

General anesthesia selectively disrupts astrocyte calcium signaling in the awake mouse cortex

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Edited by Thomas C. Südhof, Stanford University School of Medicine, Stanford, CA, and approved October 4, 2012 (received for review June 22, 2012)

Calcium signaling represents the principle pathway by which astrocytes respond to neuronal activity. General anesthetics are routinely used in clinical practice to induce a sleep-like state, allowing otherwise painful procedures to be performed. Anesthetic drugs are thought to mainly target neurons in the brain and act by suppressing synaptic activity. However, the direct effect of general anesthesia on astrocyte signaling in awake animals has not previously been addressed. This is a critical issue, because calcium signaling may represent an essential mechanism through which astrocytes can modulate synaptic activity. In our study, we performed calcium imaging in awake head-restrained mice and found that three commonly used anesthetic combinations (ketamine/xylazine, isoflurane, and urethane) markedly suppressed calcium transients in neocortical astrocytes. Additionally, all three anesthetics masked potentially important features of the astrocyte calcium signals, such as synchronized widespread transients that appeared to be associated with arousal in awake animals. Notably, anesthesia affected calcium transients in both processes and soma and depressed spontaneous signals, as well as calcium responses, evoked by whisker stimulation or agonist application. We show that these calcium transients are inositol 1,4,5-triphosphate type 2 receptor (IP₃R2)-dependent but resistant to a local blockade of glutamatergic or purinergic signaling. Finally, we found that doses of anesthesia insufficient to affect neuronal responses to whisker stimulation selectively suppressed astrocyte calcium signals. Taken together, these data suggest that general anesthesia may suppress astrocyte calcium signals independently of neuronal activity. We propose that these glial effects may constitute a nonneuronal mechanism for sedative action of anesthetic drugs.

Astrocytes display both spontaneous and evoked increases in cytosolic calcium (Ca²⁺) in response to a variety of stimuli (1). These calcium transients are thought to represent a fundamental type of astrocyte signaling involved in modulating a range of vital brain functions, including cerebral blood flow, synaptic activity, cell volume, and extracellular ion homeostasis (2–10). General anesthesia is known to both depress and alter normal neuronal firing patterns in the cortex (11, 12). Conversely, the effects of anesthesia on astrocyte function have not been systematically explored.

The molecular mechanisms by which anesthetic drugs suppress consciousness are incompletely understood. Almost every general anesthetic has numerous molecular targets in the brain, yet they all produce a remarkably similar suppression and synchronization of neuronal activity (13). In our study, we chose to test three widely used anesthetics thought to act on quite different neuronal targets: (i) isoflurane, (ii) a combination of ketamine and xylazine, and (iii) urethane. Isoflurane is believed to potentiate γ -aminobutyric acid type A receptor (GABA_AR) chloride currents and decrease glutamatergic activity both in the cortex and subcortical centers (14). Ketamine principally suppresses *N*-methyl-D-aspartic acid (NMDA)-mediated excitatory signaling in the cortex and subcortical centers. Ketamine is frequently used in combination with the α_2 -adrenoreceptor agonist xylazine to

enhance sedative effects by suppressing sympathetic activity (15). Finally, urethane (ethyl carbamate) has diffuse effects on both inhibitory and excitatory neurotransmission, as well as a range of ion channels (16). However, it is currently not known whether nonneuronal targets, such as astrocytes, may mediate some of the sedative effects of these anesthetics.

Our study addresses whether general anesthetics also affect astrocyte calcium signaling. To explore this question, we applied general anesthesia to awake head-restrained mice while simultaneously measuring calcium signals in neocortical astrocytes by two-photon laser-scanning microscopy (2PLSM) and neuronal activity with an extracellular electrocorticogram (ECoG) electrode (11, 17). We found that all three anesthetics potently suppressed astrocyte calcium transients in both the cell soma and fine processes. Moreover, the three anesthetics also fundamentally altered the pattern of calcium signals, by selectively desynchronizing astrocyte calcium transients in different cells. Additionally, we show that low doses of anesthetics selectively suppressed astrocyte calcium response to whisker stimulation, before having any effect on the amplitude of the neuronal responses. In fact, we found that local neuronal activity may not be necessary for spontaneous astrocyte calcium signaling, as blocking neuronal responses with either sodium-channel blocker tetrodotoxin (TTX), NMDA antagonist amino-5-phosphonovaleric acid (AP5), or AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not eliminate astrocyte signals. Finally, by microinjecting adenosine triphosphate (ATP), we showed that anesthesia also reduces astrocyte calcium responses to direct agonist application. In conclusion, our study demonstrates that astrocyte calcium signals are highly sensitive to anesthetics, suggesting that the sedative actions of anesthetics could be mediated, in part, by a suppression of astrocyte calcium signaling.

Results

General Anesthetics Suppress Calcium Transients in Neocortical Astrocytes. We adapted a method for 2PLSM brain imaging in awake head-restrained mice to characterize the effects of anesthesia on astrocyte calcium signaling in the neocortex (imaged at 75–150 μ m depth; Fig. 1*A*) (11, 17). Astrocytes were identified by enhanced green fluorescent protein (eGFP) expressed under the *Glt1* promoter, which helped us avoid the use of the epileptogenic dye sulforhodamine (SR)101 (18, 19). This labeling also

Author contributions: A.S.T., V.R.T., E.A.N., and M.N. designed research; A.S.T., V.R.T., E.A.N., N.L., and Q.X. performed research; A.S.T. and V.R.T. analyzed data; and A.S.T., V.R.T., E.A.N., and M.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1209448109/-DCSupplemental.

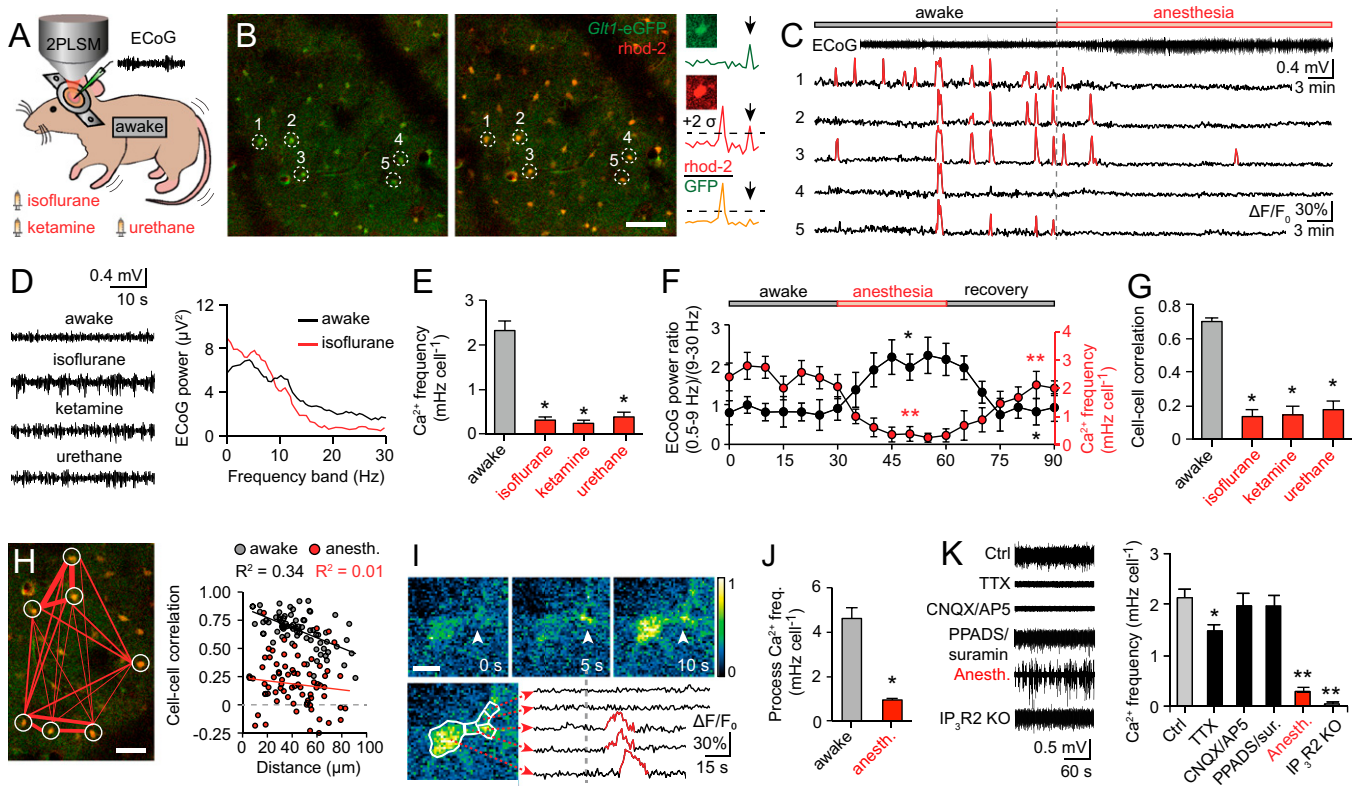


Fig. 1. General anesthesia depresses and desynchronizes spontaneous calcium transients in cortical astrocytes. (A) Two-photon laser scanning microscopy (2PLSM) was used to image spontaneous astrocyte calcium transients in awake and anesthetized head-restrained mice. Neuronal activity was recorded using an ECoG microelectrode. (B) Cortical astrocytes were identified by eGFP expressed under the *Glut1* promoter and loaded with calcium indicator rhod-2. Representative images are shown before (Left) and during (Center) a spontaneous calcium transient. (Scale bar: 60 μm .) Rhod-2 signals were normalized to eGFP fluorescence to reduce movement artifacts (dotted line represents $+2$ SDs) (Right). (C) ECoG and $\Delta F/F_0$ traces of spontaneous calcium transients illustrate the effects of anesthetic induction (isoflurane 1.5%) on neuronal and astrocyte signaling, respectively. (D, Left) Representative ECoG recordings from awake and anesthetized mice. (Right) Representative ECoG power spectra illustrating the shift to slower frequency neuronal activity induced by anesthesia. (E) Bar graph summarizing the effects of different anesthetics on spontaneous calcium signals. $*P < 0.0001$ [$n = 75$ (awake), $n = 30$ (isoflurane), $n = 25$ (ketamine), and $n = 20$ (urethane) cells from 13 animals; paired t test]. (F) Reciprocal changes in ECoG power ratio and astrocyte calcium-transient frequency following anesthesia induction (isoflurane 1.5%). $*P < 0.01$ ($n = 13$ animals; Kruskal–Wallis test); $**P < 0.001$ ($n = 75$ cells; paired t test). (G) Pearson product-moment correlation for $\Delta F/F_0$ in different astrocytes (cells). $*P < 0.0001$ [$n = 75$ (awake), $n = 30$ (isoflurane), $n = 25$ (ketamine), and $n = 20$ (urethane) cells; paired t test]. (H) Cell–cell correlation increases as a function of proximity. (Left) Representative astrocytes joined by lines whose thicknesses represent the strength of their $\Delta F/F_0$ correlation. (Scale bar: 30 μm .) (Right) Regression of cell–cell correlation of astrocyte pairs on distance [$R^2 = 0.34$ (awake) and $R^2 = 0.0091$ (anesthetized); $n = 75$ pairs for each group]. (I, Upper) False-color images illustrating calcium transient in an astrocyte process. (Lower) Rhod-2-intensity traces ($\Delta F/F_0$) for subcellular regions of interest (ROI) are shown. (Scale bar: 10 μm .) (J) Mean frequency of calcium transients in astrocyte processes is decreased in anesthetized mice. $*P < 0.001$ [$n = 32$ (awake), $n = 12$ (isoflurane), $n = 9$ (ketamine), and $n = 11$ (urethane) cells from 13 animals; paired t test]. (K, Left) Representative ECoG recordings following drug application, anesthetic induction, or $\text{IP}_3\text{R2}$ deletion. (Right) Spontaneous calcium activity following TTX (100 μM), CNQX (200 μM)/AP5 (500 μM), and PPADS (100 μM)/suramin (300 μM) application and in the same animals after isoflurane anesthesia (1.5%) and in $\text{IP}_3\text{R2}$ KO mice. $*P < 0.01$, $**P < 0.001$ [$n = 212$ (control), $n = 80$ (TTX), $n = 79$ (CNQX/AP5), $n = 66$ (PPADS/suramin), $n = 149$ (anesthesia), and $n = 88$ ($\text{IP}_3\text{R2}$ KO) cells from 17 animals; paired t test (before vs. after drug) and unpaired t test (WT vs. KO)]. Data are shown as means \pm SEM.

enabled us to control for movement artifacts in the awake mice by normalizing changes in the intensity of calcium indicator rhod-2 ($\Delta F/F_0$) to eGFP (Fig. 1B) (7). Animals were imaged for 30 min while awake and 30 min while they were anesthetized (Fig. 1C). Relatively low doses of anesthesia were used to ensure the results were consistent with previous literature on imaging of calcium transients in anesthetized rodents (1.0–1.5% isoflurane, 0.12 $\text{mg}\cdot\text{g}^{-1}$ ketamine combined with 0.01 $\text{mg}\cdot\text{g}^{-1}$ xylazine or 1.25 $\text{mg}\cdot\text{g}^{-1}$ urethane) (4, 20, 21).

We used ECoG microelectrodes in conjunction with the 2PLSM imaging to record neuronal response and the depth of anesthesia. The different anesthetics caused distinctive “sleep-like” changes in the ECoG discharge, which could be detected both qualitatively and quantitatively (Fig. 1D) (22). A spectral analysis revealed that the three anesthetics significantly changed the predominant bandwidth of activity in favor of slower-frequency

spiking (0.5–9/9–30 Hz power ratio: awake 0.80 ± 0.09 , isoflurane 2.88 ± 0.71 , ketamine 1.65 ± 0.31 , urethane 2.245 ± 0.426) (Fig. S1 A and B), similar to previous reports (15).

Next, we analyzed astrocyte calcium signaling before and after anesthesia. The most notable effect of anesthesia was a 10-fold reduction in calcium-transient frequency, from 2.33 ± 0.16 mHz per cell in the awake state to between 0.24 – 0.39 ± 0.08 mHz per cell when anesthetized (Fig. 1E). This reduction was partly attributable to a decrease in the proportion of “active astrocytes” (cells having ≥ 1 transients in a 15-min period, 82.67 ± 3.35 vs. $24.67 \pm 3.06\%$) (Fig. S1C). Notably, the suppression of calcium transients was largely reversible when the anesthesia was removed (Fig. 1F). Additionally, the anesthetics altered the kinetics of the calcium transients, reducing amplitude from $41.75 \pm 2.29\%$ to $29.87 \pm 2.47\%$ and increasing duration from 21.94 ± 1.43 to 28.47 ± 1.57 s (Fig. S1 D and E). It has been shown

previously that 2PLSM imaging can evoke calcium transients through phototoxicity or trauma induced by the cranial window (3, 4, 20). We, therefore, used a low laser power (<20 mW), a small craniotomy (1.5 mm), and controlled that the frequency of calcium transients did not increase in the latter half of recordings as one would expect with phototoxicity (Fig. S1F). Taken together, our data indicate that three anesthetics routinely used for in vivo studies and with very different molecular targets all strongly suppressed astrocyte calcium signaling.

Anesthesia Masks Normal Patterns of Spontaneous Calcium Signaling.

We subsequently studied the effect of anesthesia on the pattern of calcium signals in the cortex (Movies S1, S2, and S3). Astroglia in the motor cortex, hippocampus, and cerebellum of awake or lightly urethane-anesthetized mice have recently been shown to display fast coordinated calcium activity across numerous cells (3, 17, 23). Astrocyte “synchronization” has previously been quantified in urethane-anesthetized rats by comparing Pearson product–moment correlation coefficient (r) for $\Delta F/F_0$ recordings of adjacent astrocyte “pairs” (20). Using this method, we were able to show that awake animals display a high degree of correlation between spontaneous calcium transients in neocortical astrocytes (0.696 ± 0.016), which is virtually eliminated following anesthesia administration (0.148 ± 0.026) (Fig. 1G). Moreover, the astrocyte–astrocyte correlation appeared to be dependent on how close the cells were to each other, and this proximity correlation was again reduced by anesthesia (Fig. 1H).

Astrocytes are known to generate calcium transients also in their fine processes, which contact both synapses and blood vessels, and are, thus, ideally placed for signal transduction (1). Previous studies in anesthetized animals and in situ preparations have revealed that calcium transients in astrocytic processes are faster and more frequent than transients in the soma, perhaps indicating a greater relevance for the fast kinetics of synaptic activity (4, 20, 24). Consistent with these studies, we also found that calcium transients in astrocytic processes were more frequent and of lower amplitude and shorter duration than transients in the soma (4.38 ± 0.57 mHz per cell, $15.30 \pm 1.73\%$, and 8.81 ± 0.75 s, respectively) (Fig. 1I and Fig. S1G–I). However, the higher signal frequency in the processes was again suppressed by anesthesia to 0.91 ± 0.07 mHz per cell (Fig. 1J). Transients from adjacent processes in a single cell were also somewhat correlated, likely because of calcium-transient propagation within individual cells (20, 24). We found that anesthetics appear to alter this process–process relationship, reducing the correlation (r) between the two from 0.229 ± 0.012 to 0.162 ± 0.013 , perhaps indicating an interference with calcium propagation or mobilization (Fig. S1J).

In conclusion, we found that general anesthesia consistently disrupted the normal pattern of spontaneous calcium transients observed in awake mice, most prominently impairing fast synchronized astrocyte signaling that appear to be a sensitive hallmark of wakefulness in ours and other studies (8, 17, 25).

Astrocyte Signals Are Dependent on IP₃R2 but Not Local Synaptic Transmission.

Multiple endogenous agonists or cues can trigger astrocyte calcium transients in vitro, including ATP, glutamate, ionic or osmotic change, acetylcholine, and norepinephrine (4, 7, 8, 10, 25). The mechanisms that mediate astrocyte calcium signals in vivo are incompletely understood and have not been addressed previously in the cortex of awake animals (1, 3, 17). Previous studies in the cortex and hippocampus of anesthetized mice have indicated that calcium activity is dependent on local synaptic activity, as well as purinergic signaling (3, 4, 26). To further address this issue, we first examined what effect blocking local synaptic activity in the cortex had on astrocyte signals. Surprisingly, blocking action potentials with TTX or inhibiting glutamatergic activity with CNQX and AP5 had only minor effects on astrocyte calcium

signals, despite causing a >90% reduction in ECoG amplitude (awake: 2.13 ± 0.16 mHz per cell; TTX: 1.47 ± 0.15 mHz per cell; CNQX/AP5: 1.96 ± 0.28 mHz per cell) (Fig. 1K). Additionally, blocking purinergic signaling with a combination of pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and suramin did not significantly reduce astrocyte signals (PPADS/suramin: 1.95 ± 0.21 mHz per cell; isoflurane: 0.27 ± 0.09 mHz per cell) (Fig. 1K). Interestingly, recent studies in awake cerebellum and lightly anesthetized ferret visual cortex revealed a similar insensitivity of astroglial calcium activity to neuronal or purinergic blockade (21, 23). Because of the relative insensitivity of calcium signals to blockers of local neuronal activity, we next asked whether anesthetics might interfere directly with astrocyte calcium mobilization. Astrocyte calcium transients have been shown to critically depend on inositol 1,4,5-triphosphate type 2 receptors (IP₃R2) in vitro, in situ, and in anesthetized mice in vivo (8, 27, 28). However, the role of IP₃ receptors has not been examined previously in awake animals. We, therefore, examined calcium signaling in the cortex of awake IP₃R2 knockout (KO) mice. Consistent with the literature, we found that IP₃R2 deletion completely abrogated astrocyte calcium signaling (Fig. 1K).

Sensory-Evoked Calcium Responses Decrease After Anesthetic Induction.

Whisker deflection in lightly anesthetized rodents is known to trigger robust astrocyte calcium transients in the barrel cortex (4, 8). To explore the effects of anesthesia on sensory-evoked calcium transients, we compared whisker responses before and after anesthesia application (Fig. 2A and Movies S4 and S5). Several key differences were observed: first, we found that all three anesthetics suppressed calcium responses in astrocytes in a dose-dependent fashion before having any effect on the neuronal ECoG response ($\Delta F/F_0$: $45.13 \pm 1.94\%$, $37.54 \pm 2.21\%$, and $18.18 \pm 1.53\%$ for 0%, 1.0%, and 1.5% isoflurane, respectively) (Fig. 2B–D). Similar findings have been reported using visual stimulation in lightly anesthetized ferrets (21). This impairment of evoked calcium responses was detectable in all three anesthetics tested [P (active): $0.78 \pm 0.04\%$, $0.41 \pm 0.06\%$, $0.50 \pm 0.05\%$, and $0.41 \pm 0.06\%$ per 3 min for awake vs. isoflurane, ketamine/xylazine, and urethane, respectively] (Fig. 2E). Second, whisker responses in anesthetized but not awake animals were highly sensitive to the frequency and location of whisker stimulation (Fig. 2F). Similar to previously published data, we found no calcium responses in anesthetized mice when the stimulation frequency was ≤ 1 Hz or “off-target” (incorrect whisker) (4). Conversely, whisker responses in awake mice were much less dependent on stimulation location or frequency ($\Delta F/F_0$ for 1 Hz on-target: $36.23 \pm 2.13\%$; 5 Hz on-target: $45.13 \pm 1.94\%$; 5 Hz off-target: $42.85 \pm 2.49\%$). Third, there was a decreased correlation between the amplitude of the astrocyte calcium response and the neuronal ECoG response in awake animals, again indicating that local neuronal responses may not be the direct mediator of the signals (Fig. 2G). Fourth, whisker responses in awake animals were more variable and interspersed by more spontaneous activity. Awake mice, for instance, showed increased calcium activity for several minutes after the stimulus, likely representing increased arousal or voluntary whisker movement (Fig. S2A and B) (17). Finally, we found that sensory-evoked calcium transients in anesthetized animals were more delayed (awake: 3.56 ± 0.19 s; vs. anesthetized: 5.77 ± 0.72 s), with a slower rise time (awake: 3.06 ± 0.15 s; vs. anesthetized: 3.93 ± 0.34 s), and longer duration (awake: 19.42 ± 0.41 s; vs. anesthetized: 29.96 ± 2.39 s) (Fig. S2C–F). The anesthetics did not, however, cause any significant delay of the neuronal ECoG response (10.88 ± 0.44 ms vs. 10.57 ± 1.02 ms) (Fig. S2G). All of these effects were partly reversed after anesthetic withdrawal, mirroring the well-established lingering effects of general anesthetics on neuronal activity and animal behavior in the immediate recovery period (Figs. 2H and 1H) (29, 30).

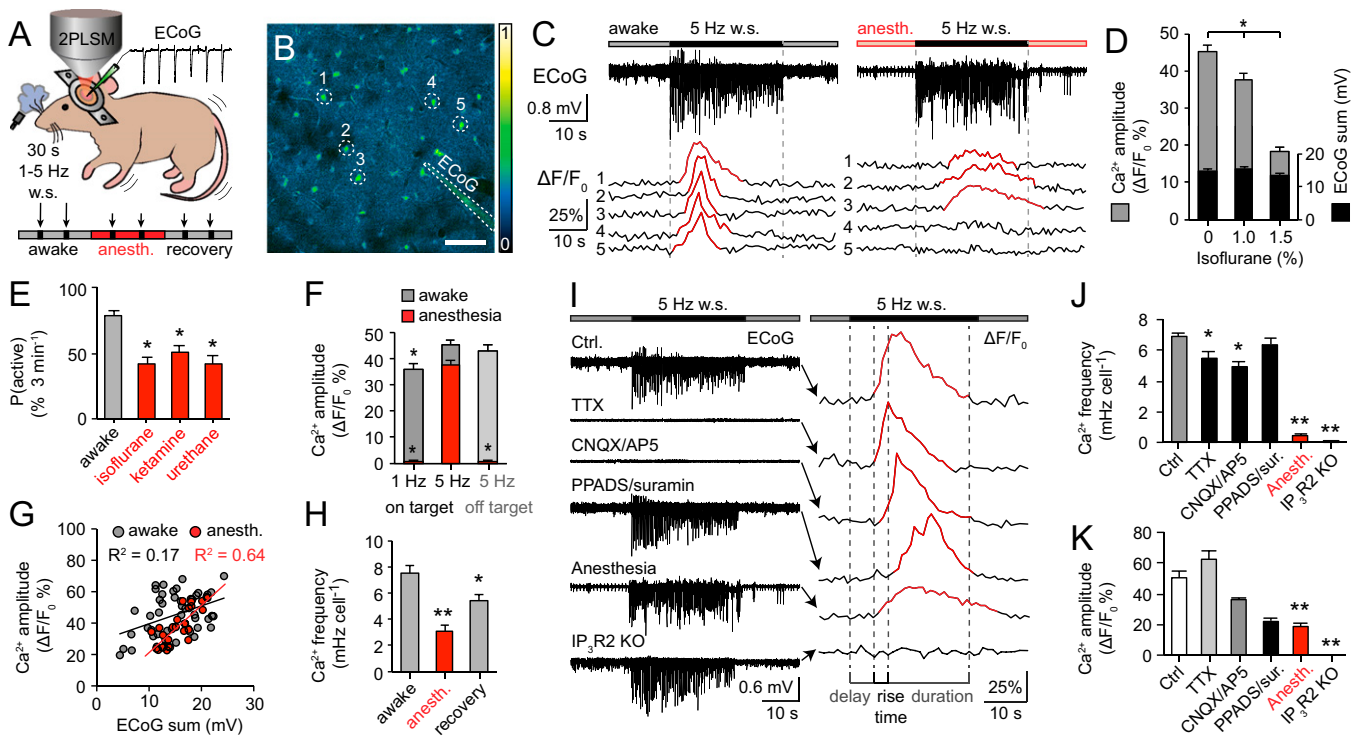


Fig. 2. Astrocyte calcium responses evoked by whisker stimulation are diminished by general anesthesia. (A) Astrocyte calcium responses and ECoG neuronal responses to whisker stimulation (w.s.) were recorded before, during, and after general anesthesia. (B) False-color image illustrating a calcium response to whisker stimulation. (Scale bar: 60 μm .) (C) Representative ECoG and $\Delta F/F_0$ calcium responses to whisker stimulation before and after anesthetic induction (1.0% isoflurane). (D) Mean calcium-transient frequency and ECoG response sum induced by whisker stimulation when increasing doses of isoflurane are administered. $*P < 0.001$ [$n = 53$ (awake), $n = 28$ (0.5% isoflurane), and $n = 25$ (1.5% isoflurane) cells from 6 animals; one-way ANOVA]. (E) Effect of different anesthetics on the probability of finding an active astrocyte (≥ 1 transient in the first 3 min) following whisker stimulation. $*P < 0.001$ [$n = 12$ (awake), $n = 12$ (isoflurane), $n = 4$ (ketamine), and $n = 4$ (urethane) animals; Wilcoxon signed-rank test]. (F) Mean calcium-transient frequency induced by different types of whisker stimulation in awake (gray bars) and anesthetized mice (red bars), including air puffs to the C6 whisker (on target) or the incorrect whisker (off-target). $*P < 0.001$; $P = 0.389$ (off vs. on-target; $n = 53$ cells for each group; one-way ANOVA). (G) Astrocyte calcium response amplitude ($\Delta F/F_0$) regressed on the neural response sum in awake and anesthetized mice. ($R^2 = 0.17$ awake, $R^2 = 0.64$ anesth.). (H) The calcium response to whisker stimulation partially recovers after anesthetic withdrawal. $*P < 0.01$, $**P < 0.001$ ($n = 53$ cells; paired t test). (I) Representative ECoG and $\Delta F/F_0$ traces in awake WT mice exposed to TTX (100 μM), CNQX (200 μM)/AP5 (500 μM), and PPADS/suramin (100 μM)/suramin (300 μM) or isoflurane anesthesia (1.5%) and in IP₃R2 KO mice. (J and K) Bar graphs summarizing the effect of drugs and IP₃R2 KO on calcium-transient frequency and amplitude, respectively. $*P < 0.01$, $**P < 0.001$ [$n = 212$ (control), $n = 80$ (TTX), $n = 79$ (CNQX/AP5), $n = 66$ (PPADS/suramin), $n = 149$ (anesthesia), and $n = 88$ (IP₃R2 KO) cells from 17 animals; paired t test (before vs. after drug) and unpaired t test (WT vs. KO)]. Data are shown as means \pm SEM.

Anesthetic Impairment of Astrocyte Whisker Responses Is Largely Independent of Neuronal Drug Effects.

A consistent but paradoxical feature of sensory-evoked astrocyte responses in anesthetized mice is that they occur 0.5–5 s after activation of adjacent neurons (Fig. 2C and Fig. S2D) (4, 8, 21, 26). This astrocyte-neuronal delay is interesting because it could indicate that cues other than synaptic spillover of neurotransmitters generate astrocyte calcium transients in vivo. To explore this hypothesis, we compared astrocytic responses to whisker stimulation before and after drug application to either block neuronal or purinergic signaling (Fig. S2C). Consistent with our previous observations, we found that sensory-evoked calcium transients in awake mouse cortex were largely unaffected when local synaptic or purinergic activity was blocked ($\Delta F/F_0$: awake, $45.75 \pm 1.27\%$; TTX, $48.34 \pm 2.65\%$; CNQX/AP5, $45.82 \pm 2.98\%$; PPADS/suramin, $41.69 \pm 2.56\%$; anesthesia, $16.23 \pm 1.52\%$; frequency: awake, 6.89 ± 0.25 mHz per cell; TTX, 5.43 ± 0.47 mHz per cell; CNQX/AP5, 4.94 ± 0.34 mHz per cell; PPADS/suramin, 6.40 ± 0.41 mHz per cell; anesthesia, 0.47 ± 0.10 mHz per cell) (Fig. 2I–K, Fig. S2D and E, and Movies S6 and S7). Moreover, similar to spontaneous calcium activity, astrocyte whisker responses were also completely abrogated in IP₃R2 KO mice, despite normal neuronal responses (Fig. 2I and J and Movie S8). Taken together, these data indicate that the astrocyte-specific anesthetic effects observed in our study may be related to a direct interference with

calcium-transient generation, and not simply occur secondary to the neuronal effects of these drugs.

General Anesthesia Impairs Astrocyte Responses to ATP Stimulation.

To separate the effects of anesthesia on astrocytes from those on neurons, we used ATP to stimulate astrocytes directly. ATP is believed to be an important mediator of astrocytic calcium signaling and reproducibly triggers astrocyte calcium mobilization by activating purinergic receptors in the cell membrane, including P2Y purinoreceptor 1, 2, and 4 (2, 31). A glass microelectrode was used to microinject ATP into the cortex, allowing us to compare astrocyte calcium responses before and after anesthetic application (Fig. 3A) (7). We found that anesthesia reduced both the probability of ATP-application triggering calcium transients (awake: $67.05 \pm 3.43\%$; vs. anesthetized: $31.80 \pm 2.47\%$) and also the overall number of transients within individual astrocytes (awake: 7.33 ± 0.54 mHz per cell; vs. anesthetized: 2.11 ± 0.38 mHz per cell) (Fig. 3B–D, Fig. S3A and B, and Movies S9 and S10). Additionally, the amplitude of the calcium transients (awake: $92.35 \pm 6.03\%$; vs. anesthetized: $61.06 \pm 6.74\%$) and the correlation (r) between transients were diminished when the animals were anesthetized (awake: 0.472 ± 0.013 ; vs. anesthetized: 0.211 ± 0.022) (Fig. 3E and F and Fig. S3C). Finally, anesthesia also shortened the overall duration of

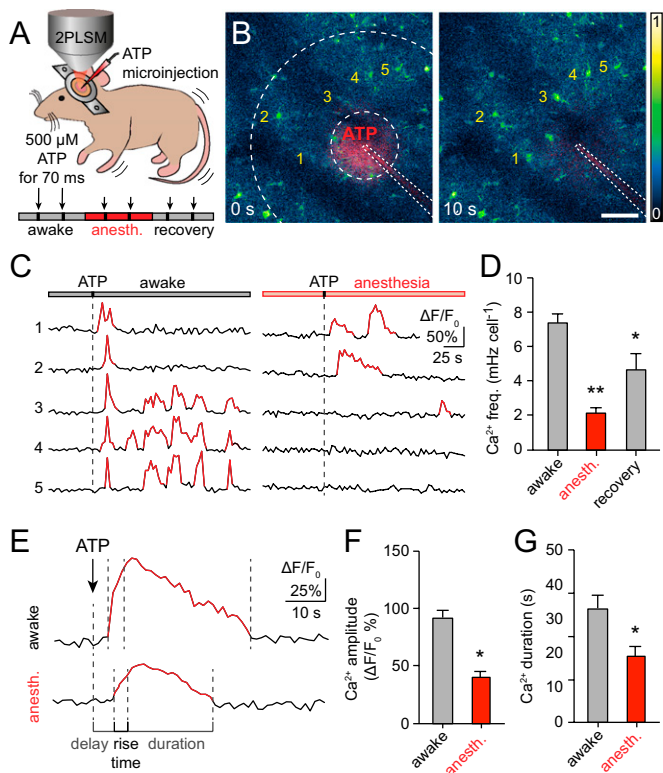


Fig. 3. Astrocyte calcium mobilization in response to ATP stimulation is impaired in anesthetized animals. (A) ATP was microinjected into the cortex in vivo using a fine glass electrode, and astrocyte calcium responses were recorded using 2PLSM before, during, and after general anesthesia. (B) Representative false-color images illustrate the calcium response to ATP application. To standardize the distance from ATP microinjection to the astrocytes chosen for analysis, two concentric circles were drawn (radius, 25 and 100 μm), and cells within this range were used. (Scale bar: 30 μm .) (C) Representative $\Delta F/F_0$ traces of ATP-evoked calcium transients in awake and anesthetized mice. (D) Bar graph summarizing the effect of anesthesia on ATP-evoked calcium signals. * $P < 0.01$ (awake vs. anesthesia); ** $P < 0.001$ (anesthesia vs. recovery) ($n = 137$ cells; paired t test). (E) Representative individual ATP-responses illustrating the detailed effects of anesthesia. (F and G) Mean calcium-response amplitude and duration, respectively, before and after anesthesia induction. * $P < 0.01$ ($n = 137$ cells; paired t test). Data are shown as means \pm SEM.

the calcium transients (awake: 26.51 ± 3.06 s; vs. anesthetized: 15.28 ± 2.13 s) (Fig. 3G and Fig. S3 D–F). Calcium response suppression was partly reversed following withdrawal of the anesthetic (4.63 ± 0.96 mHz per cell), analogous to our observations with whisker stimulation. In conclusion, our data show that anesthesia directly impairs astrocyte responses to nonneuronal stimulation, illustrating the selective effect of anesthesia on astrocytes.

Discussion

We developed a method to assess the effects of anesthesia on astrocyte calcium signaling using 2PLSM imaging of the neocortex in awake head-restrained mice. Astrocytic calcium transients have been linked to many important physiological functions, including blood flow regulation, ion and water homeostasis, and synaptic modulation (5, 7, 8, 10, 24). Most of our current understanding of astrocyte calcium signals is derived from in vivo studies using the three anesthetics tested in our study (isoflurane, ketamine/xylazine, and urethane). Moreover, these and related general anesthetics are widely used in clinical practice (3–5, 8, 9, 21, 25, 32, 33). Our data show that anesthesia rapidly and selectively impairs astrocyte calcium signaling in awake animals, with important implications for future experimental and clinical work.

All of the three anesthetics we tested are thought to exert their sedative action on different neuronal targets. Recent studies have also highlighted that these anesthetics produce neurotoxic effects (34). However, the exact mechanism by which these drugs induce unconsciousness or neurotoxicity is not clearly understood, and their effects on nonneuronal cells are not widely appreciated (21, 23). We found that all three anesthetics markedly disrupted the frequency, kinetics, and pattern of astrocyte calcium signaling. First, we observed a pronounced 10-fold reduction in the frequency of spontaneous astrocyte calcium activity both in the soma and processes following anesthetic administration. Second, we show that astrocyte calcium transients evoked by whisker stimulation are suppressed in a dose-dependent fashion by anesthesia. Third, we demonstrate that selective astrocyte calcium mobilization induced by ATP application is decreased in anesthetized animals. Finally, we found that anesthetic induction rapidly abolished a form of spontaneous, fast, and highly synchronized calcium signals that may represent a nonneuronal hallmark of wakeful cortical activity.

There is an ongoing debate whether global calcium responses across numerous astrocytes or individual localized transients are more relevant for normal brain function (35). We found that synchronized signals accounted for the majority of calcium activity in the somatosensory cortex of awake mice. Similar observations have recently been made in the motor cortex, cerebellum and hippocampus of awake or lightly anesthetized mice (3, 17, 23). In our study, we show that the synchronized calcium signals evoked by whisker stimulation and those occurring spontaneously display remarkably similar kinetics. Conversely, ATP-evoked responses display slower kinetics and spread more like the calcium “waves” that have been described in vitro and in situ (7, 31). Interestingly, previous studies using anesthetized animals have only been able to generate synchronized calcium responses by using light levels of sedation and stimulating the animals in ways that are known to elicit arousal (4, 21, 25, 26). We found that general anesthesia caused an almost complete suppression of synchronous spontaneous calcium activity. It is, therefore, conceivable that these synchronous signals are more relevant for normal brain function than the individual calcium transients studied previously in anesthetized animals or using in situ and in vitro preparations.

Perhaps the most important implication of our study is that general anesthetics can cause a direct impairment of calcium mobilization in astrocytes. We found that three anesthetics with different putative neuronal targets and effects on ECoG activity produced a remarkably similar impairment of astrocyte calcium signaling. When anesthetic doses insufficient to affect neuronal responses were used, sensory-evoked astrocyte calcium transients were still markedly reduced (21). Astrocyte responses to nonneuronal stimulation in the form of ATP microinjections were also impaired in the anesthetized mice. Moreover, we demonstrated that when synaptic activity was blocked using TTX or CNQX/AP5, astrocyte signaling was largely unaffected. Conversely, $\text{IP}_3\text{R}2$ deletion completely suppressed astrocytic signaling. A significant proportion of the anesthetic effect on astrocytes must, therefore, be mediated by an $\text{IP}_3\text{R}2$ -signaling pathway that is nominally independent of local neuronal responses (8, 23). In addition, sensory-evoked responses in astrocytes were actually less correlated with the magnitude of the neuronal response in awake than anesthetized mice. This could, perhaps, indicate that astrocytes are not simply responding to local neuronal activity but, rather, integrating multiple local and global cues. Increased animal arousal attributable to noradrenergic or cholinergic output is, for instance, capable of directly eliciting large-scale calcium activity in cortical astrocytes similar to the widespread synchronized activity we observed (8, 9, 17, 25). Supporting our results, previous in vitro studies have shown that anesthetic drugs can directly impair calcium mobilization in astrocytes (36–38). Our study, thus, provides comprehensive evidence of a direct effect of anesthesia on astrocytes in vivo.

Taken together, our data suggest that three anesthetics with markedly different neuronal targets may exert sedative or noxious effects through a direct suppression of astrocyte calcium signaling. These results call for further studies to explore the anesthetic potential of nonneuronal pharmacological targets.

Materials and Methods

Animal Preparation. *Glt-1-eGFP* and *IP₃R2* KO mice were bred as described previously, and males from 6 to 12 wk were used (7, 27). Mouse preparation was modified from published protocols (11, 17). Briefly, mice were anesthetized using isoflurane via nose cone (1.0–1.5% mixed with 1–2 L/min O₂), head-restrained with a custom-made miniframe, and habituated over 2 d in multiple 1-h sessions. A 1.5-mm craniotomy (with the dura carefully removed) was opened over the somatosensory cortex, and calcium indicator rhod-2 acetoxymethyl (Invitrogen, 2 mM) was loaded onto exposed cortex for 30–45 min before applying agarose [1.5% (wt/vol); type III-A; Sigma] and a coverslip. Animals were then head-restrained and placed on the stage located in a dark quiet room. All animal experiments were approved by the Animal Care and Use Committee of the University of Rochester.

Two-Photon Laser-Scanning Microscopy. A Mai Tai laser (SpectraPhysics) attached to a confocal scanning system (Fluoview 300; Olympus) and an upright microscope (IX51W; Olympus) was used. Calcium transients were imaged in cortex 75–125 μm below the pial surface, as described previously, using a 20× (0.95NA) lens (7). Dual channel (rhod-2 and eGFP) frames were collected at 0.2 or 1 Hz. A low sampling rate and <20-mW laser were used to avoid phototoxicity. A calcium transient was defined as an event where the change in rhod-2 intensity ($\Delta F/F_0$) normalized to eGFP deviated >2 SDs (σ) from baseline. Recordings were analyzed using previously described custom-made software (MatLab) and ImageJ (National Institutes of Health) (7).

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ECoG Recordings and Whisker Stimulation. Recordings were obtained from layer II somatosensory cortex using glass microelectrodes as described previously (4). Briefly, signals were bandpass-filtered at 1–100 Hz and digitized at 9 kHz (Digidata 1440A; Axon Instruments). Recordings were analyzed offline using pClamp version 10.2. Power spectra were obtained by using pClamp (Molecular Devices) to perform a Fourier transformation on 10 randomly selected 12-s epochs of the ECoG recordings (15). Spectral power within the slow frequency (δ and θ) and higher frequency (β) was calculated, and the mean for each animal was recorded. Whisker stimulation was delivered as described previously using a PicoSpritzer III (Parker Instrumentation) and Master 8 (A.M.P.I.). Stimuli consisted of 10-ms air pulses delivered at 1–5 Hz lasting for 30 s in total (4).

Drug Application. TTX, CNQX, AP5, PPADS, and suramin (Sigma) were added to the artificial cerebrospinal fluid bathing the craniotomy (approximate volume, 500 μL) and allowed to diffuse underneath the coverglass and through the agarose as described previously (23). For cortical microinjection, 500 μM ATP was delivered using a fine glass electrode connected to a picospritzer (15–20 psi; 70 ms; Parker Instrumentation) and visualized using 100 μM Alexa 488 (Invitrogen). The electrode was always kept in the imaging field.

Statistical Analyses. All analysis was performed using IBM SPSS Statistics 19, and all tests were two-tailed, where significance was achieved at $\alpha = 0.05$ level (for details, see *SI Materials and Methods*).

ACKNOWLEDGMENTS. This work was supported National Institutes of Health Grants NS075177 and NS078304 (to M.N.), the Research Council of Norway, Nordic Center of Excellence Program, the Letten Foundation, and the Fulbright Foundation.

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