Astrocytic Ca$^{2+}$ signaling has been intensively studied in health and disease but has not been quantified during natural sleep. Here, we employ an activity-based algorithm to assess astrocytic Ca$^{2+}$ signals in the neocortex of awake and naturally sleeping mice while monitoring neuronal Ca$^{2+}$ activity, brain rhythms and behavior. We show that astrocytic Ca$^{2+}$ signals exhibit distinct features across the sleep-wake cycle and are reduced during sleep compared to wakefulness. Moreover, an increase in astrocytic Ca$^{2+}$ signaling precedes transitions from slow wave sleep to wakefulness, with a peak upon awakening exceeding the levels during whisking and locomotion. Finally, genetic ablation of an important astrocytic Ca$^{2+}$ signaling pathway impairs slow wave sleep and results in an increased number of microarousals, abnormal brain rhythms, and an increased frequency of slow wave sleep state transitions and sleep spindles. Our findings demonstrate an essential role for astrocytic Ca$^{2+}$ signaling in regulating slow wave sleep.
We spend approximately one third of our lives sleeping, yet the purpose of sleep remains one of the greatest unsolved mysteries in biology. Recent studies have shown that not only neurons, but also glial cells are essential for sleep. Still, the nighttime of the main glial cell in the brain—the astrocyte—is poorly characterized. There is evidence that astrocytes regulate sleep drive, promote sleep-dependent brain waste clearance, and facilitate cortical oscillations that are important for learning and memory, but the signaling mechanisms that astrocytes employ to mediate these sleep-dependent functions remain elusive.

Astrocytic Ca²⁺ signals have been extensively studied in vitro and in vivo in anesthetized and, more recently, in awake animals and are considered to orchestrate neuronal circuit activity by regulating extracellular ion concentration and promoting the release of signaling substances. In addition, astrocytes not only sense local synaptic activity, but also respond with Ca²⁺ signals to neuromodulators that are involved in sleep-wake state regulation. Furthermore, astrocytic Ca²⁺ signals have been characterized under urethane anesthesia, a state that has been intensively studied and has a well-characterized circadian nature of astrocytic Ca²⁺ release of signaling substances, and are considered to orchestrate neuronal circuit activity by

Recent advances in optical imaging and genetically encoded activity sensors have enabled high-resolution imaging of astrocytic Ca²⁺ signals in unanesthetized mice, revealing an exceedingly rich repertoire of astrocytic Ca²⁺ signals. Conventional tools for analysis of astrocytic Ca²⁺ signals are based on static regions-of-interest (ROIs) placed over morphologically distinct compartments. However, the highly complex and dynamic spatiotemporal nature of astrocytic Ca²⁺ signals that has become apparent using ultrasensitive genetically encoded Ca²⁺ sensors, is not captured by static ROI analyses.

Here, we characterize astrocytic Ca²⁺ signaling in mice during natural head-fixed sleep using an automated activity-based analysis tool. We employ dual-color two-photon Ca²⁺ imaging to capture the activity of neocortical astrocytes and neurons simultaneously, combined with electrophysiological (EEG), electromyography (EMG), and behavioral monitoring. We show that Ca²⁺ signaling in astrocytes is reduced during sleep compared to wakefulness and exhibits distinct characteristics across sleep states. Strikingly, an increase in astrocytic Ca²⁺ signaling precedes transition from slow wave sleep (SWS)—but not rapid eye movement (REM) sleep—to wakefulness. Finally, we demonstrate that the inositol triphosphate (IP₃)-mediated astrocytic Ca²⁺ signaling regulates SWS by maintaining uninterrupted SWS, and affecting SWS state dynamics and sleep spindles. Taken together, our data indicate a role for astrocytic Ca²⁺ signaling in regulating SWS.

Results

Two-photon imaging of awake and naturally sleeping mice. To explore the characteristics of Ca²⁺ signaling in astrocytes and neurons across sleep-wake states, we employed dual-color two-photon Ca²⁺ imaging of neurons and astrocytes in layer II/III of the mouse barrel cortex by viral delivery of the green Ca²⁺ indicator GCaMP6f to astrocytes and the red Ca²⁺ indicator jRGECO1a to neurons (Fig. 1a). We chose barrel cortex because it has been intensively studied and has a well-characterized circuitry, and because the sensory input is easily monitored by whisker tracking. The glial fibrillary acidic protein (GFAP) and the human synapsin1 (SYN) promoters were used to target astrocytes and neurons, respectively. Imaging was performed at a frame rate of 30 Hz, in accordance with recent reports underscoring the importance of high image acquisition rates for capturing fast populations of astrocytic Ca²⁺ events. Concomitantly, we recorded mouse behavior with an infrared (IR)-sensitive camera, ECoG and EMG for classification of sleep-wake states (Fig. 1a). The transduced SYN-jRGECO1a and GFAP-GCaMP6f specifically labeled neurons and astrocytes without inducing astrogliosis or microglial activation (Supplementary Fig. 1).

We identified three different behavioral states of wakefulness by analyzing mouse movements on the IR video footage (Fig. 1b): locomotion, spontaneous whisking, and quiet wakefulness. Since locomotion in mice is tightly associated with natural whisking, our locomotion behavioral state comprises both movement and whisking. Using standard criteria on ECoG and EMG signals, we identified three sleep states (Fig. 1c, d): non-rapid eye movement (NREM) sleep, intermediate state (IS) sleep, and REM sleep. NREM sleep and IS sleep are sub-states of SWS, where IS sleep is a transitional state from NREM to REM sleep, found at the end of an NREM episode and characterized by an increase in sleep spindle frequency, increase in sigma (10–16 Hz) and theta (5–9 Hz) ECoG power and a concomitant decrease in delta (0.5–4 Hz) ECoG power. To verify that the head-fixed situation did not perturb sleep we compared sleep characteristics between freely moving and head-fixed mice. We found nearly identical sleep characteristics between the two conditions, except less time spent in REM sleep and higher ECoG power in delta and theta range in the head-fixed condition (Supplementary Fig. 2). The increase in delta and theta power could at least partially be explained by delayed sleep onset and consequently higher sleep pressure in head-fixed mice (Supplementary Fig. 2a), which has been shown to increase both delta and theta ECoG power in NREM and REM sleep.

To capture the high spatiotemporal complexity of astrocytic Ca²⁺ signaling, we developed an automated activity-based analysis algorithm (Supplementary Fig. 3). The algorithm utilizes three-dimensional filtering and noise-based thresholding on individual pixels over time to detect fluorescence events. Connecting adjacent active pixels in space and time results in regions-of-activity (ROAs) that can subsequently be combined with conventional, manually drawn ROIs or analyzed separately. The specificity of the algorithm was tested by applying the ROA method on time series from control mice expressing a Ca²⁺ insensitive fluorescent indicator (enhanced green fluorescent protein, eGFP) in cortical astrocytes (Supplementary Fig. 4). Furthermore, we compared the characteristics of Ca²⁺ event detection with ROI and ROA analyses (Supplementary Fig. 5 and Supplementary Movies 1 and 2). Ca²⁺ signals in astrocytic somata and processes were much more complex than what could be captured by static ROIs. Notably, small, low-amplitude events in microdomains remained undetected with ROI analysis (Supplementary Fig. 5c, d), resulting in up to 90% fewer detected events (Supplementary Fig. 5e, f).

In all, 13 h of wakefulness and over 15 h of natural sleep (7 h NREM, 5 h IS, 3 h REM) (Fig. 1f) in 6 wild type (WT) mice were analyzed. Representative wakefulness and sleep trials are shown in Supplementary Figs. 6 and 7, and Supplementary Movies 3 and 4.

Ca²⁺ signaling is reduced during sleep and is state specific. We used the ROA analysis to explore astrocytic Ca²⁺ signaling during wakefulness and natural sleep (Fig. 2). Astrocytic Ca²⁺ signals across the sleep-wake cycle displayed a broad repertoire of size, duration and volume (Supplementary Fig. 8). The spatial extent of ROAs ranged from ~0.9 μm² (lower detection limit) to the full field-of-view (FOV) (Supplementary Fig. 8a), whereas the
duration of the events ranged from 0.05 s to 100 s (Supplementary Fig. 8b). The majority of astrocytic Ca\textsuperscript{2+} events were small and short-lasting across all sleep-wake states (~80% events <10 \textmu m\textsuperscript{2} and <1 s) (Supplementary Fig. 8d, e, f). On average, Ca\textsuperscript{2+} events were of largest area and volume during active wakefulness (locomotion and whisking) (Supplementary Fig. 8g, h), and of longest duration during sleep (Supplementary Fig. 8i).

Since wakefulness encompasses a spectrum of sub-states that serve distinct perceptual and behavioral functions\textsuperscript{25}, we investigated astrocytic Ca\textsuperscript{2+} signaling across the different states of wakefulness. Voluntary locomotion and spontaneous whisking were associated with increased percentage of active voxels (x-y-t) (11-fold during locomotion, 2.5-fold during whisking) and increased ROA frequency (3-fold during locomotion, 1.5-fold during whisking) compared to quiet wakefulness (Fig. 2c, d, middle), similar to previous reports\textsuperscript{18,19}. By contrast, astrocytic Ca\textsuperscript{2+} activity was reduced during sleep compared to overall wakefulness, with a 94% reduction in the mean percent of active voxels (x-y-t) and a 77% reduction in the frequency of ROAs (Fig. 2c, d, left). The reduction in astrocytic Ca\textsuperscript{2+} activity was consistent for all sleep states, not only compared to active waking states, but also compared to quiet wakefulness (Fig. 2c, d, right). Although astrocytic Ca\textsuperscript{2+} activity was substantially reduced during sleep compared to wakefulness, further analyses revealed that the remaining activity during sleep nonetheless varied between states. The percentage of active voxels (x-y-t) and ROA frequency was lower during IS sleep than during NREM or REM sleep (Fig. 2c, d, right).

Importantly, our dataset yielded same trends when analyzed by another recently published astrocytic Ca\textsuperscript{2+} event analysis tool\textsuperscript{26} (AQuA), but not when analyzed by conventional ROI analysis (Supplementary Figs. 9 and 10). To conclude, we demonstrate that astrocytic Ca\textsuperscript{2+} signaling is reduced during sleep compared to wakefulness, and is state-specific.

**Ca\textsuperscript{2+} signals in sleep are most frequent in processes.** Since Ca\textsuperscript{2+} transients in astrocytic somata and processes may have different underpinnings and functional roles\textsuperscript{18,27}, we investigated the subcellular compartmentalization of Ca\textsuperscript{2+} signals during natural sleep. As previously shown for wakefulness and anesthesia\textsuperscript{18}, activity maps indicated that astrocytic Ca\textsuperscript{2+} signals were most frequent in astrocytic processes in neuropil also in natural sleep (Fig. 3a). We then quantified the subcellular distribution of the astrocytic Ca\textsuperscript{2+} signals by running the ROA algorithm within manually drawn ROIs over astrocytic somata and neuropil (Fig. 3b). This analysis also confirmed that astrocytic Ca\textsuperscript{2+} signals were of higher frequency within neuropil ROIs than within...
astrocyclic somata ROIs across all sleep-wake states (Fig. 3b). Still, both astrocytic somata and processes displayed similar magnitude and direction of change in Ca\textsuperscript{2+} signaling across sleep-wake states (Fig. 3b and Supplementary Fig. 11). To conclude, astrocytic Ca\textsuperscript{2+} signals are more frequent in processes than somata not only during wakefulness, but also during sleep.

**Spatial stability of astrocytic Ca\textsuperscript{2+} signals.** If astrocytic Ca\textsuperscript{2+} signals are specifically integrated in sleep-wake dependent circuitry, one would expect to find some stability of active regions specific to sleep-wake states. We found that generally the overlap of active areas between the two episodes of the same state was relatively low (ca. 5%) except during episodes of locomotion (ca. 25%), where typically most of the FOV was active (Fig. 4a). To evaluate whether some of the astrocytic Ca\textsuperscript{2+} signals occurred at sleep or wakefulness specific locations, we first created individual heatmaps representing the level of Ca\textsuperscript{2+} activity of every episode of all of the sleep-wake states within a FOV. Then, we analyzed the distances between heatmaps, here defined as 1 minus the Jaccard similarity coefficient (see "Methods") by performing a permutational multivariate analysis of variance\textsuperscript{28}. We first checked whether there was state-specific overlap within sub-states of wakefulness (locomotion, whisking, quiet wake) and within sub-states of sleep (NREM, IS, REM) (Fig. 4b). Here, we found that 25% of FOVs (19 of 76), including only wakefulness sub-states, exhibited state-specific activation—i.e., within these FOVs there was a smaller overlap between episodes of different states.

Fig. 2 Astrocytic Ca\textsuperscript{2+} signaling is reduced during sleep and is sleep state specific. a and b Representative x-y-t rendering of ROAs during wakefulness (a) and sleep (b). c Percentage of active voxels (x-y-t) during overall wakefulness and overall sleep (left), during locomotion, whisking and quiet wakefulness (middle), and during NREM, IS, and REM sleep as compared to quiet wakefulness (right). d Same as c but for ROA frequency expressed as number of ROAs per 100 \(\mu m^2\) per minute. Data represented as estimates ± SE and \(p\)-values (two-sided test, no adjustment for multiple comparisons) derived from linear mixed effects models statistics, \(n = 6\) mice, 283 trials. For details on statistical analyses, see “Methods.” See also Supplementary Figs. 8–10.
states (locomotion-whisking, locomotion-quiet wake, whisking-quiet wake), compared to episodes of the same state (locomotion-locomotion, whisking-whisking, quiet wake-quiet wake) (*p*-values under 0.05 as indicated by the dashed line, Fig. 4b, left). In these FOVs, the degree of overlap explained by sub-states of wakefulness was still relatively low, as indicated by the $R^2$ of ~0.3 (Fig. 4b, right). $R^2$ reflects the total overlap within episodes of the same state relative to the total overlap between episodes of same and different states. A high $R^2$ value indicates that episodes within the same state are very similar, while episodes from different states are very different. No state-specific activation was found between the sleep states (Fig. 4b, left).

Next, we assessed whether there could be activity patterns specific to either sleep or wakefulness. For FOVs with both sleep and wakefulness, 50% of FOVs (43 of 86) showed a significant level of state-specific activation (Fig. 4c, left). $R^2$ of FOVs with both sleep and wakefulness states (Fig. 4c, right) was generally higher than $R^2$ of FOVs with only wakefulness or only sleep states (Fig. 4b, right), suggesting that active areas in sleep are somewhat different from areas that are active during wakefulness.

Taken together, these data show a low degree of overlap of astrocytic $Ca^{2+}$ activity across sleep-wake states, but indicate a moderate degree of sleep and wakefulness specific spatial activation patterns.

**Astrocytic $Ca^{2+}$ signals increase prominently upon awakening.** We observed that astrocytic $Ca^{2+}$ activity was not evenly temporally distributed within a given brain state, but rather was clustered at transitions from one state to another (Supplementary Figs. 6 and 7, red squares). To explore the relationship between state transitions and astrocytic $Ca^{2+}$ activity, we plotted ROA frequency aligned to the beginning of states, i.e., from quiet wakefulness to locomotion or whisking, and from either NREM, IS, or REM sleep to wakefulness (Fig. 5a). The start of locomotion and whisking was detected by movement of the whisker or wheel in the surveillance video, whereas the start of wakefulness during sleep-to-wake transitions was manually determined as the first sign of ECoG desynchronization (Fig. 5b). Astrocytic $Ca^{2+}$ events were strongly clustered around specific brain state transitions (Fig. 5a, c, d). As expected, transitioning from wakefulness to sleep was associated with a decrease in $Ca^{2+}$ signals (Supplementary Fig. 12e). However, all other state transitions started with small $Ca^{2+}$ events that eventually merged to form larger and longer events (Supplementary Fig. 13). During transitions from
quiet wakefulness to locomotion or whisking, increases in astrocytic Ca\(^{2+}\) signaling followed the transition in the ECoG signal, muscle activity, and mouse movement (Fig. 5a, c, e). The onset of astrocytic Ca\(^{2+}\) did not differ between transitions to locomotion or whisking, but the peak Ca\(^{2+}\) response was larger for locomotion (Fig. 5f, g).

We then investigated the relationship between astrocytic Ca\(^{2+}\) activity and the transition from sleep to wakefulness (Fig. 5a, b, d). Waking up from REM sleep was associated with larger peak Ca\(^{2+}\) signals than other transitions (Fig. 5g), but was temporally more similar to state transitions of wakefulness, as astrocytic responses were delayed compared to the transition in ECoG.
Fig. 5 Astrocytic Ca\(^{2+}\) signals increase prominently upon awakening. a Temporal raster plots of normalized ROA frequency during the transitions (top to bottom): quiet wakefulness to locomotion (n = 199 transitions); quiet wakefulness to whisking (n = 370 transitions); NREM sleep to wakefulness (n = 59 transitions); IS sleep to wakefulness (n = 140 transitions); REM sleep to wakefulness (n = 87 transitions). b Examples of transitions from NREM, IS, and REM sleep to wakefulness in the ECoG traces. c Mean time-course of (top to bottom) astrocytic Ca\(^{2+}\) signals and z-scores of ECoG power, wheel or whisker motion and EMG activation during transitions from quiet wakefulness to locomotion or whisking. Data represented as mean ± SEM, n = 6 mice. d Same as c but for transitions from NREM, IS, or REM sleep to wakefulness. e Percentage of transitions with preceding astrocytic Ca\(^{2+}\) signals. Data represented as mean ± SEM, n = 6 mice, number of samples for each transition described in a. f Astrocytic Ca\(^{2+}\) signal onset relative to state transition. Data represented as estimates ± SE and p-values (two-sided test, no adjustment for multiple comparisons) derived from a linear regression model, n = 6 mice, number of samples for each transition described in a. g Peak frequency of ROAs during state transitions. Data represented as estimates ± SE and p-values (two-sided test, no adjustment for multiple comparisons) derived from linear regression models, n = 6 mice, number of samples for each transition described in a. For details on statistical analyses, see "Methods". See also Supplementary Figs. 12 and 13.
signal, muscle activity, and mouse movement (Fig. 5a, d, f). However, to our surprise, astrocytic Ca\(^{2+}\) signals typically preceded the awakenings from SWS (NREM and IS sleep states). We observed a prominent increase in astrocytic Ca\(^{2+}\) signal frequency 1–2 s before the shift in ECoG, EMG, and mouse movement in 60% of NREM sleep to wakefulness and 72% of IS sleep to wakefulness transitions (Fig. 5d–f). As astrocytic Ca\(^{2+}\) signaling did not precede the transition from NREM or IS sleep to wakefulness in all cases, we investigated if ECoG power could determine the temporal profile of astrocytic Ca\(^{2+}\) signal onset (Supplementary Fig. 12). We found that high delta ECoG power was associated with earlier astrocytic Ca\(^{2+}\) onset in NREM sleep (Supplementary Fig. 12a).

Our observation of enhanced astrocytic Ca\(^{2+}\) signaling upon awakenings—particularly preceding transitions from SWS to wakefulness—suggests that astrocytic Ca\(^{2+}\) signaling may play a causal role in modulating sleep-to-wake transitions.

**Correlation of astrocytic and neuronal Ca\(^{2+}\) activity.** Having identified sleep-wake state-specific spatial and temporal features of astrocytic Ca\(^{2+}\) activity, we then sought to investigate sleep-wake state-specific relationships between astrocytes and local neurons. To capture astrocytic Ca\(^{2+}\) activity, we measured jRGECO1a fluorescence in hand-drawn ROIs over neuronal somata and neuropil (Fig. 6a). The frequency of Ca\(^{2+}\) signals in neuronal somata, like in astrocytes, was lower during sleep compared to wakefulness, although the reduction was modest (Fig. 6b). In order to examine the temporal relationship between astrocytic and neuronal Ca\(^{2+}\) signals across sleep-wake states, we calculated the onset of astrocytic Ca\(^{2+}\) signals in neuropil ROIs relative to Ca\(^{2+}\) events in soma ROIs of neurons (Fig. 6c). During locomotion and spontaneous whisking, and to a lesser extent during quiet wakefulness, a population of astrocytic Ca\(^{2+}\) signals displayed a modest degree of temporal alignment with nearby neuronal somatic Ca\(^{2+}\) events (Fig. 6c), indicating some level of astrocyte-neuron synchrony during wakefulness, similar to previous reports. By contrast, during all sleep states astrocytic Ca\(^{2+}\) signals displayed a broad distribution of onset time differences relative to neighboring neurons (Fig. 6c). Next, we analyzed whether astrocytic signals were synchronized to neuronal activity in the neuropil. We quantified the correlation between astrocytic and neuronal Ca\(^{2+}\) signals within the same neuropil ROIs (Fig. 6d). Similar to the temporal relationship with the neuronal somata, astrocytic Ca\(^{2+}\) signals displayed a modest correlation with neuronal signals in neuropil during wakefulness, but were significantly decorrelated during sleep (Fig. 6e).

**Astrocytic IP\(_3\)-mediated Ca\(^{2+}\) signaling regulates SWS.** Our observations of astrocytes during sleep revealed a spatiotemporal specificity of Ca\(^{2+}\) activity, particularly during SWS, that could indicate causal roles for astrocytic signaling in sleep regulation. To identify these roles of astrocytic Ca\(^{2+}\) signals in sleep, we employed the I\(\text{tpr}2\)/−/− mouse model, in which astrocytic Ca\(^{2+}\) signaling is strongly attenuated, but not abolished (as shown in experiments on awake and anesthetized mice). In agreement with previous reports, we found that I\(\text{tpr}2\)/−/− mice exhibited reduced astrocytic Ca\(^{2+}\) signaling in all states of wakefulness as measured by ROA frequency and active voxels (x-y-t) (Supplementary Fig. 14a, b). However, astrocytic Ca\(^{2+}\) activity measured by both the percentage of active voxels (x-y-t) and ROA frequency did not significantly differ between WT and I\(\text{tpr}2\)/−/− mice during sleep (Fig. 7b, c). Even so, I\(\text{tpr}2\)/−/− mice exhibited Ca\(^{2+}\) signals with disrupted spatiotemporal features—namely, longer duration and smaller spatial extent (Fig. 7a, d, e).

This finding was observed in all states of wakefulness, but during sleep was restricted to SWS (NREM and IS states).

Next, we investigated whether IP\(_3\)-dependent astrocytic Ca\(^{2+}\) signaling had any effect on sleep dynamics. We compared sleep architecture and spectral ECoG properties between the two genotypes and found that I\(\text{tpr}2\)/−/− mice exhibited more frequent NREM and IS bouts that were of shorter duration than in the WT mice (Fig. 8a, b). More fragmented SWS sleep could be a consequence of more frequent microarousals (short wakefulness intrusions characterized by a reduction of low-frequency ECoG power) and awakenings, which interrupt the sleep states, or more frequent NREM-to-IS and IS-to-NREM transitions. The number of awakenings did not differ between the genotypes in any of the sleep states (Fig. 8d). However, we found that I\(\text{tpr}2\)/−/− mice have ~20 more microarousals per hour than WT mice (Fig. 8i). Such microarousals were associated with abrupt increases in astrocytic Ca\(^{2+}\) signaling in WT mice, whereas no such response was observed in I\(\text{tpr}2\)/−/− mice (Fig. 8g). Surprisingly, I\(\text{tpr}2\)/−/− mice were completely devoid of the prominent astrocytic Ca\(^{2+}\) increases seen upon awakenings in WT mice (Fig. 8e).

NREM-to-IS and IS-to-NREM transitions were more frequent in I\(\text{tpr}2\)/−/− mice compared to WT mice (Fig. 8b), indicating abnormal SWS state dynamics in the knockouts. Interestingly, in WT mice, NREM-to-IS transitions were preceded by a decrease in astrocytic Ca\(^{2+}\) signaling, whereas IS-to-NREM transitions were followed by an increase in astrocytic Ca\(^{2+}\) signaling. This was not the case in I\(\text{tpr}2\)/−/− mice (Fig. 8i), and it is tempting to hypothesize that IP\(_3\)-mediated astrocytic Ca\(^{2+}\) signaling is important to sustain uninterrupted SWS by regulating NREM and IS state transition- and possibly preventing microarousals.

Finally, we assessed the spectral ECoG properties of NREM, IS, and REM sleep between the genotypes. We detected a decrease in delta power during NREM sleep, an increase in theta during REM sleep and an increase in sigma power during IS sleep in I\(\text{tpr}2\)/−/− mice (Fig. 9a). ECoG activity in the sigma frequency range is indicative of sleep spindles—bursts of neuronal activity linked to memory consolidation. As I\(\text{tpr}2\)/−/− mice displayed higher sigma power in IS, we next evaluated the occurrence of sleep spindles in WT and I\(\text{tpr}2\)/−/− mice (Fig. 9b). The frequency of sleep spindles in IS sleep was indeed considerably higher in I\(\text{tpr}2\)/−/− mice than in WT mice (Fig. 9c). Intriguingly, sleep spindles were followed by an IP\(_3\)-dependent increase in astrocytic Ca\(^{2+}\) signals (Fig. 9d). These data indicate a role for astrocytic IP\(_3\)-mediated Ca\(^{2+}\) signaling pathway in regulating the architecture and brain rhythms of SWS.

**Discussion**

Astrocytes are emerging as crucial components of neural circuits that actively take part in signal processing in the brain. In anesthetized and awake state Ca\(^{2+}\) signaling has been shown to be the central signaling mechanism in astrocytes, and our study here reports on astrocyte Ca\(^{2+}\) activity in natural sleep. We have demonstrated that astrocytic Ca\(^{2+}\) signaling changes prominently across the sleep-wake cycle, being reduced during sleep while abruptly becoming elevated upon awakening, often before behavioral and neurophysiological signs of the sleep-to-wake transition. Genetic ablation of IP\(_3\)R2, an important astrocytic Ca\(^{2+}\) signaling pathway, led to abnormal sleep architecture, state dynamics and brain rhythms of SWS. Taken together, our data show that astrocytes are essential for normal slow-wave sleep through mechanisms involving intracellular Ca\(^{2+}\) signals. The concept that a non-neuronal cell type is indispensable for appropriate SWS will guide future studies aimed at deciphering sleep regulatory mechanisms and identifying novel treatment strategies for sleep disorders.
The use of ultrasensitive genetically encoded Ca\(^{2+}\) indicators and two-photon microscopy has revealed that astrocytes display a wide range of complex Ca\(^{2+}\) signals. These signals predominantly occur in fine astrocytic processes in neuropil, which contact thousands of neuronal synapses\(^9,17,18,31\). Deciphering the spatiotemporal dynamics of astrocytic Ca\(^{2+}\) signals in vivo can reveal how astrocytes coordinate and/or react to highly localized changes in neuronal and metabolic activity\(^17,32\). However, conventional analytic methods based on static ROIs are insufficient in capturing the true complexity of astrocytic Ca\(^{2+}\) signaling. Here, we developed and employed an activity-based algorithm that enabled us to probe the complexity of astrocytic Ca\(^{2+}\) signals during sleep and sleep-wake transitions. Our ROA analysis was able to capture sleep and wake state-dependent modulations in astrocytes that were undetected using static ROIs. Further, analysis of our data with the ROA tool resulted in almost

**Fig. 6 Correlation of astrocytic and neuronal Ca\(^{2+}\) signals.** a Representative image of SYN-jRGECO1a fluorescence in neurons and GFAP-GCaMP6f fluorescence in astrocytes, and ROIs over neuron somata (pink) and neuropil (black circles of 10 μm in diameter). ROIs were manually segmented in 92 time series from 6 mice. Scale bar 20 μm. b Frequency of Ca\(^{2+}\) signals in neuron somata across sleep-wake states . Data represented as estimates ± SE, and p-values (two-sided test, no adjustment for multiple comparisons) derived from linear mixed effects models statistics, n = 6 mice, 92 trials. c Raster plots and histograms (1 s bins) of the onset time difference for astrocytic Ca\(^{2+}\) events in neuropil ROIs (black circular ROIs in a) relative to a Ca\(^{2+}\) event in neuron somata ROIs (pink ROIs over neuron somata in a). Each black dot represents the temporal location of one astrocytic Ca\(^{2+}\) event relative to a Ca\(^{2+}\) event in a neuronal soma. d Example traces of continuous astrocytic Ca\(^{2+}\) event frequency in neuropil ROIs (number of ROAs per 100 μm\(^2\) per minute), and neuronal Ca\(^{2+}\) signal ΔΨ/F\(_0\) in neuropil ROIs during locomotion, whisking, quiet wakefulness, NREM sleep, IS sleep, and REM sleep. e Correlation coefficient between continuous traces of astrocytic Ca\(^{2+}\) event frequency in neuropil, measured as number of ROAs per 100 μm\(^2\) per minute, and neuronal Ca\(^{2+}\) signal ΔΨ/F\(_0\) in neuropil across sleep-wake states. Data represented as estimates ± SE and p-values (two-sided test, no adjustment for multiple comparisons) derived from linear mixed effects models statistics, n = 6 mice, 81 trials. For details on statistical analyses, see “Methods”.

The use of ultrasensitive genetically encoded Ca\(^{2+}\) indicators and two-photon microscopy has revealed that astrocytes display a wide range of complex Ca\(^{2+}\) signals. These signals predominantly occur in fine astrocytic processes in neuropil, which contact thousands of neuronal synapses\(^9,17,18,31\). Deciphering the spatiotemporal dynamics of astrocytic Ca\(^{2+}\) signals in vivo can reveal how astrocytes coordinate and/or react to highly localized changes in neuronal and metabolic activity\(^17,32\). However, conventional analytic methods based on static ROIs are insufficient in capturing the true complexity of astrocytic Ca\(^{2+}\) signaling. Here, we developed and employed an activity-based algorithm that enabled us to probe the complexity of astrocytic Ca\(^{2+}\) signals during sleep and sleep-wake transitions. Our ROA analysis was able to capture sleep and wake state-dependent modulations in astrocytes that were undetected using static ROIs. Further, analysis of our data with the ROA tool resulted in almost
Fig. 7 Astrocytic Ca\(^{2+}\) signaling during sleep is dependent on the Ip3 pathway. a Representative x-y-t rendering of ROAs during NREM, IS, and REM sleep in WT and Itpr2\(^{-/-}\) mice. b–e Mean ROA frequency expressed as number of ROAs per 100 \(\mu m^2\) per minute (b), the percentage of active voxels (x-y-t) (c), ROA duration (s) (d), and ROA area (\(\mu m^2\)) (e) during NREM, IS, and REM sleep in WT and Itpr2\(^{-/-}\) mice. Data represented as estimates ± SE and \(p\)-values (two-sided test, no adjustment for multiple comparisons) derived from linear mixed effects models statistics, \(n = 6\) mice, 196 trials for WT, \(n = 6\) mice, 100 trials for Itpr2\(^{-/-}\). For details on statistical analyses, see “Methods.” See also Supplementary Fig. 14.
Fig. 8 Astrocytic IP₃-mediated Ca²⁺ signaling pathway regulates SWS. 

- **a–c** Bout duration (a), bouts per hour (b), and percentage recording time (c) of NREM, IS, and REM sleep in WT and Itpr2²⁻/⁻ mice. For WT mice: n = 3 mice, 1140 NREM episodes, 787 IS episodes, 86 REM episodes. For Itpr2²⁻/⁻ mice: n = 3 mice, 1140 NREM episodes, 787 IS episodes, 86 REM episodes. 

- **d** Awakening probability calculated as awakenings from NREM, IS, or REM sleep divided by total number of NREM, IS, or REM episodes. For WT mice: n = 4 mice, 806 NREM episodes, 617 IS episodes, 92 REM episodes; for Itpr2²⁻/⁻ mice: n = 3 mice, 1140 NREM episodes, 787 IS episodes, 86 REM episodes. 

- **e** Mean ± SEM of astrocytic Ca²⁺ signals during transitions from NREM, IS, or REM sleep to wakefulness. For WT mice: n = 6 mice, 59 NREM awakenings, 141 IS awakenings, 87 REM awakenings; for Itpr2²⁻/⁻ mice: n = 6 mice, 23 NREM awakenings, 56 IS awakenings, 27 REM awakenings. 

- **f** Frequency of microarousals during NREM sleep. For WT mice: n = 6 mice, 355 microarousals; for Itpr2²⁻/⁻ mice: n = 6 mice, 240 microarousals. 

- **g** Mean ± SEM of (top to bottom) astrocytic Ca²⁺ signals, z-scores of EMG activation and whisker motion aligned to the beginning of a microarousal. For WT mice: n = 6 mice, 303 microarousals; for Itpr2²⁻/⁻ mice: n = 5 mice, 191 microarousals. 

- **h** Number of NREM-to-IS, IS-to-NREM, and IS-to-REM transitions. For WT mice: n = 4 mice, 386 NREM-to-IS, 187 IS-to-NREM, 68 IS-to-REM transitions; for Itpr2²⁻/⁻ mice: n = 3 mice, 440 NREM-to-IS, 267 IS-to-NREM, 61 IS-to-REM transitions. 

- **i** Mean ± SEM of astrocytic Ca²⁺ signals during NREM-to-IS, IS-to-NREM, and IS-to-REM transitions in WT and Itpr2²⁻/⁻ mice. For WT mice: n = 6 mice, 90 NREM-to-IS, 35 IS-to-NREM, 25 IS-to-REM transitions; for Itpr2²⁻/⁻ mice: n = 6 mice, 62 NREM-to-IS, 14 IS-to-NREM, 8 IS-to-REM transitions. Data represented as estimates ± SE and p-values (two-sided test, no adjustment for multiple comparisons) derived from linear regression models unless otherwise stated. For details on statistical analyses, see “Methods”.

More frequent microarousals and more frequent transitioning between NREM and IS states. These results suggest a potentially critical role of astrocytic silencing during the transitional state between NREM and REM, or alternatively, a critical role for elevated astrocytic signaling in order to maintain uninterrupted SWS sleep.

In Itpr2²⁻/⁻ mice, SWS was not only fragmented, but ECoG power characteristics were also affected, as there was a reduced power in the delta frequency range during NREM sleep. Altogether with the finding of shorter NREM bout duration detected in Itpr2²⁻/⁻ mice, this sleeping pattern resembles one of a mouse line with astrocyte specific impairment of vesicular exocytosis, the dnSNARE mice². Astrocytes are the source of the sleep pressure agent and somnogen adenosine²,37. The release of adenosine is blocked in dnSNARE mice, resulting in impaired sleep homeostasis as measured by reduced ECoG delta power and time spent in sleep, and shorter NREM bout duration after sleep deprivation². These data suggest that one of the downstream effects of IP₃-mediated Ca²⁺ signaling during SWS could be the release of adenosine. IS sleep in Itpr2²⁻/⁻ mice was associated with increased sigma ECoG power and considerably more sleep spindles, compared to WT mice. Similar to microarousals and SWS state transitions, sleep spindles in WT mice were associated with particular Ca²⁺ signaling pattern, which was not observed in Itpr2²⁻/⁻ mice. Although sleep spindles are known to be important for learning and memory, too many sleep spindles can be...
maladaptive. Excessive sleep spindles have been observed in humans with learning disabilities and have been shown to predict poor avoidance performance in rats\(^\text{39-41}\). Our data indicates that the IP\(_3\)-mediated astrocytic Ca\(^{2+}\) signaling plays a key role in stabilizing and maintaining uninterrupted SWS and modulating SWS brain rhythms critical for learning and memory. However, since the gene knockout was global, we cannot rule out that altered astrocytic Ca\(^{2+}\) signaling in non-neocortical (e.g., brain-stem) regions affects neurons regulating SWS. Similarly, we did not study whether the manipulation of astrocytic Ca\(^{2+}\) signaling affected specific cortical neurons thought to regulate SWS and spindles\(^\text{22,23}\). Future studies should delineate those specific pathways and circuits responsible for astrocytic IP\(_3\)-mediated modulation of sleep and investigate whether aberrant Ca\(^{2+}\) signaling in astrocytes underlies sleep disorders.

The dynamics of brain state shifts are intimately linked to the brain-wide release of the neuromodulators noradrenaline and acetylcholine\(^\text{44}\). In recent years, astrocytes have been portrayed as one of the most important players in neuromodulator-driven state shifts in awake or anesthetized animals\(^\text{42,43}\). In our experiments, astrocytic Ca\(^{2+}\) signaling typically preceded transitions from SWS (both NREM and IS sleep), but not from REM sleep. This finding is consistent with the temporal profile of cortical nor-epinephrine (NE) release from locus coeruleus (LC) neurons upon awakening. The firing of LC noradrenergic (NA) neurons upon SWS-to-wake transitions has been shown to precede the onset of EEG activation by 0.3–1 s, whereas this predictive activity was not seen for REM sleep to wake transitions\(^\text{29}\), suggesting that astrocytic Ca\(^{2+}\) signals upon awakening are triggered by NE. NE acts on Gq-coupled \(\alpha_1\)-adrenergic receptors on astrocytes, leading to Ca\(^{2+}\) release from the endoplasmic reticulum through IP\(_3\)R2\(^\text{12,18,44}\). This might explain the absence of Ca\(^{2+}\) surges upon awakening in the Itpr2\(^{-/-}\) mice, which lack IP\(_3\)R2 (Fig. 8e). It is important to mention that astrocytic Ca\(^{2+}\) signals did not precede all SWS-to-wake transitions (Fig. 5a, e). The reason for this variance in Ca\(^{2+}\) onset time is not entirely clear, and as we show in Supplementary Fig. 12, there is some dependency on the depth of the preceding NREM sleep, suggesting that the prevailing neurochemistry of the brain tissue is important for how astrocytes respond to their triggers. These findings pose the intriguing question of what comprises an awakening and whether astrocytic awakening could be a new marker of the transition to wakefulness.

An unexpected finding was the lack of increase in astrocyte Ca\(^{2+}\) signaling during REM sleep compared to NREM sleep. REM sleep is characterized by high cortical levels of extracellular acetylcholine compared to NREM sleep\(^\text{45}\) and astrocytes have been shown to respond to acetylcholine with increased Ca\(^{2+}\) signaling upon direct application or stimulation of cholinergic nuclei\(^\text{13}\). Therefore, we expected a strong increase in astrocytic Ca\(^{2+}\) activity during REM sleep. This was, however, not the case, implying that acetylcholine alone is not sufficient to trigger astrocyte Ca\(^{2+}\) elevations.

In recent years, astrocytes have been established not only as passive supporters of neurons, but also as active participants in the bidirectional communication between the two cell types. Firing patterns of cortical neurons vary across sleep-wake states and are of higher frequency during wakefulness than sleep\(^\text{46}\). We found that astrocytic Ca\(^{2+}\) signaling was somewhat synchronized to neuronal activity during wakefulness, whereas very little correlation was found during sleep. It is tempting to hypothesize that during states of wakefulness, the observed astrocyte-neuron correlation reflects local neuronal activation specifically related to sensory input to the barrel cortex, whereas in sleep, astrocytic Ca\(^{2+}\) signals are activated by subcortical brain state-specific circuitry, in line with our Ca\(^{2+}\) activity overlap analysis showing that there were brain state-specific regions of Ca\(^{2+}\) activity.

Neuronal activity data obtained from fluorescent Ca\(^{2+}\) sensors as a proxy for neuronal firing should be interpreted with caution. Neuronal firing patterns are at least partially state-dependent\(^\text{47}\), indicating that due to the sensitivity and kinetics of the sensor, Ca\(^{2+}\) readouts could be affected in a non-linear manner, hence not faithfully reflecting neuronal firing across various states.

---

**Fig. 9 Astrocytic IP\(_3\)-mediated Ca\(^{2+}\) signaling during sleep spindles.**

a Mean ECoG power of delta (0.5–4 Hz) frequency during NREM sleep, theta (5–9 Hz) frequency during REM sleep, and sigma (10–16 Hz) frequency during IS sleep, normalized to 0.5–30 Hz total power. For WT mice: \(n = 4\) mice, 785 NREM episodes, 668 IS episodes, 102 REM episodes; for Itpr2\(^{-/-}\) mice: \(n = 3\) mice, 957 NREM episodes, 648 IS episodes, 74 REM episodes.

b Representative example of a sleep spindle in ECoG trace and power spectrogram.

c Mean number of spindles per minute during IS sleep. For WT mice: \(n = 3\) mice, 516 IS episodes; for Itpr2\(^{-/-}\) mice: \(n = 3\) mice, 560 IS episodes.

d Mean ± SEM (top to bottom) astrocytic Ca\(^{2+}\) signals, whisker movement z-score and ECoG power spectrograms aligned to the beginning of a sleep spindle. For WT mice: \(n = 6\) mice, 243 sleep spindles; for Itpr2\(^{-/-}\) mice: \(n = 6\) mice, 93 sleep spindles. Data represented as estimates ± SE and \(p\)-values (two-sided test, no adjustment for multiple comparisons) derived from linear regression models unless otherwise stated. For details on statistical analyses, see “Methods”.

---


Moreover, as the neuronal Ca$^{2+}$-event detection is threshold-based, it is likely that neuronal activity is only detected above a certain spiking rate, possibly contributing to an underestimation of neuronal activity levels. Even so, our neuronal Ca$^{2+}$ data across sleep-wake states (Fig. 6b) is in line with other Ca$^{2+}$- imaging and electrophysiological studies showing reduced neuronal firing during sleep compared to wakefulness$^{22,46}$. Future electrophysiological studies using unit recordings will have to confirm our observations.

In conclusion, our results indicate that even though reduced during sleep, astrocytic IP$_3$-mediated Ca$^{2+}$ signaling serves as a regulatory pathway to sustain uninterrupted SWA and maintain sleep spindles that are important for learning and memory.

**Methods**

**Animals.** Male WT (C57BL/6), Janvier Labs. Iprr2$→$–/Iprr2$^{1n1C18en}$, MGL3649079$^{38}$ and Gli1$^{+}\text{GeFP}$ mice$^{48}$ were housed on a 12:12 light/dark cycle (lights on at 8:00 a.m. – 4:00 p.m. cage. Iprr2$→$–/Iprr2$^{1n1C18en}$ mice were backcrossed into a C57BL/6 background for at least 15 generations. Each animal underwent surgery at the age of 8–10 weeks, followed by accommodation to being head-restrained and two-photon imaging sessions (2–3 times per week) for up to 2 months. Adequate measures were taken to minimize pain and discomfort. Sample sizes were determined based on our previous studies using similar techniques (no power calculations were performed). No randomization or blinding was performed. All procedures were approved by the Norwegian Food Safety Authority (project number: FOTS 11983).

**Cloning and virus production.** The DNA sequences for the genetically encoded fluorescent Ca$^{2+}$- indicators GCaMP6f$^{50}$ and rJRECO1a$^{51}$ were first amplified by PCR from pGVP-CMV-GCaMP6f and pGVP-CMV-NE5-JRECO1a (Addgene) with 5’ BstUI and 3’ HindIII, and sub-cloned into the recombined adeno-associated virus (rAAV) vector pAAV-6P-SEW8$^{52}$ for generating pAAV-SYN-GCaMP6f and pAAV-SYN-rJRECO1a, respectively. The human glial fibrillary acidic protein (GFAP) promoter$^{53}$ was inserted with MluI and BamHI into pAAV-SYN- GCaMP6f construct for obtaining pAAV-GFAP-GCaMP6f. Serotype 2/1 rAAVs from pAAV-GFAP-GCaMP6f and pAAV-SYN-JRECO1a were produced$^{54}$, and purified by AVB Sepharose affinity chromatography$^{55}$, following titration with real-time PCR (rAAV titers about 10$^{10}$–10$^{12}$ viral genomes/mL, TaqMan Assay, Applied Biosystems). For cortical rAAV-transduction of both astrocytes and neurons, rAAV-GFAP-GCaMP6f and rAAV-SYN-JRECO1a were mixed 1:1.

**Surgical procedures and intrinsic imaging.** Mice were anesthetized with isoflurane. Two silver wires (200 μm, non-insulated, GoodFellow) were inserted epidurally into two burr holes overlying the right parietal hemisphere for ECoG recordings, and two stainless steel wires (50 μm, insulated except 1 mm tip, GoodFellow) were implanted in the nuchal muscles for EMG recordings. The skull over the left hemisphere was thinned for intrinsic imaging, a custom-made titanium head-bar was glued to the skull and the implant sealed with a dental cement cap. After 2 days, representations of individual whiskers in the barrel cortex were mapped using intrinsic optical imaging. The brain region activated by single-whisker deflection (10 Hz, 6 s) was identified by increased red light absorption. After 2 days, chronic window implantation and virus injection was performed$^{56}$. A round craniotomy of 2.5 mm diameter was made over the barrel cortex using the intrinsic optical imaging map as a reference. A dental drill was used to carefully cut the skull with intermittent soaking and removal of debris with hemostatic sponges (Spongostan Dental, Ethicon) until only ~0.1 mm of the bone thickness was left. The bone flap was subsequently removed. The virus mixture (70 nL at 35 nL/min) was injected at two different locations in the barrel cortex at a depth of 400 μm. A window made of two circular coverslips of 2.5 and 3.5 mm was glued together by Spongostan Dental, Ethicon) until only ~0.1 mm of the bone thickness was left. The emitted photons were detected with Pelteir cooled photomultiplier tubes (model 7422PA-40 by Hamamatsu Photonics K.K.). Images (512 x 512 pixels) were acquired at 30 Hz in layer 2/3 of barrel cortex.

**Head-fixed sleep protocol.** To assist in sleep a head-fixed position, we adjusted the running disc position to mimic the body’s natural position observed during unrestrained sleep$^{59}$. We observed that locking the movement of the wheel once the mouse showed first signs of sleep, such as delta waves in ECoG and eyes closing, facilitated falling asleep. The imaging sessions of sleeping mice started at 9–10 a.m. (ZT –1), the beginning of light phase, and lasted until 3–6 p.m. (ZT 7–10). The mice did not have access to food or water while sleeping under the microscope, however, in natural conditions mice feed almost exclusively during the dark phase, ZT 12–24$^{40}$–$^{41}$. First signs of drowsiness, such as high delta power in the ECoG signal and eyes closing, were observed 15–45 min after head-fixation, and typically mice spent 90–120 min head-fixed under the microscope before falling asleep. Mice that did not show any signs of sleep within the first 2 h of head-fixation were removed from the microscope. The mice had an exact replica of the microscope running disc in their home cage, and in our hands this made a large difference in aiding the mice to fall asleep (i.e., initially we tried various types of stages, like a spherical treadmill and a tube for immobilization with little success). Mice were not sleep deprived or manipulated in any other way before imaging to induce sleep.

**Behavior and electrophysiology recording.** ECoG and EMG signals were recorded using a Multicam 700B amplifier with headstage CV-7B, and digitized by Digidata 1440 (both Molecular Devices). Mouse behavior was recorded by an IR-sensitive surveillance camera. Data acquisition was synchronized by a custom-written LabVIEW software (National Instruments).

**Sleep–wake state scoring.** Sleep states were identified from filtered ECoG (0.5–30 Hz) and EMG signals (100–1000 Hz) based on standard criteria for rodent sleep$^{22}$ (Fig. 1c, d): NREM sleep was defined as high-amplitude delta (0.5–4 Hz) ECoG activity and low EMG activity; IS was defined as an increase in theta (5–9 Hz) and sigma (9–16 Hz) ECoG activity, and a concomitant decrease in delta ECoG activity; REM sleep was defined as low-amplitude theta ECoG activity with delta/delta ratio >0.5 and low EMG activity. Wakefulness states were identified using the IR-sensitive surveillance camera video by drawing ROIs over the running wheel and mouse snout (Fig. 1b). The signal in the wheel and snout ROIs was used to calculate pixel-by-pixel time-averaged frames in the respective ROIs. Voluntary locomotion was identified as signals above a threshold in the wheel ROI. Spontaneous whisking was defined in the snout ROI. Quiet wakefulness was defined as wakefulness with no signal above threshold in both ROIs. Short and long quiet wakefulness episodes could represent different behavioral states, such as restful quiescence or freezing, and be influenced by the degree of habituation, which could affect astrocytic Ca$^{2+}$-signaling patterns. In our dataset >90% of quiet wakefulness bouts were shorter than 10 s and there was no difference in estimated Ca$^{2+}$ signaling levels when including long-lasting bouts compared to omitting them (Supplementary Fig. 15). Sleep episodes of >30 s duration were analyzed.

**Sleep–wake state transition and microarousal scoring.** For the transition from NREM and IS to wakefulness, onset of wakefulness was determined by the first sign of ECoG desynchronization$^{52}$ (activation) (Fig. 5b). During the transitions from REM to wakefulness, end of REM was identified by the interruption of sustained theta waves and the onset of desynchronized ECoG (Fig. 5b). Microarousals were defined as periods of above-threshold signal in the whisker imaging camera video (see also the above paragraph Sleep–wake state scoring) within NREM episodes with a duration of at least 0.3 s but <3 s.

**Comparison of sleep between freely behaving and head-fixed mice.** Male C57BL/6 mice were implanted with ECoG, EMG electrodes and a head-bar as described in the above section “Surgical procedures and intrinsic imaging”. After recovering from the surgery (3 days), mice were habituated to both custom-built head-fixed and custom-built unrestrained sleep setups. The head-fixed setup consisted of head-bar clamps, a running wheel and IR-sensitive surveillance camera (as illustrated in Fig. 1a). The unrestrained sleep setup consisted of a transparent cage, a custom-built pulley system for the ECoG and EMG electrodes, and an IR-sensitive surveillance camera. After habituation, mice were placed in either the head-fixed or the unrestrained sleep setups and experimental sessions of sleeping mice started at 9–10 a.m. (ZT 1–2), the beginning of light phase, and lasted until
3–6 p.m. (ZT 7–10). Electroencephalogram (EEG) and electrooculogram (EOG) signals were recorded using a Neuroscan SynAmps2 system, amplified, digitized at 2-kHz sampling rate, and analyzed using Neuroscan Synaps and Brainwave software. The EEG signal was referenced to the linked mastoids. The EOG signal was referenced to the right eye. Eye blinks were identified by a custom algorithm and were digitally removed from the EEG data. Activity-related potentials (ARPs) were defined as increases in the fluorescence intensity inside the retinal ganglion cells (RGCs). The peak ARP frequencies were estimated using the custom algorithm implemented in MATLAB. The overlap between the binary images of different sleep-wake states was then calculated as the area of the intersection divided by the area of the union.

**ROA overlap analysis.** The overlap of active areas between episodes was evaluated by creating ROA heatmaps of the active voxels in each episode and estimating the overlap of these by calculating the Jaccard similarity coefficient. The Jaccard similarity coefficient is a number between 0 and 1, with 0 indicating no overlap and 1 indicating identical activation and was defined as $J(x, y) = \frac{s_{\text{x,y}}}{s_x + s_y - s_{\text{x,y}}}$, where $x$ and $y$ are vectors of pixels in each heatmap being compared.

Conversely, one minus the Jaccard similarity coefficient is a measure of dissimilarity between episodes and is also known as the Jaccard distance. We then calculated the distance matrix between all episodes from all FOVs, and compared these using the permutational multivariate analysis of variance—PERMANOVA—implemented in PRIMER v6. This method was used in the analysis, resulting in a three-dimensional (3D) matrix of active or inactive voxels producing the time series ($F_0$) was calculated by smoothing the pre-processed time series ($F$) using a moving average filter, $F_0 = \frac{1}{T} \sum_{t=1}^{T} F_t$, where $T$ is the length (number of frames) of the moving average filter. A standard deviation of the noise in $S$ was estimated as $\sigma_S = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (S_i - \mu_S)^2}$, where $N$ is the total number of voxels in that episode, while excluding the ignored areas. 3D vessel masks were created to visualize the distribution of vessels in the brain, resulting in a three-dimensional (3D) matrix of active or inactive voxels. Active voxels were defined as increases in the fluorescence intensity inside the respective ROIs. Relative fluorescence was calculated as $\Delta F/F_0 = (F - F_0)/F_0$, where $F$ and $F_0$ are the fluorescence intensities in and outside the ROI, respectively. The relative change in fluorescence was then calculated using the following formula:

$$\Delta F/F_0 = (F_{\text{max}} - F_{\text{min}})/F_0$$

where $F_{\text{max}}$ and $F_{\text{min}}$ are the maximum and minimum values of the ROA frequency traces. ROA frequency was then normalized using $z$-score transformation, which are the mean and standard deviation was calculated from the first 10 s ($-5$ to $5$ s from the transition).
onset of Ca$^{2+}$ activity was then measured at the first point the traces crossed a threshold of 0.5 standard deviations.

**Ca$^{2+}$ signal correlation analysis.** For astrocytic Ca$^{2+}$ signals, an average ROA frequency trace was extracted from all neuropil ROIs per FOV from the GFP-GCaMP channel. For the neuronal Ca$^{2+}$ signal, an average ΔF/F0 trace was extracted from all neuropil ROIs per FOV from the SYN-jRGCeO1a channel. The baseline for neuronal signal was estimated by the mode of the trace. Both neuronal and astrocytic traces were subsequently smoothed with a gaussian filter (σ = 0.25 s). The maximum Pearson cross-correlation was calculated between astrocytic and neuronal Ca$^{2+}$ traces for each episode. The mean lag in all comparisons were less than a single frame.

**ECoG and sleep spindle analysis.** ECoG power spectrograms were calculated using the MATLAB function spectrum() with default parameters from 0 to 256 Hz. To compare ECoG power across genotypes and different mice, raw data were normalized to the average total power in the 0.5–30 Hz frequency range during NREM sleep per mouse and average power was calculated using the MATLAB bandpower() function. To detect sleep spindles the ECoG signal was first normalized as above, followed by bandpass filtering in the frequency range 10–16 Hz (second order zero-phase Butterworth filter). The analytic signal was then found by using the Hilbert transform, as a measure of the instantaneous power of the bandpass filtered signal, and smoothed by a gaussian filter with a sigma of 0.2 s. Peaks in the smoothed data (found by the MATLAB function findpeaks()) were treated as putative sleep spindles. Peaks with a width at threshold of <0.5 s or >5 s were discarded.

**Statistical analyses.** Statistical analyses were conducted in R (version 3.6.0) and MATLAB using mixed effect regression models. As fixed effects, we included brain state (the overall above sleep/overall wake, or the sub-states REM/SIS/NREM/quiet wakefulness/walking/locomotion), genotype (WT/Itpr−/−), function. No corrections for multiple comparisons were applied. The raw data that support the findings of this study are available from the corresponding author upon request. The source data underlying Figs. 2c–d, 3b, 4b–c, 5e–g, 6b, c, 7b–e, 8a–d, h, 9a, c, and Supplementary Figures 2a–d, 4b–c, 5e–f, 8g, 9b–l, 11, 14a–e, 15 a–c, are provided as Source Data files.

**Data availability**

The raw data that support the findings of this study are available from the corresponding author upon request. The source data underlying Figs. 2c–d, 3b, 4b–c, 5e–g, 6b, e, 7b–e, 8a–d, h, 9a, c, and Supplementary Figures 2a–d, 4b–c, 5e–f, 8g, 9b–l, 11, 14a–e, 15 a–c, are provided as Source Data files.

**Code availability**

The code supporting the current study along with a small test dataset is available as Supplementary Software. Source data are provided with this paper.

Received: 27 September 2019; Accepted: 9 June 2020; Published online: 06 July 2020

References

1. Frank, M. G. The role of glia in sleep regulation and function. *Handb. Exp. Pharmacol.* **253**, 83–96 (2019).
2. Halassa, M. M. et al. Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. *Neuron* **61**, 213–219 (2009).
3. Xie, L. et al. Sleep drives metabolite clearance from the adult brain. *Science* **342**, 373–377 (2013).
4. Poskanzer, K. E. & Yuste, R. Astrocytes regulate cortical state switching in vivo. *Proc. Natl Acad. Sci. USA.* **113**, E2675–E2684 (2016).
5. Szabó, Z. et al. Extensive astrocyte synchronization advances neuronal coupling in slow wave activity in vivo. *Sci. Rep.* **7**, 6018 (2017).
6. Bazargani, N. & Attwell, D. Astrocyte calcium signaling: the third wave. *Nat. Neurosci.* **19**, 182–189 (2016).
7. Verkhratsky, A. & Niedergerg, M. Physiology of astroglia. *Physiol. Rev.** **98**, 239–289 (2018).
8. Volterra, A., Liaudet, N. & Savtchouk, I. Astrocyte Ca$^{2+}$ signalling: an unexpected complexity. *Nat. Rev. Neurosci.* **15**, 327–335 (2014).
9. Haustein, M. D. et al. Conditions and constraints for astrocyte calcium signaling in the hippocampal mossy fiber pathway. *Neuron* **82**, 413–429 (2014).
10. Panatier, A. et al. Astrocytes are endogenous regulators of basal transmission at central synapses. *Cell* **146**, 785–798 (2011).
11. Araque, A., Martin, E. D., Perea, G., Arenillo, J. I. & Buño, W. Synchronically released acetylcholine evokes Ca$^{2+}$ elevations in astrocytes in hippocampal slices. *J. Neurosci.* **22**, 2443–2450 (2002).
12. Palkovits, M. & Noreuil indicates that astroglial responsiveness to local circuit activity. *Neuron* **82**, 1263–1270 (2014).
13. Takata, N. et al. Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo. *J. Neurosci.* **31**, 18155–18165 (2011).
14. Lee, S. H. & Dan, Y. Neuromodulation of brain states. *Neuron* **76**, 209–222 (2012).
15. Scammell, T. E., Arrigoni, E. & Lipton, J. O. Neural circuitry of wakefulness and sleep. *Neuron* **93**, 747–765 (2017).
16. Pagliardini, S., Funk, G. D. & Dickson, C. T. Breathing and brain state: urethane anesthesia as a model for natural sleep. *Respir. Physiol. Neurobiol.* **188**, 324–332 (2013).
17. Bundocci, E. et al. Neuroscience: three-dimensional Ca$^{2+}$ imaging advances understanding of astrocyte biology. *Science* **356**, 28524740 (2017).
18. Srinivasan, R. et al. Ca$^{2+}$ signaling in astrocytes from *Ipr32−/−* mice in brain slices and during startle responses in vivo. *Nat. Neurosci.* **18**, 708–717 (2015).
19. Stobart, J. L. et al. Cortical circuit activity evokes rapid astrocyte calcium signals on a similar timescale to neurons. *Neuron* **98**, 726–735.e4 (2018).
20. Feldmeyer, D. et al. Barrel cortex function. *Prog. Neurobiol.* **103**, 3–27 (2013).
21. Sofroniew, N. J., Cohen, J. D., Lee, A. K. & Svoboda, K. Natural whisker-guided behavior by head-fixed mice in tactile virtual reality. *J. Neurosci.* **34**, 9537–9550 (2014).
22. Niethard, N. et al. Sleep-state-specific regulation of cortical excitation and inhibition. *Cereb. Biol.* **26**, 2739–2749 (2015).
23. Seibt, J. et al. Cortical dendritic activity correlates with spindle-rich oscillations during sleep in rodents. *Nat. Commun.* **8**, 1–12 (2017).
24. Dijk, D. J., Brunner, D. P., Beersma, D. G. M. & Borbély, A. A. Electroencephalogram power density and slow wave sleep as a function of prior waking and circadian phase. *Sleep* **13**, 430–440 (1990).
25. McGinley, M. J. et al. Waking state: rapid variations modulate neural and behavioral responses. *Neuron* **87**, 1143–1161 (2015).
26. Wang, Y. et al. Accurate quantification of astrocyte and neurotransmitter fluorescence dynamics for single-cell and population-level physiology. *Nat. Neurosci.* **22**, 1936–1944 (2019).
27. Khalk, B. S. & Sofroniew, M. V. Diversity of astrocyte functions and phenotypes in neural circuits. *Nat. Neurosci.* **18**, 942–952 (2015).
28. Anderson, M. J. A new method for non-parametric multivariate analysis of variance. *Austral. Ecol.* **26**, 32–46 (2001).
29. Takahashi, K., Kayama, Y., Lin, J. S. & Sakai, K. Locus coeruleus neuronal activity during the sleep-waking cycle in mice. *Neuroscience* **169**, 1113–1126 (2010).
30. Diekelmann, S. & Born, J. The memory function of sleep. *Nat. Rev. Neurosci.* **11**, 114–126 (2010).
31. Stobart, J. L. et al. Long-term in vivo calcium imaging of astrocytes reveals distinct cellular compartment responses to sensory stimulation. *Cereb. Cortex* **28**, 184–198 (2018).
32. Agarwal, A. et al. Transient opening of the mitochondrial permeability transition pore induces microdomain calcium transients in astrocyte processes. *Neuron* **39**, 587–605.e7 (2017).
33. Nimmerjahn, A., Mukamel, E. A. & Schnitzer, M. J. Motor behavior activates Bergmann glial networks. *Neuron* **62**, 400–412 (2009).
34. Thrane, A. S. et al. General anesthesia selectively disrupts astrocyte calcium signaling in the awake mouse cortex. *Proc. Natl Acad. Sci. USA.* **109**, 18974–18979 (2012).
35. Sorensen, G. L., Knudsen, S. & Jenum, P. Sleep transitions in hypocretin-deficient narcolepsy. Sleep 36, 1173–1177 (2013).

36. Astori, S., Wimmer, R. D. & Lüthi, A. Manipulating sleep spindles-expanding views on sleep, memory, and disease. Trends Neurosci. 36, 738–748 (2013).

37. Pasqual, O. et al. Neurobiology: astrocytic purinergic signaling coordinates synaptic networks. Science 310, 113–116 (2005).

38. Manoach, D. S. & Stickgold, R. Abnormal sleep spindles, memory consolidation, and schizophrenia. Annu. Rev. Clin. Psychol. 15, 451–479 (2019).

39. Fogel, S. M., Smith, C. T. & Beninger, R. J. Too much of a good thing? Elevated baseline sleep spindles predict poor avoidance performance in rats. Brain Res. 1319, 112–117 (2010).

40. Shibagaki, M., Kiyono, S. & Watanabe, K. Sommeil nocturne chez des enfants très retardés: patterns EEG anormaux des cycles de sommeil. Electroencephalogr. Clin. Neurophysiol. 49, 337–344 (1980).

41. Gibbs, E. L. & Gibbs, F. A. Extreme spindles: correlation of electroencephalographic sleep pattern with mental retardation. Science 138, 1106–1107 (1962).

42. Kjaerby, C., Rasmussen, R., Andersen, M. & Nedergraad, M. Does global astrocytic calcium signaling participate in awake brain state transitions and neuronal circuit function? Neurochem. Res. 42, 1810–1822 (2017).

43. O’Donnell, J., Ding, F. & Nedergraad, M. Distinct functional states of astrocytes during sleep and wakefulness: is noxinephrine the main regulator? Curr. Sleep. Med. Rep. 1, 1–8 (2015).

44. Ding, F. et al. a1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. Cell Calcium 54, 387–394 (2013).

45. Jasper, H. H. & Tessier, J. Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep. Science 172, 601–602 (1971).

46. Kanda, T., Ohyama, K., Muramoto, H., Kitajima, N. & Sekiya, H. Promising techniques to illuminate neuromodulatory control of the cerebral cortex in sleeping and waking states. Neurosci. Res. 118, 92–103 (2017).

47. Miyawaki, H., Watson, B. O. & Diba, K. Neuronal activity. J. Neurosci. 27, 6607–6619 (2007).

48. Chen, T.-W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499, 295–300 (2013).

49. Regan, M. R. et al. Variations in promoter activity reveal a differential regulator? Mol. Cell 54, 1810–1822 (2010).

50. Shibagaki, M., Kiyono, S. & Watanabe, K. Sommeil nocturne chez des enfants très retardés: patterns EEG anormaux des cycles de sommeil. Electroencephalogr. Clin. Neurophysiol. 49, 337–344 (1980).

51. Sorensen, G. L., Knudsen, S. & Jenum, P. Sleep transitions in hypocretin-deficient narcolepsy. Sleep 36, 1173–1177 (2013).

52. Shevtsova, Z., Malik, J. M., Michel, U., Bahr, M. & Kugler, S. Promoters and serotypes: targeting of adenovirus-associated vectors for gene transfer in the rat central nervous system in vitro and in vivo. Exp. Physiol. 90, 53–59 (2005).

53. Hirrlinger, J. et al. Split-Cre complementation indicates coincident activity of different genes in vivo. PLoS ONE 9(11), e1000426 (2014). https://doi.org/10.1371/journal.pone.0004268

54. Tang, W. et al. Stimulation-evoked Ca2+ signals in astrocytic processes at hippocampal CA3-CA1 synapses of adult mice are modulated by glutamate and ATP. J. Neurosci. 35, 3016–3021 (2015).

55. Smith, R. H., Levy, J. R. & Kotin, R. M. A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. Mol. Ther. 17, 1888–1896 (2009).

56. McNeil, C. J. & Shetty, A. K. Zika virus: a serious global health threat. J. Trop. Pediatr. 63, 242–248 (2017).

57. Huber, D. et al. Multiple dynamic representations in the motor cortex during sensorimotor learning. Nature 484, 473–478 (2012).

58. Yüzer, O., Pra, M., Zimmermann, R. & Huber, D. Pupil size coupling to cortical states protects the stability of deep sleep via parasympathetic modulation. Curr. Biol. 28, 392–400.e3 (2018).

59. Greenwell, B. J. et al. Rhythmic food intake drives rhythmic gene expression more potently than the hepatic circadian clock in mice. Cell Rep. 27, 649–657.e5 (2019).

60. Possidente, B. & Birnbaum, S. Circadian rhythms for food and water consumption in the mouse, Mus musculus. Physiol. Behav. 22, 657–660 (1979).

61. Vollm, C. et al. Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. Proc. Natl Acad. Sci. USA. 106, 21453–21458 (2009).

62. Takahashi, K., Lin, J. S. & Sakai, K. Characterization and mapping of sleep-waking specific neurons in the basal forebrain and preoptic hypothalamus in mice. Neuroscience 161, 269–292 (2009).

63. Pneumatikakis, E. A. & Giovannucci, A. NoRMCorre: an online algorithm for piecewise rigid motion correction of calcium imaging data. J. Neurosci. Methods 291, 83–94 (2017).

64. Oksanen, J. et al. Community ecology package: vegan: R A Language and Environment for Statistical Computing. Available at: https://cran.r-project.org/web/packages/vegan/index.html (2010).

65. Pinheiro, J., Bates, D. & DebRoy, S. S. Linear and nonlinear mixed effects models. R package version 3.1.127. Available at: https://CRAN.R-project.org/package=nlme (2016).

66. Carey, V. J. & Wang, Y. Mixed-Effects Models in S and S-Plus. J. Am. Stat. Assoc. 96, 1135–1136 (2001).

67. Rizopoulos, D. GLMMadaptive: Generalized Linear Mixed Models Using Adaptive Gaussian Quadrature (2019). Available at: https://cran.r-project.org/package=GLMMadaptive.

Acknowledgements

Professor Erlend A. Nagelhus tragically died on 10th January 2020. We will always remember him as an inspiring leader, a good friend and a great scientist. We thank Gustavo Borges Moreno e Mello for his contribution to the establishment of the setup for awake animal imaging and monitoring, Arild Njå for guidance on electromyography, Xiaoyi Zhang and Mina Martine Frey for assistance with immunohistochemistry, and Johannes Helm for continuous technical support on the two-photon microscope. We thank Janne Gronli and Jelena Mrdalj for valuable help on establishing rodent sleep scoring. We acknowledge the support by UNINETT Sigma2 AS for making data storage available through NIRD, project NS9021K. This work was supported by the Research Council of Norway (grants #249988, #240476, and #262552), the South-Eastern Norway Regional Health Authority (grant #2016070), the European Union’s Seventh Framework Program for research, technological development, and demonstration under grant agreement no. 601055, The National Association of Public Health, The Olav Thon Foundation and the Letten Foundation.

Author contributions

Conceptualization: E.A.N., K.A.G.V.; Methodology: L.B., R.E., K.A.G.V., E.A.N., W.T., R. S.; Software: D.M.B., K.S.Å., R.E., K.H.P.; Formal analysis: D.M.B., R.E., C.C., G.H.H., K. H.P.; L.B.; Investigation: L.B.; Resources: W.T., E.A.N., R.S.; Writing—original draft: L.B., E.A.N., Writing—review and editing: L.B., R.E., E.A.N., A.C.; Visualization: L.B., R. E.; Supervision: R.E., E.A.N., K.A.G.V.; Funding acquisition: E.A.N.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17062-2.

Correspondence and requests for materials should be addressed to R.E.

Peer review information Nature communications thanks Michael Halassa, Thomas Papoun, and Dmitri Rusakov for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020