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# Selective optogenetic activation of orexinergic terminals in the basal forebrain and locus coeruleus promotes emergence from isoflurane anaesthesia in rats

Dan Wang[†], Yongxin Guo[†], Huiming Li, Jiannan Li, Mingzi Ran, Juan Guo, Lu Yin, Shiyi Zhao, Qianzi Yang[**] and Hailong Dong[*]

Department of Anaesthesiology and Perioperative Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, China

[**]Corresponding author. E-mails: qianziyang@hotmail.com, hldong6@hotmail.com

[†]These authors contributed equally to the paper.

## Abstract

**Background:** The neuropeptide orexin promotes arousal from general anaesthesia, however the neuronal circuits that mediate this effect have not been defined. We investigated whether orexinergic neurones modulate the basal forebrain (BF) and locus coeruleus (LC) in emergence from anaesthesia.

**Methods:** *Hcrt*$^{cre}$ rats were generated using a CRISPR/Cas9-based approach. Viruses encoding optogenetic probes were injected into the perifornical lateral hypothalamic (PeFLH) area, optogenetic fibres were embedded in the PeFLH, BF, or LC, and changes in anaesthesia state under 1.4 vol% or 0.8 vol% isoflurane were determined.

**Results:** In the PeFLH, 98.8% (0.4%) of orexin-A-positive cells expressed tdTomato, and 91.9% (2.2%) of tdTomato cells were orexin-A-positive. Under 1.4 vol% isoflurane anaesthesia, compared with control groups, burst suppression ratio was less, and emergence time was shorter in groups with optogenetic activation of orexinergic cell bodies in the PeFLH (923 [162] *vs* 493 [68] s, $P$=0.0003) or orexinergic terminals in the BF (937 (122) *vs* 674 (108) s, $P$=0.0049) or LC (913 [128] *vs* 742 [76] s, $P$=0.022). Optical stimulation of orexinergic terminals in the BF and LC also improved the movement scores of rats under 0.8 vol% isoflurane anaesthesia.

**Conclusions:** Activation of orexinergic terminals in the FB or LC mediates facilitation of emergence from anaesthesia by orexinergic neurones during isoflurane anaesthesia.

**Keywords:** basal forebrain; isoflurane; locus coeruleus; mechanisms of anaesthesia; optogenetics; orexin

### Editor's key points

- Orexin can promote arousal from general anaesthesia, but the neuronal pathways that mediate this effect are not clear.
- The involvement of orexinergic neurones in modulating the basal forebrain and locus coeruleus in emergence from isoflurane anaesthesia were studied using optogenetic approaches.
- Optogenetic activation of orexinergic neurones in the perifornical lateral hypothalamic area or their terminals in the basal forebrain or locus coeruleus facilitated emergence from anaesthesia.
- These findings provide additional evidence for a selective role of orexinergic signalling in emergence from anaesthesia.

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A number of neurotransmitters, including dopamine, histamine, and glutamate, mediate the transition from anaesthesia to wakefulness.[1–4] Among these neural pathways, orexinergic neurones in the perifornical lateral hypothalamic (PeFLH) area, also called hypocretin (Hcrt) neurones, potentially trigger emergence.[5–8] Orexinergic neurones project widely to many brain areas, with particularly dense innervation of nuclei containing monoaminergic and cholinergic neurones that constitute the ascending activating system.[9–12] These nuclei include the noradrenergic (NA) neurones of the locus coeruleus (LC), serotonergic neurones of the dorsal raphe (DR) nucleus, histaminergic neurones of the tuberomammillary nucleus, dopaminergic neurones of the ventral tegmental area (VTA), and cholinergic neurones of the basal forebrain (BF). The LC and BF are two key arousal nodes that have crucial effects on the general anaesthetic state.

Located anterior to the hypothalamus and ventral to the basal ganglia, the BF expresses high levels of orexin receptors,[13] and the BF regulates emergence from propofol and sevoflurane anaesthesia via orexin modulation.[6,14,15] Microinjection of orexin into the BF during isoflurane anaesthesia shortened emergence by increasing cortical acetylcholine release,[5] indicating a potential circuit in which orexin is involved in promoting arousal.

The LC is an NA structure in the brainstem known to promote wakefulness and arousal that receives dense afferent projections from orexinergic neurones.[16,17] LC-NA stimulation can be sufficient to facilitate recovery from isoflurane anaesthesia.[18] Delivery of orexin-A directly into the LC can increase wakefulness from a sleep state.[19] However, unlike in natural sleep, whether the LC also mediates the emergence-promoting effect of orexin in anaesthesia has not been clarified.[20]

Previous evidence has relied heavily on pharmacological approaches, which are unable to determine the role of interactions between orexinergic neurones and other neurones during arousal *in vivo*. We took advantage of optogenetics in a transgenic rat ($Hcrt^{cre}$) model to investigate arousal from isoflurane anaesthesia by orexinergic pathways. By activating orexinergic cell bodies in the PeFLH or their axon terminals in the BF and LC, we confirmed the regulatory role of orexinergic neurones in the BF and LC during emergence from anaesthesia.

# Methods

## Animals

$Hcrt^{cre}$ knock-in rats were generated using the CRISPR/Cas9-based approach.[21] Briefly, a single-guide RNA (sgRNA) was designed using the CRISPR design tool (http://crispr.mit.edu) to target exon 1 of the *Hcrt* gene. On-target activity of the sgRNA was screened using a Universal CRISPR Activity Assay (UCA™; Biocytogen Inc., Beijing, China). The T7 promoter sequence was added to the Cas9 or sgRNA template by PCR amplification *in vitro*. A circular targeting vector containing Cre recombinase and WPRE bGH pA was mixed with Cas9 mRNA and sgRNA and then co-injected into the cytoplasm of one-cell stage fertilised Sprague–Dawley (SD) rat eggs. The injected zygotes were transferred into oviducts of SD pseudopregnant females to generate F0 rats. F0 rats with the expected genotype confirmed by tail genomic DNA PCR and sequencing were mated with SD rats to establish germline-transmitted F1 founders. F1 founders were genotyped by tail genomic PCR/DNA sequencing, and Southern blot examination was performed to confirm the genotype.

We crossed rats with Cre-dependent tdTomato reporter knock-in (B-Tdtomato cKI rats, SD-Gt (ROSA26)$^{tm1(CAG-LSL-Tdtomato)}$/Bcgen) provided by Beijing Biocytogen Inc. (Beijing, China) for verification of Cre expression. $Hcrt^{cre}$ homozygotes were mated with wild-type female SD rats to produce heterozygotes.

All wild-type SD rats us were provided by the Animal Center of Fourth Military Medical University (Xi'an, China). Animals were housed with a constant temperature of 24°C (0.5°C) and relative humidity of 60% (2%) with a light-controlled schedule (lights on between 07:00 and 19:00) with free access to food and water. We selected only male transgenic rats for behavioural consistency.[12] Male 10- to 12-week-old $Hcrt^{cre}$ rats were used for virus microinjection in the PeFLH (AP −2.95 mm, ML +1.35 mm, DV −8.2 mm), BF (AP 0.00 mm, ML +1.45 mm, DV −7.80 mm), or LC (AP −9.75 mm, ML +1.45 mm, DV −5.30 mm).[22] Rats were randomised by random serial numbers. Sample size was calculated as described.[12] The experimental protocol was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of our institutes and ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

## EEG recording

Rats were placed in a horizontal Plexiglas® cylinder (45 cm long, 30 cm in diameter). Anaesthesia was induced and maintained by 1.4 vol% or 0.8 vol% isoflurane with 100% $O_2$ at 1.5 L $min^{-1}$. An anaesthesia monitor (Philips G60; Philips Goldway, Shenzhen, Guangdong, China)) was connected to detect the concentration of isoflurane in the cylinder. EEG signals were continuously recorded through three stainless steel screws anchored on the skull surface (AP +2.0 mm, ML −3.0 mm; AP −7.0 mm, ML +5.0 mm), by a Powerlab 16/35 amplifier system (PL3516; ADInstruments, Bella Vista, NSW, Australia) and Labchart Pro version 8.0.10 (MLU60/8; ADInstruments). Raw EEG data were digitised at a rate of 1000 Hz and bandpass filtered at 0.3–50 Hz for further analysis. The total power spectrum, burst suppression ratio (BSR), and power distribution in each frequency band were completed by MATLAB (The MathWorks, Inc., Natick, MA, USA). Details of EEG analysis are presented in the Supplementary Methods.

## Optical stimulation

Optical stimulation was performed with blue light (473 nm) from a laser (MBL–III–473-100 mV; Thinker Tech Nanjing Biotech, China). Laser intensity was measured using an optical power metre (SANWA Electric Instrument, Tokyo, Japan) to which the fibre was attached to maintain intensity at 10–15 mV. Based on the optimal stimulation frequency from the *in vitro* electrophysiological test (Fig. 1g and h), optical activation was performed by a 473 nm laser at 20 Hz for 10 ms. Each stimulation cycle was 120 s on and 30 s off. The optical stimulation protocol used for the cell body of orexinergic neurones was the same as that applied for axon terminals in the BF and LC.

## Examination of induction and emergence times

The methods for determining the durations of anaesthesia induction and emergence were as previously described.[15]

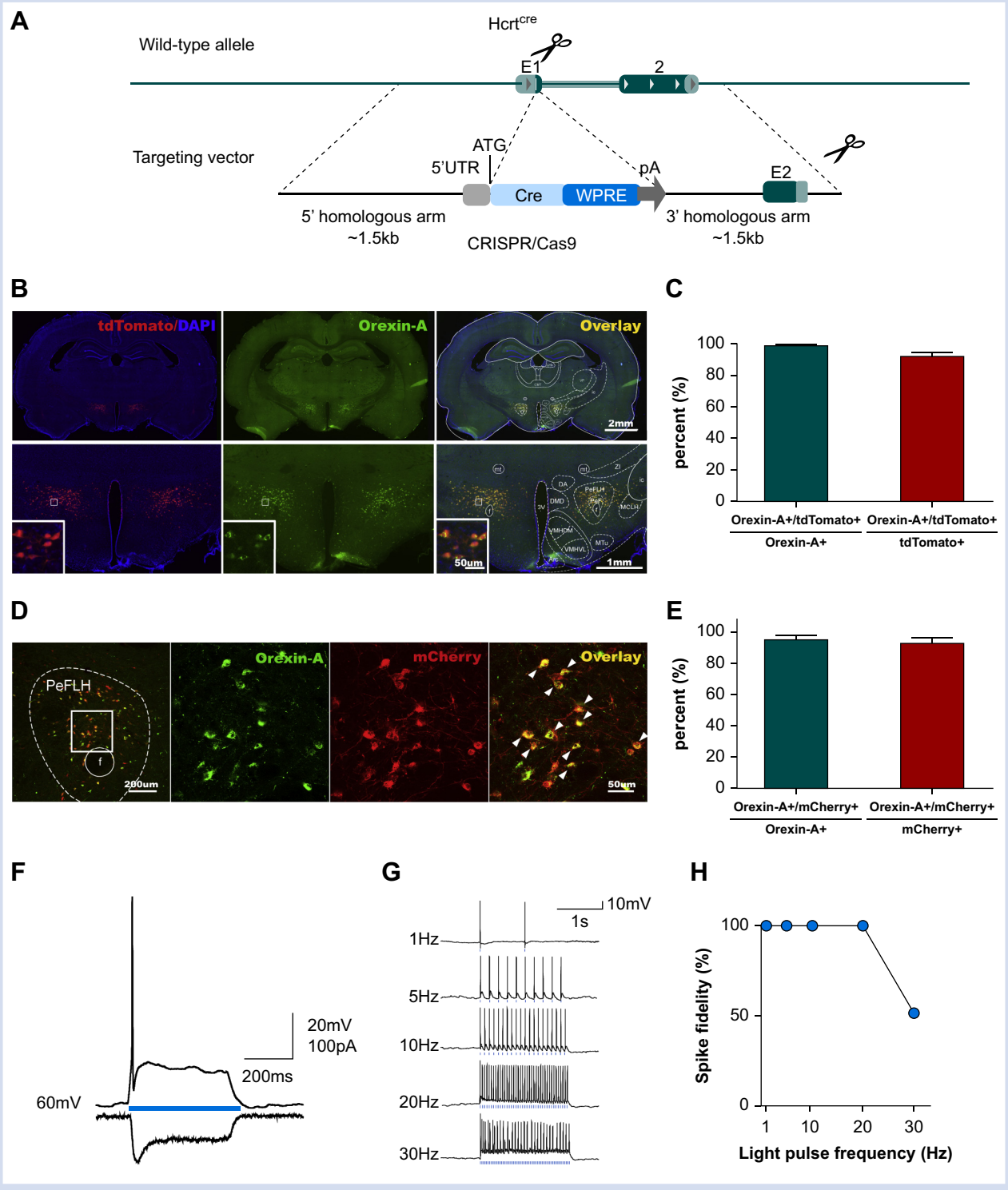



Fig 1. Validation of *Hcrt*$^{cre}$ rats. (a) Schematic overview of the strategy to knock in the Cre cassette immediately after the target gene by CRISPR/Cas9 technology. (b) Co-expression of tdTomato and orexin-A in the perifornical lateral hypothalamic area (PeFLH) area. Whole brain sections (top) show both tdTomato (red) and orexin-A (green) expression in the hypothalamus, especially in the PeFLH area. The insets show co-localisation of tdTomato and orexin-A in the PeFLH area. (c) Quantification of orexin-A-positive (orexin-A+) and tdTomato-positive (tdTomato+) cells (three sections per rat, $n$=3 rats). (d) Co-expression of ChR2-mCherry (red) and orexin-A immunoreactivity (green) in the PeFLH. Arrowheads indicate double labelling of orexin-A and ChR2-mCherry in PeFLH cells. (e) Quantification of orexin-A-positive (orexin-A+) and ChR2-mCherry (mCherry+) cells (three sections per rat, $n$=3 rats). Data are expressed as mean (standard deviation [SD]). (f) Depolarisation and spiking of ChR2-expressing orexinergic neurones after illumination (at 473 nm) in current-clamp mode. This depolarisation coincided with an inward current in voltage-clamp mode (bottom). (g) Stimulation by blue light at different frequencies in brain slices *in vitro* activated orexinergic neurones ($n$=5 cells in five different slices, $n$=4 rats); blue bars represent 10 ms light pulses. (h) Fidelity of responses of ChR2-expressing orexinergic neurones to light pulses. E1, exon 1; E2, exon 2; PeFLH, perifornical lateral hypothalamic area; 5′UTR, 5′ untranslated region.

Briefly, we counted the time from onset of anaesthetic administration to loss of righting reflex (LORR) and the time from the end of anaesthesia administration to recovery of righting reflex (RORR). When rats were placed in the horizontal cylindrical observation cage under anaesthesia (Fig 2b), LORR was checked by turning the cage 90° every 15 s. The time of LORR was determined when a rat did not turn itself prone onto all four limbs. After termination of anaesthesia, RORR was checked by turning the cage 90° every 30 s until the animals placed all four feet on the floor. A heating pad was taped to the bottom of the cage and was maintained at 37.5°C.

### Arousal scoring

Arousal responses were scored using the adaptation method.[23,24] Movement of whiskers, head, and limbs were marked as 0 (without), 1 (slightly), or 2 (forcefully), respectively, according to the intensity of movement. Righting scores were marked as 0 and 2, respectively, if prone or extremities were touching the floor. Walking scores were marked as 0, 1, and 2, referring to rats that stood but did not walk, rats that crawled but without their abdominals leaving the floor, and rats that walked with their abdominals off the floor, respectively.

### Statistical analysis

Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Data are expressed as the mean (standard deviation). Statistical significance was assessed using Student's *t*-test for comparison of two groups, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni's test for three or more groups, and Mann–Whitney *U*-test for behavioural scoring. A $P$ value <0.05 was considered statistically significant.

The methods for surgical procedures, *in vitro* electrophysiological recording, EEG analysis, and immunohistochemistry are provided in the Supplementary Methods.

## Results

### Generation of *Hcrt*$^{cre}$ knock-in rats

*Hcrt*$^{cre}$ rats, in which *Cre* is driven by an ~108 kb fragment of the *Hcrt* gene promoter, were generated using CRISPR/Cas9 technology (Fig. 1a). To verify the selectivity of this transgenic rat, we crossed *Hcrt*$^{cre}$ rats with Cre-dependent tdTomato reporter knock-in rats (B-Tdtomato cKI rats). Of their offspring, 98.8% (0.45%) of orexin-A-positive cells expressed tdTomato in the lateral hypothalamus ($n$=3), whereas 91.9% (2.2%) of tdTomato cells were orexin-A-positive ($n$=3; Fig. 1b and c). Most of the orexin-A-positive cells were located in the PeFLH. Stereotactic injection of ChR2-mCherry inducible adeno-associated virus rAAV-EF1f-DIO-ChR2-mCherry into the lateral hypothalamus in *Hcrt*$^{cre}$ rats, and immunohistochemical staining of the hypothalamic sections with an antibody against orexin were performed (Fig. 1d–1e). We found that 95.1% (2.8%) of orexin-A-positive cells co-expressed ChR2-mCherry ($n$=3). In contrast, 93.2% (3.0%) of ChR2-mCherry-expressing cells were orexin-A-positive ($n$=3). No mCherry fluorescence was observed in wild-type rats after virus transduction, confirming that expression was exclusive to Cre-expressing neurones.

After 3 weeks of virus expression in *Hcrt*$^{cre}$ rats, blue light (473 nm) stimulation of the PeFLH in brain slices *in vitro* effectively activated light-sensitive channel-expressing neurones. ChR2-expressing orexinergic neurones showed robust depolarisation and spiking after 500 ms illumination (473 nm) in current-clamp mode and an inward current in voltage-clamp mode (Fig. 1f). Optical stimulation at 1–30 Hz evoked spiking at different frequencies in current-clamp mode (Fig. 1g). The fidelity of the response of orexinergic neurones to light pulses at frequencies reached up to 20 Hz (Fig. 1h).

### Optical stimulation of cell bodies of orexinergic neurones in the PeFLH promotes arousal from anaesthesia

To activate the cell bodies of orexinergic neurones, the ChR2-mCherry virus or its control were injected into the PeFLH, and an optical fibre was inserted above the injection site (Fig. 2a). Animals were placed into a horizontal cylindrical cage for isoflurane anaesthesia (Fig. 2b), and 10 min optical stimulation was delivered at 40 min after the start of anaesthesia (Fig. 2c). During optical stimulation, there was a significant change in the EEG pattern and power spectrum in the ChR2-mCherry group. EEG burst suppression during 1.4 vol% isoflurane maintenance was largely and promptly decreased by optical stimulation (Fig. 2d). Statistical analysis of a total of 20 min of EEG recordings (optical stimulation for 10 min and 5 min before and after photostimulation) demonstrated a significant decline in the BSR during optical activation (from 59.6% [10.3%] to 4.9% [5.0%], $F_{(19,190)}$=9.655, $P$ < 0.0001; Fig. 2e). Photostimulation of orexinergic neurones in the PeFLH was also given at the same time as the onset of isoflurane and ended until LORR, which had no effect on anaesthesia induction time. However, when stimulation was started at the end of isoflurane inhalation and continued until RORR, optical activation of the orexinergic neurones shortened arousal time (923 [162] s *vs* 493 [68] s, $t_{(10)}$=5.469, $P$=0.0003; Fig. 2f).

### Orexinergic neurones have strong projections to BF and LC

To verify projections of orexinergic neurones, we injected ChR2-mCherry virus into the PeFLH. After 3 weeks, the virus was densely expressed in the BF, LC, and some other areas, indicative of strong orexinergic innervations to these nuclei (Fig. 3a). To stimulate orexinergic terminals, we injected ChR2-mCherry virus into PeFLH and inserted optical fibres into BF or LC. Four weeks after virus injection, fluorescence-labelled varicosities were observed in BF and LC (Figs 3b and 4a, Supplementary Fig. 2A). However, statistical comparison indicated that the optical density in the LC was slightly less than that in the BF (194 [12] *vs* 231 [2], $P$=0.0167, $t_{(8)}$=3.014; Supplementary Fig. 2b, left).

### Optical stimulation of orexinergic terminals in the BF facilitates emergence from isoflurane anaesthesia

Photoactivation of orexinergic terminals in the BF during 1.4 vol% isoflurane maintenance caused a significant decline in BSR (Fig. 3c). Averaged BSR gradually decreased during the first 2 min of optogenetic stimulation, remained at a significantly low level for 6 min, and then gradually returned to baseline (from 66% [8%] to 5% [6%], $F_{(19,200)}$=4.765, $P$ < 0.0001; Fig. 3d). Photostimulation of orexinergic terminals in the BF promoted arousal, shown as a shorter time to RORR compared with the control group (937 [122] s *vs* 674 [108] s, $t_{(10)}$=3.594, $P$=0.0049)

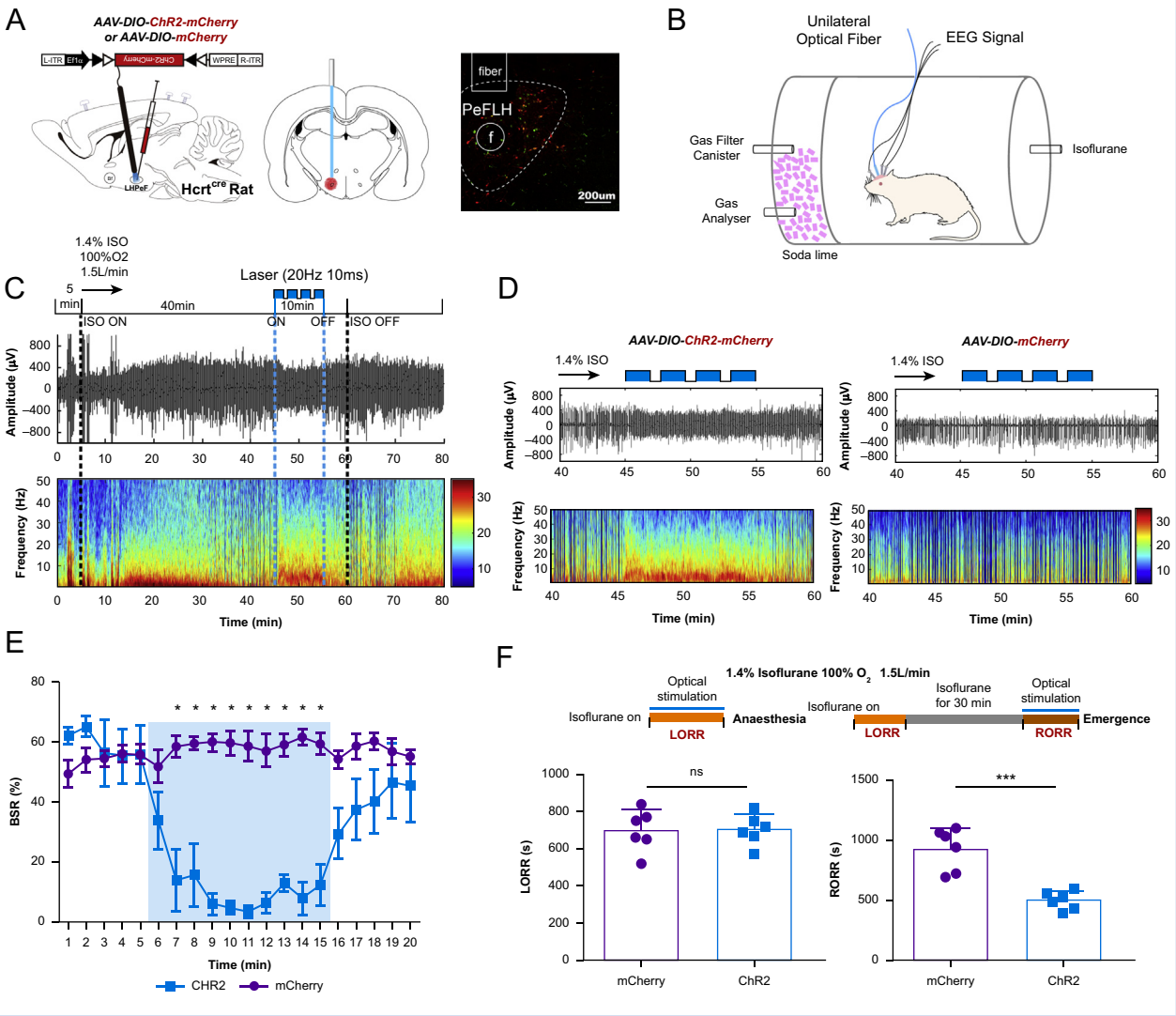
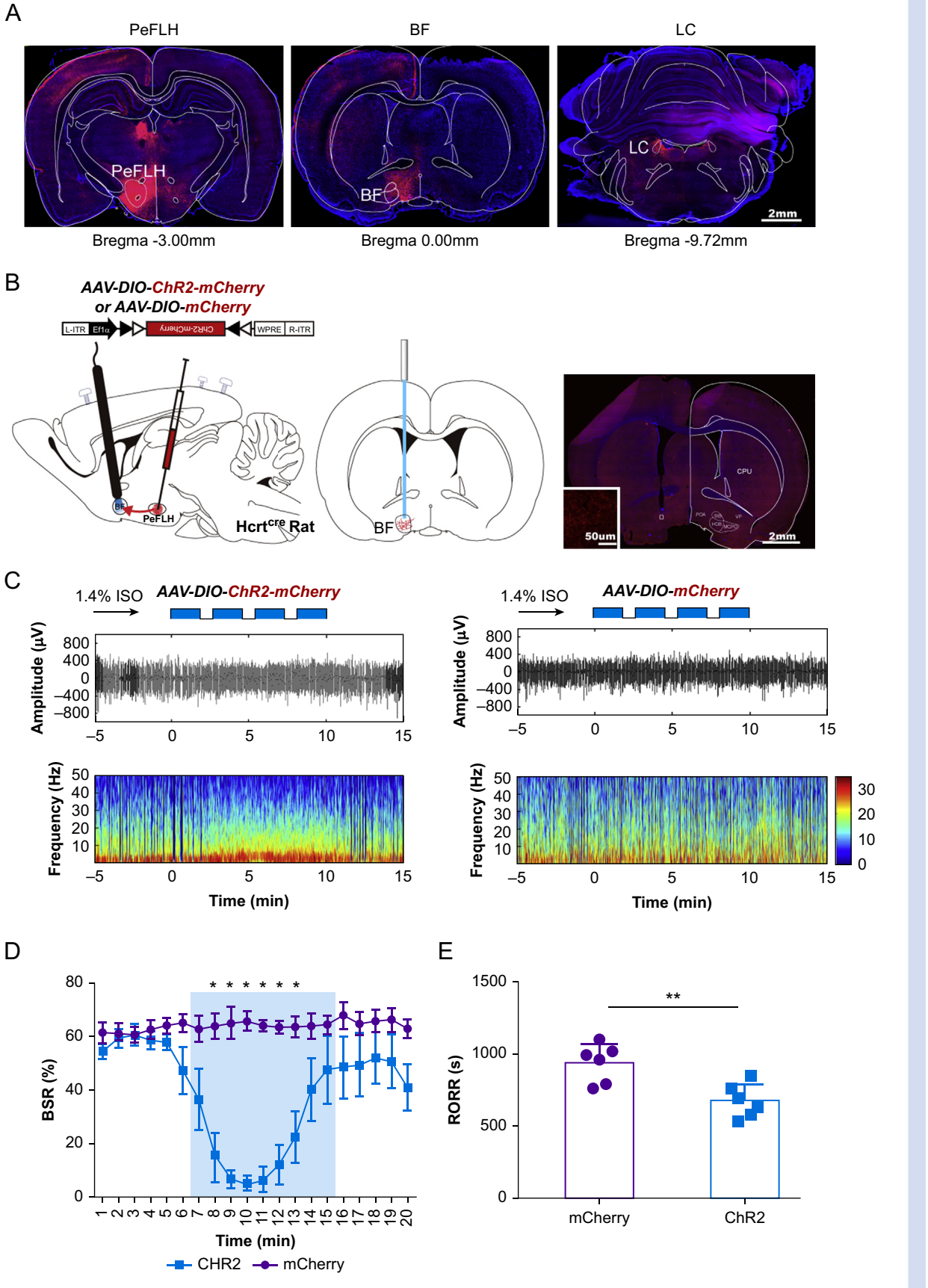



Fig 2. Optical stimulation of orexinergic cell bodies under 1.4 vol% isoflurane anaesthesia accelerates wakefulness. (a) Diagrams of sagittal (left) and coronal (middle) brain sections showing the site of virus injection and fibre embedding. A representative photomicrograph shows microinjection and virus expression locations. (b) Schematic diagram of the apparatus used for behavioural observation, optical fibre stimulation and EEG signal recording during anaesthesia. (c) Optical stimulation protocol during anaesthesia maintenance. After a 5 min wakefulness state, the rat was exposed to isoflurane (1.4 vol%) for 40 consecutive min, with optical stimulation for 10 min. A representative raw EEG trace and corresponding power spectrogram throughout the recording process of a rat expressing ChR2-mCherry. (d) Representative EEG traces and power spectrograms during optical stimulations in a mCherry-expressing rat (left) and ChR2-expressing rat (right). (e) BSR at 5 min before, 10 min during and 5 min after optical stimulation in mCherry and ChR2 rats. BSR is plotted at each minute ($n$=6). Using two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's multiple comparisons: $F_{(19,190)}$=9.655, $P < 0.0001$. (f) Schematic of protocols for optical stimulation during anaesthesia induction and emergence (top). LORR and RORR after optical stimulation of orexinergic neurones in mCherry control (grey) and ChR2 (blue) groups (bottom) ($n$=6). Using two-tailed unpaired Student's $t$-test: $t_{(10)}$=0.087, P=0.932 for LORR, $t_{(10)}$=5.469, P=0.0003 for RORR. *P<0.05, ***P<0.001 *vs* mCherry. Data are presented as mean (SD). ISO, isoflurane; BSR, burst suppression ratio; LORR, loss of righting reflex; RORR, recovery of righting reflex; PeFLH, perifornical lateral hypothalamic area; SD, standard deviation.

but had no effect on isoflurane anaesthesia induction (Fig. 3e and Supplementary Fig. 1A).

To determine whether stimulation of orexinergic terminals in the BF under lighter anaesthesia could induce a visible behavioural manifestation of emergence, we administered 0.8 vol% isoflurane (100% 1.5 L $min^{-1}$ in $O_2$) (Fig. 3f). During optical stimulation of BF, EEG recordings of the ChR2-mCherry group were indicative of a change from an anaesthesia state to an awake-like state (Fig. 3g), which returned to an anaesthesia state after cessation of stimulation. We compared the power distribution in each frequency band during the 5 min period of anaesthesia before optical stimulation and the last 5 min during stimulation. Optical stimulation in ChR2-mCherry rats produced a decrease in delta power (63% [3%] *vs* 42% [8%]; P=0.0002), whereas the power in theta (16% [2%] *vs* 22% [4%]; P=0.0172), beta (4.3% [0.8%] *vs* 11% [4%; P=0.0045), and gamma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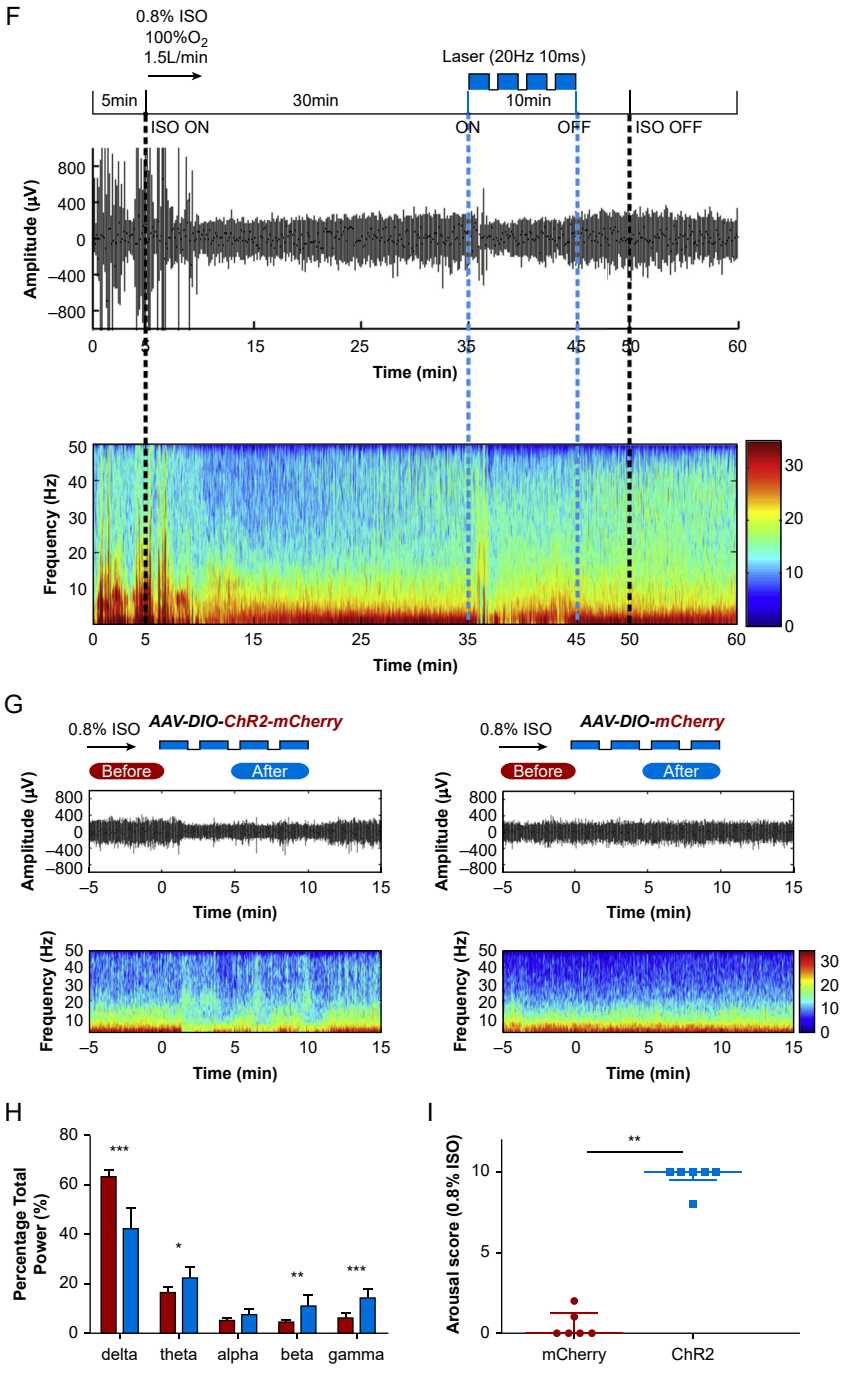
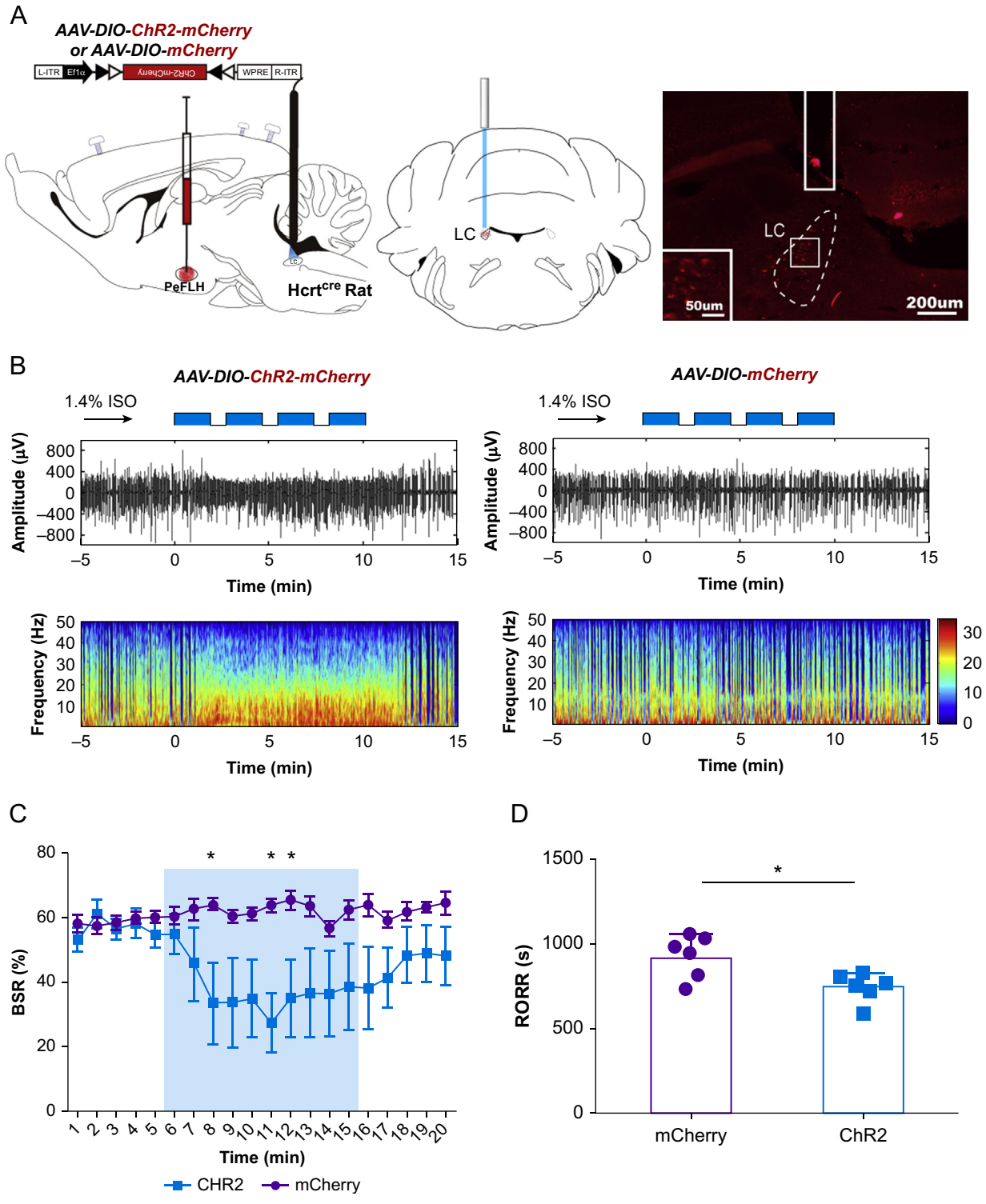



Fig 3. Optical stimulation of orexinergic terminals in the basal forebrain promotes emergence. (a) Red virus expression indicates projections from orexinergic neurones in the PeFLH to the BF and LC. (b) Diagrams of sagittal (left) and coronal (middle) brain sections showing virus injection and fibre location. The virus AAV-DIO-ChR2-mCherry and its control were injected into the PeFLH area of *Hcrt*$^{cre}$ rats, and stimulating fibres were embedded in the BF. The right photomicrograph shows orexinergic terminals expressing mCherry in the BF. (c) Representative EEG traces and power spectra during optical stimulation (blue bars) in a ChR2-mCherry (left) and mCherry (right) rat during 1.4 vol% isoflurane anaesthesia. (d) BSR at 5 min before, 10 min during, and 5 min after optical stimulation in mCherry and ChR2 rats. BSR is plotted at each minute ($n$=6). Using two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's multiple comparisons test: $F_{(19,200)} = 4.765$, $P < 0.0001$. (e) RORR after optical stimulation of orexinergic terminals in the BF in mCherry control (grey) and ChR2 (blue) groups ($n$=6). Using two-tailed unpaired Student's *t*-test: $t_{(10)}$=3.594, $P$=0.0049 for RORR. (f) Optical stimulation protocol during 0.8 vol% isoflurane anaesthesia. After 30 min inhalation of isoflurane, the rat was optically stimulated for 10 min (blue bars). A representative raw EEG trace and the corresponding power spectrogram show the marked changes in power and frequency induced by photostimulation in ChR2-mCherry animals. (g) Representative EEG oscillations and EEG power spectra during optogenetic activation of orexinergic terminals in the BF under 0.8 vol% isoflurane anaesthesia (blue bars) in ChR2-mCherry (left) and mCherry (right) rats during 0.8 vol% isoflurane anaesthesia. (h) EEG power percentage of different frequency bands at 5 min before (red) and after (blue) optical stimulation in ChR2 animals. Using two-way ANOVA followed by *post hoc* Bonferroni's multiple comparisons: $F_{(4,50)}$=29.66, $P$<0.0001. (i) Behavioural responses during optogenetic activation of orexinergic terminals in the BF under 0.8 vol% isoflurane anaesthesia. Using the two-tailed Mann–Whitney *U*-test, $P$=0.002. *$P$<0.05, **$P$<0.01, and ***$P$<0.001 *vs* mCherry. Data are presented as the mean (SD). ISO, isoflurane; BSR, burst suppression ratio; RORR, recovery of righting reflex; BF, basal forebrain; LC, locus coeruleus; PeFLH, perifornical lateral hypothalamic area; SD, standard deviation.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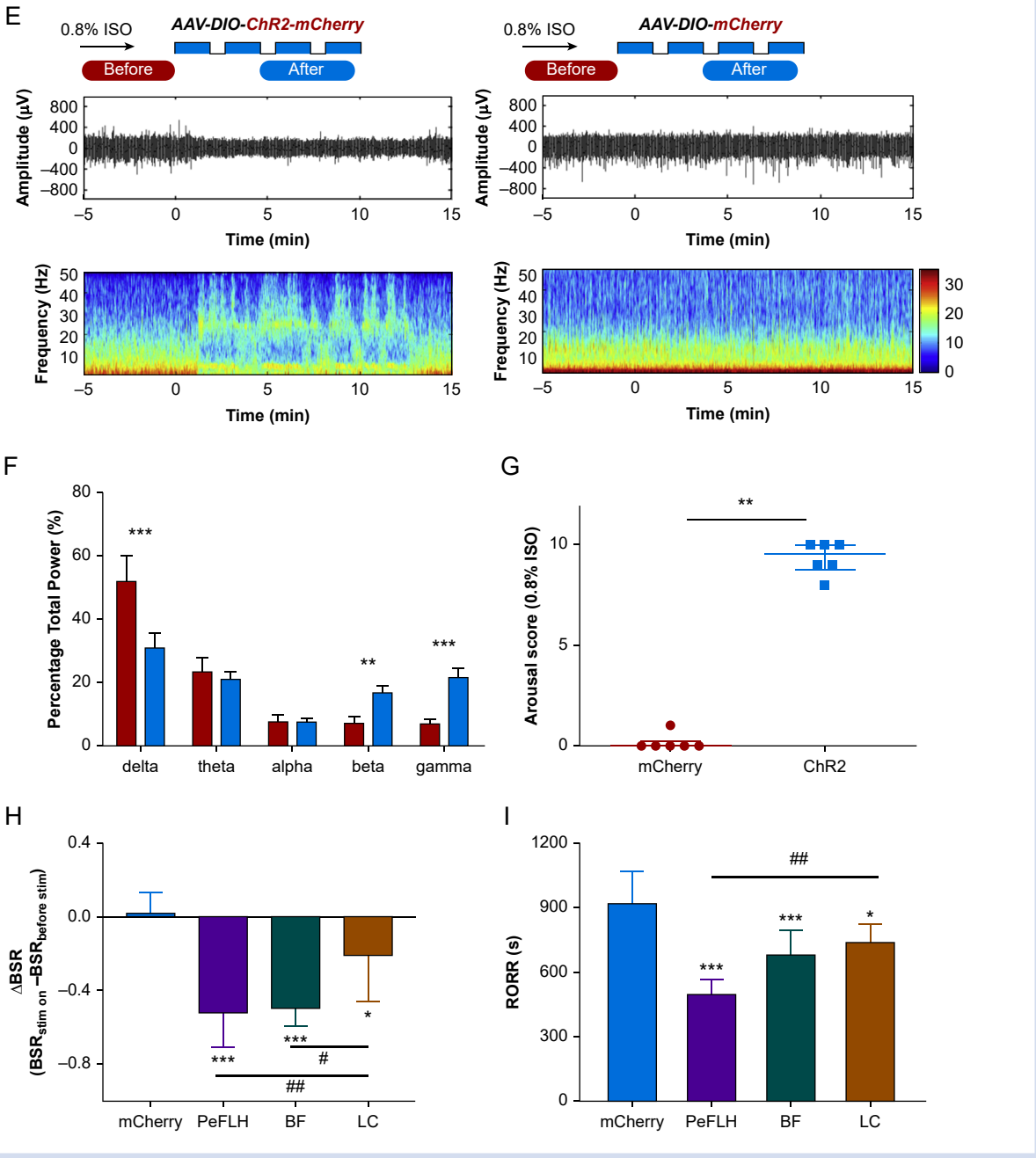


Fig 4. Optical stimulation of orexinergic terminals in the locus coeruleus facilitates arousal. (a) Diagrams of sagittal (left) and coronal (middle) brain sections showing virus injection and fibre location in the LC. The right photomicrograph shows orexinergic terminals expressing mCherry in the LC. (b) Representative EEG traces and power spectra during optical stimulation (blue bars) of the LC in a ChR2-mCherry (left) and mCherry (right) rat during 1.4 vol% isoflurane anaesthesia. (c) BSR at 5 min before, 10 min during, and 5 min after optical stimulation. BSR is plotted at each minute ($n$=6). Using two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's multiple comparisons: $F_{(19,180)}=1.939$, $P$=0.034. (d) RORR after optical stimulation of orexinergic axon terminals in the LC in the mCherry control (grey) and ChR2 (blue) groups ($n$=6). Using two-tailed unpaired Student's *t*-test: $t_{(10)}=2.586$, $P$=0.027 for RORR. (e) Representative EEG oscillations and EEG power spectra during optogenetic activation of orexinergic terminals in the LC under 0.8 vol% isoflurane anaesthesia (blue bars) in ChR2-mCherry (left) and mCherry (right) rats. (f) Power percentage of different frequency bands at 5 min before (red) and after (blue) optical stimulation in ChR2 animals. Using two-way ANOVA followed by *post hoc* Bonferroni's multiple comparisons: $F_{(4,50)}=40.24$, $P<0.0001$. (g) Behavioural responses during optogenetic activation of orexinergic terminals in the LC under 0.8 vol% isoflurane anaesthesia. Using two-tailed Mann–Whitney *U*-test, $P$=0.002. (h) The decreases in BSR during optical stimulation. One-way ANOVA followed by *post hoc* Bonferroni's test, $F_{(3,32)}=21.87$, $P<0.0001$. (i) RORR in four groups. Time to righting reflex was analysed by using one-way ANOVA followed by *post hoc* Bonferroni's test: $F_{(3,32)}=21.02$, $P<0.0001$. $n$=6, in the PeFLH, BF, LC groups, and $n$=18 in the mCherry group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* mCherry; $^{\#}P<0.05$, $^{\#\#}P<0.01$. Data are presented as the mean (SD). ISO, isoflurane; BSR, burst suppression ratio; RORR, recovery of righting reflex; BF, basal forebrain; LC, locus coeruleus; PeFLH, perifornical lateral hypothalamic area; SD, standard deviation.

(6.1% [1.7%] *vs* 14.2% [3.3%]; $P$=0.0006) bands markedly increased (Fig. 3h). There was no difference in the EEG power spectrum in the mCherry animals with stimulation (Supplementary Fig. 1B). Upon optical stimulation, the arousal score was much higher in the ChR2 than in the mCherry group ($P$=0.002, Fig. 3i). Movements of the legs, head, and whiskers of ChR2 animals were clearly observed, whereas those behaviours were hardly observed in mCherry animals upon stimulation. Righting was observed in all ChR2 animals but not in mCherry animals. Moreover, five out of six ChR2 animals walked on their paws with their abdomen off the floor, whereas no walking was seen in the control group (Table 1).

### Optical stimulation of orexinergic terminals in the LC promotes arousal from isoflurane anaesthesia

Photostimulation of orexinergic terminals in the LC also reduced BSR during 1.4 vol% isoflurane anaesthesia (from 63% [5%] to 27% [21%], $F_{(19,180)}$=1.939, $P$=0.034; Fig. 4c). However, reduction in BSR was less than that induced by activation of BF orexinergic terminals or orexinergic cell bodies (−2.0 [0.2] *vs* −0.5 [0.1], $t_{(32)}$=3.187, $P$=0.019; −2.0 [0.2] *vs* −0.5 [0.2], $t_{(32)}$=3.442, $P$=0.009; Fig. 4h). Photostimulation of orexinergic terminals in the LC also facilitated arousal from general anaesthesia (913 [128] s *vs* 742 [76] s, $t_{(10)}$=2.586, $P$=0.0217), but no effect on induction of anaesthesia was observed (Fig. 4d and Supplementary Fig. 1C). The reduction of RORR induced by LC activation, comparable with that induced by activation of BF orexinergic terminals, was less than that of orexinergic cell body activation (493 [68] s *vs* 742 [76] s, $t_{(32)}$=3.514, $P$=0.008; Fig. 4i).

During anaesthesia maintenance induced by 0.8 vol% isoflurane, optical stimulation of LC orexinergic terminals altered EEG patterns in the ChR2-mCherry group from an anaesthesia state to an awake-like state (Fig. 4e). EEG recordings returned to baseline, indicative of an anaesthesia state, after the stimulation. Specifically, optical stimulation in ChR2-mCherry rats produced a decrease in delta power (52% [7%] *vs* 31 [4%], $P$=0.0003), whereas the power in the beta (7.0% [2.0%] *vs* 16.7% [2.0%], $P$<0.001) and gamma (6.8% [1.8%] *vs* 21.7% [2.7%], $P$<0.0001) bands increased (Fig. 4f). There was no significant difference in the EEG power distribution in the mCherry animals with stimulation (Supplementary Fig. 1C). Upon optic stimulation, rat movements were increased in the ChR2-mCherry rats ($P$=0.002; Fig. 4g). Generally, optical stimulation increased leg, head, and whisker movements in ChR2-mCherry rats and induced the righting response. Unlike in the BF terminal activation group, however, only three out of six animals in the LC activation group stood and walked on their paws (Table 2).

### PeFLH-LC and PeFLH-BF pathways have different efficacies in facilitating emergence

Compared with the BF, the LC is smaller with fewer but clustered neurones. To avoid insufficient stimulation of terminals, we tested the arousal efficacy of the PeFLH-LC and PeFLH-BF pathways by a retrograde approach. We injected $AAV_{retro}$-ef1a-DIO-ChR2-mCherry virus into the LC or BF of *Hcrt-cre* rats (300 nl per rat, $n$=5), which provided retrograde access to orexin cell bodies. We put optical fibres into the PeFLH, which specifically activates the orexinergic PeFLH-LC or PeFLH-BF projections (Fig. 5a). Four weeks after virus microinjection, photostimulation at either PeFLH-LC or PeFLH-BF orexin innervations reduced the BSR (Fig. 5b–e). Activation of PeFLH-BF innervations was more powerful than of PeFLH-LC in reducing burst suppression waves (−0.34 [0.04] *vs* −0.18 [0.09], $P$ < 0.001, $F_{(2,17)}$=75.17; Fig. 5f).

## Discussion

Orexin is an important excitatory transmitter[25] involved in various neural functions such as sleep, food intake, depression, and facilitation of arousal from general anaesthesia.[8,26,27] To further confirm the regulatory effects of orexinergic neurones on the BF and LC during anaesthesia emergence, we used optogenetic approaches in transgenic $Hcrt^{cre}$ rats. Activation of orexinergic neuronal cell bodies or their terminals in the BF and LC shortened the time to RORR and induced a decrease in the BSR under 1.4 vol% isoflurane anaesthesia. Under 0.8 vol% isoflurane anaesthesia, optical stimulation of orexinergic terminals in the BF and LC reduced the delta power percentage in EEG recordings and promoted movement recovery in anaesthetised rats.

A variety of experiments using pharmacological methods have revealed the emergence-promoting effects of orexinergic neurones in general anaesthesia induced by sevoflurane, isoflurane, propofol, or ketamine.[6,14,15,28,29] Before optogenetics, selectively stimulating specific orexinergic neural elements with a temporal resolution relevant to anaesthesia or wakefulness episodes and achieving spatial selectivity to probe those terminals surrounding cells were difficult tasks. The current experiments did not concentrate on the ligands or receptors of the orexinergic system, but rather observed high-resolution EEG changes during anaesthesia and accurately activated both orexinergic neurones in the PeFLH and their terminals in the BF and LC by optogenetic approaches during maintenance and recovery of anaesthesia. We found that orexinergic terminals in the BF and LC were not involved in the induction process of isoflurane anaesthesia under current conditions, which is consistent with previous research.[15] suggesting that the neural substrates governing transitions into and out of the anaesthetised state are not identical.

Although stimulation of either the orexinergic cell body or its terminals in the BF and LC was able to shorten emergence time and decrease BSR, the emergence-promoting efficacy of orexinergic cell body activation was more obvious. Compared with terminal activation, orexinergic cell body activation resulted in a longer-lasting decline in EEG burst suppression and shorter time to RORR, suggesting that there could also be other nuclei or neural circuits involved in the effect. Indeed, orexin neurones also project to the ventral tegmental area, pedunculopontine tegmental nucleus (PPTg), central nucleus of the amygdala (CeA) etc.,[9] some of which are also involved in emergence from general anaesthesia. How these various pathways correlate with each other remains unknown.

Promotion of emergence efficiency was different between BF and LC activation. Under 1.4 vol% isoflurane anaesthesia, the BSR of BF orexinergic terminal optical stimulation decreased to a greater extent than that of LC orexinergic terminal optical stimulation. Furthermore, the RORR reduction revealed that rats with BF terminal stimulation took less time to recover from anaesthesia than those with LC stimulation. To investigate whether fibreoptic light effectively activated a sufficient number of neurones in the BF and LC, we assessed c-Fos expression in these two areas after optical stimulation. As shown in Supplementary Fig. 2C–E, c-Fos expression in BF and LC was significantly enhanced by optical activation. In

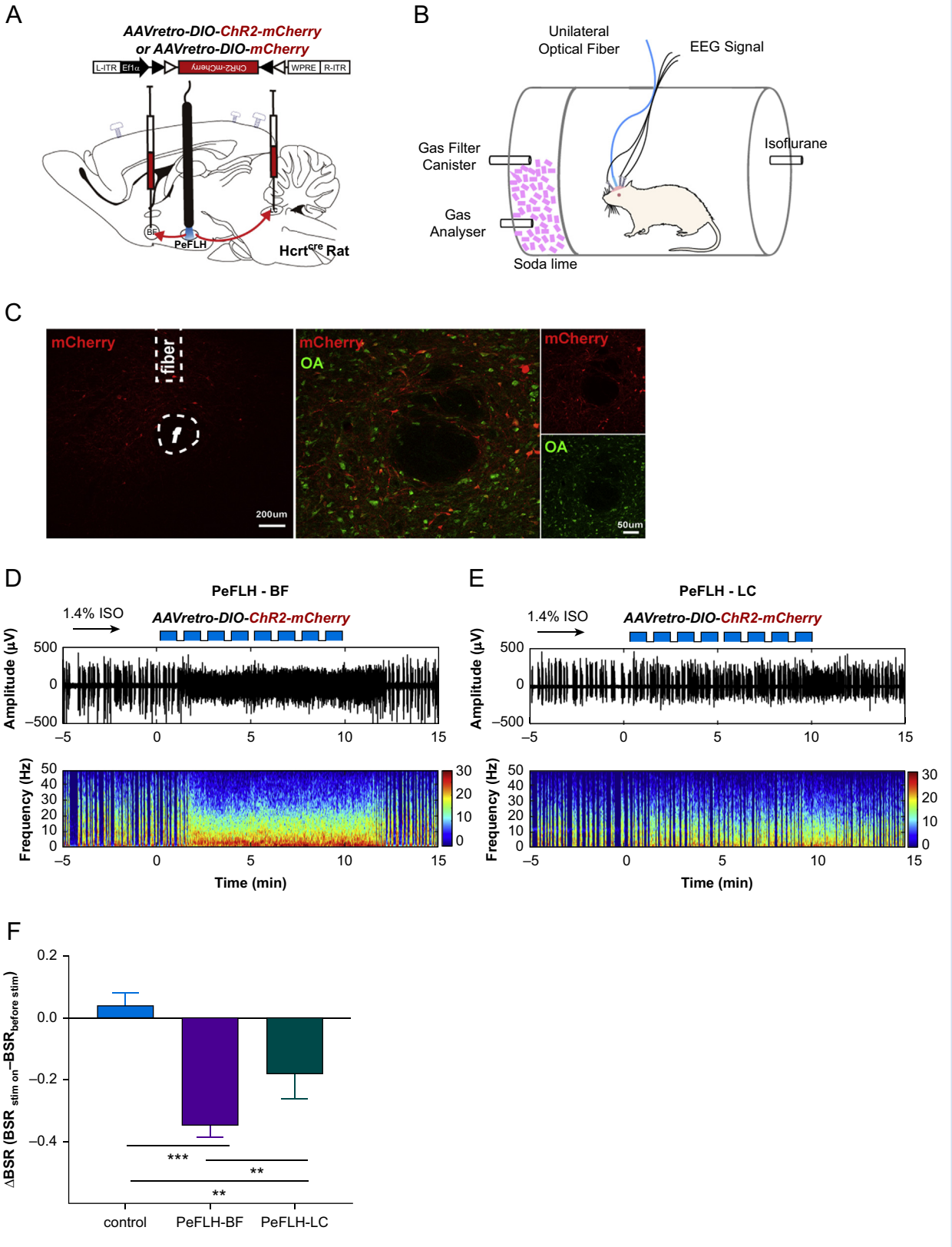


Fig 5. Optical stimulation of orexinergic cells projecting to basal forebrain and locus coeruleus elicit differential promotion of arousal. (a) Diagram of virus injection and fibre embedding. (b) Schematic diagram of the apparatus used for behavioural observation, optical fibre stimulation and EEG signal recording during anaesthesia. (c) Representative photomicrographs of virus expression and fibre location. (d–e) Representative EEG traces and power spectrograms during optical stimulations in an $AAV_{retro}$-CHR2 expressed rat. (f) The decreases of BSR during optic stimulations ($n$=5). One-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's test was used, $F_{(2,17)}$=75.17, $P$<0.0001. Data are presented as mean (SD). ISO, isoflurane; BSR, burst suppression ratio; BF, basal forebrain; LC, locus coeruleus; PeFLH, perifornical lateral hypothalamic area; SD, standard deviation.

Table 1 Arousal behaviour scores after stimulation of orexin terminals in the basal forebrain. The arousal behaviour scores. Leg, head, and whisker movements, and the righting and walking status were scored for each animal. Comparison of the total scores of two groups, ChR2 *vs* mCherry, using two-tailed Mann–Whitney *U*-test, $P=0.0022$.

| Animals | Leg movement | Head movement | Whisker movement | Righting | Walking | Total score |
|---|---|---|---|---|---|---|
| 1-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 2-ChR2 | 2 | 2 | 2 | 2 | 0 | 8 |
| 3-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 4-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 5-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 6-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 1-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 2-mCherry | 0 | 1 | 0 | 0 | 0 | 1 |
| 3-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 4-mCherry | 1 | 0 | 1 | 0 | 0 | 2 |
| 5-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 6-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |

Table 2 Arousal behaviour scores after stimulation of orexin terminals in the locus coeruleus. The arousal behaviour scores. Leg, head, and whisker movements, and the righting and walking status were scored for each animal. Comparison of the total scores of two groups, ChR2 *vs* mCherry, using two-tailed Mann–Whitney *U*-test, $P=0.0022$.

| Group | Leg movement | Head movement | Whisker movement | Righting | Walking | Total score |
|---|---|---|---|---|---|---|
| 1-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 2-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 3-ChR2 | 2 | 2 | 2 | 2 | 0 | 8 |
| 4-ChR2 | 2 | 2 | 2 | 2 | 1 | 9 |
| 5-ChR2 | 2 | 2 | 2 | 2 | 1 | 9 |
| 6-ChR2 | 2 | 2 | 2 | 2 | 2 | 10 |
| 1-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 2-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 3-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 4-mCherry | 0 | 0 | 1 | 0 | 0 | 1 |
| 5-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |
| 6-mCherry | 0 | 0 | 0 | 0 | 0 | 0 |

comparison, there were more c-Fos-expressing cells in the BF than in the LC (Supplementary Fig. 2F). We also assessed c-Fos expression after optostimulation in the AAVretro experiment. As shown in Supplementary Fig. 3A and B, more c-Fos-positive cells were found in the BF than in the LC (Supplementary Fig. 3C). Compared with activation of the PeFLH-LC orexin pathway, photostimulation at PeFLH-BF induced a larger number of c-Fos-expressing cells in the PeFLH. As the PeFLH to LC is 3–4 times farther than the PeFLH to BF in distance, and there were fewer virus-positive terminals in the LC (Supplementary Fig. 2B), the difference in activation of BF and LC could be attributed to the discrepancies in the quantity of orexin fibres and distance from the PeFLH. This experiment provides additional evidence for the differential efficacy of arousal between BF and LC.

In addition to the difference in the quantity of projections from PeFLH to BF and LC, the downstream regulation of these two nuclei may also contribute to the discrepancy in arousal. Both the BF and LC, known to play important roles in attention and arousal, have widespread outputs to the cerebral cortex. The LC is a NA structure in the brainstem known to promote wakefulness, and cholinergic neurones in the BF also send ascending projections to activate the cortex. However, anatomical and functional differences of the projections from these two areas into cerebral cortex have been identified.[30] The BF projections are highly selective to individual cortices, whereas the LC projects diversely into multiple cortices, indicating that they may modulate cortical activity to a different extent. A recent study from Pal and colleagues[31] noted the differential roles of cholinergic *vs* NA processes in the promotion of arousal from sevoflurane anaesthesia. They demonstrated that cholinergic stimulation of the prefrontal cortex, but not of the parietal cortex, restored wake-like behaviour during continuous exposure to sevoflurane anaesthesia, whereas NA stimulation of the prefrontal and parietal areas resulted in EEG activation but failed to produce any signs of wake-like behaviour. As in our experiment, although photostimulations of orexinergic terminals either in BF or LC induced a pro-arousal effect under isoflurane anaesthesia, the effect of stimulation of BF was stronger than that of LC. The differential activation efficacy of the cortices and the small quantity of neurones of the LC may also increase the variability of response to optical stimulation. Taken together, these results imply that different nuclei activating diverse cortices lead to different awakening effects.

We cannot exclude the possibility of retrograde depolarisation of hypocretin cell bodies after photostimulation in either BF or LC terminals. It is worth emphasising that we applied the same duration and intensity of optical stimulation at the PeFLH, BF, and LC, whereas the strength of pro-arousal effects was different among diverse stimulation targets, indicating the reliability of our results. Nevertheless, whether optical stimulation of the terminal projection area might involve the excitability of their perikarya is an important question. To stimulate the terminals and electrophysiologically record the change in excitability could answer this question.

The BF contains three types of neurones: cholinergic, glutamatergic, and gamma-aminobutyric acid (GABA)ergic,[32] which play different roles in sleep-arousal regulation.[33–37] Although we have confirmed the role of the PeFLH-BF circuitry in facilitating emergence from anaesthesia, we have not revealed the cell type of BF neurones in this regulatory pathway. Given the differences in the distance from PeFLH to BF and to LC, AAV virus transfected in the PeFLH may take different times to get full expression in these two areas. We did not check the time-dependent increase of AAV virus expression in LC terminals or AAV retrovirus expression in PeFLH cell bodies. *Hcrt-cre* rats were killed in the fifth week after virus injection for confirmation of virus expression and fibre location, immediately after completion of photostimulation experiments. Whether the virus has reached full expression in both BF and LC at this time point was not clear. These limitations of the current study deserve further exploration.

In summary, our results show that the BF and LC are effectors for orexin-mediated anaesthesia-to-wake transitions during isoflurane anaesthesia, and that BF possibly plays a more potent role in facilitation of emergence.

## Authors' contributions

Study conception: HD
Behavioural tests: YG
Optogenetic experiments: YG, DW
Immunofluorescence experiments: MR
Electrophysiological recording: HL
EEG recording: DW, LY
Data assimilation: JL
Data analysis: JG, SZ
Data interpretation: QY, HD
Drafting of manuscript: YG
Writing of manuscript: YG
Critical revision of manuscript: QY, HD

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## Declarations of interest

The authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2020.09.037.

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