal was continuously recorded for another 10 min. The calcium signal intensity was expressed as $\Delta F/F = (F_1 - F_0)/F_0\text{-}F_{offset}$. We used MATLAB (R2020a, MathWorks, USA) to analyze the change in $\Delta F/F$ 2 min before and 4 min after turning 2.0% sevoflurane on or off.

Immunohistochemistry

Mice were deeply anesthetized with pentobarbital sodium and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. Brains were successively dehydrated with 20% and 30% sucrose in PBS. Then, 15-μm-thick coronal slices of the brain samples were made using a Leica freezing microtome. Frozen slices were washed by PBS three times and were incubated with 5% donkey serum in PBST (containing 0.3% Triton X-100) for 2 h subsequently primary antibody in antibody diluent at 4°C overnight. The sections were then incubated for 2 h with a secondary antibody. Finally, the slices were cover-slipped using DAPI Fluoromount-G (Southern Biotech, USA) and then observed and captured using a fluorescence confocal microscope (Olympus FV1000, Japan). For c-fos staining, three animals were used in each group, and two brain slices were used for the statistical analysis of each mouse. C-fos/mCherry or c-fos/GFP co-labelled neurons were counted at 500 μm × 500 μm of the target nucleus, which was blinded. Primary antibody: GABA antibody (GeneTex, GTX125988, rabbit, 1:200), anti-c-fos antibody (Abcam, ab208942, mouse, 1:1000), secondary antibody: Alexa Fluor® 488-labelled goat anti-mouse IgG, Alexa Fluor® 488-labelled goat anti-rabbit IgG, Cy3-labelled donkey anti-mouse IgG, and Cy3-labelled donkey anti-rabbit IgG (Servicebio, Wuhan, China).

Temperature measurement

An infrared thermal camera (FOTRIC 348X+, China) was used to measure the temperature inside the ear canal (highest body surface temperature) of mice. In the optogenetic experiment, (*rAAV2/9-GAD67-ChR2-GFP/rAAV2/9-GAD67-GFP* into DMH), body temperature was monitored before anesthesia (0 min), 2 min before illumination (15 min), after illumination (19 min) and after anesthesia (30 min) (Figure S1).

QUANTIFICATION AND STATISTICAL ANALYSIS

The evaluation criteria for anesthesia induction and emergence were based on the loss and recovery time of the righting reflex. Changes in consciousness during maintenance of anesthesia using electroencephalogram (EEG) and arousal behavioral scores under 1.5% and 2.0% sevoflurane anesthesia. Regulation of neuronal activity by optogenetics, chemogenetics and immunofluorescence staining was used to verify the reliability of the virus.

GraphPad Prism 8.0 (GraphPad Software, USA) and SPSS 25.0 Software (IBM, USA) were used for statistical analysis of behavioral results and immunofluorescence counting results. Two-tailed Student's t tests were chosen for comparisons between the two groups. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used. The BSR per minute during anesthesia was measured using two-way ANOVA followed by Post Hoc Bonferroni's multiple order. The two-tailed Mann-Whitney U-test was adopted for behavioral scoring in light stimulation. All measurement data are expressed as the mean ± the standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.