Sensory Transmission is Bi-directionally Modulated by Astrocytic Ca\textsuperscript{2+} in Barrel Cortex ofBehaving Mice

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Abstract: Different effects of astrocyte during sleep and awake have been extensively studied, especially for metabolic clearance by the glymphatic system, which works during sleep and stops working during waking states. However, how astrocytes contribute to modulation of sensory transmission during sleep and awake animals remain largely unknown. Recent advances in genetically encoded Ca\textsuperscript{2+} indicators have provided a wealth of information on astrocytic Ca\textsuperscript{2+}, especially in their fine perisynaptic processes, where astrocytic Ca\textsuperscript{2+} most likely affects the synaptic function. Here we use two-photon microscopy to image astrocytic Ca\textsuperscript{2+} signaling in freely moving mice trained to run on a wheel in combination with \textit{in vivo} whole-cell recordings to evaluate the role of astrocytic Ca\textsuperscript{2+} signaling in different behavior states. We found that there are two kinds of astrocytic Ca\textsuperscript{2+} signaling: a small long-lasting Ca\textsuperscript{2+} increase during sleep state and a sharp widespread but short-long-lasting Ca\textsuperscript{2+} spike when the animal was awake (fluorescence increases were 23.2 ± 14.4% for whisker stimulation at sleep state, compared with 73.3 ± 11.7% for at awake state, paired t-test, \(p < 0.01\)). The small Ca\textsuperscript{2+} transients decreased extracellular K\textsuperscript{+}, hyperpolarized the neurons, and suppressed sensory transmission; while the large Ca\textsuperscript{2+} wave enhanced sensory input, contributing to reliable sensory transmission in aroused states. Locus coeruleus activation works as a switch between these two kinds of astrocytic Ca\textsuperscript{2+} elevation. Thus, we show that cortical astrocytes play an important role in processing of sensory input. These two types of events appear to have different pharmacological sources and may play a different role in facilitating the efficacy of sensory transmission.

Keywords: glymphatic system, Ca\textsuperscript{2+} wave, \textit{in vivo}, sensory transmission, sleep, wake
Dynamic changes of astrocytic Ca\(^{2+}\) during synaptic transmission have been extensively studied in the last decades\(^1\text{-}^6\); however, the topic remains controversial\(^1\text{-}^7\). As stated in a recent review: “Few topics in neuroscience are as controversial as the idea that calcium concentration elevations in astrocytes release transmitters that regulate neuronal and vascular function”\(^1\). One reason might be that we were expecting logic input-output functions, but astrocytes are more complicated and a given input may have different output depending on brain states. For example, one study has demonstrated that the Ca\(^{2+}\) elevation evoked by sensory stimulation can be separated into fast and slow components; fast Ca\(^{2+}\) signals may initiate the cerebral blood flow response, while the slower and longer-lasting astrocytic Ca\(^{2+}\) elevations may contribute to the sustained hemodynamic response\(^8\). We were expecting only one type of astrocytic Ca\(^{2+}\) signaling: the induction is due to mGluR receptors\(^1\), their mechanisms are due to IP3R\(_2\) induced Ca\(^{2+}\) release from endoplasm reticulum (ER)\(^9\), and the function is to release gliotransmitters\(^10\text{-}^{14}\). Different kinds of astrocytic Ca\(^{2+}\) with different spatial location and temporal dynamics have started to be reported recently\(^15\text{-}^{17}\). And new mechanisms involved in astrocytic Ca\(^{2+}\) are reported, such as glutamate transporters\(^18\), Na\(^+\)/Ca\(^{2+}\) exchangers\(^19\), TRPA1 channels\(^1\). And new functions are appearing too\(^4\), such as increasing the fatty acid metabolism\(^18\), enhancing clearance of extracellular materials like the glymphatics\(^20\text{-}^{21}\), affecting the circadian rhythm\(^4\text{-}^{22}\).

Recently, the role of astrocyte in controlling global cortical network is a hot topic in many recent studies\(^4\text{-}^{23}\), such as affecting the circadian rhythm\(^4\text{-}^{24}\), controlling the slow oscillation\(^12\), affecting the neural circuits at different behavior states\(^25\text{-}^{27}\). Previous experiments in our lab and other labs have found that the glymphatic system, the network of perivascular spaces through which cerebrospinal fluid and interstitial fluid can move through the brain\(^21\text{-}^{22}\), is regulated by sleep and norepinephrine. In addition, it is reported that sensory input can induce local astrocytic Ca\(^{2+}\) during anesthesia state\(^26\text{-}^{27}\), whereas they can trigger widespread Ca\(^{2+}\) signals in cerebral cortex mediated by alpha1 adrenergic receptor activation at waking states\(^28\). However, the local astrocytic Ca\(^{2+}\) at the fine processes, especially at tripartites area, which got most of the Ca\(^{2+}\) activity in an astrocyte, and the one most likely related to synaptic function\(^2\), are largely unknown. Recent advances in genetically encoded Ca\(^{2+}\) indicators have provided a wealth of information on astrocytic Ca\(^{2+}\)\(^7\). We here used genetically encoded Ca\(^{2+}\) indicators (GCaMP6f) to monitor astrocytic Ca\(^{2+}\) signaling in live free moving mice on a wheel in combination with dual whole-cell recordings in single neurons and local field potential (LFP) recordings in layer
2/3 of barrel cortex. We found that astrocytic Ca\(^{2+}\) signaling can be differently induced and exert two different effects on sensory transmission at different behavior states. We also found that astrocytic Ca\(^{2+}\) signaling have two opposing effects on sensory input: The small Ca\(^{2+}\) increase in sleep mice suppresses sensory input, while the larger Ca\(^{2+}\) transients enhances sensory input in alert awake mice. This is similar to the frequently described function of norepinephrine (NE) in increasing sensory input\(^{29}\). Thus, our study offers an explanation for the complex role of astrocytes in sensory processing.

Results

Astrocytic processes demonstrate two kinds of Ca\(^{2+}\) signaling from sensory transmission

In order to probe into the astrocytic Ca\(^{2+}\) signal at fine processes in sensory inputs in free moving mice, we used the genetically encoded calcium indicator (GCaMP6f) that is selectively expressed in cortical astrocytes to monitor the Ca\(^{2+}\) transients with two-photon microscopy\(^{2}\) (Fig.1A-B, Figure S1). Two weeks after virus microinjections, GCaMP6f-expressing astrocytes can be seen under two-photon microscopy, similarly as our previous reports\(^{31}\). On the day of the experiment, a cranial window was prepared under 1.5% isoflurane anesthesia. The animals totally recovered after 30 min prior to *in vivo* whole-cell recordings of single neurons and monitoring astrocytes in layer 2/3 of barrel cortex (Fig. 1A)\(^{9,28,31}\). The sleep states were defined as periods in which the animals closed eyes, but spectral analysis of electroencephalography (EEG) recordings showed that most of its power resides in slow waves of 0.5~4Hz\(^{32}\) (Fig. 1C); concurrently with relatively low amplitude in electromyography (EMG) recordings in the neck. In both sleep and waking mice, we were able to identify some spontaneous Ca\(^{2+}\) signals in fine processes. Representative fine processes were randomly chosen to analyze the fluorescence changes (Fig. 1D). As expected, low frequency of whisker simulation (5 Hz) significantly increased astrocytic Ca\(^{2+}\) transient in the astrocytic fine processes at both sleep and waking states. However, the whisker stimulation induced Ca\(^{2+}\) transients during sleep state are much smaller compared with that during the waking state (peak fluorescent peak increased 23.2 ± 14.4% for whisker stimulation at sleep state, compared with 73.3 ± 11.7% for at arousal states; \(p < 0.01\), t-test, \(n = 24\) processes in 6 animals, Fig.1D-F; Figure S2). Awake states were characterized as
eyes opening, spontaneously whisker movement, and accompanied with typical electromyography (EMG) recordings (Fig. 1C). Whisker stimulation induced much larger Ca^{2+} transients at arousal state than those during sleep state, with a fast latency (3.8 ± 2.1 s compared with 7.5 ± 1.6 s at sleep state, n = 24 processes in 6 animals, Fig. 1G), a fast rise of fluorescence increase (5.8 ± 2.4 %/s compared with 22.3 ± 4.8 %/s at sleep state, p < 0.01, paired t-test, n = 24 processes in 6 animals, Fig. 1H). However, the decay was also faster for the astrocytic Ca^{2+} signals at arousal state (4.3 ± 0.8 %/s compared with 0.8 ± 0.7 %/s at sleep state, p < 0.01, paired t-test, n = 24 processes in 6 animals, Fig. 1I); while the duration were similar (with 12.3 ± 3.5 s compared with 18.6 ± 4.5 s for sleep, n = 24 processes in 5 animals; paired t-test, p = 0.56).

The small Ca^{2+} transients at sleep state derive from influx of extracellular Ca^{2+}

Then we tested if the small Ca^{2+} transients are similar to that reported before, we applied the traditional agonist, ATP (adenosine 5’-triphosphate, 100 µM), UTP (uridine 5’-triphosphate, 100 µM) to induce Ca^{2+} in astrocytes Fig. 2A), which have been shown to induce astrocytic Ca^{2+} but have no effects in neurons 20, 33. The results showed that they really induced small astrocytic Ca^{2+} (24.4 ± 2.3 % for ATP, 21.6 ± 2.2 & for UTP, n = 36 processes in 5 animals, Fig. 2B). In addition, we also tested the antagonist for the whisker stimulation induced Ca^{2+}, and the results showed that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaoine-2,3-dione (CNQX, 10 µM) with ionophresis in the field of imaging in layer 2. It is surprising that application of CNQX (10 µM) had little effect on the small Ca^{2+} transients (p = 0.34, paired t-test, n = 5 mice). The N-methyl-D-aspartate receptor (NMDA) receptor antagonist D-2-Amino-5-phosphono-pentanoic acid (APV, 50 µM) did not significantly alter the small transients either (p = 0.37, paired t-test, n = 25 processes in 5 mice). However, mGluR receptor antagonist 6-(phenylethynyl)-pyridine (MPEP, 50 µM) decreased the small Ca^{2+} transients (10.5 ± 4.2, p < 0.01, paired t-test n =18 cells in 5 mice, Fig. 2C). Then we used injected GCaMP6f in mice with genetic deletion of IP3 receptor (Type 2), whisker stimulations are still able to induce Ca^{2+} transients (18.5 ± 4.2 %, n = 4 animals, 18 cells, Fig. 2C). This suggested that the small Ca^{2+} is independent on the intracellular Ca^{2+}, which is consistent with previous results9. So it might be due to entrance of extracellular Ca^{2+} through Na^{+}-Ca^{2+} exchanger (NCX)33 or Transient receptor potential channels (TRP) channels34,35. Then we applied NCX blocker SN-6 (SN-6:2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic
acid ethyl ester, 50 µM) and SEA0400 (SEA0400: 2-[4-[(2,5-difluorophenyl)methoxy]-phenoxy]-5-ethoxyaniline, 50 µM) on the surface of the brain, and found that the Ca²⁺ transients became smaller (From 18.6 ± 3.7 % to 6.8 ± 2.8 %, p < 0.01, n = 25 cells in 5 mice, paired-t-test, Fig. 2C). In addition, Kir4.1 blocker BaCl₂ (50 µM) also blocked the Ca²⁺ transients significantly, making the Ca²⁺ transients much smaller (From 18.2% ± 3.5 % to 4.8 % ± 2.7 %, p < 0.01, n = 26 cells in 5 mice, paired-t-test, Fig. 2C) 36,37. And CM-4620, the CRAC channel (calcium released induced calcium channels) blocker also blocked the Ca²⁺ signaling significantly. This suggests that the astrocytic Ca²⁺ transients are due to neuronal activity induced accumulation of extracellular K⁺, which is rapidly buffered (taken up) via Kir4.1 in astrocytes 36,37. Astrocytic processes contains many microdomains that contain the NCX, which might in turn, induce influx of Na⁺ and Ca²⁺ 19. During sleep, Ca²⁺ increased 20, would induce CRAC open to increase intracellular Ca²⁺, so the Ca²⁺ signaling is majorly derived from extracellular space (Fig. 2D).

The small Ca²⁺ transients blocked sensory transmission

To monitor the effects of the small Ca²⁺ transients on neuronal activity, we did in vivo whole-cell recordings for the neurons in layer II –III. During sleep, all neurons stereotypically oscillated between two intrinsic stable membrane potentials (1.23 ± 0.25 Hz, -75.4 ± 2.7 mV and -54.7 ± 3.2 mV, n = 7 cells in 7 animals, one cell in each animal, Figure S3). However, contrary to previous reports that the thalamus can block the sensory transmission to the cortex, whisker stimulation really elicited EPSPs during sleep, which can be recorded in both whole-cell recording (averaged at 13.4 ± 2.1 mV for the first ten EPSPs, n = 8 cells, Fig. 3A), and LFP recordings (averaged at 0.8 ± 0.4 mV for the first ten EPSPs, n = 8 cells, Fig. 3B). The whole-cell EPSPs happened at 29.8 ± 1.6 ms after air puffing (n = 8 cells, one cell in each animal), while the LFP EPSPs occurred with a latency of about 19.3 ± 1.7 ms after whisker stimulation (Fig.3C). However, the LFP EPSPs terminated much earlier than Vm EPSP (52.38 ± 6.2 ms, compared with 186.4 ± 17.4 ms for whole-cell recordings, n = 8 animals, t-test, p < 0.01; Fig.3C). Of note, the latency of the evoked response might be extremely long, which might be due the time of air puffing. Then we used ATP and UTP to induce the Ca²⁺, and found that the UTP induced Ca²⁺ transient in the astrocytes had little effects on the slow oscillation (if not removed the up-down state oscillation), but blocked the sensory transmission. After the Ca²⁺ transients, the whisker stimulation induced EPSPs decreased (Fig.3D). We also screened whisker
stimulation induced Ca\textsuperscript{2+} transients, by first apply a batch of whisker simulation (10 s, 5 Hz), and after 5 seconds, apply another batch of whisker stimulation, and the results showed that the second batch of stimulation got much smaller EPSPs (13.8 mV ± 2.3 mV as before, compared with 6.8 mV ± 1.8 mV after, paired t-test, \( p < 0.01 \), \( n = 5 \) animals). Similarly, the EPSPs recorded in LFP also decreased from 0.82 mV ± 0.18 mV to 0.38 mV ± 0.14 mV. In addition, the EPSP decrease can be reversed by blocker SN-6 (50 µM) and SEA0400 (50 µM), and BAPTA-am (10 µM) during the sleep states (One-way ANOVA, \( p < 0.01 \), \( n = 5-7 \) animals, Fig. 3E). To ensure the astroglial origin of the EPSP enhancement, agonist-induced Ca\textsuperscript{2+} signals were studied in MrgA1\textsuperscript{+} mice\textsuperscript{33}. In these mice, the Gq-linked MrgA1 receptor is expressed in astrocytes under the control of the glial fibrillary acidic protein (GFAP) promoter; the MrgA1 receptor can be selectively stimulated with Phe-Met-Arg-Phe-NH\textsubscript{2} (FMRF) amide. Injection of FMRF (10 µM) evoked [Ca\textsuperscript{2+}] transient with an average increase of 19.7 ± 4.1 during sleep period. Furthermore, FMRF induced [Ca\textsuperscript{2+}] transients enhanced whisker stimulation induced EPSPs, with an amplitude decreasing from 14.4 ± 2.5 mV before FMRF to 7.1 ± 2.0 mV after FMRF (whole cell recording, paired-t-test, \( n = 6 \), \( p < 0.01 \), Fig. 3F).

**Big astrocytic Ca\textsuperscript{2+} transients at arousal states enhanced sensory transmission**

Then we investigated the mechanisms of astrocytic Ca\textsuperscript{2+} signals at awake states. Whisker stimulation induced much larger Ca\textsuperscript{2+} transients at fine processes during arousal state than those during sleep state, the fluorescence increase was 73.3 ± 11.7%, with a duration to be 18.6 ± 4.5 s (\( n = 24 \) cells in 5 animals, Fig. 4A-B.). The characteristics of astroglial Ca\textsuperscript{2+} transients was tested in the presence of mGluR antagonist MPEP (50 µM), and it is found that the Ca\textsuperscript{2+} transients, \( \Delta F/F_0 \) decreased to 17.7 ± 9.2 % during waking states (21 cells, in 5 animals, Fig. 4B). This suggested that the astroglial Ca\textsuperscript{2+} transients at waking states are due to glutamate release. In addition, when the mice with genetic deletion of IP3 receptor (Type 2), whisker stimulation induced Ca\textsuperscript{2+} transients decreased significantly to 25.5 ± 5.4 % (\( n = 19 \) cells in 4 animals, Fig. 4B). Contrary to that of sleep, NCX blocker SN-6 (50 µM) and SEA-0400 (50 µM) has little effects on whisker stimulation induced Ca\textsuperscript{2+} transient, with an averaged peak fluorescence of 58.6 ± 5.1 % (36 processes, in 5 animals, Fig. 4B). In addition, CRAC channel blocker CM-4620 has little effects either. These data suggest that the big Ca\textsuperscript{2+} transient is due to IP3 dependent Ca\textsuperscript{2+} release from endoplasmic reticulum (ER).
Numerous studies have shown that astrocytic Ca\textsuperscript{2+} enhanced EPSP \textit{in vitro} \textsuperscript{7,39}. To test the effects of astrocytic Ca\textsuperscript{2+} on sensory transmission in free moving mice, we first induce the Ca\textsuperscript{2+} transient with a batch of whisker simulation (10 s, 5 Hz), and after 5 seconds, apply another batch of whisker stimulation to test the effects of astrocytic Ca\textsuperscript{2+} on EPSPs (Fig. 4C). The results showed that the second batch of whisker stimulations was enhanced by first batch of whisker simulation induced Ca\textsuperscript{2+} in astrocytes. EPSPs were increased after astrocytic Ca\textsuperscript{2+} from 14.2 ± 2.7 mV (n = 5 animals) for the first batch, to 21.8 ± 3.4 mV, (n = 7 animals, **, \textit{p} < 0.01, Fig. 4D). The EPSPs recorded with LFP also increased from 0.91 ± 0.22 mV (n = 5 cells, or animals) to 1.48 ± 0.27 mV, (n = 7 animals, **, \textit{p} < 0.01, Fig. 4D). In addition, ATP (100\textmu M) also induced Ca\textsuperscript{2+} in astrocytes, and found that astrocytic Ca\textsuperscript{2+} also induced even bigger EPSPs, from 14.5 mV ± 2.3 mV before ATP to 17.6 ± 1.8 mV after ATP (36 processes in 5 mice, t-test, * \textit{p} < 0.05, Fig. 4E) or UTP induced EPSPs, from 14.1 ± 1.5 mV before UTP to 16.9 ± 2.1 mV (32 cells in 5 mice, Fig. 4E). As the animals aroused, and the membrane potential depolarized from a resting 70.6 ± 2.3 mV to 64.6 ± 2.1 mV (n = 5 mice, Fig. 4C, F), possibly due to the extracellular K\textsuperscript{+} increases \textsuperscript{20,33}. The membrane potential also depolarized by agonist (ATP, UTP and FMRF) (One–way ANOVA, \textit{p} < 0.01, n = 4-5 mice, Fig. 4F). Most importantly, the EPSP induced Action potentials (APs) also increased, with the reliability increased from 45.6 ± 6.3 % to 73.8 ± 7.5 % (n = 5 mice, Fig. 4G),

**Adrenergic activity is a prerequisite for big Ca\textsuperscript{2+} waves**

As the whisker stimulation induced smaller Ca\textsuperscript{2+} signaling during sleep state and bigger Ca\textsuperscript{2+} signaling during arousal waking state, and their functions seem to make the sleep brain even quieter, the waking brain even more aroused. We next try to see if they can switch from one to the other, like previous report, which suggested that the astrocytic Ca\textsuperscript{2+} needs the norepinephrine (NE) in locus cereus (LC) to be primed\textsuperscript{28}. First we stimulated LC during sleep, and then applied a batch of whisker stimulation, and compared the whisker stimulation induced Ca\textsuperscript{2+} transients before and after LC stimulation. Whisker stimulation during sleep states induced small increases in Ca\textsuperscript{2+} transients; and after LC stimulation, the animals really woke up, and then whisker stimulation induced much larger Ca\textsuperscript{2+} transients (fluorescence transient was 27.2 ± 4.3% for whisker stimulation before LC stimulation; after LC stimulation, Ca\textsuperscript{2+} transients increased by 68.8 ± 6.1%, n = 27 cell processes in 5 animals, ** one-way ANOVA, \textit{p} < 0.01) (Fig. 5A-B). To
further prove the LC stimulation was really through NE, we injected NE (100 µM) to the barrel cortex, and found that whisker stimulation induced Ca\textsuperscript{2+} signaling really increased from 23.6 ± 3.8% to by 61.8 ± 4.1%, n = 29 cell processes in 5 animals, ** one-way ANOVA, p < 0.01). We then used different adrenergic antagonists to the Barrel cortex, such as \(\alpha_1\)-AR antagonist terazosin (100 µM). Terazosin significantly blocked whisker stimulation induced Ca\textsuperscript{2+} signalling (peak of \(\Delta F/F_0\) in the presence of the drug was 31.2 ± 4.9%, p < 0.01, t-test, n = 31 cells in 4 animals). Conversely, \(\alpha_2\)-AR antagonist metoprolol (10 µM) was not significantly effective (\(\Delta F/F_0\) peaked at 52.7 ± 7.6%, n = 28 cells in 4 animals) at LC induced Ca\textsuperscript{2+} signaling.

We also compared whisker stimulation evoked EPSPs before or after LC stimulation, and found that LC significantly increased the amplitude of whisker stimulation induced EPSPs (from 0.51 ± 0.12 mV for the first batch of whisker stimulation, to 1.1 ± 0.19 mV for the second batch of whisker stimulation, n = 5, paired t-test, \(p < 0.01\) (Fig. 5B). To make sure the enhancement of EPSPs was due to Ca\textsuperscript{2+} transients induced by first batch whisker stimulation, we applied BAPTA/AM (10 µM), which was majorly taken up by astrocytes, and has been often used to block astrocytic Ca\textsuperscript{2+} transients in many previous reports\textsuperscript{33}. The results showed that \(\Delta F/F_0\) decreased to 12.7 ± 1.2 % in BAPTA/AM loaded cells after LC stimulation, and the EPSPs were also decreased (from 0.78 ± 1.2 mV to 0.3 ± 0.2 mV, **\(p < 0.01\), One-way ANOVA, 5 animals, Fig. 5C). In addition, \(\alpha_1\)-AR antagonist terazosin (100 µM) significantly reduced EPSPs (before 0.91 ± 0.12 mV to 0.41 ± 0.19 mV, n = 5 animals. Fig. 5D-E). Reversely, we also found that when the animals turned from arousal state to sleep states, the EPSPs got smaller (Fig. S4).

**Discussion**

Increasing body of evidence has proved that astrocytes are major players in the modulation of neurons under both physiological and pathological conditions\textsuperscript{40}. Beyond the local astrocytic control of synaptic activity, the role of astrocytic modulation in large scale of neuronal ensembles, such as the global sleep and waking states, is gradually recognized too\textsuperscript{40}. For example, the astrocytes help sleep through connexin 43\textsuperscript{24}, or changing extracellular metabolites\textsuperscript{31}, or ions\textsuperscript{20}. In addition, astrocyte has been suggested to be involved in attention control\textsuperscript{41}. As far as we know, these two kinds of astrocytic Ca\textsuperscript{2+} signaling during sleep state and the aroused waking states have not been reported before. Here we further found that the LC stimulation acts switch
between the small Ca$^{2+}$ signaling at sleep state to the higher Ca$^{2+}$ signaling, which might be the mechanism for LC/NE modulating brain arousal state$^{42}$.

**Small Ca$^{2+}$ transient facilitates sleep like slow oscillation during sleep state**

During sleep, whisker stimulation induced K$^+$ release from neurons, will be taken up by astrocytes, K$^+$ influx would in turn induced Ca$^{2+}$ influx; this kind of Ca$^{2+}$ influx or ATP agonist induced Ca$^{2+}$ release can open Ca$^{2+}$ release activated Ca$^{2+}$ channels (CRAC) to evoke Ca$^{2+}$ transients (Figure 6). Astrocytic processes contains many microdomains that contains the NCX and Na$^+$ pumps with high ouabain affinity α2, and the increase of Ca$^{2+}$ would in turn increase NCX activity, thus increase sytosolic Na$^+$ and Ca$^{2+}$ concentrations; and increased sytosolic Na$^+$ in turn activate Na$^+$ pump to increase K$^+$ take-up by astrocytes$^{19}$. Thus astroglial Ca$^{2+}$ transients would decrease extracellular K$^+$, to hyperpolarize neurons and block sensory transmission$^{20}$. In all, astrocytes block the sensory transmission possibly by the membrane potential hyperpolarization during sleep.

**Large Ca$^{2+}$ wave enhances sensory transmissions**

Here we might be the first to report that the astrocytes in the barrel cortex exhibit two types of Ca$^{2+}$ events. These two types of events appear to have different pharmacology/sources and may play a different role in facilitating the efficacy of synaptic transmission during different behavior states. During last three decades, it has been found that astroglial cells regulate synaptic connectivity through multiple mechanisms$^{40-44}$, including the concept of "active" astrocytes capable of releasing 'gliotransmitters'$^{45-47}$. Overwhelming evidence for such physiological properties have confirmed the effects of gliotransmitters on synaptic activities$^{48}$. Therefore, the reported results about function of Ca$^{2+}$ signaling in this study are possibly due to glutamate release to regulate synaptic connectivity. Even though some studies about astrocytic Ca$^{2+}$ suggests that astrocyte calcium transients are not involved in synaptic transmissions in hippocampus$^7$. The “third wave” of astrocytic calcium signaling research in astrocytic fine processes has now been surged up, and focused on the calcium transients occurring in fine astrocyte processes not resolved in earlier studies$^{44}$. Here our result report that the large Ca$^{2+}$ wave is really a prerequisite for reliable sensory transmission at waking states.

**Adrenergic activation acts as a switch to evoke large Ca$^{2+}$ wave**
Previous studies suggested that astrocyte responds with robust calcium elevations during arousal/startle due to the release of noradrenaline from noradrenergic projections via a mechanism likely involving α1 adrenoceptors on astrocytes17. Here we also report that astrocytic calcium transients are normally quiet27 and very hard to be activated without synergetic activation by NE/LC system together whisker stimulation. NE, an important neurotransmitter in the circadian rhythm, can prime astrocytic Ca²⁺ signaling, which in turn induces many astrocytic activities such as gliotransmitter releases (Figure 6). NE acts on astrocytes through α- and β-adrenergic receptors (α/β−ARs), with the former being linked to InsP₃ production and intracellular Ca²⁺ release⁴⁶. Our data, together with our previous data also showed that it is through α- adrenergic receptors (α/β−ARs) that the astrocytic Ca²⁺ wave is primed⁴⁹-⁵¹. One of the major functions of NE induced astrocytic Ca²⁺ wave is increasing extracellular K⁺. After LC stimulation or NE application, the extracellular K⁺ can increase immediately approximately 1 mM²⁰. The increased K⁺ can depolarize the neurons to let them leave the slow oscillation and be ready to fire action potentials at EPSPs. The increased extracellular K⁺ can also prime astrocytic Ca²⁺ to increase its function at clearance and recycle of the neurotransmitters, help the synaptic transmission last longer. In all, astrocytic calcium signaling is a prerequisite for reliable sensory transmission, especially during waking state when the neurons need more work for astrocytes⁵³.

In addition, the glymphatic system is a glial-dependent perivascular network that plays a pseudolymphatic role in the brain. The perivascular spaces form a complex brain-fluid transport system that supports fast exchange with interstitial fluid and clearance of waste products from the brain from the intricate environment of the neuropil. Sleep is necessary for the normal function of the glymphatic system; while norepinephrine release blocks the glymphatic system. Norepinephrine release decreases during sleep, resulting in an expansion of the interstitial space and a subsequent potentiation of glymphatic-fluid transport. A recent study suggested that the astrocytic Ca²⁺ elated with the glymphatic systems³, which suggested that neural slow waves are followed by hemodynamic oscillations, which in turn are coupled to CSF flow. These results demonstrate that the sleeping brain exhibits waves of CSF flow on a macroscopic scale, and these CSF dynamics are interlinked with neural and hemodynamic rhythms³.

Materials and Methods
Animal preparation for awake in vivo recordings: Adult (10 weeks old) C57Bl/6 wild type mice were used (both male and female, from Charles River Laboratories). The preparation for mouse experiments was modified from published protocols 48,49. Briefly, mice were anesthetized using isoflurane (1.5% mixed with 1-2 L/min O₂), head restrained with a custom-made mini-frame and habituated to the restraint over one week in multiple session, with a total training duration of 3-4 hours. A 1.5 mm craniotomy was then opened over the somatosensory cortex (1.5 mm in diameter, 3 mm lateral and 1.5 mm posterior to the Bregma), the dura was carefully removed, and the mice were allowed 60 min recovery prior to conducting the experiments. The craniotomy procedure lasted < 20 min to minimize anesthesia exposure on the recording day. Animals were then head-strained, placed in a behavioral tube to minimize movement and relocated to the imaging room, which was kept dark and sleep. Body temperature was maintained with a heating pad. For cortical drug surface application, artificial cerebrospinal fluid (aCSF) was perfused across the cortex of awake mice at a rate of 2 mL/min, into a custom-made well with ~200 μL volume, through tubing with ~100 μL volume, meaning the entire volume bathing the brain was exchanged every ~9 s. The aCSF solution contained 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 26 mM NaHCO₃, pH 7.4. For imaging, calcium indicator rhod-2 AM (Invitrogen) was loaded onto exposed cortex for 30-40 min before applying agarose (1.5%, type III-A, Sigma) and a coverslip 50. All animals were trained several times a day, for three days before the experiments. All procedures followed National Institute of Health guidelines and were approved by the Institution of Animal Care and Use Committee. The animals were anesthetized with 1.5% isoflurane during the surgery, and then after being mounted on the equipment, the isoflurane was stopped and the animal was left to run freely on the wheel during sleep/wake states in the dark room, except that the heads were held tightly on a frame (detailed methods refer to previous publications, e.g.9).

Chemicals: All standard chemical were purchased from Sigma-Aldrich, except mentioned otherwise. CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione, Sigma), APV (D-2-Amino-5-phosphono-pentanoic acid, Sigma), MPEP (6-(phenylethynyl)-pyridine, Tocris), terazosin (Tocris), metoprolol (Tocris), SN-6 (2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester, Tocris), SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]-phenoxy]-5-ethoxyaniline, CM-4620 (Tocris), synthesized by Taisyo Pharmaceutical Co. Ltd), BAPTA-am:(1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (Tocris), UTP (Uridine 5'-triphosphate, Tocris).

Surgical procedure for virus injection: pAAV5-GfaABC1D-cyto-GCaMP6f-SV40 (Cat# AV5-52925, UPenn vector core), which is a genetically encoded Ca²⁺ indicator driven by the astrocyte specific GfaABC1D promoter, was injected in Barrel cortex. The mice were anesthetized with 1-2% isoflurane with oxygen supply and were placed in a stereotactic head frame with a heating pad underneath. A small vertical incision was made on the skin and the craniotomy (0.5 mm × 0.5 mm) was performed with a drill. The virus was injected at a volume of 500 nl/site without dilution, 3 injection sites were utilized. A glass micropipette with a tip of 10 μm was used for injection with microinjection control.

In vivo two-photon imaging and stimulation: A custom-built microscope attached to a Tsunami/Millenium laser, Spectra Physics, Mountain View, CA) and scan box (FV300 Fluoview Software, Olympus, Center Valley, PA) was used for 2-photon imaging through a 203 objective (0.9 NA, Olympus). Excitation wavelength was in the range of 800--820 nm. Emission
wavelengths were split to detect fluo-4 and AlexaFluor 594 signals as previously described. Images of astrocytic Ca\(^{2+}\) signaling were recorded every 2–3 s, which was sufficient to capture evoked responses while limiting laser induced photo damage at a laser power of <30 mW. Prior to whisker stimulation experiments, anesthesia 0.5 mg/kg D-tubocurarine was injected to prevent small reflex movements that could distort imaging. Direct LC stimulation was applied using a bipolar concentric electrode. Stimulation consisted of a single train of 20–100 pulses (100 Hz, 50 \(\mu\)A, 0.5 ms square pulses).

**In vivo whole-cell recording:** Recordings were obtained from layer II barrel cortex using glass microelectrodes. LFP signals were externally filtered at 6 Hz (Filter Butterworth Model by Encore, Axopatch 200B by Axon Instruments), bandpass filtered at 1-100 Hz and digitized (Digidata 1440A by Axon Instruments). Recordings were analyzed offline using pClamp 10.2. Whole-cell recordings were performed with blind patching, by watching the pipette resistance. Patch electrodes were fabricated from filament thin-wall glass (World Precision Instruments) on a vertical puller; the resistance of the pipette was about 6 to 9 megohms with intracellular pipette solution added. The pipette solution contained 140 mM K-glucocinate, 5 mM Na-phosphocreatine, 2 mM MgCl\(_2\), 10 mM Hepes, 4 mM Mg-ATP, and 0.3 mM Na-GTP (pH adjusted to 7.2 with KOH). The junction potential between the patch pipette and the bath solution was zeroed before forming a gigaseal. Patches with seal resistances of less than 1 gigohm were rejected. Data were low pass–filtered at 2 kHz and digitized at 10 kHz with a Digidata 1440 interface controlled by pClamp Software (Molecular Devices). Whisker stimulation was delivered using a picospritzer III (Parken Instrumentation) and Master 8 (A.M.P.I.). The amplifier bandwidths were normally 0.5 Hz to 100 Hz. EEG recording was digitized at 100 Hz and then subjected to spectral analysis using a complex demodulation procedure.

**In vivo intracellular recording:** In vivo intracellular recordings were obtained from layer II barrel cortex using glass microelectrodes. The surgery was done the same as in vivo whole cell recordings. The electrode was made from a thick wall glass (B150F-4, World Precision Instruments). The resistance of the electrodes was 50-150 M\(\Omega\), after filled with 1.0 M potassium acetate. Intracellular recording was done with the same equipment as whole cell recordings, except that the manipulation of the pipette was done with the advancement of 1-2 \(\mu\)m step with the fastest movement mode. After penetration, the series resistance was adjusted, and the current-clamp was used to measure the membrane potentials of the astrocytes.

**EEG and EMG recording** Acquired EEG/EMG signals were amplified ((Filter Butterworth Model by Encore, Axopatch 200B by Axon Instruments), at a sampling frequency of 1K Hz. The EEG signal was filtered with high-pass: 0.5 Hz, low pass: 30 Hz, and EMG signal high-pass filtered at 10Hz. Wakefulness was subdivided into sleepfulness (DW) or arousal wakefulness (AW) using EMG peak-to-peak amplitude of all wake epochs across the 12-h recording. QW was defined as 33rd percentile or less and AW 66th percentile or higher of all wake EMG peak-to-peak amplitude values. Concurrently with EEG recordings, spectral analysis of electroencephalography (EEG) recordings showed that most of its power resides in 4–6Hz, which were interrupted intermittently with slow waves of 0.5–4Hz.

**Statistical analysis:** All analyses were performed using SPSS 19 software (IBM) and all tests were two-tailed where significance was achieved at \(\alpha = 0.01\) level. For independent samples, a \(t\)-test (\(\leq 2\) variables) or one-way ANOVA (> 2 variables) was used; for paired samples, a paired \(t\) test was used.
Author Contributions
F.W., J.H.H. planned the project. F.W., E.W., WW, S.G. wrote the manuscript. K.Z., S.G., P.W., W.W., H.W., F.W. performed in vivo electrophysiology and imaging. F.W., J.Y., L.G., W.W., J.H.H. performed data analysis. W.D., F.W., and R.F. performed animal surgery and imaging experiments.

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Competing financial interest
The authors declare that there are not competing financial interests.

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Figure legend

**Figure 1. Two kinds of Ca^{2+} transients at astrocytic fine processes are characterized as the sleep vs awaked state.** A. A schematic drawing depicts the experimental setup combing two-photon imaging of astrocytic Ca^{2+} signaling in the barrel cortex of within vivo whole-cell recordings in single neuron and local field recordings in awake behaving GCaMP6f transgenic mice. B. Representative image of cortex displaying the expression of GCaMP6f are majorly in SR100β loaded astrocyte. C. Representative recordings of EEG and EMG and power spectrum analyses (blue as sleep; red as arousal). Upper traces are representative ECoG traces from whisker stimulation. Arousal states were shown as no apparent evoked ECoG response and strong EMG activity. D. Typical representative fluorescence changes showing that whisker stimulation induced small Ca^{2+} increases during sleep states, scale bar = 20 µm. E. The analyses of fluorescence changes corresponding GCaMP6f F/F0 traces at the fine processes, which are marked as circles in D (n = 6 animals). F-J. Statistical data show the comparison the whisker stimulation induced changes of Ca^{2+} transients (***p < 0.01, paired t-test, n = 24 trails in 6 animals).

**Figure 2. The small Ca^{2+} transients derive from influx of extracellular Ca^{2+}.** A. Typical images show that agonist UTP (100µM) induced small Ca^{2+} transients as shown by increased fluorescence intensity. The scale bar is 20 µm. B. Typical traces show the Ca^{2+} transients and also changes of extracellular K^{+}. C. Statistical data show the comparison the whisker stimulation induced changes of Ca^{2+} transients with agonists, and also in IP3R2 knockout mice, and also with different manipulation of blockers (p < 0.01, one-way ANOVA, n = 4 - 6 animals). D. A schema shows the intrinsic ion channels involved in the oscillation. Whisker stimulation induced K^{+}
release from neurons, will be taken up by astrocytes via Kir4.1, K^+ influx induced Na^+ influx, which in turn induced Ca^{2+} influx via Ca^{2+} induced Ca^{2+} channels (CRAC) open to let Ca^{2+} influx, or ATP agonist induced Ca^{2+} release can activate Ca^{2+} release activated CRAC open. Consistently, NCX blocker SN-6 + SEA0400, CRAC channel blocker (CM-4620), Kir4.1 blocker BaCl2 and Ca^{2+} buffer BAPTA all blocked the Ca^{2+} signaling.

**Figure 3** Small Ca^{2+} transients blocked sensory transmission. A. Typical images showing UTP (100 µM) induced changes in fluorescence. Scale = 20 µm. B. Statistical comparisons among the effects of antagonists on whisker simulation (WS) induced Ca^{2+} transients, and ATP and UTP induced Ca^{2+} signaling. C. Typical traces of whisker stimulation induced EPSPs recorded with whole-cell recordings (Vm EPSPs) and local field recordings (LFP EPSPs) during sleep state. D. Typical traces of EPSPs before and after UTP (100 µM) induced Ca^{2+} signaling. The UTP induced Ca^{2+} transient in the astrocytes had little effects on the slow oscillation (if not removed the up-down state oscillation), but blocked the sensory transmission. Right picture shows a patched neuron loaded with Alex488 (red), together with astrocytes loaded with GCaMP6f (green). E-F. Comparisons of amplitude of EPSPs recorded with whole-cell recordings (left panel) and LFP recordings during sleep, the amplitude of EPSPs was decreased after 1st batch of WS or UTP induced Ca^{2+} signaling than before (p > 0.01, n = 8 animals).

**Figure 4** Whisker stimulation induces big Ca^{2+} transients at arousal states. A. Fluorescent images showing that whisker stimulation induced big Ca^{2+} increases during waking states. B. Comparisons of the relative changes of Ca^{2+} (ΔF/F_0) in astrocytes at whisker stimulation (**, p < 0.01, one-way ANOVA, n = 4-7 animals). C. Typical traces of whole-cell recordings show the spontaneous membrane potential changes induced by Ca^{2+} transients. D. Typical recordings shows EPSP changes after whisker stimulation (WS) induced astrocytic Ca^{2+} signaling at the Barrel cortex in live mice. E. Statistical analysis of the EPSP changes after astrocytic Ca^{2+} signaling induced by whisker stimulation and agonists (ATP, UTP and FMRF). Comparisons of amplitude of EPSPs recorded with both whole-cell recordings (upper panel), and LFP (local field potential) recordings (lower panel) during sleep and waking states. F. Analysis of membrane potential changes from in vivo whole-cell recordings show the membrane potential changes. G. Analysis of reliability of whisker stimulation induced action potentials before and after Ca^{2+} signaling.

**Figure 5.** Adrenergic activity switches small Ca^{2+} signaling to big waves. A. Fluorescent images showing that LC stimulation can induce large Ca^{2+} increases after LC stimulation. Scale bar = 20 µm. B. A scheme illustrates projections from LC stimulation (red dashed line) and whisker stimulation (green solid line and blue dashed line) to the Barrel cortex. LC stimulation was generated using bipolar concentric electrode, while whisker stimulation was generated through air puffing. C. Typical traces show the changes of Ca^{2+} transients and LFP EPSPs. Upper traces (blue trace is from sleeping state, while the red trace is after LC stimulation) are relative changes of Ca^{2+} (ΔF/F_0) in an astrocytic process located close to the recording electrode. Lower traces are LFP recorded by the recording electrode. D. Analysis shows comparisons of the relative changes of Ca^{2+} (ΔF/F_0) in astrocytic processes at whisker stimulation (**, p < 0.01, one-way ANOVA, n = 4-7 animals). E. Analysis shows comparisons of the relative changes of
LFP EPSPs at whisker stimulation (**, p < 0.01, one-way ANOVA, n = 4-7 animals). The x-axis labels indicate the type of stimulation used. Additional abbreviations used are as following: TER, α1-antagonist terazosin; METO, β-antagonist metoprolol; ** p < 0.01.

**Figure 6. A model shows the mechanisms of Ca²⁺ transients during sleep and waking states.**

**Left.** During sleep, whisker stimulation induced K⁺ release from neurons, which will be taken up by astrocytes, K⁺ influx induced Ca²⁺ influx, or ATP agonist induced Ca²⁺ release can activate Ca²⁺ release activated Ca²⁺ channels (CRAC) open to get much more Ca²⁺ influx inside the processes. The Ca²⁺ increase would in turn make feedback to increase intracellular Na⁺, which would activate Na pump to take up K⁺, and make the neurons to hyperpolarization. **Right.** During waking states, NE activates Gq receptors which primes the astrocytes, and synergistically works with mGLu to activate Gq receptors to release even more IP₃, and induce Ca²⁺ release from ER, and induced gliotransmitters release (such as ATP, or glutamate, or glutamine).

**Figure S1. Movies for Ca²⁺ signaling.**

**Figure S2. Images for GCaMP6f with NeuN staining.**

**Figure S3. The intrinsic ion channels involved in up-down state oscillation.** **A.** Typical traces showing the injection of currents (30pA) induced depolarization of down-states and up-states. The lower trace is the extension of the recordings in the square, which shows that the down-state and up-state can be reversed. **B.** The I-V curves drawn from the traces in A, show the possible ion channels involved in down-state and up-state, which might be leaking K⁺ currents and persistent Na⁺/Ca²⁺ currents respectively. **C.** A model shows the intrinsic ion channels involved in the oscillation. The leaking K⁺ currents are outward currents, and the persistent Na⁺/Ca²⁺ currents are inward, their balance constitutes the two stable states: up-states and down-states.

**Figure S4. Switches between sleep states and waking states.** **A.** Typical traces showed that whisker stimulation induced LFPs decreases significantly when the animals switches from alert waking state to sleep states. **B.** Typical recordings of whisker stimulation induced LFP show the LC stimulation increased the EPSPs in LFP.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6