0.01. Scale bar: x = 4 min, y = 10% (**D**) TMA$^+$ traces of shifts in extracellular space volume (α) following switch from sleep to awake-inducing aCSF (*upper trace*) or awake to sleep-inducing aCSF (*lower trace*) Note: higher amplitude = decreased dilution of TMA$^+$, and smaller extracellular space. Data are summarized to the right. Paired t-test (**awake-inducing**: n = 16 animals; $t(15)$ = 11.04, $P < 0.0001$; **sleep-inducing**: n = 11 animals; $t(10)$ = 8.95, $P < 0.0001$) **$P < 0.01$. Scale: x = 2 min, y = 2 mV. (**E**) Schematic of cisterna-magna infusion and wire EEG/EMG recording setup. (**F** and **G**) Representative traces showing EEG and EMG activity prior, during, and following a 0.3-0.5 μl min^{-1} infusion of modified awake-inducing (**F**) or sleep-inducing (**G**) aCSF into the cisterna magna. The 1-4 Hz relative power (% of 1-32 Hz) is presented in averaged 10 min bins below. Infusion was run between ZT 5.5-7 (gray bar) (**F**) and ZT15-16.5 (purple bar) (**G**), during sleep and awake periods, respectively. Scale bar: x = 30 min, y = 0.5 mV (**F**), 1mV (**G**).

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Supplementary Materials and Methods

All data were collected in male C57/BL6 mice (Charles River, 8-12 weeks) housed on a 12-hour light/dark cycle. A subset of mice were acclimated for > 2 weeks to a reverse-light cycle in order to facilitate awake-recording experiments. All experimental times are normalized to the diurnal cycle as zeitgeber (ZT) times, with ZT0 = the time of lights on (beginning of the sleep period), and ZT12 = the time of lights off (beginning of the awake period). The experiments were approved by the Institution of Animal Care and Use Committee of University of Rochester and performed according to guidelines from the National Institutes of Health. Efforts were taken to minimize the number of animals used.

Preparation of Ion-Sensitive and ECoG Microelectrodes

Ion-sensitive and ECoG microelectrodes (ISM) for K^+, Ca^{2+}, Mg^{2+}, and H^+ were pulled from single-barreled pipette glass (TW150-4) with a tip of < 2-3 μm with a puller. Pipettes were silanized using dimethylsilane. K^+, Ca^{2+}, and H^+ ion-sensitive microelectrodes (ISMs) were loaded with a ~300 μm column of valinomycin-based K^+ ion-exchange resin (Potassium Ionophore I-Cocktail B), Ca^{2+} ion-exchange resin (Calcium Ionophore II- Cocktail A), or H^+ ion –exchange resin (Hydrogen Ionophore I-Cocktail A) and backfilled with 150 mM KCl (*25*), 100 mM $CaCl_2$ (*25*), and phosphate-buffered saline (PBS) with a pH of 7.4 (*26)*, respectively. A reference glass ECoG recording electrode was backfilled with 150 mM NaCl and placed < 50 μM from the ISM electrode. Mg^{2+} ISMs were prepared as described previously with minor modifications (*27*). Briefly, the Mg^{2+} ionophore was prepared using Mg^{2+} ion-exchange resin (Magnesium Ionophore IV). The original solvent (tetrahydrofuran) was replaced with a mixture prepared with cyclohexanone, o-NPOE (2-Nitrophenyl octyl ether), ETH500 (Tetradodecylammonium tetrakis (4-chlorophenyl) borate), KTPC1PB (Potassium tetrakis (4-chlorophenyl) borate), PVC (Poly (vinyl chloride)). The solution was vortexed for at least 3 hours, or until all the solutes were dissolved.

Ion-sensitive Electrode Calibration for in vivo Recordings

Electrodes were calibrated immediately prior to and following each experiment using either awake or sleep aCSF solutions and a stepwise gradient of K^+ (2.5 mM, 3.5 mM, 4.5 mM), Ca^{2+} (0.5 mM, 1.0 mM, 1.5 mM), or H^+ (pH (6.5, 7.5, 8.5), titrated with 2 M HCl/1 M NaOH in the presence of 10 mM HEPES using a pH meter). Calibration data were fitted to the Nikolsky equation to determine the electrode slope and interference (28). Mg^{2+}-ISMs were calibrated in 3 groups, each containing 0 mM, 1.0 mM, or 1.5 mM Ca^{2+} in addition to a stepwise gradient of Mg^{2+} (0.5 mM, 1.0 mM, and 1.5 mM). In a subset experiments, Mg^{2+} microelectrodes were also tested in 0 mM Mg^{2+} and a gradient of Ca^{2+} (0.5 mM, 1.0 mM, and 1.5 mM) for calculation of the Ca^{2+}-selectivity coefficient.

While H^+, K^+, and Ca^{2+} were highly selective, Mg^{2+} electrodes exhibited more variable Ca^{2+} sensitivity. To correct for this, the selectivity coefficient of the electrode for Ca^{2+} (K_{MgCa}) was estimated in Mg^{2+} using the separate solution method (29), where the potentials observed at equal activity for Ca^{2+}-free and Mg^{2+}-free solutions are used to estimate K_{MgCa}. 5 of 22 electrodes exhibited a K_{MgCa} outside the range of [0.1-0.6] and were excluded. The remaining values were averaged and used to correct for Ca^{2+} interference in sleep/wake and microdialysis studies, with $[Ca^{2+}]_e$ shifts between states recorded with the Ca^{2+} ISM subtracted from $[Mg^{2+}]_e$ shifts during analysis. As an independent control analysis, the K_{MgCa} for the Mg^{2+} electrodes was used to calculate the change in $[Ca^{2+}]_e$ when awake mice were anesthetized with isoflurane.

Slice Preparation and recording of $[K^+]_e$

All the slices used for $[K^+]_e$ recordings were prepared from adult mice (8-10 weeks) as described previously (30). Briefly, mice were anesthetized using 1.5% isoflurane in a closed chamber, were sacrificed, and brains were rapidly immersed in ice-cold cutting solution containing 230 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 10 mM glucose (pH 7.2-7.4). Coronal slices (350 μm) were prepared using a vibratome from ~bregma 1.0-1.5 mm, roughly somatosensory cortex. Slices were then transferred to an oxygenated aCSF containing 126 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 10 mM glucose (pH 7.2-7.4, osmolality = 300 mOsm). Slices were incubated in aCSF for 1 to 5 hours at room temperature before recording. Throughout recordings, slices were superfused with aCSF containing 2.5 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM

$MgCl_2$ gassed with 5% CO_2 and 95% O_2 at room temperature. For measurement of $[K^+]_e$, K^+-ISM and saline backfilled reference electrodes were prepared as described above and inserted into the layer II-III somatosensory cortex, approximately 200 μm below the pial surface, within 50 μm of each other. Following acquisition of a 10-20 minute stable baseline recording, the perfusion aCSF was switched with an aCSF with the same ionic concentrations in addition to a neuromodulator cocktail consisting of 40 μM NE (norepinephrine), 10 μM Ach (acetylcholine), 10 μM DA (dopamine), 50 μM HA (histamine), and 400 μM ascorbic acid to prevent oxidative degradation. Concentrations were chosen based off of previous slice studies, and are based on effective concentrations used to see neuronal effects. These are higher than basal extracellular concentrations seen with in vivo microdialysis studies as 1) Microdialysis concentrations are the average concentration seen over extended timeframes, reducing their ability to measure transient changes resulting from burst-release events, 2) NE is highly susceptible to oxidation, potentially reducing both the effective concentration within slice as well as the level measured with microdialysis, 3) to our knowledge no slice studies have shown changes at microdialysis-derived concentrations, and 4) Significant metabolic, gene expression, and inflammatory changes accompanying slice preparation may reduce latent responsiveness to these modulators. A subset of slices were perfused with a cocktail solution containing 0.1 μM Orexin A and B, and, showing no significant difference in $[K^+]_e$ increase (unpaired, two-tailed t-test of all cocktail ± TTX with versus without Orexin A/B; n = 7 slices with Orexin A/B, mean change = 0.342, SEM = 0.038 mM $[K^+]_e$, n = 27 slices without, mean change = 0.363, SEM = 0.039 mM $[K^+]_e$: t(32) = 0.257, P = 0.799) were grouped with the remaining slices. Following a 20 minute recording period to determine transitions between solutions, the perfusion-aCSF was switched back to the baseline solution to permit recording of a 10-20 minute recovery period. Two separate groups of TTX recordings were performed. In the first set, perfusion aCSF was replaced with an aCSF containing 1 μM TTX in addition to the neuromodulator cocktail. In a second series of experiments, aCSF containing TTX was perfused 15 minutes prior to perfusion with TTX + neuromodulator cocktail. This group was added to completely block synaptic transmission prior to exposure to the neuromodulator cocktail. However, the groups exposed to TTX exhibited a comparable increase in $[K^+]_e$ when exposed to the neuromodulator cocktail (one-way ANOVA of all groups relative to initial aCSF without TTX baseline: F (4,45) = 19.8, P < 0.0001], no significant difference was found using the Tukey post-hoc test between TTX groups where TTX

1 was added with the neuromodulator cocktail (mean ± SEM) [0.31 ± 0.04] or 15 minutes prior

2 [0.33 ± 0.04]) Tukey multiple comparison post hoc test, $P > 0.9$. For clarity both TTX groups

3 were pooled and shifts in $[K^+]_e$ for each change in solution (eg, aCSF to TTX or aCSF to

4 neuromodulator cocktail + TTX) were compared. To evaluate peak amplitude changes in $[K^+]_e$,

5 a subset of slices were exposed to the inhibitor of glycolysis iodoacetate (3.5 mM) (*31*) for 10

6 minutes after administration of the neuromodulator cocktail and prior to switching back to the

7 baseline solution.

8

9 **Animal Preparation for *in vivo* recordings**

10

11 Mice were prepared as described previously (*32*). Briefly, animals were anesthetized using

12 isoflurane (1.5%) two days prior to recordings. A custom-made headplate was then affixed to

13 the skull using dental cement to permit head-restraint. Over the following days, animals were

14 gradually acclimated to head-restraint on the microscope stage for a total duration of 4-5 hours.

15 The mice were resting in a padded container in a quiet dark room. Animals that were not

16 sleeping during the last training sessions were excluded. To mimic recording sessions, efforts

17 were taken to ensure minimal noise and light interference during training. On the morning of

18 recordings animals were again anesthetized with isoflurane (1.5%), and a 1-1.25 mm cranial

19 window was prepared over somatosensory cortex (-1.5 mm Anterior/Posterior, 3 mm

20 Medial/Lateral). Custom-made EMG recording electrodes (From DSI, portable transmitter) were

21 implanted within the neck musculature. Body temperature was maintained at 37°C. Animals

22 were permitted to recover for a minimum of 30 minutes prior to recording.

23

24 **In vivo recordings of extracellular ion concentrations and ECoG**

25

26 In vivo recordings of extracellular cation concentrations were obtained from layer II-III

27 somatosensory cortex (200 μm below the pial surface). For all experiments ECoG electrodes

28 and ISMs were prepared as described above and inserted within 50 μm of each other. ECoG and

29 extracellular ion concentrations ($[K^+]_e$, $[Ca^{2+}]_e$, $[Mg^{2+}]_e$, $[H^+]_e$) were recorded in the awake, sleep,

30 and 2% isoflurane anesthetized states. EMG recordings were obtained to further verify shifts

31 between sleep, awake, and anesthetized states, in a subset of animals. ECoG and extracellular

ion concentrations data were sampled at 10 kHz and filtered from 0.1-2 kHz using an MultiClamp 700A /700B. Data were then digitized using Digidata 1322A, recorded using clampex 9.2 or clampex 10.2, and analyzed using Clampfit 9.2 or 10.2. EMG measurements were recorded using a DP-311 Differential Amplifier.

Natural Sleep/Wake Transitions and Isoflurane Recordings

Natural transitions between sleep and wakefulness were recorded during the animals sleep period between ZT4-8 with the cranial window covered by the sleep aCSF (table S1). Efforts were taken to allow the animals to fall asleep, including contact with the same person and minimal noise interference. To compare natural transitions from sleep to awake states, sleeping mice were woken up by gentle air puffs directed at the tail or eyes. Isoflurane experiments were run during the awake period (ZT16-20) using awake aCSF to cover the cranial window. After obtaining a 30-50 minute baseline recording, 2% isoflurane anesthesia was administered using an SAR 830/P ventilator, SurgiVet vaporizer, and custom-made, rubber nose-cone. Anesthesia was maintained for 30-50 minutes to assess the stability of state-dependent shifts in extracellular ion concentrations, and recordings were continued for 20-60 minutes after discontinuing anesthesia. 10 mM HEPES was excluded from the awake aCSF solution for recordings of awake to isoflurane anesthesia using the H^+-ISM.

For analysis of ion concentrations, DC shifts were adjusted for through subtraction of the reference trace from the value obtained with the ISM. Resulting traces were then reduced to a 10 Hz sampling interval using and exported into excel. Data (mV) were calculated as median voltage for each experiment, and were converted to mM values using the Nikolsky equation and individual electrode calibration curves. Sleep/wake transitions displayed a significant reduction in power averaging 30% (Fig. S1D-F), with increases in EMG activity, visually observed mouse activity and 1-4 Hz delta power.

To identify stable state-dependent shifts, state changes lasting a minimum of 1-minute were used. Four-separate 30 s epochs were used for each sleep/wake transition, with baseline sleep values being defined as the 30-60 s prior to the stimulation or clear sleep-wake transitions; non-overlapping awake periods followed 30-240 s post stimulation, as the ion trace appeared to reach a stable level, and recovery concentrations were calculated at least 5 minutes following the

return to sleep. For isoflurane recordings, two-minute bins were analyzed from immediately prior to isoflurane induction, cessation, and following stabilization of the ion trace minutes after discontinuation of anesthesia. In figures, the average of the 1-4 Hz power was binned and presented at 10-20 s (wake/sleep) or 3 min (Isoflurane traces) in order to illustrate transitions.

ECoG power was analyzed using a custom-written Matlab code. In brief, data were exported from Clampex into Matlab, and spectrograms were derived for each transition. The average power density and prevalence for each segment corresponding to each sleep or awake state was taken over 4 or 10s epochs and used for raw power analyses. Relative power was calculated by normalizing traces to the total 1-32 Hz power. CNQX raw power analysis of EMG was performed similarly using the sum of 1-100 Hz frequencies. To reduce movement-related noise, epochs exhibiting >2x the mean awake value were excluded from analysis (Avg. 2.97% of CNQX ECoG epochs). For CNQX spectral analysis, data were exported to matlab and converted to power density using the fast fourier-transform function, normalized to 1-32 Hz power to derive relative prevalence, and plotted using Prism.

To determine the duration of shifts between states, data were similarly reduced to 10-100 Hz bins, and state shifts were defined as the time taken for an ion to increase or decreased from its pre-transition baseline to 90% of its stable post-transition concentration. To better gauge sleep-wake shifts, these shifts were matched to shifts in ECoG total power, as well as shifts in 1-4 Hz prevalence and EMG power when possible to determine correlations between electrophysiological and ionic measures of wakefulness. Further, isoflurane-induction was measured from the beginning of isoflurane administration. To adjust for noise, a 0.02-2 s rolling average was taken, and shifts were determined as the earliest point crossing the 90% threshold - *provided* that the increase/decrease was sustained for a minimum of 0.5 s.

Representative ion traces were presented as the 2 s (sleep/wake traces, 10 ms bins) or 20 s (isoflurane and CNQX traces, 100 ms bins) rolling average of the raw data. Data were graphed and analyzed using Prism 5. For each group, a one-way, repeated-measures ANOVA was performed and groups were individually compared using a Tukey post-Hoc test for transitions between sleep/wake, CNQX, and isoflurane.

Recordings evaluating the effect of CNQX on extracellular ion concentrations

1   To define the role of excitatory transmission on state-dependent changes in extracellular

2   ion concentrations, the AMPA receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione,

3   CNQX was added to the bath aCSF in a subset of experiments. In these studies, 30 minute

4   baseline recordings were obtained using awake mice with awake aCSF covering the cranial

5   window (table S1). Following this period, the awake aCSF solution was substituted with the

6   same solution containing 200 µM CNQX as described previously (*33*). ECoG and ion levels

7   typically stabilized within 10 minutes, and recordings under CNQX were continued for an

8   addition 10-20 minutes. ECoG power was analyzed using the power spectrum function available

9   in Clampfit 10.2. Data were exported to excel and power values were summed over the 1-32 Hz

10   range to determine total power. In order to guarantee substantial reduction of ECoG power

11   under CNQX for determination of the effects of excitatory neuronal activity on shifts in

12   extracellular ion concentration, animals exhibiting less than a 60% reduction in ECoG power

13   under CNQX were excluded from analysis. 2% isoflurane was subsequently administered

14   through a nose cone and recordings were continued in the presence of both CNQX and isoflurane

15   for an additional 40-60 minutes.

16

17   **Defining the effects of changing aCSF solutions on Extracellular Ion Concentrations**

18

19   To recapitulate state-dependent shifts in $[K^+]_e$, $[Ca^{2+}]_e$, $[Mg^{2+}]_e$, and $[H^+]_e$ in extracellular space,

20   we conducted a series of exploratory experiments to determine the magnitude of shifts in ion

21   concentration at 200 µM below the cortex resulting from changing the aCSF solution over the

22   cortex. Using this, we designed a series of aCSF solutions to mimic previous *in vivo* recordings

23   of awake and sleep states as well as "awake-inducing" and "sleep-inducing" aCSFs (table S1),

24   which were designed to drive cation concentration shifts similar to natural sleep/awake

25   transition. For all of the following experiments, the solutions in *table S1* were used to either

26   maintain the current brain-state dependent ionic milieu or drive concentrations of $[K^+]_e$, $[Ca^{2+}]_e$,

27   $[Mg^{2+}]_e$, and $[H^+]_e$ to the opposing behavioral state (eg, shifting the brain locally from sleep to

28   awake in sleeping mice by removing sleep aCSF and replacing it with awake-inducing aCSF

29   (table S1, Group 2). These transitions were all validated with ISMs as described above and as

30   shown in Fig. S4.

31   Transitions for sleeping mice were recorded from ZT4-8 during the mouse's natural sleep

cycle. In these experiments, sleep aCSF was placed over the cortex of sleeping mice, and was later replaced with awake-inducing aCSF in order to determine the magnitude of the shift for each ion measured. Likewise, transitions for awake mice were recorded between ZT16-20, during the mouse's natural awake period. Following a baseline period using awake aCSF solution over the cortex, aCSF was changed to the sleep-inducing solution and resulting ion changes were measured. 30-40 minute recordings were taken for each solution to permit ion equilibration and to test the stability of shifts in interstitial ion concentration (20-30 minutes). $[Ca^{2+}]$ was held constant in $[Mg^{2+}]_e$-experiments to eliminate the effect of changes in $[Ca^{2+}]$-interference resulting from changing the aCSF solution. Efforts were taken to carefully change the solutions in surface pool without touching either electrode and to minimize disturbing the mice during the process. The median of a 1-minute stable epoch was selected following equilibration, usually 10-20 minutes after applying a solution, and converted into mM using the Nikolsky equation. Data from these experiments were plotted and statistics were analyzed using Prism 5 using two-tailed, paired t-tests and one-way repeated measures ANOVA with post-hoc Tukey multiple comparisons tests to determine differences in 2 and 3 group experiments, respectively.

Effects of manipulating extracellular ion concentration on local ECoG Activity

To evaluate the effect of imposing changes in the extracellular ion concentration, 1.0-1.25 mm cranial windows were prepared symmetrically over both hemispheres (-1.25 mm Anterior/Posterior, 3 mm Medial/Lateral), and a glass ECoG recording electrode was inserted symmetrically 200 μm deep in each hemisphere. In the first set of experiments, recordings were obtained between ZT4-8 to study the effects of awake-inducing aCSF on sleeping mice. In this experiment, sleeping mice were prepared as described above, with sleep aCSF over both the left and right cranial windows (table S1). Concentrations were chosen to elicit shifts in interstitial ion composition at 200 μM below the pial surface comparable to those seen in natural sleep/wake transitions, and did not vary by more than 1-2.5 mM of baseline levels for both sleep and awake states in any formulation in order to minimize gradient effects occurring between the surface of the brain and the recording depth. Following a baseline ECoG recording, the solution covering the left window was removed and replaced with awake-inducing aCSF (table S1).

ECoG recordings were converted into power-spectra using clampfit 10.2, and data were exported to excel. Raw power from each hemisphere was normalized to 1-32 Hz overall power for each trace, and the 1-4 Hz slow-wave power from the left (aCSF changed) hemisphere was normalized to the right hemisphere to account for potential differences resulting from natural, whole-brain state fluctuations. In the second set of experiments, awake animals were recorded between ZT16-20. Following a baseline period where awake aCSF was placed over both hemispheres, the solution over the left hemisphere was removed and replaced with sleep-inducing aCSF (table S1). ECoG was analyzed as before, data were graphed and analyzed using Prism 5, and a two-tailed, paired t-test was used to compare the difference in normalized delta power between baseline and left-window-altered groups.

Iontophoretic tetramethylammonium (TMA$^+$) quantification of the extracellular space volume

All experimental procedures were adapted from previous studies (*10, 11, 28, 34-37*). For measurements of TMA$^+$, microelectrodes with an outer diameter of 2–3 μm were fabricated from double-barreled theta-glass using a tetraphenylborate-based ion exchanger. The TMA$^+$ barrel was backfilled with 150 mM TMA-chloride and the reference barrel filled with 150 mM NaCl and 10 μM Alexa 568. All recordings were obtained by inserting the two electrodes to a depth of 200 μm below the cortical surface in somatosensory cortex. The electrode tips were imaged after insertion using 2-photon excitation to determine the exact distance between the electrodes (typically ~150 μm). The TMA$^+$ signal was calculated by subtracting the voltage measured by the reference barrel from the voltage measured by the ion-detecting barrel using a dual-channel microelectrode preamplifier. The Nikolsky equation was used for calibration of the TMA$^+$ electrodes based on measurements obtained in electrodes containing 0.5, 1, 2, 4, and 8 mM TMA-chloride in 150 mM NaCl. A series of currents of 20 nA, 40 nA, 80 nA, and 120 nA were applied using a dual-channel microelectrode preamplifier, and measurements were acquired relative to similar recordings obtained in 0.3% agarose prepared from a solution containing 0.5 mM TMA-Cl and 150 mM NaCl. A custom-made MATLAB software, 'Walter', developed by C. Nicholson was used to calculate α and λ values.

For CNQX experiments, awake mice were prepared as described above with the TMA$^+$ electrodes located in the somatosensory cortex. The first set of animals was prepared with awake

aCSF (table S1) placed over the cortex. TMA$^+$ values were estimated both prior to and after the administration of 2% isoflurane through a nose cone. For the second set of experiments awake mice were prepared starting with awake aCSF over the surface of the cortex. Following a brief baseline recording period the awake aCSF was removed and replaced with awake aCSF containing 200 μM CNQX. TMA$^+$ pulses were then given both during the CNQX/awake state as well as after the administration of 2% isoflurane (isoflurane + CNQX). For these experiments a 1-minute window of the ECoG prior to the first TMA$^+$ pulse was used for a power spectrum analysis with Clampfit 10.2. The resulting 1-32 Hz power was summated and normalized to the base pre-CNQX baseline to determine the % change in ECoG power.

To study shifts in extracellular space resulting from changes in surface aCSF, two cohorts of mice were used. In the first set of recordings, mice were prepared as described above with sleep aCSF over the surface of the brain (table S1), and were lightly anesthetized using 1% isoflurane to prevent awakening mice during frequent manipulations. Following baseline TMA$^+$ recordings, sleep aCSF was replaced with awake-inducing aCSF and a second set of TMA$^+$ recordings was conducted. In the second set of recordings, awake mice were prepared as above with awake aCSF placed over the surface of the brain. Again following recording of baseline TMA$^+$ values, awake aCSF was removed and replaced with sleep-inducing aCSF. TMA$^+$ values were collected again to determine if local ionic changes could increase the extracellular space volume similar to natural sleep. The α and λ values were compared between each pair of aCSF solutions without moving the electrodes respectively (table S1). Tortuosity (λ) was consistent with previous reports (11), with λ increasing slightly from λ =1.71 ± 0.02 to 1.77 ± 0.03, paired t-test: t (15) = 3.425, P = 0.004, in sleep aCSF to awake-inducing aCSF experiments and remaining unchanged (λ = 1.52 ± 0.07 to 1.52 ± 0.07, paired t-test, t (10) = .0844, P = 0.934) in awake aCSF to sleep-inducing aCSF experiments. A two-tailed, paired t-test was used to test for statistically significant differences between groups.

EMG and EEG recording during Cisterna Magna Infusion

For EMG and EEG recording electrodes were prepared as described previously (38). Briefly, six-channel, custom-made EEG/EMG recording electrodes were prepared by soldering small (<0.75'') segments of insulated 0.008'' silver wire into gold-pin connectors. These electrodes

were then combined into a six-channel EEG electrode holder and secured using dental cement. The EEG and electromyogram (EMG) recording wires were inserted over the surface of the skull (4 electrodes) and the neck musculature (2 electrodes). For aCSF infusion, a cisterna magna cannula was implanted (30G blunted needle) and connected with closed-end polyethylene tubing, which was pre-filled with aCSF and sealed. The cannula was sealed into the skull with cement. After recovery from anesthesia, mice were placed in a recording chamber, and the electrode holder was connected to a 6-channel commutator to permit free movement of the mouse within the chamber. Food and water were freely accessible in the recording cages.

For the first set of experiments animals were habituated overnight to permit the recordings to stabilize and all infusions/recordings were conducted during the animal's light cycle, where it has the highest proclivity toward sleep (*39*). Recording sessions were started at ZT2 to record baseline EEG/EMG activity. The cisterna magna cannula was opened and connected to a dual-channel Harvard Apparatus syringe pump and perfused at 0.3 μl min^{-1} with sleep aCSF for a minimum of 30 minutes to equilibrate the system and eliminate animals in which the baseline aCSF infusion changed EEG activity. The sleep aCSF was replaced with a modified awake-inducing solution of (10 mM KCl, 0.5 mM $CaCl_2$, 0.3 mM $MgCl_2$, pH 8.9) and the brain was perfused for approximately 1.5 hrs at 0.3 μl min^{-1}. After this period aCSF infusion was terminated and the EEG/EMG recording was continued until ZT12 for analysis of recovery. We conducted the second set of experiments between ZT13 and ZT20, where animals have the highest proclivity for wakefulness (*39*). In this set of experiments, animals were first implanted with EEG/EMG recording electrodes and CM cannula, and habituated to the recording setup. On the day of recording, animals were given 12 hours to acclimate to the recording setup and EEG/EMG recordings were started at the beginning of the dark cycle (ZT12). One-hour later, awake aCSF infusion was commenced at 0.5 μl min^{-1} to evaluate the effect of aCSF infusion upon the EEG signal. Again, the few animals that displayed a change in EEG signal in response to aCSF infusion were not used. After a minimum of 30 minutes awake aCSF-infusion, a ~1.5 hour infusion of modified sleep-inducing aCSF (1.5 mM KCl, 2.5 mM $CaCl_2$, 4.5 mM $MgCl_2$, pH 7.0) was conducted at the same rate, with EEG/EMG recordings continued for several hours afterward to permit analysis of the mouse's return to normal circadian activity. Modified aCSF ion concentrations and influx rates calculated based off several major considerations: 1) Previous studies have shown the rate of CSF production and absorption in the brain is roughly

35

0.37 µl min^{-1} (40). Infusion rates were comparable to this with slight differences in sleep and awake to account for differences in the possible concentration of infused ions. Ion concentrations were estimated first assuming constant mixing of the infused aCSF and continuous endogenous CSF production. (eg. assuming in the natural sleep state a 0.37 µl min^{-1} rate of CSF production containing 3.86 mM K$^+$ and a 0.3 µl min^{-1} infusion rate of 10 mM K$^+$-aCSF, this would mix to create a constant 6.6mM K$^+$ in CSF; not considering continuous CSF and ion clearance) 2) In addition to the continuous mixing of new CSF and the infusion aCSF, total brain volume is ~503-637 µl in adult mice (40-42). As such, with a 0.3 µl min^{-1} infusion rate, this would be approximately 0.0029-0.0036 mM min^{-1} assuming dilution into a single fluid compartment. 3) While it is possible that the dorsal surface of the brain and the spinal cord near the infusion site may be exposed to higher concentrations of the cations in these experiments, our previous work with the glymphatic system shows that CSF is transported by convective fluxes through the brain parenchyma and along the vasculature effectively, distributing changes over a large volume and preventing substantial buildup at any given point. Finally, 4) the brain is highly effective at clearance of cations, particularly K$^+$, which can be quickly cleared from the extracellular space by astrocytes and redistributed to neighboring astrocytes (43). EEG recordings were conducted using an XLTEK, 32-channel EEG system. Electrodes were referenced to one skull electrode and saved using XLTEK, and data were analyzed using Neuroscore to determine percent prevalence (Delta: 1-4 Hz, Theta: 4-8 Hz, Alpha: 8-13 Hz, Beta: 13-32 Hz) and time spent asleep/awake.

Quantification of extracellular cation concentrations using microdialysis

To determine state-dependent changes in [K$^+$]$_e$, [Ca^{2+}]$_e$, [Mg^{2+}]$_e$ in the cortex of awake, sleeping, and anesthetized mice a microdialysis guide cannula was positioned within the medial prefrontal cortex at a location of AP +2.0, ML +0.3 from Bregma, and DV -1.0 below the dura, as described previously (10). The guide cannula was secured to the skull using dental cement and animals were permitted to recover. 3-5 days later, a 2-mm microdialysis probe was inserted into the brain and perfused with filtered aCSF (For K$^+$ and Ca^{2+}: 155 mM NaCl, 4 mM KCl, 1.25 mM CaCl$_2$, 2 mM Na$_2$HPO$_4$, and 0.85 mM MgCl$_2$; For Mg^{2+}: 140 mM NaCl, 4 mM KCl, 1.25 mM CaCl$_2$, 20 mM NaHCO$_3$, and 0.85 mM MgCl$_2$, both adjusted to 300-305 mOsm and pH=7.35-
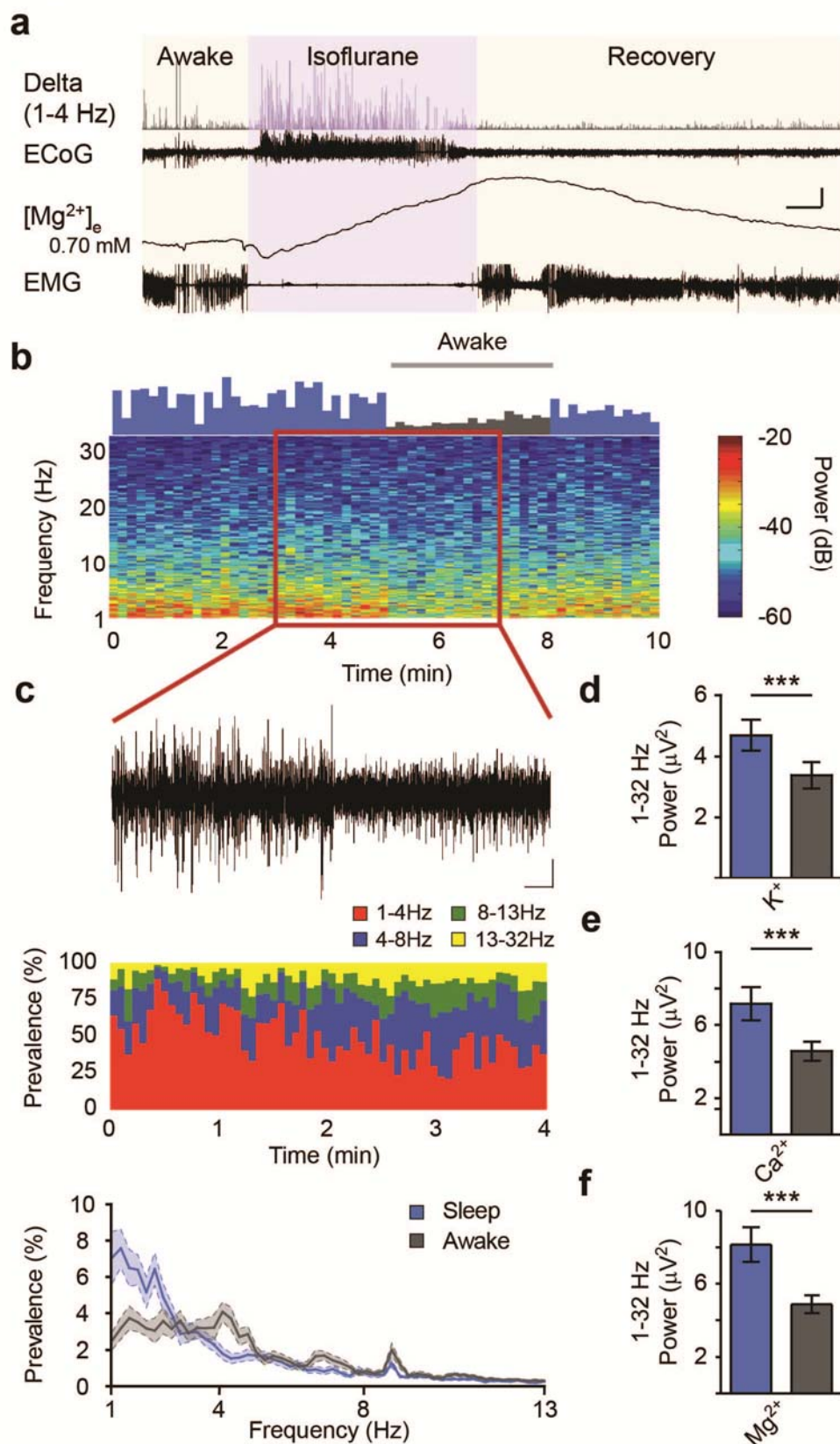
7.40). Animals were allowed to recover for a minimum of 3 hours following probe implantation, with sleep samples collected between ZT2-8 and awake samples collected between ZT14-20. Isoflurane measurements were typically performed between ZT14-20 as well, and mice were monitored to maintain body temperature throughout 1.5% isoflurane anesthesia.   For ion measurement studies, a gradient of 5-aCSF solutions was used for each ion to estimate the true brain concentration.   For each ion, the above aCSF was used with minor changes in the concentration of the ion being studied. The gradients used were: $[K^+]$ = (1.75, 2.5, 3.25, 4.0, and 4.75 mM); $[Ca^{2+}]$ = (0.5, 1.0, 1.5, 2.0, and 2.5 mM); and $[Mg^{2+}]$ = (0.5, 0.9, 1.3, 1.7, and 2.1 mM). Sets of 5-20 µl samples were collected at a rate of 1 µl min^{-1} using a dual-channel syringe pump, and tubing was washed between each sample to prevent cross-contamination.   Sample orders were randomized in each animal to prevent bias from sequential ordering, and samples were immediately frozen following collection.

For measurements of ion concentrations in sample solutions, ISMs were prepared as described above, and calibrations were performed before and after each sample or set of 2-3 samples. Calibration solutions used were taken from the same daily stock solutions used for microdialysis, with two different base aCSF solutions: For K^+ and Ca^{2+} studies, aCSF contained 4.0 mM KCl, 1.25 mM $CaCl_2$, 0.85 mM $MgCl_2$, 155 mM NaCl, and 2 mM NaH_2PO_4. Due to interference between NaH_2PO_4 and the Mg^{2+}-ionophore, Mg^{2+} microdialysis studies used aCSF containing 4.0 mM KCl, 1.25 mM $CaCl_2$, 0.85 mM $MgCl_2$, 140 mM NaCl, and 20 mM $NaHCO_3$). Solutions were titrated to pH = 7.35-7.4 using 1 M NaOH and 1 M HCl, and were adjusted to an osmolality of 300-305 mOsm using 1.45 M NaCl and a micro-osmometer. For each set of ion analyses, aCSF contained a stepwise change in the ion concentrations. K^+ calibration solutions = [1.0, 1.75, 2.5, 3.25, 4.0, 4.75, and 5.5 mM K^+]; Ca^{2+} aCSF calibration solutions = [0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mM Ca^{2+}]; and Mg^{2+} aCSF calibration solutions = [0.5, 0.9, 1.3, 1.7, 2.1, and 2.5 mM Mg^{2+}]. Calibration slopes and intercepts were calculated using the Nikolsky equation and, for Mg^{2+} electrodes, the Ca^{2+}-selectivity coefficient described above was used. Samples were matched to the nearest corresponding 4 concentrations for each calibration to optimize the fit. (eg, for 1.0 mM Mg^{2+} the calibration curve used the 0.5, 0.9, 1.3, and 1.7 mM Mg^{2+} solutions). Of note, the same two-aCSF solutions were used for the calibrations of the ion selective microelectrodes as used during the microdialysis. Calibrations and samples were tested by adding 20 µl of each solution to a glass coverslip. A ground wire, reference-electrode, and

ion-sensitive microelectrode were then inserted and allowed to stabilize. All samples were collected and analyzed by different investigators and the samples blinded prior to ion measurements.   Following acquisition, data were analyzed using ClampFit 10.2 with resulting data fit to each calibration curve using the Nikolsky equation to determine the ion concentration of the sample. $[Mg^{2+}]_e$ measurements were adjusted for background $[Ca^{2+}]$ using the $[Ca^{2+}]$ selectivity coefficient described above. Data were plotted as the concentration of the ion in the aCSF entering the microdialysis probe versus the change in concentration in the solution collected ($[ion]_{in}$ vs. $[ion]_{in} - [ion]_{out}$). Using the no-net flux method (*44, 45*), and a root-mean squared linear regression was plotted using Microsoft Excel. From this, the x-intercept was taken as the estimated point of no-net flux across the probe membrane; ie, the true brain concentration of the ion. Differences between states were identified using a one-way ANOVA and Tukey post hoc test for K^+, and an unpaired, two-tailed t-test for shifts between pre-isoflurane and isoflurane states for Ca^{2+} and Mg^{2+}.

Fig. S1

a

Awake   Isoflurane   Recovery

Delta (1-4 Hz)

ECoG

$[Mg^{2+}]_e$ 0.70 mM

EMG

b

Awake

Frequency (Hz)

Time (min)

Power (dB)

c

1-4Hz   8-13Hz
4-8Hz   13-32Hz

Prevalence (%)

Time (min)

Prevalence (%)

Sleep
Awake

Frequency (Hz)

d

***

1-32 Hz Power (μV^2)

K^+

e

***

1-32 Hz Power (μV^2)

Ca^{2+}

f

***

1-32 Hz Power (μV^2)

Mg^{2+}

Fig. S1. ECoG power and prevalence shifts in sleep to awake transitions. (**A**) Representative trace from $[Mg^{2+}]_e$ isoflurane showing the raw delta power shift in 10 s epochs throughout the baseline, isoflurane, and recovery periods. (**B**) Total Power and spectrogram of a single representative sleep → wake → sleep transition plotted from 1-32 Hz with power density given by color spectrum depicted to the right. Note relative differences between density of low-frequency between initial sleep and awake periods as well as the gradual increase in delta in early stages of sleep following wakefulness. Data presented in 10 s epochs. (**C**) Representative ECoG trace, relative power prevalence in 4 s epochs, and the average power from each sleep/wake epoch, binned by 0.25 Hz, for the 4 min period centered on the sleep/wake transition. Power by presented in lower panel is displayed as mean (dark line) ± SEM (shaded area)(**D-F**) Summary of total power shifts (1-32 Hz) from sleep → awake transitions. Paired t-test for each group: (**D**) $t(26) = 5.698$, $P < 0.0001$. (**E**) $t(27) = 4.218$, $P = 0.0002$. (**F**) $t(71) = 5.678$, $P < 0.0001$. Data are presented as mean ± SEM with sleep bars shown in blue, and awake bars in gray.

Fig. S2

a

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c

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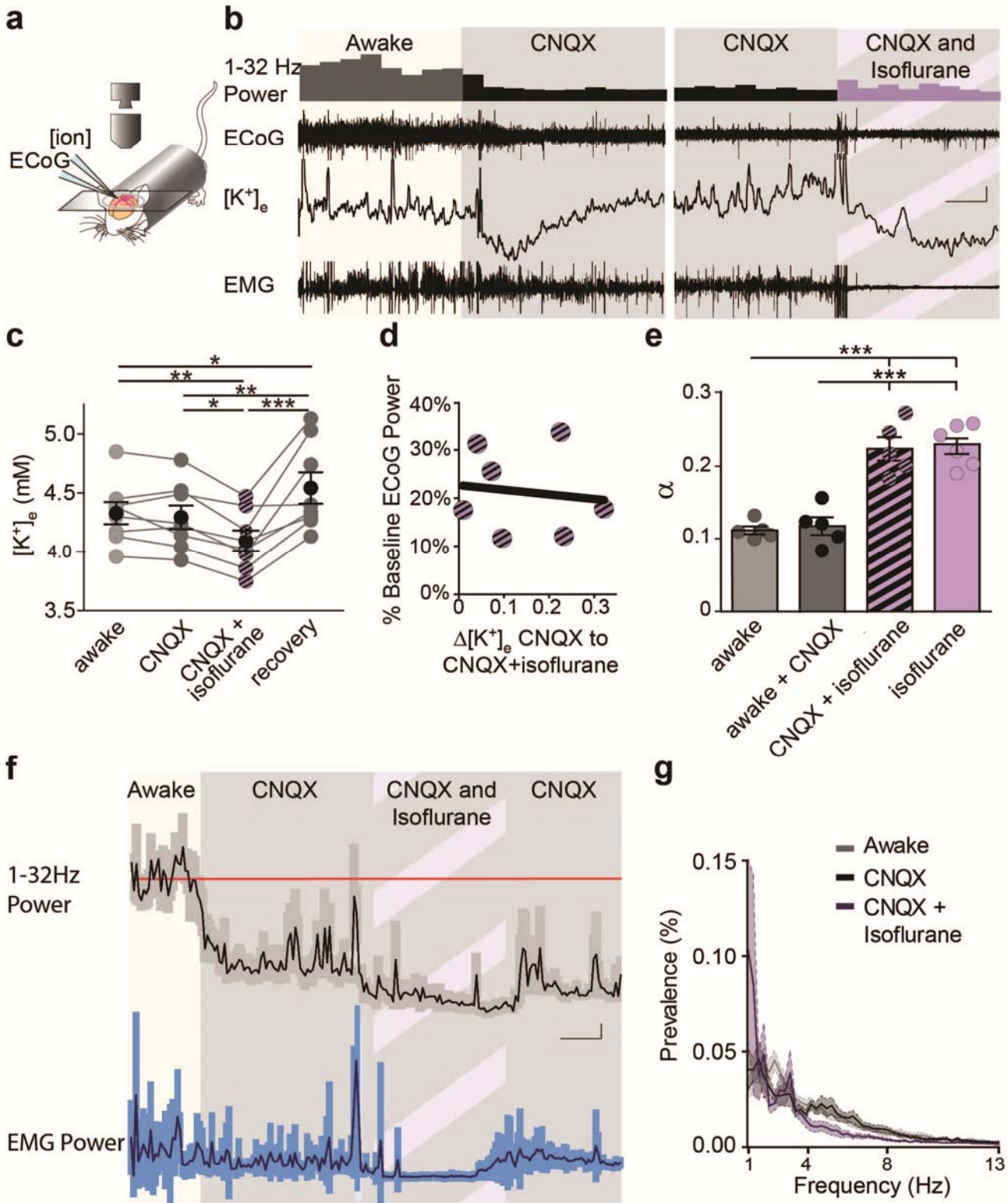
Fig. S2. Blocking local AMPA receptor activity does not alter awake $[K^+]_e$ or the isoflurane-induced decrease in $[K^+]_e$. (**A**) Schematic depicting the recording setup for *in vivo* ion concentration recordings. Mice rest in a padded tube while ECoG and ISM electrodes are placed in the somatosensory cortex. EMG recordings are conducted through wires inserted in the neck musculature. (**B**) Representative recording of ECoG, $[K^+]_e$, and EMG during the application of AMPA-receptor blocker CNQX (200 μM) to the aCSF covering the cranial window of awake mice (*Left*) and during the induction of isoflurane anesthesia in CNQX-treated mice (*Right*). Raw 1-32 Hz power is given in the histogram at the top. Note: Animals exhibiting <60% decrease in ECoG power under CNQX were excluded (**C**) Summary of analyses comparing awake, awake + CNQX, isoflurane + CNQX, and CNQX recovery after isoflurane anesthesia. *n* = 7 animals; One-way, repeated measures ANOVA: $F(3,18) = 23.75$, $P < 0.0001$. Tukey post hoc multiple comparisons test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bars: x = 5 min, y = 0.2 mM $[K^+]_e$, 0.5 mV ECoG/EMG. (**D**) Scatter plot of the change in $[K^+]_e$ in CNQX-treated animals during the induction of isoflurane anesthesia versus remaining 1-32 Hz power relative to baseline. A linear regression of the data is given in black. Note: the flat slope suggests that the reduction in neuronal activity due to CNQX and magnitude of $[K^+]_e$ shift evoked is independent of depth of CNQX-mediated inhibition of excitatory activity. (**E**) Summary of fraction of the extracellular space volume (α value, TMA^+ recording) in awake, awake with CNQX, isoflurane with CNQX, and isoflurane-only animals. *n* = 5 animals per group for (awake, awake + CNQX, CNQX +isoflurane) and 6 animals for isoflurane. One-way ANOVA: $F(3, 17) = 29.20$, $P < 0.0001$. Tukey post-hoc multiple comparisons test: $***P < 0.001$. Error bars: mean (black circle) ± SEM. (**F**) (*top)* Mean ± SEM of total 1-32 Hz power normalized to the starting 5-minute baseline pre-CNQX (*n* = 6 animals). (*Bottom*) Average of all traces for total EMG power normalized to baseline prior to application of CNQX (*n* = 5 animals). Data are presented as mean ± SEM analyzed over 4s epochs for the 20 minutes pre-CNQX and 20 minutes following CNQX, isoflurane, and cessation of isoflurane. Scale bars: x = 5 min, y = 25%, 20% baseline ECoG and EMG power, respectively. (**G**) Summary of relative power from 1-13 Hz, in 0.25 Hz bins, normalized to total 1-32 Hz power throughout recording period. Note the predominately overlapping spectra from awake and CNQX traces. Awake (gray), Awake+CNQX (black), and CNQX + Isoflurane (purple). Data are presented as mean (dark line) ± SEM.
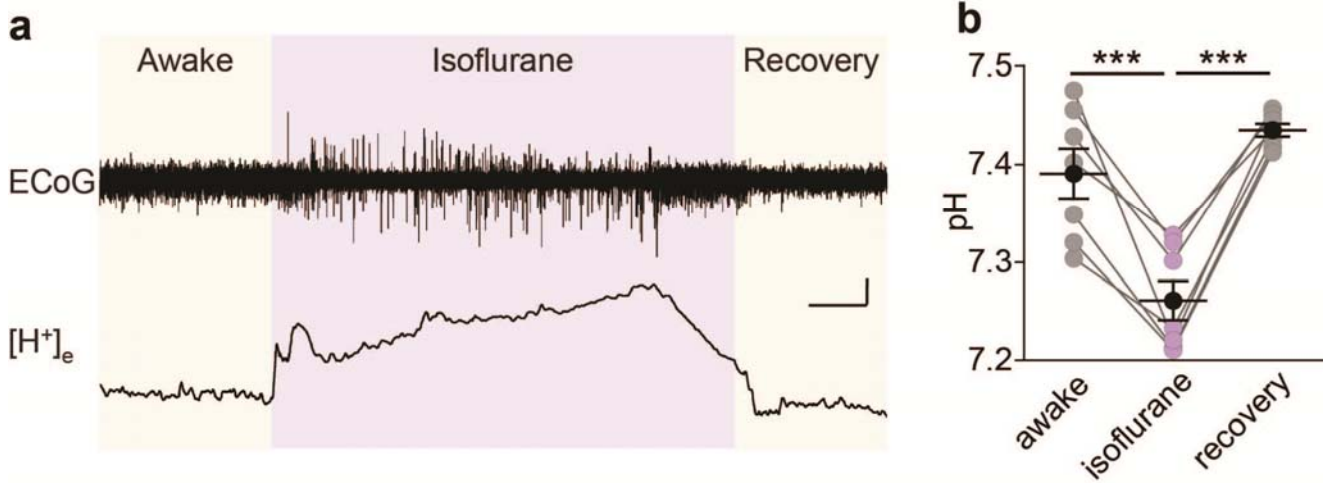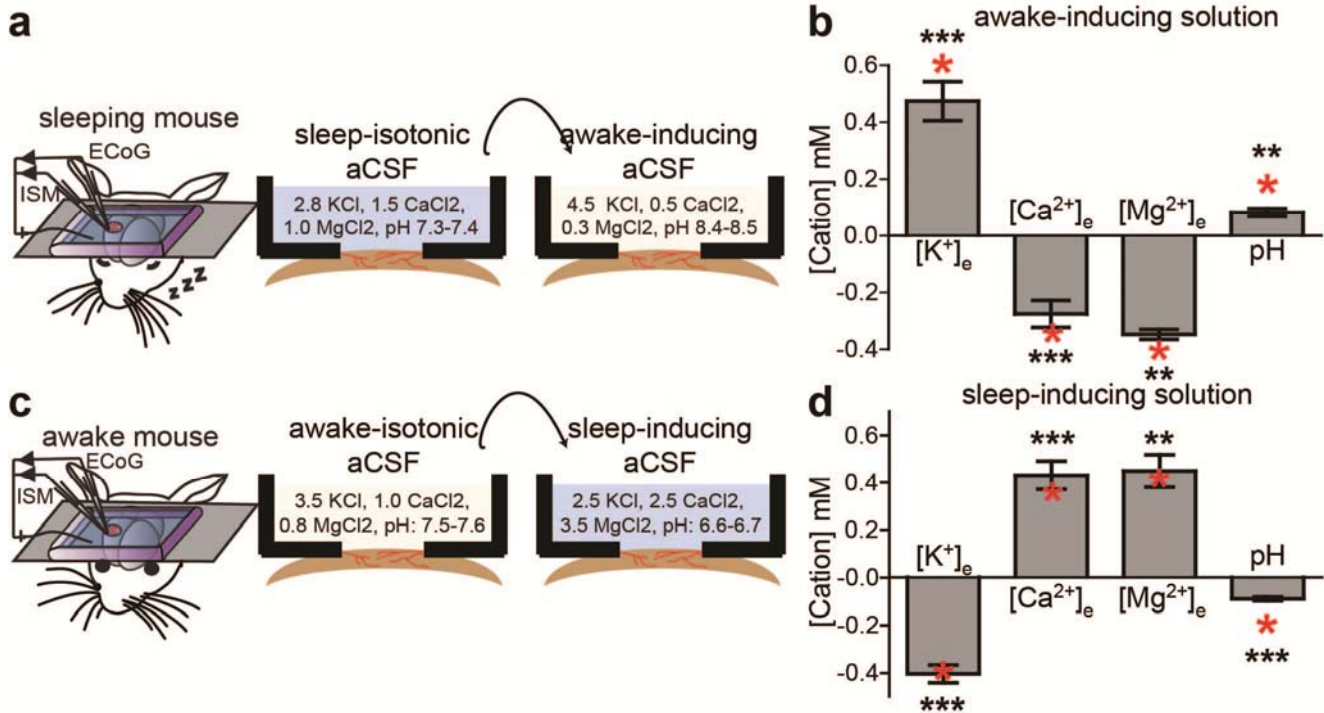
Fig. S3

Fig. S3. Isoflurane anesthesia induces a rapid increase in $[H^+]_e$ in awake mice. (**A**) Representative recording of ECoG and $[H^+]_e$ in an awake mouse before, during, and after administration of isoflurane anesthesia. Scale bar: x = 5 min, y = 0.05 pH, 0.5 mV (**B**) Summary of $[H^+]_e$ shifts, converted to pH scale for each mouse during transitions from awake to isoflurane anesthesia. n = 7 animals. One-way, repeated measures ANOVA: $F_{(2, 12)} = 40.30$, $P < 0.0001$. Tukey post-hoc multiple comparisons test: ***$P < 0.001$. Error bars: mean (black circle) ± SEM.

Fig. S4. Surface aCSF Composition alters extracellular fluid ion concentrations.

(**A**) Schematic figure depicting animal setup and experiment in which recordings were obtained in sleeping mice with sleep aCSF covering the cranial window between ZT4-8. Following baseline recordings, sleep aCSF was removed and replaced with awake-inducing aCSF while the animals remained asleep. (**B**) Summary of local ion changes 200 μM below the pial surface of the cortex following the protocol in **A**. A red asterisk denoting the magnitude of the change in each ion observed during awake to isoflurane transitions in Figs. 2-4, and Fig. S3 is superimposed on each column. Note: local changes in the ion composition of aCSF covering the 1-1.5 mm cranial window does not alter global state of the animals. For two-tailed t-test comparisons of each ion: n = 8 $[K^+]_e$ (t (7) = 6.861, P = 0.0002, 10 $[Ca^{2+}]_e$ ($t(9)$ = 5.228, P < 0.0005, 6 $[Mg^{2+}]_e$ ($t(5)$ = 19.90, P < 0.0001), and 5 pH ($t(4)$ = 5.741, P = 0.0046); ***P* < 0.01, ****P* < 0.001*; two-tailed, paired t-test. (**C**) Schematic figure depicting awake mouse recordings between ZT16-20. Following baseline acquisition, awake aCSF was replaced with sleep-inducing aCSF. Note, mice remained awake and active throughout the duration of the experiment; ion shifts represent local changes recorded at 200 μM below the pial surface. (**D**) Summary of shifts recorded in mice recorded as in **C**. Red asterisks of each ion's recorded shift from awake to isoflurane anesthesia in Figs. 2-4 and Fig. S3 are superimposed. For two-tailed t-

1   test comparisons of each ion: $n = 8$ $[K^+]_e$ ($t(7) = 10.66$, $P < 0.0001$) and $[Ca^{2+}]_e$ ($t(7) = 6.33$, $P =$

2   0.0004), 7 $[Mg^{2+}]_e$ ($t(6) = 5.586$, $P = 0.0014$), and 9 pH animals ($t(8) = 11.18$, $P < 0.0001$). **P

3   < 0.01, ***$P < 0.001$. Error bars: mean $\pm$ SEM. Solution concentrations are given in table S1.

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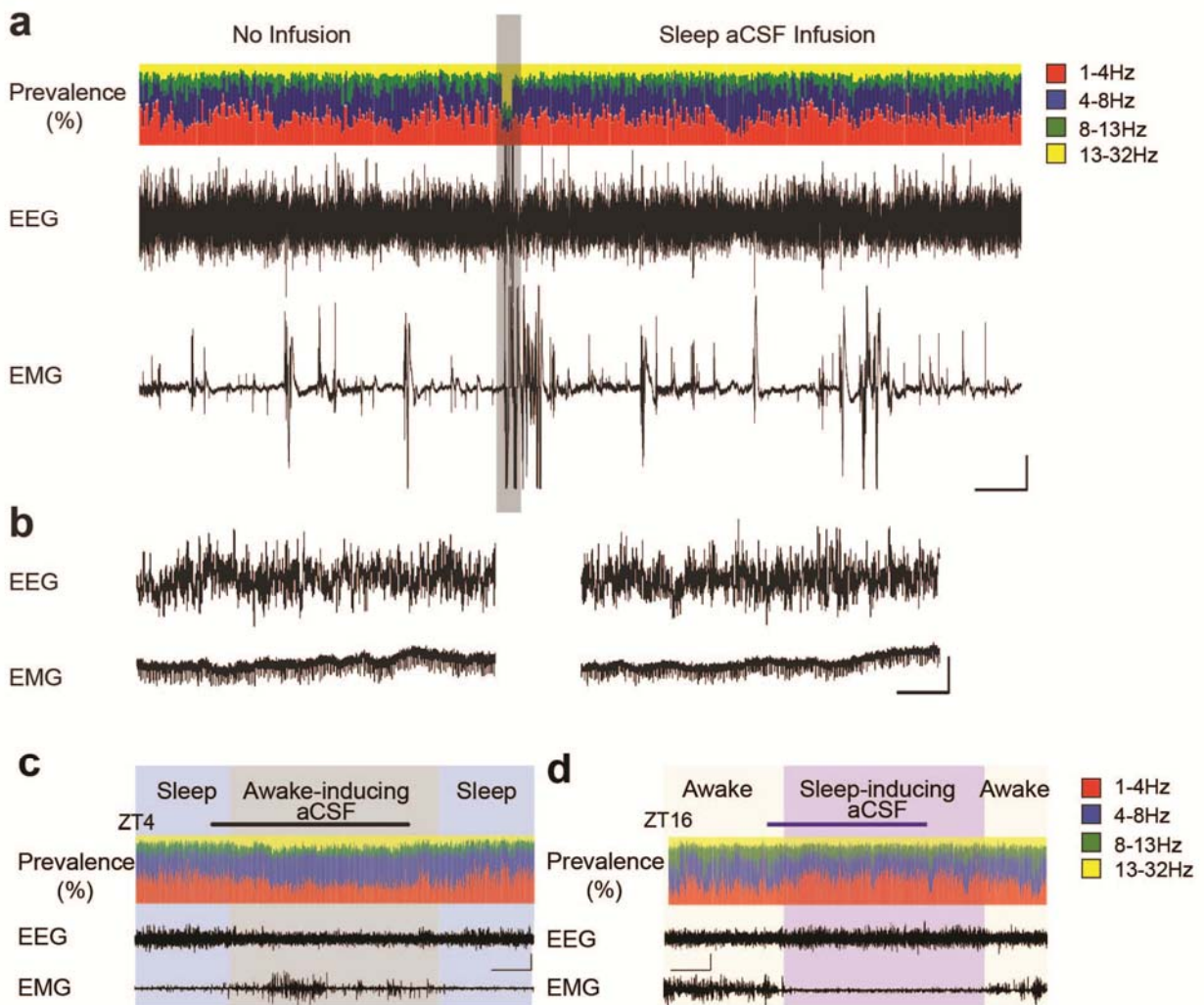
Fig. S5. Intracisternal infusion of aCSF does not alter cortical EEG. (**A**) Representative trace from a single animal illustrating recorded EEG/EMG activity before cisterna-magna infusion of sleep aCSF. Top row: Relative prevalence for delta (1-4 Hz), theta (4-8 Hz), alpha (8-13 Hz), and beta (13-32 Hz) in 10 s epochs before and after infusion of aCSF into the cisterna magna. Scale bar: x = 5 min. y = 0.14 mV EEG, 0.75 mV EMG. (**B**) High-magnification segments from **A** showing representative delta waves before and during infusion of aCSF into the cisterna magna. Scale bar: x = 5 s, y = 0.15 mV EEG, 0.07 mV EMG. (**C-D**) Relative prevalence of representative EEG recordings shown in Fig. 5F and G were plotted in 10 s epochs. Delta (1-4 Hz), theta (4-8 Hz), alpha (8-13 Hz), and beta (13-32 Hz), awake-inducing (**C**) and sleep-inducing (**D**) Scale Bars: Scale bar: x = 30 min, y = 0.5 mV (**C**), 1mV (**D**)

Table S1. aCSF solutions for *in vivo* experiments

Group 1	Awake aCSF (mM)	Sleep-inducing aCSF (mM)
	3.5 KCl, 1.0 CaCl$_2$, 0.8 MgCl$_2$ pH 7.5-7.6 10 mM HEPES	1.5-2.5 KCl, 2.5 CaCl$_2$, 2.5-3.5MgCl$_2$ pH 6.6-6.7 10 mM HEPES
Group 2	Sleep aCSF (mM)	Awake-inducing aCSF (mM)
	2.8 KCl, 1.5 CaCl$_2$, 1.0 MgCl$_2$ pH 7.3-7.4 10 mM HEPES	4.5 KCl, 0.5 CaCl$_2$, 0.3 MgCl$_2$ pH 8.4-8.5 10 mM HEPES