Consciousness can be defined by two major attributes: awareness of environment and self, and arousal, which reflects the level of awareness. The return of arousal after general anesthesia presents an experimental tool for probing the neural mechanisms that control consciousness. Here we have identified that systemic or intracerebral injection of the cannabinoid CB₁ receptor (CB₁R) antagonist AM281 into the dorsomedial nucleus of the hypothalamus (DMH) — but not the adjacent perifornical area (Pef) or the ventrolateral preoptic nucleus of the hypothalamus (VLPO) — accelerates arousal in mice recovering from general anesthesia. Anesthetics selectively activated endocannabinoid (eCB) signaling at DMH glutamatergic but not GABAergic synapses, leading to suppression of both glutamatergic DMH-Pef and GABAergic DMH-VLPO projections. Deletion of CB₁R from widespread cerebral cortical or prefrontal cortical (PFC) glutamatergic neurons, including those innervating the DMH, mimicked the arousal-accelerating effects of AM281. In contrast, CB₁R deletion from brain GABAergic neurons or hypothalamic glutamatergic neurons did not affect recovery time from anesthesia. Inactivation of PFC-DMH, DMH-VLPO, or DMH-Pef projections blocked AM281-accelerated arousal, whereas activation of these projections mimicked the effects of AM281. We propose that decreased eCB signaling at glutamatergic terminals of the PFC-DMH projection accelerates arousal from general anesthesia through enhancement of the excitatory DMH-Pef projection, the inhibitory DMH-VLPO projection, or both.
Endocannabinoid signaling in hypothalamic circuits regulates arousal from general anesthesia in mice

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Introduction

Consciousness is an essential human attribute (1), but the neural mechanism controlling consciousness remains unknown (2, 3). Consciousness consists of two major components: arousal (i.e., the level of consciousness) and awareness of the environment and self (i.e., the content of consciousness). Arousal after cessation of general anesthesia provides an experimental model to investigate the neural mechanism that controls consciousness (3–6), as yearly more than 312 million patients are placed under general anesthesia worldwide (7). Although the exact mechanism underlying arousal from anesthesia is unknown, ample evidence suggests a remarkable similarity between anesthesia-induced unconsciousness and deep sleep (1, 3, 4, 6, 8). The hypothalamus is a key brain region for regulation of sleep and wakefulness (9–13). Specifically, the dorsomedial nucleus of the hypothalamus (DMH) conveys and reorganizes circadian rhythms of sleep and wakefulness from the suprachiasmatic nucleus to both the wake-promoting perifornical area (Pef) and the sleep-promoting ventrolateral preoptic nucleus (VLPO) (9–13). The DMH receives both GABAergic innervation from the suprachiasmatic nucleus (SCN) (9–13) and glutamatergic innervation from the prefrontal cortex (PFC) (14) and then sends GABAergic axons innervating VLPO GABA-containing neurons and glutamatergic axons innervating Pef orexin-containing neurons. Two well-characterized endogenous cannabinoids (endocannabinoids [eCBs]) are 2-arachidonoylglycerol and anandamide (or N-arachidonoylthetanolamine), which are mainly hydrolyzed by monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), respectively (15). It is well established that eCB activation of presynaptic cannabinoid CB1 receptor (CB1R), the most abundant G protein–coupled receptor in the brain (16), inhibits release of glutamate and GABA from glutamatergic and GABAergic axon terminals, respectively (17, 18). However, it is not known whether and how eCB signaling at synapses of glutamatergic and GABAergic axons participates in the pro-

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cess of arousal after general anesthesia. Loss of wakefulness and arousal from anesthesia in rodents can be quantified as the time to loss or resumption of righting responses (LORR or RORR), respectively, following application or cessation of general anesthesia. A recent study showed that CB 1R blockade suppressed LORR following administration of the anesthetic propofol (19), indicating that anesthetic-increased eCB signaling enhances anesthetic induction of unconsciousness. We therefore hypothesized that decreased eCB signaling at PFC-DMH, SCN-DMH, DMH-Pef, and/or DMH-VLPO projections may participate in arousal after general anesthesia.

In the present study, we critically examine this hypothesis by employing behavioral, electrophysiological, electron microscopic, molecular biological, conditional mutagenetic, and chemogenetic strategies. General anesthesia was induced by administration of isoflurane or sevoflurane, two volatile anesthetics commonly used in clinical practice. Our findings suggest that eCB signaling at axon terminals of glutamatergic PFC-DMH projection, but not GABAergic SCN-DMH projection, and subsequent involvement of both glutamatergic DMH-Pef and GABAergic DMH-VLPO projections participate in arousal after general anesthesia.

Results

eCB modulation of arousal from anesthesia through the DMH. Anesthesia was delivered to mice in a standard anesthetic chamber (Supplemental Figure 2) (20). We quantified RORR time (i.e., emergence or recovery time) following a 30-minute exposure to isoflurane or sevoflurane anesthesia, with pharmacological interventions performed 15 minutes before the end of anesthesia. We first quantified recovery time in association with a continuous EEG recording following isoflurane anesthesia. When rats showed RORR, their EEG spectrum displayed a sudden change from a generally high-power to low-power state (Supplemental Figure 3), clearly suggesting the reliability of measuring RORR time for the quantification of brain arousal after cessation of general anesthesia.

To examine whether increased eCB signaling modulates arousal after anesthesia, we injected JZL195 (20 mg/kg, i.p.), an inhibitor of both MAGL and FAAH (15). JZL195 significantly prolonged recovery time after isoflurane anesthesia (Figure 1A). We reasoned that if this longer recovery time resulted from increased eCB signaling, blocking eCB signaling should shorten recovery time. Indeed, an i.p. injection of the CB 1R antagonist AM281 shortened recovery time after isoflurane anesthesia in a dose-dependent manner, with the maximal effect produced by 3 mg/kg (Figure 1B). AM281 is a CB 1R antagonist/inverse agonist; however, the CB 1R neutral antagonist NESS0327 (21) also significantly shortened recovery time after isoflurane anesthesia. All summary graphs show mean ± SEM; n, number of rats in each group. *P < 0.05 and **P < 0.01 vs. sham or Veh, Tukey’s post-hoc test after 1-way ANOVA (A: F_{2,13} = 46.17, P < 0.05; B: F_{2,13} = 46.17, P < 0.01; D: F_{2,11} = 12.13, P < 0.01) or Student’s t test (C and E–G).

Next, we investigated the hypothesis that systemic AM281 accelerates arousal by a direct blockade of CB 1R in the wake-promoting Pef, the sleep-promoting VLPO, or both. This hypothesis is unlikely to be supported, as no significant effects on recovery time were detected after bilateral microinjection of AM281 (0.1 μg/0.3 μl/side) into either the Pef (Figure 1E and Supplemental Figure 4) or the VLPO (Figure 1F and Supplemental Figure 4). Because the DMH plays an essential role in the integration of sleep and wakefulness (9–12), we then probed whether intra-DMH application of AM281 could mimic the effects of systemically injected AM281. Indeed, bilateral intra-DMH application of AM281 (0.1 μg/0.3 μl/side) (Supplemental Figure 4) significantly shortened recovery time (Figure 1G).

Anesthetics enhance eCB signaling at DMH glutamatergic but not GABAergic synapses. To reveal how anesthetics modulate eCB signaling in the DMH, we performed whole-cell patch clamp on hypothalamic slices. We identified two types of DMH neurons with distinct electrical fingerprints, i.e., neurons with or without...
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2297

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The DMH contains a moderate density of CB₁R-immunoreactive axons/terminals (24). Notably, our electron microscopic study showed that approximately 32% of DMH inhibitory synapses and 8% of DMH excitatory synapses were CB₁R-immunopositive (Figure 2, C–F). We then recorded depolarization-induced suppression of inhibition (DSI) or excitation (DSE) at DMH GABAergic or glutamatergic synapses, respectively, because both DSI and DSE are specifically associated with eCB signaling (17, 25, 26). Bath application of isoflurane did not significantly affect DSI in either LTS⁺ or LTS⁻ neurons in the DMH (Figure 3, A, B, and E), suggesting that isoflurane does not significantly affect eCB signaling at GABAergic synapses onto either GABAergic or glutamatergic DMH neurons. In contrast, isoflurane significantly increased DSE in both LTS⁺ and LTS⁻ neurons (Figure 3, C–E), suggesting that isoflurane significantly increased eCB signaling at glutamatergic synapses in both GABAergic and glutamatergic DMH neurons. eCB activation of glutamatergic presynaptic CB₁R inhibits presynaptic release of glutamate, inducing deactivation (i.e., inhibition) of postsynaptic neurons (17, 18). We therefore hypothesized that general anesthesia would suppress both LTS⁺ and LTS⁻ neurons in the DMH in a CB₁R-dependent manner. Indeed, bath application of either isoflurane or sevoflurane onto hypothalamic slices significantly reduced the firing rate of both LTS⁺ and LTS⁻ neurons, which returned to pretreatment levels after washout of isoflurane (Figure 3F) or sevoflurane (Supplemental Figure 7A). In the presence of AM281, however, isoflurane did not significantly affect the firing rate of either LTS⁺ or LTS⁻ neurons (Supplemental Figure 7B).

Blocking CB₁R at DMH glutamatergic synapses accelerates arousal through VLPO and Perl. Based on our electrophysiological findings, we reasoned that if systemic AM281 shortened recovery time via increased release of presynaptic glutamate but not GABA in the DMH, then intra-DMH application of the glutamate N-methyl-d-aspartate receptor (NMDAR) antagonist MK801, but not the GABA receptor (GABAR) antagonist bicuculline, would block the effects of systemic AM281. As expected, bilateral intra-DMH administration of MK801 (0.005 μg/0.3 μl/side), but not bicuculline (0.2 μg/0.3 μl/side) (Supplemental Figure 4), abolished the shortened recovery time induced by systemic AM281 (Figure 4, A and B).

Figure 2. Electrophysiological and ultrastructural characteristics of DMH GABAergic and glutamatergic neurons. (A) Sample current clamp traces (>20 repetitions) show two distinct types of DMH neurons with or without low-threshold spikes, i.e., LTS⁺ or LTS⁻, respectively, elicited when the membrane is depolarized from negative potentials. (B) Sample current clamp traces (>20 repetitions) of LTS⁺ or LTS⁻ (top) were recorded from fluorescent DMH neurons (bottom photos) of GABAergic (GABA) and glutamatergic neurons (Glutamate) from Gad67-GFP and vGlut2-icreERT2 mutant mice, respectively. (C–F) Electron microscopic images (3 mice) show CB₁R-immunoreactive peroxidase reaction product in DMH GABAergic axonal terminals (iT in C and D) or glutamatergic axonal terminals (eT in E and F). Black arrowheads identify symmetric and asymmetric synaptic contacts of presumed GABAergic and glutamatergic nature, respectively, with the neuronal soma profile (Soma) or dendritic profile (D). Scale bars: 0.25 μm.

low-threshold spikes (LTS), referred as LTS⁺ or LTS⁻ neurons, respectively (22) (Figure 2A). Glutamic acid decarboxylase 67 (GAD67) is a GABA biosynthesis enzyme, so we employed transgenic mice expressing EGFP selectively in GAD67-synthesizing neurons (i.e., Gad67-GFP mice) (23) to identify GABAergic neurons under fluorescence microscopy. To identify DMH glutamatergic neurons under fluorescence microscopy, we injected a double-floxed (DIO) Cre recombinase-dependent adenovirus vector fused with mCherry to form AAV-mCherry into the DMH of mice with knockin of improved CreERT2 (iCreERT2) into the Vglut2 locus (hereafter referred to as vGlut2-iCreERT2 mice; Supplemental Figures 5 and 6). Fluorescent DMH cells in Gad67-GFP and vGlut2-iCreERT2 mutant mice were LTS⁺ and LTS⁻ neurons, respectively (Figure 2B), thus representing DMH GABAergic and glutamatergic neurons, respectively.
The DMH sends GABAergic and glutamatergic axons innervating the VLPO and Pef, respectively (9–13). If systemic AM281 selectively increases release of presynaptic glutamate but not GABA in the DMH, both GABAergic DMH-VLPO and glutamatergic DMH-Pef projections should be activated. We therefore hypothesized that blocking either VLPO GABAergic or Pef glutamatergic synaptic neurotransmission would abolish the shortened recovery time induced by systemic AM281. In support of this hypothesis, bilateral intra-VLPO injection of bicuculline (0.2 μg/0.3 μl/side) (Supplemental Figure 4) or bilateral intra-Pef injection of MK801 (0.005 μg/0.3 μl/side) (Supplemental Figure 4), doses that did not significantly affect recovery time on their own, prevented systemic AM281 from shortening recovery time (Figure 4, C and D). Interestingly, systemic injection of either bicuculline (0.5 mg/kg, i.p.) or MK801 (0.02 mg/kg, i.p.), at doses that did not significantly affect recovery time on their own, also prevented sys-
CB1R deletion from PFC glutamatergic neurons accelerates arousal. Our electrophysiological and behavioral results together suggest that activation of CB1R at DMH glutamatergic input participates in arousal from anesthesia. Because DMH neurons receive strong innervation directly from glutamatergic neurons in the prefrontal and infralimbic areas of the PFC (14), we examined the contributions of CB1R at glutamatergic PFC-DMH projection to arousal by taking advantage of conditional mutagenesis to selectively delete CB1R from glutamatergic neurons. Glutamatergic neurons in the cerebral cortex, including the PFC, contain the glutamate transporter vGLUT1 (27). CBIR-floxed mice (CBIRfl/fl) (28) were crossed with vGLUT1-iCreERT2 mutant mice with knockin of iCreERT2 into the Vglut1 locus (Supplemental Figures 8 and 9) to generate a vGLUT1-CB1R-KO mouse line. Electron microscopy studies confirmed the absence of CB1R immunoreactivity selectively in vGLUT1-immunopositive glutamatergic terminals in the DMH (Figure 5, A–C), suggesting an inducible deletion of CB1R from glutamatergic axon terminals of the PFC-DMH projection in vGLUT1-CB1R-KO mice. Relative to vGLUT1-CB1R-WT mice, vGLUT1-CB1R-KO littermates recovered significantly faster after anesthesia (Figure 5D).

These results are not conclusive, however, because of vGLUT1’s widespread expression in glutamatergic neurons throughout the cerebral cortex, including the PFC. To overcome this potential limitation, we further examined the effects of selective deletion of CB1R from glutamatergic PFC neurons on arousal from anesthesia. AAV-CaMKII-iCre-GFP was injected bilaterally into prelimbic and infralimbic PFC in CB1R-floxed mice and their WT littermates (Supplemental Figure 10), i.e., PFC-CB1R-flox and PFC-CB1R-WT littermates. Electron microscopy study confirmed the absence of CB1R immunoreactivity in many vGLUT1-immunopositive glutamatergic terminals in the DMH (Figure 5, E–G), suggesting an effective deletion of CB1R in axon terminals of the glutamatergic PFC-DMH projection. Relative to PFC-CB1R-WT mice, PFC-CB1R-flox littermates recovered significantly faster after anesthesia (Figure 5H).

Our initial findings that intra-Pef application of the CB1R antagonist AM281 did not significantly affect arousal from anesthesia suggest that CB1R in the glutamatergic DMH-Pef projection from glutamatergic axon terminals of the PFC-DMH projection accelerates arousal through VLPO and Pef. Both high and low doses of the GABA antagonist bicuculline and the NMDAR antagonist MK801 were used in order to reveal their dose-response effects on arousal after anesthesia. (A–D) Relative to vehicle (Veh), AM281 (AM, 3.0 mg/kg, i.p.) significantly shortens recovery time, which is not significantly affected by bilateral intra-DMH microinjection of bicuculline (A: 0.2 μg(side/injection)). Bilateral intra-DMH microinjection of MK801 (B: 0.005 μg(side/injection)), intra-VLPO microinjection of bicuculline (C: 0.2 μg(side/injection)), and intra-Pef microinjection of MK801 (D: 0.005 μg(side/injection)) – which do not significantly affect recovery time on their own – prevents AM281 (3 mg/kg, i.p.) from shortening recovery time. (E and F) Relative to Veh, AM significantly shortens recovery time, which is blocked by an i.p. injection of 0.5 mg/kg bicuculline (E) or 0.02 mg/kg MK801 (F) that does not significantly affect recovery time on its own. All summary graphs show mean ± SEM; n, number of rats in each group. *P < 0.05 and **P < 0.01 vs. Veh, Tukey’s post-hoc test after 1-way ANOVA: A: F4,23 = 22.6, P < 0.01; B: F4,32 = 29.101, P < 0.01; C: F4,24 = 32.101, P < 0.01; D: F4,25 = 15.025, P < 0.01; E: F4,26 = 34.053, P < 0.01; F: F4,27 = 19.273, P < 0.01.)
is not required for arousal after anesthesia. To confirm these findings, we further studied the effects of selective deletion of CB1R from vGLUT2-containing glutamatergic neurons in the hypothalamus (27). The CB1R-floxed mice (28) were crossed with vGLUT2-iCreERT2 mice as described above to generate the vGLUT2-CB1R–KO mouse line. Electron microscopy study confirmed the absence of CB1R immunoreactivity in vGLUT2-immunopositive glutamatergic terminals in the Pef (Figure 5, I–K). vGLUT2-CB1R–KO and vGLUT2-CB1R-WT littermates showed similar recovery time after anesthesia (Figure 5L).
Our electrophysiological and behavioral results suggest that systemic AM281-accelerated arousal does not require eCB signaling in either DMH GABAergic synapses or GABAergic DMH-VLPO projection. To confirm this observation, we examined the effects of a selective deletion of CB1R from GABAergic neurons in the brain. GAD65 is a GABA biosynthetic enzyme in GABAergic neurons. CB1R-floxed mice (28) were crossed with GAD65-iCreERT2 mutant mice with knockin of iCreERT2 into the GAD65 locus (Supplemental Figure 11) to generate a GAD65-CB1R-KO mouse line. Electron microscopy study confirmed the absence of CB1R signaling in GABAergic neurons.
immunoreactivity in GABAergic axon terminals in the DMH of GAD65-CR-R-KO mice (Figure 5, M and N). GAD65-CR-R-KO and GAD65-CR-R-WT littermates showed similar recovery times after anesthesia (Figure 5O), thus supporting our electrophysiological and behavioral data obtained in WT rats.

**PFC-DMH-Pef/VLPO inactivation counteracts AM281's effects on arousal.** Our behavioral and electrophysiological studies suggest that AM281-mediated activation of PFC-DMH, DMH-Pef, and DMH-VLPO projections accelerates arousal from anesthesia. Thus, selective inactivation of these projections could counteract AM281's effects. To examine this hypothesis, we employed a combination of designer receptors exclusively activated by designer drugs (DREADD) technology and the pseudorabies virus (PRV) vector expressing Cre recombinase (PRV-Cre). To generate a DREADD, we mutated muscarinic receptors to be activated by clozapine N-oxide (CNO) but not by their natural ligands (29–31). DREADD was then delivered by an AAV expressing a doubly floxed inverted open reading frame, which was fused with mCherry to form AAV-DREADD-mCherry. Thus, DREADD expression could be monitored, and stable transgene inversion was achieved with the FLEX Switch (Figure 6A) (32). After intra-VLPO injection, PRV-Cre entered axonal terminals and then underwent retrograde transport (33, 34) to DMH neurons (Figure 6B). As a thymidine kinase gene–null viral vector, the PRV-Cre used here.
is unable to replicate in neurons or to be transmitted trans-synaptically and does not produce detectable deleterious effects on infected neurons or animals (33). When AAV-DREADD-mCherry was injected into the DMH, mCherry was detected in those DMH neurons projecting to the VLPO that had received PRV-Cre injection (Figure 6, B and C).

To inactivate DMH-VLPO projection, we employed Gq-coupled hM4Di receptors, whose activation by CNO stimulates G protein–coupled inwardly rectifying potassium channels, resulting in membrane hyperpolarization and subsequent neuronal silencing (29–31). Rats received intra-VMH injection of PRV-Cre and intra-DHM injection of AAV-hm4Di-mCherry (Supplemental Figure 12). Patch clamp recording revealed that bath application of CNO to hypothalamic slices significantly decreased the firing rate of mCherry-labeled DMH neurons (Figure 6D), suggesting that CNO activation of AAV-hm4Di-mCherry inactivates the DMH neurons innervating the VLPO. As expected, an i.p. injection of CNO in these rats prevented systemic AM281 from shortening recovery time (Figure 6E). Similar results were observed in rats receiving intra-PFC injection of PRV-Cre and intra-DHM injection of AAV-hm4Di-mCherry (Figure 6F and Supplemental Figure 13) or intra-DHM injection of PRV-Cre and intra-PFC (i.e., prelimbic and infralimbic areas of the PFC) injection of AAV-hm4Di-mCherry (Figure 6G and Supplemental Figure 14).

**PFC-DMH-Pef/VLPO activation enhances arousal.** We next examined whether selective activation of DMH-VLPO, DMH-Pef, or PFC-DMH projection could mimic the effects of AM281 on arousal from anesthesia. To activate DMH-VLPO projection, we employed Gq-coupled hM3Dq receptors, as their activation by CNO induces membrane depolarization and subsequent neuronal activation (29–31). Rats received intra-VMH injection of PRV-Cre and intra-DHM injection of AAV-hm3Dq-mCherry (Supplemental Figure 12). Patch clamp recording revealed that bath application of CNO to hypothalamic slices significantly increased the firing rate of mCherry-labeled DMH neurons (Figure 7A), suggesting CNO activates DMH-VLPO projection. As expected, an i.p. injection of CNO significantly shortened recovery time (Figure 7B). Similar results were found in rats receiving either intra-PFC injection of PRV-Cre and intra-DHM injection of AAV-hm3Dq-mCherry (Figure 7C and Supplemental Figure 13) or intra-DHM injection of PRV-Cre and intra-PFC injection of AAV-hm3Dq-mCherry (Figure 7D and Supplemental Figure 14). However, rats treated with AM281 (3 mg/kg, i.p. or its vehicle after receiving intra-DHM injection of PRV-Cre and intra-PFC injection of AAV-hm3Dq-mCherry did not show a significant difference in recovery time (Figure 7E and Supplemental Figure 14), indicating the bottom or floor effects produced by AM281 injection or DREADD-mediated activation of PFC-DMH projection.

These results are inconclusive for two reasons. First, because the same DMH neurons innervate both the Pef and other brain regions (13), CNO stimulation of these neurons could activate the Pef and other brain regions. Second, while approximately 59% of DMH-Pef projections were glutamatergic immunoreactive (13), CNO activation of DMH neurons retrogradely labeled by intra-Pef-injected PRV-Cre could activate both glutamatergic and non-glutamatergic DMH neurons. To overcome these potential problems, we studied the effects of selective activation of axon terminals of the glutamatergic DMH-Pef projections. When AAV-hm4Di was injected into a brain region, it entered neuronal cell bodies and then was anterogradely transported to their axon terminals where CNO was administered to activate hM4Di, hence inhibiting presynaptic release of neurotransmitters (35, 36). After intra-DHM injection of AAV-hm3Dq-mCherry in vGLUT2-iCreERT2 mice, mCherry was detected in DMH cells (Figure 7F), suggesting iCre-mediated inversion and expression of the hm3Dq in glutamatergic neurons. Ten days later, the mice were killed for patch clamp recordings of glutamatergic DMH-Pef projection. Bath application of isoflurane onto hypothalamic slices significantly reduced the amplitude of the first excitatory postsynaptic currents (EPSCs) and significantly increased the paired pulse ratio (PPR), changes that were reversed by CNO (Figure 7G and H). In another group of vGLUT2-iCreERT2 mice receiving intra-DHM injection of AAV-hm3Dq-mCherry, bilateral intra-Pef injection of CNO significantly shortened recovery time (Figure 7I and Supplemental Figure 15). Collectively, the morphological, electrophysiological, and behavioral data shown in Figure 7, F–I, suggest that after intra-DHM injection in vGLUT2-iCreERT2 mice, hM3Dq was expressed in DMH glutamatergic neurons and then anterogradely transported to their axon terminals in the Pef, where CNO activation of presynaptic hM3Dq increased release of glutamate at glutamatergic DMH-Pef synapses. As expected, we also observed that bilateral intra-DHM injection of CNO significantly shortened recovery time in vGLUT1-iCreERT2 mice receiving bilateral intra-PFC injection of AAV-hm3Dq-mCherry 2 weeks before CNO injection (Figure 7J and Supplemental Figure 16).

**Discussion**

It is unknown whether eCB signaling participates in arousal from general anesthesia. We observed here that increased eCB signaling by the eCB degradation inhibitor JZL195 delayed arousal after general anesthesia, whereas the CB1R antagonists AM281 and NESP0327 accelerated arousal. Application of AM281 into the DMH mimicked the effects of systemic injection, suggesting that decreased eCB signaling in the DMH participates in arousal from anesthesia. GABAergic SCN-DMH projection is required for circadian rhythms of sleep and wakefulness (9, 11–14). Feeding behavior requires integration through eCB activation of CB1R at GABAergic and excitatory synapses in the DMH. Thus, DMH CB1Rs from glutamatergic DMH neurons, as both GABAergic and DSE from glutamatergic DMH neurons, as both GABAergic and glutamatergic DMH neurons are all likely targets of eCB. This idea is supported by induction of DSI from GABAergic and DSE from glutamatergic DMH neurons, as both DSI and DSE are mediated by eCB signaling (17, 25, 26).

Surprisingly, isoflurane did not significantly affect DSI recorded from either GABAergic or glutamatergic DMH neurons, suggesting that general anesthetics do not significantly affect eCB signaling at GABAergic inputs onto DMH neurons, including the GABAergic SCN-DMH projection. Therefore, arousal from anesthesia is unlikely to require decreased eCB signaling at GABA-
ergic inputs onto DMH neurons. Indeed, intra-DMH injection of the GABAR antagonist bicuculline did not significantly affect systemic AM281-accelerated arousal. The insignificant contributions of eCB signaling at DMH GABAergic synapses, including the GABAergic SCN-DMH projection, to arousal is further supported by our observation that an inducible deletion of CBIR selectively from forebrain GABAergic neurons in GAD65-CB,R-KO mice did not significantly affect arousal.

In contrast to DSI, DSE recorded from both GABAergic and glutamatergic DMH neurons was significantly enhanced by isoflurane, strongly suggesting that anesthetics enhance eCB signaling at DMH glutamatergic synapses, although the mechanisms underlying isoflurane's increase of DSE, but not DSI, are unknown. Notably, intra-DMH injection of the NMDAR antagonist MK801 blocked systemic AM281-accelerated arousal, suggesting that decreased eCB signaling in the DMH during arousal from anesthe-
sia occurs in glutamatergic inputs into the DMH. Because the DMH receives strong glutamatergic innervation from the prelim-
bic and infralimbic areas of the PFC (14), we hypothesized that decreased eCB signaling during arousal occurs at axon terminals of glutamatergic PFC-DMH projection. Indeed, we observed that a selective deletion of glutamatergic CBIR either from the entire cerebral cortex or from the PFC mimicked the effects of system-
ic and intra-DMH application of AM281. Because decreased eCB signaling at axon terminals of glutamatergic PFC-DMH projection could increase release of glutamate, inactivation or activation of PFC-DMH projection, respectively, would counteract or mimic the arousal-accelerating effects of AM281. This idea is supported by data showing that AM281's effects on arousal were counteracted by inactivation of neurons of the PFC-DMH projection and mimicked by activation of either somata or glutamatergic axon terminals of the PFC-DMH projection.

The DMH is the largest source of inputs to both the VLPO and Pef (9, 11, 12). Approximately two-thirds of DMH-VLPO projec-
tions are GABAergic, and approximately two-thirds of DMH-Pef projections are glutamatergic (13). Our findings that intra-VLPO or intra-Pef injection of AM281 did not significantly affect arousal exclude a significant role of eCB signaling at either GABAergic VLPO or glutamatergic Pef synapses in arousal. This idea is further supported by the finding that arousal was not significantly affected by inducible deletion of CBIR from either GABAergic neurons in GAD65-CB,R-KO mice or hypothalamic glutamatergic neurons in vGLUT2-CBIR-KO mice. These results also suggest that the very low level of CB,R in somata of DMH neurons, as indicated by immunohistochemical staining (37), plays an insignificant role in arousal from anesthesia.

However, our findings that systemic AM281-accelerated arousal was abolished after either intra-VLPO injection of bicuculline or intra-Pef injection of MK801 suggest the association of GABAergic DMH-VLPO and glutamatergic DMH-Pef projections with arousal from anesthesia. This idea is also supported by the finding that inactivation of either DMH-VLPO or DMH-Pef projection counteracted systemic AM281-accelerated arousal. GABAergic neurons in the VLPO promote sleep through inhibition of the main ascending arousal nuclei in the brainstem, whereas orexinergic neurons in the Pef promote wakefulness via widespread excitation throughout the brain, including the brainstem ascending arousal system (1, 9). We therefore propose that activation of the sleep-promoting inhibitory VLPO neurons and wake-promoting excitatory Pef neurons suppresses and promotes arousal, respectively. If activation of excitatory PFC-DMH projection participates in arousal from anesthesia, the accelerated arousal could result from enhancement of GABAergic DMH-VLPO projection, glutamatergic DMH-Pef projection, or both. This is because enhancement of inhibitory DMH-VLPO projection could inhibit the arousal-suppressing VLPO, hence accelerating arousal, which could also result from activation of the arousal-promoting Pef following enhancement of excitatory DMH-Pef projection. Indeed, we found that activation of DMH-VLPO projection or activation of either cell bodies or glutamatergic axon terminals of the DMH-Pef projection significantly accelerated arousal.

Our results clearly suggest that during the natural process of arousal after cessation of anesthesia, any driving force to pro-
mote arousal, such as enhancement of PFC-DMH, DMH-VLPO, or DMH-Pef projection alone, is able to accelerate arousal. How-
ever, AM281-accelerated arousal may require a simultaneous enhancement of multiple brain circuitries, including PFC-DMH, DMH-VLPO, and DMH-Pef projections. Therefore, systemic AM281-accelerated arousal was abolished after systemic or intra-VLPO injection of bicuculline; after systemic or intra-Pef injection of MK801; or after chemogenetic-mediated inactivation of PFC-DMH, DMH-VLPO, or DMH-Pef projection. If activation of glutamatergic PFC-DMH, glutamatergic DMH-Pef, and GABAergic DMH-VLPO projections accelerates arousal, blockade or inactivation of each projection may delay arousal after anesthe-
sia. Unexpectedly, we found recovery time after anesthesia was not significantly prolonged after systemic or intra-VLPO injection of bicuculline, after systemic or intra-Pef injection of MK801, or after chemogenetic-mediated inactivation of PFC-DMH, DMH-VLPO, or DMH-Pef projection. After the cessation of anes-
thesia, multiple mechanisms, such as the simultaneous involve-
ment of multiple brain circuitries, may be necessary for arousal. Therefore, blockade or inactivation of the PFC-DMH-Pef/ VLPO circuitry alone may not strong enough to prolong arousal time after anesthesia. Accordingly, our finding that increased brain eCB signaling after a systemic injection of JZL195 prolonged recovery time indicates that the increased eCB signaling to pro-
long arousal may occur in multiple brain circuitries including the PFC-DMH-Pef/VLPO projection.

Here we employed a combination of behavioral, ultrastructural, electrophysiological, conditional mutagenesis, and chemogenetic strategies to investigate the questions of whether and how eCB signaling in PFC-DMH-VLPO/Pef circuitry modulates arousal. We provide evidence suggesting that after cessation of anesthesia, the driving force to increase eCB signaling disappears, leading to decreased eCB signaling at a specific hypothalamic node, i.e., the DMH glutamatergic synapses connecting the PFC-DMH, DMH-VLPO, and DMH-Pef circuitries. Decreased eCB signaling increases release of glutamate from axon termi-

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In this study, we employed iCreERT2 mutant mice for electrophysiological, behavioral, and chemogenetic studies. The transgenic strategy has been used over the past couple decades for generation of traditional Cre mice, but it is now known that both the transgenic strategy and traditional Cre have potential problems. For example, the traditional bacteriophage-derived Cre is not optimal for eukaryotes due to its codon usage of prokaryotes, and its 65 CpG dinucleotides may induce epigenetic silencing (38). To overcome these potential problems, we generated vGLUT1-iCreERT2, vGLUT2-iCreERT2, and GAD65-iCreERT2 mutant mice with a knockin of iCreERT2 (38, 39) into the Vglut1, Vglut2, or GAD65 locus. Mice received 8 daily injection of tamoxifen 1 week before use (40). In the case of intra-DMH injection of AAV-mCherry into vGLUT2-iCreERT2 mice, red fluorescent cells were detected in the DMH, signifying the successful inversion and expression of the mCherry and EYFP by Cre. This finding was confirmed with in situ hybridization of Vglut1 or Vglut2 and iCre mRNAs in the PFC and DMH.

The present study relies heavily on a large number of experiments with intracerebral microinjections of various receptor agonists or antagonists, AAV vectors, or vehicle. In order to minimize the potential problem of acute reaction following intracerebral insertion of injection needle, we collected behavioral data either acutely after an intracerebral administration of various receptor agonists or antagonists or vehicle through the guide cannula implanted 1–2 weeks in advance, or 1–2 weeks after intracerebral application of AAV vectors or vehicle. To achieve the goal of restricting the treatment within the boundaries of the VLPO, Pef, DMH, or PFC, a well-trained experimenter steadily injected a 0.3 μl volume in 5 minutes. We excluded all animals with an injection site outside the target brain region. Although we cannot exclude the possibility that a small amount of injected agent spread outside the target brain region, the major effects were likely achieved through the effective action of injected agents within the target brain region. This idea is supported by our finding that the same amount of AM281 produced significant behavioral effects after intra-DMH injection but not after intra-Pef injection, even though these two hypothalamic regions are adjacent (Supplemental Figure 4).

Methods

Animals. Behavioral and electrophysiological studies employed male Sprague-Dawley rats (Charles River) weighing 250–300 g and 75–100 g, respectively. Electron microscopy studies used adult male C57BL/6 mice (Charles River) weighing 25–30 g. Rats and mice were housed in groups of 2 and 4, respectively, in standard cages under standard laboratory conditions (12-hour light/12-hour dark cycle, 22 ± 2°C, food and water ad libitum). Animals were allowed to adapt to laboratory conditions for at least 1 week and adapt to the testing room for at least 30 minutes before behavioral tests.

Generation of mutant mice. CBIR-floxed mice were produced as described previously (29). vGLUT1-iCreERT2, vGLUT2-iCreERT2, and GAD65-iCreERT2 mutant mice were generated by Biocytogen.
with the same procedures. Briefly, to make the targeting construct, the appropriate regions were designed and cloned from BAC library to be the homologous arms. For vGLU1 targeting construct, the 5.8-kb of genomic fragment from the upstream of exon 2 to the downstream of exon 11 and the 6.7-kb fragment beginning from the 3' UTR of the Vglut1 gene (also known as Slc17a7) were cloned. For vGLU2 targeting construct, the 5' homologous region is a 9.0-kb fragment containing Vglut2 exons 9, 10, and 11 and the intron sequences, and the 3' homologous region is a 4.9-kb one containing the Vglut2 3' UTR. For GAD65-iCreERT2 mice, the 5' and 3' homologous regions are an 8.5-kb fragment spanning exons 13, 14, and 15, and a 4.0-kb fragment beginning from the 3' UTR of GAD65 gene, respectively. To avoid disruption of normal gene expression, 2A-CreERT2 was introduced between the coding sequence of the last exon of each gene and its 3' UTR. The final targeting vector included the 5' homologous region, a neomycin phosphotransferase expression (Neo) cassette with two FRT sites flanked, the coding sequence of the gene, respectively. To avoid disruption of normal gene expression, the linearized targeting construct obtained with the restriction endonuclease indicated in Supplemental Figures 5, 8, and 11 was electroporated into embryonic stem (ES) cells (Biocytogen) with a C57BL/6 background. Ran-sor integrations will be negatively selected by diphtheria toxin A (Invitrogen, AM2546) in buffer contained 50% (vol/vol) deionized formamide (Sigma-Aldrich, 1× Denhardt’s solution (Sigma-Aldrich, D2532), and 10% wt/vol dextrose sulfate (Sigma-Aldrich, D8906). After washes with a series of saline sodium citrate (SSC) solutions, the sections were counterstained with 1 μg/ml DAPI (Thermo Fisher Scientific, D1306) for 10 minutes and were coverslipped with SlowFade Gold antifade reagent (Invitrogen, MP 32956). Photographs were taken with an Olympus BX51 microscope or Zeiss confocal microscope. No hybridization signal was detected when sense RNA probes were used. The distribution of Vglut1 and Vglut2 mRNA in the mouse brain was compared with that reported in a recent study (43).

**Quantification of recovery time from anesthesia.** Five minutes before anesthesia, an animal was placed into a rotating Plexiglas anesthesia chamber (Supplemental Figure 2) (20) connected to an isoflurane or sevoflurane vaporizer (Ohmeda). Anesthesia was 1.4% isoflurane or 2.4% sevoflurane in 100% oxygen, which was continuously delivered into the chamber at 1.5 l/min for 30 minutes. Immediately after cessation of anesthesia, pure oxygen was continuously delivered at 1.5 l/min into the chamber, where the rat was placed in a dorsal recumbent position on the floor of the chamber for quantification of RORR or recovery time, i.e., the time from cessation of isoflurane administration to resumption of a prone position.

**EEG recording.** Rats were anesthetized with 1.5%–2% isoflurane (vol/vol) and placed in a stereotaxic frame. Body temperature was kept constant throughout the operation by using a heating pad. After anesthesia was established, the skin was incised to expose the skull and the overlying connective tissue removed. Two pairs of stainless steel screws were implanted bilaterally over the frontal cortex (antero-posterior [AP] 3.9, medial-lateral [ML] ±2 mm) and occipital cortex (AP–7.4 mm, ML±5 mm) (44). The screws and electrodes were secured to the skull using dental cement. After surgery, rats were allowed to recover for at least 1 week before experiments. Rats were acclimatized to the EEG recording cable for 30 minutes, during which EEG was recorded with a sampling frequency of 0–150 Hz using Powerlab 16/35 amplifier system (PL3516, ADInstruments) and Labchart Pro v8.0.10 (ML160/8, ADInstruments). Then the rats were transferred into the Plexiglas anesthesia chamber for isoflurane anesthesia for 30 minutes as described above. Vehicle for AM281 was injected 15 minutes before the end of anesthesia. EEG was recorded continuously until 15 minutes after RORR. EEG spectrum analysis and spectrograms were performed by using MATLAB software (Mathworks).

**Chemical/viral treatment.** For consciousness recovery study, animals received an i.p. injection or an intra-hypothalamic (i.h.) injection of a chemical, virus vector, or vehicle 15 minutes before the cessation of isoflurane anesthesia, with or without i.p. or i.h. administration of another chemical or vehicle 25 minutes before anesthesia cessation. The following chemicals were purchased from Tocris Bioscience and dissolved in physiological saline, unless otherwise stated: AM281 (0.3, 1.5, or 3 mg/kg, i.p.; 0.1 μg/0.3 μl/side, i.h.; dissolved in 5% Tween-80, 5% DMSO, and 90% physiological saline); NESS0327 (Cayman Chemical; 0.3 mg/kg, i.p.; dissolved in 5% Tween-80, 5% DMSO, and 90% physiological saline) (21); MK801 (0.02 or 0.2 mg/kg, i.p.; 0.02 or 0.005 μg/0.3 μl/side, i.h.); bicuculline (0.5 or 2 mg/kg, i.p.; 0.2, 0.4, or 4 μg/0.3 μl/side, i.h.); JZL195 (20 mg/kg, i.p.; dissolved in 5% Tween-80, 5% DMSO, and 90% physiological saline). All AAV viruses, including AAV8-hSyn-DIO-mCherry (AAV-mCherry), AAV8-hSyn-DIO-hM4Di-mCherry (AAV-hM4Di-mCherry), and

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**Research Article**

**GAD65-iCreERT2 mutant mice to generate vGLUT1-CB1R-KO, -floxed mice**

CB1R into C57BL/6 blastocysts. Chimeric mice were generated and bred...
AAV8-hSyn-DIO-hM3Dq-mCherry (AAV-hM3Dq-mCherry), were purchased from the University of North Carolina Gene Therapy Center, Vector Core. Aliquots of virus vectors were stored at –80°C before an i.h. microinjection. PRV-Cre stocks were prepared and titrated as described previously (45), except that each 10-cm dish of virus stock was resuspended in 500 μl and stored in 50-μl aliquots at –80°C. On the day of surgery, stocks were thawed, cell debris was pelleted by centrifugation at high speed for 1 minute in a benchtop centrifuge, and the supernatant was carefully taken for injection.

Under isoflurane anesthesia, two guide cannulae (33-gauge, Plastics One) were implanted into the bilateral DMH (AP −3.2, ML ±0.75, dorsal-ventral [DV] −8.8 mm), VLPO (AP −0.4, ML ±1, DV −9 mm), PeF (AP −3.1, ML ±1.4, DV −8.6 mm), or PFC (AP +3.7, ML ±0.7, DV −4.8 mm), after which the cannulae were affixed to the skull with screws and dental acrylic and then filled with stylettes. One week later, animals received isoflurane anesthesia for quantification of recovery time, during which an i.h. injection of 0.3 μl of the chemical or vehicle was performed in a 5-minute period. For chemogenetic study, rats were anesthetized to receive an i.h. injection of 0.5 μl PRV-Cre (6.0 × 10^6 to 7.0 × 10^6 genome copies per ml), AAV-hM4Di-mCherry or AAV-hM3Dq-mCherry (4.6 × 10^12 genome copies per ml) (46), or AAV-mCherry into bilateral DMH, VLPO, PeF, or PFC at a speed of 0.1 μl/min. Seven or 14 days after surgery, rats were given CNO (Cayman Chemical; 5 mg/kg, i.p.; dissolved in DMSO), followed 1 hour later by isoflurane anesthesia for quantification of recovery time. Finally, all rats were perfused transcardially with 4% PFA. Brains were sectioned to identify cannula placement and/or the location of mCherry fluorescence expression.

The vGLUT2-iCreERT2 and vGLUT1-iCreERT2 mice used for chemogenetic study of the activation of DMH-Pef or PFC-DMH terminals received bilateral intra-DMH injection (AP −1.7, ML ±0.5, DV −5.6 mm) or intra-PFC injection (AP +1.7, ML ±0.4, DV −3.35 mm) of AAV-hM3Dq-mCherry and implantation of cannulae into the PeF (AP −1.9, ML ±1.0, DV −4.8 mm) or DMH (AP −1.7, ML ±0.5, DV −4.6 mm) as described above. Two weeks after surgery, vGLUT2-iCreERT2 mice received intra-Pef administration of CNO (10 μM/0.2 μl/side) and vGLUT1-iCreERT2 mice received intra-DMH administration of CNO 4 hours before experiments to study consciousness recovery after anesthesia.

The CBIR-fixed mice used for AAV-mediated deletion of CBIR in PFC glutamatergic neurons received bilateral intra-PFC injection (AP +1.7, ML ±0.4, DV −3.35 mm) of AAV-CaMKII-iCre-GFP (a gift from Liping Wang, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China) and 6 weeks later underwent electron microscopic study or behavioral study to quantify the recovery time after isoflurane anesthesia.

Electron microscopy analysis. See our recent study (29) for detailed protocols. Briefly, animals were transcardially perfused under anesthesia with 100 ml of 0.1% glutaraldehyde, 4% formaldehyde, and 15% saturated picric acid in phosphate buffer (PB, pH 7.4). Coronal hypothalamic vibrosections were cut at 50 μm. After being freeze-thawed with liquid nitrogen, the sections were incubated for 1 hour in 15% saturated picric acid in phosphate buffer (PB, pH 7.4). Coronal hypothalamic vibrosections were cut at 50 μm. After being freeze-thawed with liquid nitrogen, the sections were incubated for 1 hour in 15% saturated picric acid in phosphate buffer (PB, pH 7.4). Coronal hypothalamic vibrosections were cut at 50 μm. After being freeze-thawed with liquid nitrogen, the sections were incubated for 1 hour in 15% saturated picric acid in phosphate buffer (PB, pH 7.4). Coronal hypothalamic vibrosections were cut at 50 μm. 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reversible, isoflurane or sevoflurane solution was replaced with normal ACSF at 4 minutes after application. To test whether isoflurane inhibition of firing rate was CB1R dependent, we applied ACSF solution containing AM281 (1 μM) onto brain slices, and AM281 solution was present throughout the whole experiment. To identify DMH glutamatergic neurons in the vGLUT2-iCreERT2 mice, young mice received a microinjection of AAV-mCherry into the DMH at a rate of 0.15 μl/min for 2 minutes; 1 week later hypothalamic slices were prepared as described above. To reveal chemogenetic functioning, young rats received a microinjection of AAV-hM3Dq-mCherry into the DMH (AP +3.0, ML ±0.6, DV –8.9 mm) and PRV-Cre into the VLPO (AP –0.3, ML ±0.9, DV –8.1 mm) at a rate of 0.15 μl/min for 2 minutes. One week later, rats were killed for preparation of hypothalamic slices as described above, and mCherry-positive DMH neurons were identified and recorded. After acquisition of stable recordings for 5–7 minutes, slices were perfused with ACSF solution containing 1 μM CNO. Changes in membrane potential and spike activity were recorded, measured, and plotted with a pCLAMP 10.0 and GraphPad Prism 6.0. To reveal terminal release properties of DMH-PeF projection in vGLUT2-iCreERT2 mice, a stable whole-cell recording configuration was established on a PeF neuron, and a second stimulation glass electrode (2–6 MΩ) filled with 2 M NaCl was placed about 100 μm away from the patched neuron. Paired pulse facilitation (PPF) or depression (PPD) was collected by voltage clamp recordings using a stimulus pulse with a single square wave every 10 seconds. Paired-pulse responses were performed at intervals of 100 ms. PPR was measured as the amplitude ratio of the second to the first postsynaptic response from an average of 10 pairs of pulses. The data were acquired with pCLAMP 10.4 (Molecular Devices), and measured and plotted with Clampfit 10.4 and GraphPad Prism 6.0.

Statistics. Results are reported as mean ± SEM. Statistical analysis of the data was performed using 2-tailed Student’s t test, 1-way ANOVA, followed by Tukey’s post-hoc test. Statistical significance was set at P < 0.05.

Study approval. The present studies in animals were reviewed and approved (ACC-2012-004) by the Animal Care Committee of the University of Ottawa Institute of Mental Health Research at the Royal, Ottawa. Similar procedures were also approved by the Animal Care Committee of the Fourth Military Medical University, Xi’an.

Author contributions. HZ, LT, NG, FG, YL, RX, XL, JL, RB, LEP, FW, CXL, YR, XW, and JL collected the data and performed the analyses. HZ, LT, NG, FG, RX, RB, LEP, KM, SXW, ZX, LX, JL, HD, LX, and XZ wrote the manuscript. ZX, LX, and HD supervised the project.

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