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Does Global Astrocytic Calcium Signaling Participate in Awake Brain State Transitions and Neuronal Circuit Function?

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Abstract

We continuously need to adapt to changing conditions within our surrounding environment, and our brain needs to quickly shift between resting and working activity states in order to allow appropriate behaviors. These global state shifts are intimately linked to the brain-wide release of the neuromodulators, noradrenaline and acetylcholine. Astrocytes have emerged as a new player participating in the regulation of brain activity, and have recently been implicated in brain state shifts. Astrocytes display global Ca²⁺ signaling in response to activation of the noradrenergic system, but whether astrocytic Ca²⁺ signaling is causative or correlative for shifts in brain state and neural activity patterns is not known. Here we review the current available literature on astrocytic Ca²⁺ signaling in awake animals in order to explore the role of astrocytic signaling in brain state shifts. Furthermore, we look at the development and availability of innovative new methodological tools that are opening up for new ways of visualizing and perturbing astrocyte activity in awake behaving animals. With these new tools at hand, the field of astrocyte research will likely be able to elucidate the causal and mechanistic roles of astrocytes in complex behaviors within a very near future.

Keywords

Astrocytes; Calcium; Brain state; Neuronal circuit; Wakefulness

Introduction

Every day, we continuously shift between different behavioral contexts, such as zoning out while waiting for the bus or getting a kick from high intensity workout at the gym. During these shifts in brain state, the internal dynamics of the brain and its responsiveness to

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incoming external sensory stimuli vary greatly [1–6] (Figure 1). The internal brain state can oscillate and shift even in the absence of marked behavioral changes of which the transition between different phases of sleep and from sleep to awake are the most well-recognized [7–11].

The neuronal activity patterns during sleep and anesthesia have been interrogated in detail and much is known about both intrinsic and extrinsic properties governing these patterns. By comparison, neuronal activity pattern transitions in the awake brain are subtler, and the mechanisms orchestrating these transitions are less studied. However, an increasing body of literature are starting to emerge [3, 4, 12–16].

Different stages of sleep and wakefulness are characterized by specific patterns of electroencephalographic (EEG) activity: during non-rapid-eye-movement (NREM) sleep, slow 1–4 Hz oscillations are dominant, whereas REM sleep is characterized by desynchronized high-oscillatory activity. The awake brain state has been categorized into quiet wakefulness and active behavior with active behavior most often being associated with periods of locomotion or active whisking [13, 17]. Seminal studies have shown that quiet wakefulness is characterized by synchronized large-amplitude low-frequency fluctuations in the local field potential or membrane potential [3, 4, 12]. During the active state, the brain activity desynchronizes and low-frequency fluctuations are suppressed, while higher-frequency oscillations become more apparent [3, 4, 12]. Even though the shifts in brain activity patterns that occur during sleep (NREM to REM) and wakefulness (quiet wakefulness to active state) are quite similar, there are large differences in muscle tone between sleep and awake states.

In this review, we will focus on brain state shifts in the awake behaving brain. Functionally, the switch from quiet wakefulness to an active state have been associated with changes in sensory information processing and representations, such as an increased gain of neurons in primary visual cortex and modulated sound-evoked responses in primary auditory cortex [3, 4, 16, 18], but almost nothing is known about the role of astrocytes in these transitions.

Glial cells are the most abundant cell type in the human brain [19]. In the human forebrain, ~20% of all cells are astrocytes [20], and in the mammalian cerebral cortex the number of astrocytes exceeds that of neurons [21]. Despite this striking high cell number, we are just starting to comprehend and investigate the diversity and importance of astrocyte functions (*reviewed in* Khakh and Sofroniew, 2015). It is now accepted that astrocytes are involved in homeostatic functions such as ion regulation, transmitter clearance as well as neurovascular coupling. In sleep, evidence suggests that astrocytes might play a role in inducing and maintaining the different sleep phases. A study found that astrocytes oscillated in phase with slow-wave activity during sleep [22] through redistributing of extracellular K^+ thus maintaining the depolarization (UP state) [23]. Another group found that by electrically stimulating single astrocytes thereby inducing Ca^{2+} fluctuations, they could induce cortical UP state synchronization [24, 25]. Several studies have also implicated astrocytes in sensory information processing in both anesthetized [26] and awake behaving animal [27] as well as in cortical state transitions in anesthetized animals [25], suggesting that astrocytes might participate in controlling the ongoing brain state and the shift between states.

If we are to understand how brain state transitions occur during wakefulness, and how this influences the representation and processing of the external world in cortical circuits, we need to start including astrocytes in our conceptualizations and hypotheses. Here we review recent work, largely in awake behaving animals, investigating the role of large global Ca^{2+} responses of astrocytes in brain state changes and arousal. Determining the functional role of these brain-wide astrocytic fluctuations in shaping neuronal circuits, sensory input, and ultimately decision making are important for deciphering the behavioral circuits in normal and pathological conditions.

Neuromodulator Systems Involved in Cortical State Transitions

The causal mechanisms underlying awake cortical state transitions are still debated and not fully understood, but an increasing amount of work has implicated central neuromodulator systems including norepinephrine (NE) and acetylcholine (ACh) (Eggermann et al., 2014; Fu et al., 2014b; Polack et al., 2013; Schiemann et al., 2015; *reviewed in* McGinley et al., 2015b) (Figure 2). NE is produced in the brain stem nuclei, locus coeruleus (LC), which projects widely throughout the brain and provides the sole source of NE. ACh on the other hand originates from several brain stem clusters, including the pedunculopontine tegmental and the lateral dorsal tegmental nuclei, as well as from basal forebrain nuclei, including nucleus basalis. Both LC and cholinergic basal forebrain neurons ramp up their firing rate in response to behavioral states associated with desynchronized cortical neuronal activity, such as attention, arousal, and spontaneous locomotion, pointing to their involvement in state transitions [28–30].

A large body of literature suggests that NE and ACh modulate neuronal activity via several mechanisms. Earlier work have established that NE both excite and inhibit neurons [31–34]. Though, it appears that NE is mostly excitatory on cortical pyramidal neurons via its activation of metabotropic G_q -coupled noradrenergic α_1 -receptors [35, 36]. This effect is mostly ascribed to inhibition of the Ca^{2+} -activated K^+ current I_{AHP} [37, 38], and a reduction in other K^+ currents [36]. Experiments investigating the functional mechanisms of ACh on neuronal properties have demonstrated that ACh most often triggers prolonged and marked excitation of cortical pyramidal neurons via combined activation of cholinergic metabotropic muscarinic and ionotropic nicotinic receptors [39–41]. This is thought to be due to a general decrease in neuronal K^+ conductance [42]. Thus, recordings from pyramidal neurons have shown that ACh block the voltage-dependent K^+ current I_{M} , the I_{AHP} , and with and to some extend a leak K^+ current [43–46]. Further evidence for key roles of both NE and ACh for the transition between awake states is based on elegant *in vivo* experiments using pharmacological blockage of NE and ACh receptors locally in the cortex [12, 14, 15, 28]. This has demonstrated that both NE and ACh are strongly involved in mediating the cortical state transition and neuronal activity modulation associated with locomotion, leading to the hypothesis that NE and ACh reduce low-frequency and facilitate higher-frequency oscillations during states of increased arousal [17]. Furthermore, NE and ACh can change the composition of extracellular ions and in this way control the electrocorticographic patterns [7].

Taken together, substantial evidence supports a prominent role for NE and ACh in mediating the state transition between quiet wakefulness and the active state. Given their widespread projection targets and ability to globally shift the levels of NE and ACh throughout the cerebral cortex, they are perfectly situated to regulate cortical state changes in the awake brain. However, several other systems, such as thalamo-cortical and cortico-cortical pathways, have also been linked to awake state shifts, and suggesting that brain state transitions and stabilization likely requires both global, as well as local signaling (*reviewed in* Lee et al., 2013; Lee and Dan, 2012; Zaghera and McCormick, 2014).

Astrocytes Respond to Neuromodulators

Astrocytes respond to transmitter release with increases in their intracellular Ca^{2+} concentration via several mechanisms [21, 49]. This astrocytic Ca^{2+} signaling has attracted tremendous attention within the last 20 years, because it likely represents a form of signaling that astrocytes may employ for communication in the brain. A large variety of astrocytic Ca^{2+} responses have been uncovered that may play a role in regulating local neuronal activity (*reviewed by* Bazargani and Attwell [50]). However, many of these experiments have been conducted in preparations from immature animals, proving to be problematic due to developmental regulation of astrocytic receptor expression [51]. Furthermore, anesthesia significantly blunts astrocytic Ca^{2+} signaling *in vivo* [52, 53]. The most intriguing experiments are thus performed *in vivo* in awake animals. Initially, a clear link between peripheral sensory stimulations and astrocytic Ca^{2+} responses was reported: in anesthetized animals, Ca^{2+} transients within cortical astrocytes could be elicited by whisker stimulation, foot shock, and mechanical stimulation [26, 54, 55]. Early work in cultured astrocytes revealed that NE is a potent activator of astrocytic Ca^{2+} signaling [56–58]. A link between LC and NE-mediated activation of astrocytes was confirmed by Bekar *et al.* (2008) that demonstrated that in anesthetized animals, when LC is electrically stimulated, cortical astrocytes strongly respond with Ca^{2+} increases, which could be blocked by noradrenergic antagonists. LC activation is clearly connected to startle responses, which can be viewed as states of hyperarousal. Ding *et al.* (2013) confirmed and extended the findings from Bekar *et al.* (2008) in awake animals and showed that startle-induced LC activation caused long-ranging astrocytic cortical Ca^{2+} signals that were triggered by endogenous NE release from noradrenergic fibers acting on cortical astrocytic α 1-adrenergic receptors. This has led to a novel concept within the field that astrocytes in the awake animal respond to arousing stimuli by elevating their Ca^{2+} levels globally via NE-mediated α 1-receptor activation, potentially making astrocyte signaling an inherent player in global state transitions [27, 59, 60]. Thus, we need to consider astrocytes as NE mediators, when interpreting downstream-effects of LC activation. There are several phenomena that are necessary to consider and discuss with regards to this. First, neurons in LC and astrocytes display very different activity time courses: sensory stimuli elicits short-lasting LC bursting activity *in vivo* (less than a couple of 100 msec) followed by intervals of silence [61], whereas astrocytic Ca^{2+} responses rise and peak slowly (over the course of seconds). Therefore, one could speculate that NE is not solely responsible for astrocyte activation, but may permit the involvement of other sources of astrocyte activation. Second, it is a possibility that certain of the neuronal responses to NE might occur indirectly through astrocytes. Due to the slow time course of

astrocytes, any immediate impact on neuronal responses would be expected to follow that time scale, or possibly create more long-term neuronal changes. This is interesting in context to a newly published study showing that astrocytic Ca^{2+} signals, mediated by fly analog to neuromodulators, are directly involved in the regulation of neuronal activity and sensory-driven behaviors in drosophila flies [62]. However, direct comparisons to rodent studies should be undertaken with caution, since there are large differences in the species-specific cell and transmitter characteristics.

In addition to NE-mediated astrocytic Ca^{2+} signaling, evidence for ACh-mediated astrocytic signaling also exists [63–67]. In anesthetized animals, stimulating the nucleus basalis of Meynert, a main source of cholinergic innervation to the cortex, triggered pronounced astrocytic Ca^{2+} signaling in sensory cortex, which was blocked by a muscarinic cholinergic receptor antagonist [63]. Complementing work was done in the visual cortex *in vivo*, where stimulation of the nucleus basalis of Meynert in anesthetized mice resulted in astrocytic Ca^{2+} signals that were mediated by direct activation of cholinergic muscarinic receptors on astrocytes [64]. Further work implicating ACh in astrocytic Ca^{2+} signaling has been performed in hippocampal slices showing that astrocytes respond to cholinergic muscarinic receptor stimulation with Ca^{2+} signaling [65, 67]. Most recently, experiments performed *in vivo* and *ex vivo* showed that hippocampal hilar astrocytes respond to optogenetical stimulation of septohippocampal fibers with Ca^{2+} signaling, and this was blocked with nicotinic ACh receptor antagonists [66]. Interestingly, stimulation of the nucleus basalis of Meynert elicited astrocytic Ca^{2+} responses that were persistent throughout the stimulation [63], whereas astrocytic Ca^{2+} responses induced by whisker stimulation returned to baseline even during stimulation [26] indicating that the origin of stimulation elicit responses mediated by different transmitter systems.

It is important to note, that the noradrenergic and cholinergic neuromodulators are not two separated signaling systems. They both play a significant role in arousal and vigilance and the signaling systems in the awake state are closely linked through reciprocally presynaptic modulation of transmitter release: NE inhibits the release of ACh through expression of inhibitory G_i -coupled α_2 -noradrenergic receptors on cholinergic axon terminals [68, 69], and ACh facilitates Ca^{2+} -dependent NE release through activation of G_q -coupled mAChRs [70]. Taken together, a substantial amount of work points to astrocytes as a target of noradrenergic and cholinergic transmission, leading to broad global astrocytic Ca^{2+} signaling positioning them optimally for contributing to global brain state changes in the awake brain.

Are Astrocytes involved in Brain State Changes?

Whether astrocytes causally participate in the transition between quiet wakefulness and the active behaving state, associated with improved sensory processing [4, 12, 15, 18], is currently unresolved. Their strong and faithful activation by NE and ACh might indicate that they very well could be, but conflicting data also exist. The few studies in the awake behaving mouse that have investigated the involvement of astrocytes in brain state shifts have focused on the transition between still to a moving state [27, 71, 72], a behavioral transition that is also associated with cortical neuronal state changes [3, 4, 12, 15, 17, 18].

Dombeck *et al.* (2007) [71] and Nimmerjahn *et al.* (2009) [72] used a chemical Ca^{2+} indicator in combination with the astrocyte-specific fluorescent indicator, sulforhodamine 101, to distinguish between neuronal and astrocytic Ca^{2+} signals, whereas Paukert *et al.* (2014) [27] used a genetically encoded Ca^{2+} indicator specifically expressed in astrocytes. The use of genetically encoded Ca^{2+} indicators might be preferred over chemical indicators because it has been shown that sulforhodamine 101 in higher dosages can induce abnormal cortical neuronal activity in both anesthetized and awake mice [73]. Dombeck *et al.* (2007) reported that in layer 2/3 of sensory cortex, only 11% of astrocytes show strong Ca^{2+} signaling correlation to spontaneous locomotion activity, whereas 60% showed little correlation. In contrast, in cerebellar Bergmann glia, Nimmerjahn *et al.* (2009) found consistent increases in astrocytic Ca^{2+} levels with every spontaneous locomotor episode. However, a later study by Paukert *et al.* (2014) found that only around 30% of Bergmann glia responded to spontaneous locomotor episodes, confirming the observation from sensory cortex. Thus, it seems like spontaneous locomotion in mice does not trigger reliable activation of Ca^{2+} signaling in astrocytes. However, neither of these experiment did concomitantly record pupil dilations, often used as an “online” correlative readout of LC-mediated noradrenergic activity [3, 6, 17]. Thus, whether LC firing and noradrenergic signaling was co-occurring consistently together with locomotion onset in these experiments is unknown. The lack of correlation between astrocytic Ca^{2+} signaling and locomotion onset might potentially be explained by a lag of correlation between LC firing and locomotion onset in these behavioral contexts.

These three studies all show that astrocytic Ca^{2+} signals develop ~1–2 seconds following spontaneous locomotion onset [27, 71, 72], whereas neuronal activity pattern changes precede locomotion onset [3, 6, 12]. Hence, these data suggests that global astrocytic Ca^{2+} signaling is not required for initiation of awake cortical state shifts, but instead mainly depends on neuronal activity. Evidence suggests that neuronal activity also plays a role in the activation of locomotor-induced astrocyte Ca^{2+} activity: Nimmerjahn *et al.* (2009) showed that the locomotor-induced astrocytic Ca^{2+} transients in Bergmann glia were abolished after blocking neural activity [72], whereas Paukert *et al.* (2014) showed that AMPA and NMDA receptor antagonists attenuated, but did not completely block the locomotor-induced astrocytic Ca^{2+} responses [27]. This might suggest that glutamatergic and noradrenergic act synergistically to promote astrocytic Ca^{2+} activity.

Paukert *et al.* (2014) found that enforced locomotion on a treadmill, in contrast to spontaneous locomotion, elicited reliable astrocytic Ca^{2+} responses [27], which illustrates one potential caveat and concern regarding the behavioral contexts often used to trigger the global astrocytic noradrenergic-mediated Ca^{2+} signaling. Enforced locomotion is likely perceived as a startle-stimulation by the animal, similar to the astrocytic Ca^{2+} signals elicited by other types of startle-stimulation [59, 60]. Since spontaneous locomotor shifts do not reliably activate astrocytic Ca^{2+} fluctuations, one could speculate that the episodes leading to increased Ca^{2+} signaling involved some degree of startle, since initiating movement when head fixed might involve some discomfort and stress for the animal. One way to avoid this problem could be monitoring locomotor patterns employing a setup allowing real spontaneous state shifts, such as a floating ball [4, 71, 74].

Startle-stimulation is linked to NE release, and Paukert *et al.* (2014) showed that by pairing enforced locomotion, and thus global astrocytic NE-mediated signaling, with visual stimuli presentation, the astrocytes markedly enhanced their Ca^{2+} signaling in response to visual stimulation. In contrast, when mice were stationary, astrocytes did not seem to respond much to visual stimuli presentation in primary visual cortex. These results may suggest that startle-induced widespread noradrenergic signaling shifts the gain of neocortical astrocytic networks, allowing the astrocytes to respond to local sensory-evoked neuronal activity [27]. However, the study did not provide data documenting that visual stimulus alone in the absence of forced locomotion induced astrocytic Ca^{2+} signaling. Most importantly, it remains to be defined whether astrocytic Ca^{2+} signaling is required for enhancing the gain of primary visual cortex neurons during spontaneous locomotion as has been observed [4, 12, 15, 18].

Recent work in primary auditory cortex, and earlier concepts in humans, has shown that states of hyper-arousal are associated with reduced sensory discrimination and diminished cognitive abilities [3, 29, 75]. Thus, one might speculate if the global astrocytic startle-driven Ca^{2+} response is associated with improved or decreased (fight-or-flight response) cortical cognitive functions. Just as beneficial as short-lasting LC burst activation is for attention and arousal, just as harmful is chronic activation; stress is strongly linked to altered LC functionality [76–81] and can lead to depression or posttraumatic stress disorder [82, 83]. Thus, alterations in LC firing and NE release may cause these pathophysiological changes through astrocyte activation. In an animal model of epilepsy, hyper-synchronous neuronal activity depended on astrocyte activity, and treatment with antiepileptic drugs reduced astrocytic Ca^{2+} signaling, indicating that astrocyte activation might underlie dysfunctional neuronal circuit activity [84]. It is known that during states of attention, LC-mediated NE amplifies behavioral relevant information [85–87]. Thus, it could be interesting to evaluate the role of astrocytic Ca^{2+} signals in brain state shifts involving a transfer from passive to active attention performance. In order to delineate the function of astrocytes in more subtle cognitive brain state changes, it would be essential to have an experimental set-up that does not involve startle or stress-triggering components and that depends on active decision-making of the animal. An interesting recently accepted publication showed that in brain slices, wash-in of subthreshold NE sensitized α_1 -adrenergic receptor-dependent astrocytic Ca^{2+} responses to local short-lasting exposure to concentrated NE [88]. The increased responsiveness of astrocytes to low concentration of NE was mediated by activation of β -adrenergic receptors. Thus, tonic LC firing, resulting in low NE levels, may prime astrocytes to LC burst activity, leading to shorter-lasting but higher peak NE levels [89].

Future experiments should aim to answer the causal and mechanistic association between astrocytic noradrenergic Ca^{2+} signaling and changes in awake brain states and the consequent effect on cortical sensory information processing. Currently, the up- and downstream roles of astrocytes in brain state shifts remains unsolved. Downstream effects of astrocyte activation could involve maintaining and stabilizing the ongoing current brain state, as well as involvement in mediating down-stream priming of the neural networks during the active behaving state (Figure 3).

Down-stream effects of astrocyte activation

One of the central questions in the field of astrocyte research is, what are the down-stream effects of astrocytic Ca^{2+} signaling? And how does this, if it does, contribute to cortical circuit processing? Astrocytes are globally distributed within the brain and in areas containing a high density of dendrites and axons, like the cerebral cortex, the number of astrocytes exceeds that of neurons [20, 21] providing a near-complete cortical coverage [90–92]. Thus, astrocytes are perfectly situated to mediate brain-wide activity changes, but at present, we still do not understand the down-stream consequences of astrocyte activation on neuronal synapses, circuits, and behaviors.

A wealth of down-stream functions of astrocytic Ca^{2+} activation have been presented during the past two decades including modulation of synaptic strength through release of so-called gliotransmitters such as glutamate, ATP, adenosine, or D-serine, which are mainly based on slice preparations from immature animals. These results are extensively covered in other reviews such as Haydon and Nedergaard, 2014 [93].

Studies suggest that interactions between astrocytic processes and synapses correlate with synaptic strength; synapses closely enwrapped with astrocytic processes appear larger compared to those without [94] and the motility of astrocytic processes mediated by intracellular Ca^{2+} fluctuations increases in response to sensory and synaptic activity, and primes the astrocytic coverage and general structural organization of synapses [95–97]. Furthermore, stimuli that elicits long-term potentiation of neuronal responses also increased motility of astrocytic processes [96, 98]. One study explored the consequences of increasing the proximity of astrocytic processes to synapses, and found that the reduced distance between hippocampal synapses and astrocytic glutamate transporters reduced the availability of glutamate thereby compromising AMPA-mediated long term potentiation and thereby generation of fear-memory [99]. Several other studies have previously established this link between astrocytic glutamate transporter expression and regulation of neuronal function [100, 101].

In contrast to glutamate, the extracellular level of GABA neurotransmitter was enhanced following astrocytic Ca^{2+} signaling due to reduced functional expression of GABA transporters on astrocytes [102]. Thus astrocytes seem to perform differential regulation of extracellular excitatory and inhibitory neurotransmitters indicating a role for astrocytes in the regulation of neuronal network state.

Another possible way for astrocytes to modulate neuronal networks is through modulation of extracellular K^+ levels. Increased extracellular K^+ increases the membrane potential of neurons enhancing the neuronal network excitation [103, 104]. Astrocytes have been shown to be responsible for K^+ buffering: astrocytic Ca^{2+} signaling activates the astrocytic Na^+/K^+ ATPase resulting in uptake of K^+ and lowering of extracellular K^+ concentration [105–108] (Figure 3). This astrocytic Ca^{2+} -regulated K^+ uptake reduced the frequency of excitatory input to neurons, while reducing synaptic failures, indicating that Ca^{2+} signaling in astrocytes might increase the signal-to-noise ratio of synaptic processing [104]. Another way K^+ is cleared from the extracellular environment is via a co-transport system transporting

Na⁺ and K⁺ ions in conjunction with Cl⁻ ions into the astrocytes, and occurs in astrocytes following astrocytic Ca²⁺ influx caused by K⁺-mediated depolarization [109, 110].

Glycogen is important for energy production, and decomposition of glycogen has been shown to deliver energy for astrocytic K⁺ uptake [111, 112]. In brain, glycogen is stored mainly in astrocytes [113]. Astrocyte Ca²⁺ increases activates enzymes necessary for glycogenolysis, and Ca²⁺ influx into astrocytes may be driven by astrocytic K⁺ uptake [114]. In addition, astrocytic morphological remodeling has been shown to depend on activation of noradrenergic β-receptors [115–117] likely through activation of astrocytic cAMP-pathways, which initiates rapid degradation of glycogen for energy metabolism [118, 119]. In addition, intracellular increases in Ca²⁺ levels lead to mitochondrial ATP production across all cell types (reviewed in [120, 121]). Thus, NE-mediated astrocytic Ca²⁺ responses might prepare for an increased demand for K⁺ and glutamate buffering, which would be expected when shifting to an active brain state associated with desynchronization.

Tools for delineating down-stream effects of astrocyte activation

In order to assess the functional role of astrocyte activation on brain state shifts and the involved neuronal network activity, it is critical to be able to interfere with astrocyte Ca²⁺ activity in awake animals without affecting neuronal activity. However, specific modulation of astrocyte activity has proven difficult due to the fact that astrocytic Ca²⁺ fluctuations are mediated almost exclusively by G-protein coupled receptors that are not selectively expressed on astrocytes limiting the use of pharmacological approaches. Here we propose certain approaches that could prove useful for modulating astrocytic Ca²⁺ levels or G-protein coupled receptor activity.

For direct modulation of the intracellular concentration of Ca²⁺ in astrocytes, several groups has employed ultra-violet light flash photolysis, in which astrocytes are specifically loaded with caged Ca²⁺ or IP₃ [122, 123]. However, for interrogation of circuit changes underlying brain state changes, a large number of astrocytes need to be modulated simultaneously making this approach insufficient. In addition, photolysis of caged Ca²⁺, but not physiological receptor-mediated activation of the Ca²⁺ signal, has been shown to trigger glutamate release, suggesting that Ca²⁺ uncaging triggers non-physiological effects [123].

A possible solution to modulating astrocyte activity on a global scale came with the development of genetically encoded modulators [124]. Designer receptors exclusively activated by designer drugs (DREADDs) are engineered G-protein coupled receptors that respond to a biological inert molecule, clozapine-N-oxide (CNO) [125, 126] and specific genetic targeting allows expression in selective cell populations such as astrocytes. Since neuromodulators enhance astrocytic Ca²⁺ activity through G_q-protein coupled receptors, this approach resembles the endogenous physiological response of astrocytes to aroused brain states. However, since DREADDs are activated by the systemically administered drug, CNO, there is limited spatial and temporal control over DREADD activation, which may lead to non-physiological high amplitudes, long-lasting Ca²⁺ increases within the stimulated cells. Activation of G_q-coupled DREADDs expressed brain-wide in astrocytes elicited pronounced phenotypic effects such as increases in blood pressure, heart rate, and saliva

production, while reducing body temperature and increasing sedation in the presence of a GABA receptor agonist [124]. It remains to be determined whether these observations are physiological effects of peripheral or brain-wide astrocyte activity or might reflect some degree of pathological Ca^{2+} signaling in astrocytes.

Optogenetics is based on light-induced activation of ion fluxes with high temporal control, which is a very powerful tool for modulating neuronal activity, but since changes in voltage across the membrane does not reflect the primary G-protein coupled receptor mediated activation of astrocytes, the physiological relevance of this approach in astrocytes can be discussed. However, a number of studies have used optogenetic modulation of astrocytes and was able to elicit light-induced increases in the concentration of astrocytic Ca^{2+} following expression of channelrhodopsin in astrocytes [127–129]. Interestingly, a study also reported increased astrocytic Ca^{2+} fluctuations following light-induced activation of the proton pump, Archaeorhodopsin (Arch) that mediate an hyperpolarizing outward current of H^+ (hyperpolarization) [25].

The field of chemo- and optogenetics target genetically defined cell populations by transgenic approaches. The transgene often employ a cell-specific promoter and within astrocyte research, the GFAP promoter has been commonly used to obtain specific expression in astrocytes. However, a recent study has shown that a construct placed under control of the GFAP promoter (dominant-negative domain of vesicular SNARE) resulted in neural as well astrocytic expression [130]. Thus, when employing transgenic approaches, careful assessment and control needs to be undertaken.

G_q -coupled receptor activation of astrocytes is believed to initiate Ca^{2+} fluctuations through activation of IP_3 receptor regulated intracellular Ca^{2+} stores. Astrocytes express only one of three subtypes of the receptor, the IP_3 receptor type 2 (IP3R2), and germline IP3R2 knockout mice display reduced G_q -mediated astrocytic Ca^{2+} fluctuations, however, with no apparent behavioral alterations or impact on synaptic plasticity (Agulhon et al., 2010; reviewed in Xie et al., 2015). This same lack of behavioral changes was observed in a conditional knockout of IP3R2 restricted to GFAP positive cells within the CNS [133] indicating that astrocytic Ca^{2+} fluctuations may not be important for behavioral function. However, studies have found that Ca^{2+} signaling remains in the IP3R2 knockout mice indicating that astrocytic Ca^{2+} signaling can occur independently of IP3R-mediated internal release [134, 135] possibly through transient receptor potential (TRP) channels located in the membrane [136]

Conditional knockout approaches can prove highly useful in the evaluation of astrocyte responses in brain state changes. Conditional knockouts are most commonly based on the Cre-lox system, and for temporal control of the conditional knockout, inducible Cre recombinase can be employed. Interestingly, an inducible and conditional G-protein ($\text{G}_{\alpha q}/\text{G}_{\alpha 11}$) conditional knockout mouse strain [137] are available, and could be crossed with astrocyte-specific Cre recombinase mouse lines to selectively knockout G_q -coupled signaling pathways in astrocytes.

Imaging astrocyte Ca²⁺ activity during brain state shifts

Genetically encoded Ca²⁺ indicators (GECIs) have recently become a strong tool for visualizing astrocytic Ca²⁺ fluctuations with targeted delivery to both the membrane or cytosol of astrocytes [136] using viral delivery or the use of transgenic mice. This approach has been widely used for *in vivo* studies, where Ca²⁺ fluctuations in awake head-fixed mice are visualized through cranial windows using two-photon microscopy. In addition, the use of transgenic mouse strains having a global expression of GECIs allow for macroscopic low-resolution imaging of large brain regions perfect for monitoring global Ca²⁺ signals [138]. However, these approaches are largely limited to imaging of upper cortical layer, and in order to determine the global brain changes underlying brain state shifts, access to deeper brain regions involved in the circuit of interest is needed as well. Recent developments of more sophisticated red-shifted GECIs opens up for imaging in deeper layers due to the tissue-penetrating effect of infrared light [139]. In addition, red-shifted GECIs may allow less invasive imaging approaches such as transcranial imaging, which would be very valuable for longer-lasting behavioral studies of astrocytic Ca²⁺ signals. Other approaches to gain access to deeper brain regions through implanted imaging cannulas have emerged due to recent development of head-mounted miniaturized microscopes as well as fiber photometry [140–143]. This also allows investigation of freely moving animals in contrast to head-fixed animals skipping the need for unnatural fixation and subsequent increased stress levels of the animal that will likely affect the behavior of the animals.

Conclusion

In this review, we have strived to present and discuss the current literature on awake cortical state transitions in light of the potential involvement of astrocytes and their Ca²⁺ signaling. The awake brain state is transitioning between periods of quiet wakefulness and states of active behavior governed by neuromodulators such as NE and ACh. Astrocytes are ubiquitously in the brain and display widespread global Ca²⁺ signals in the awake behaving animal, when activated by NE or ACh. This links them as an inherent and important player for brain-wide neuronal state transitions, but we still do not understand their role in these circuitries. An exciting new literature have started to investigate astrocytic Ca²⁺ signaling patterns, when mice spontaneously transition into the active brain state, but the tools for determining the causative role of astrocytes have been lacking. Several new transgenic modulating and imaging tools have within the last years have pioneered the field of neuronal circuits and their causality for behavior. We propose that the astrocyte research field starts to develop and implement these techniques in order to delineate the role of astrocytes in brain state transitions and ultimately their causality in behavior.

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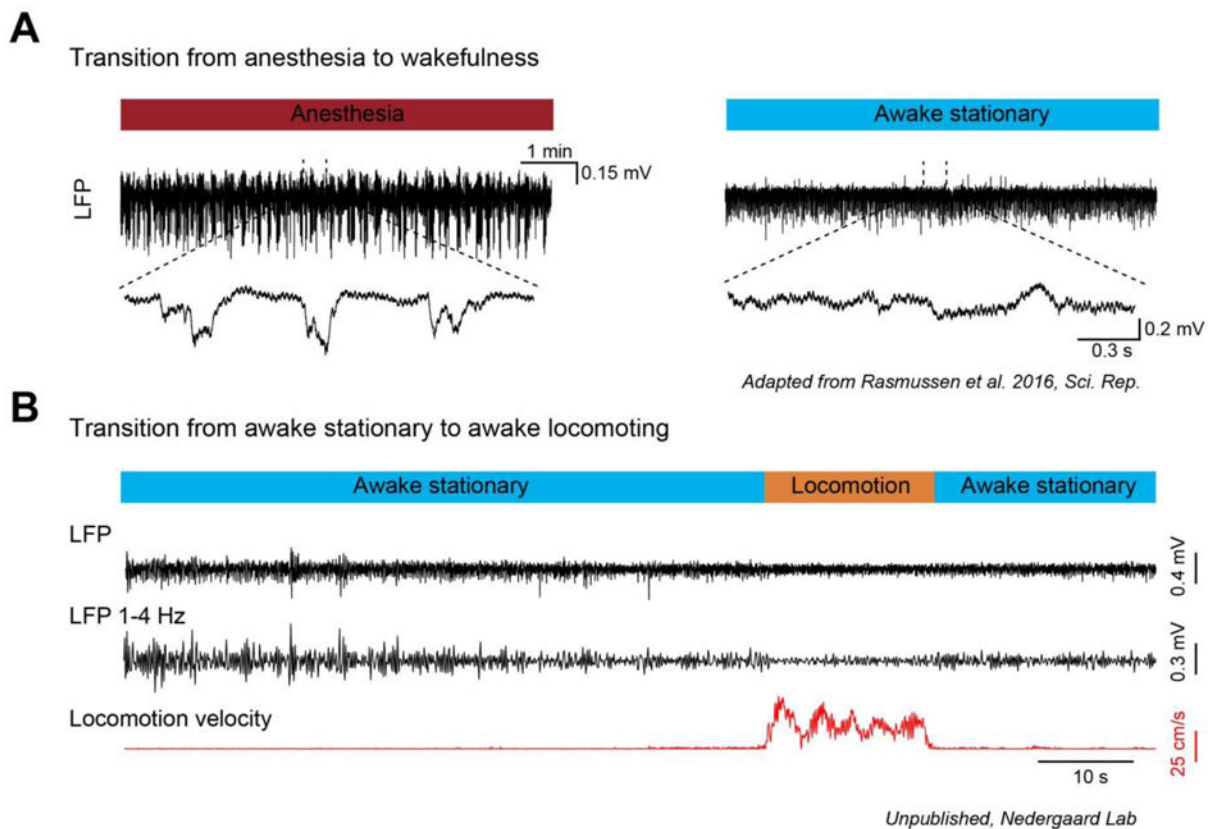


Fig. 1. Neuronal cortical state changes

Local field potential (LFP) characteristics of cortical state changes in mice. (A) LFP recording from layer 2/3 of mouse sensori-motor cortex during the transition from anesthesia-induced slow-wave patterns to waking. During anesthesia and sleep, slow wave fluctuations are prominent in the LFP signal and appear as synchronized summed population up and down states. The transition from anesthesia, or sleep, to waking is associated with a suppression of slow fluctuations in the LFP and a loss of synchronized down states. (B) LFP recording (raw broadband and band-pass filtered 1–4 Hz) from layer 2/3 of mouse visual cortex during the transition from stationary (quiet wakefulness) to voluntary locomotion (active behavior) on a running wheel. Voluntary locomotion is associated with a substantial suppression of LFP low-frequency 1–4 Hz fluctuations. Panel A is from Rasmussen et al., 2016 [73] and panel B is from unpublished experiments from the Nedergaard laboratory.

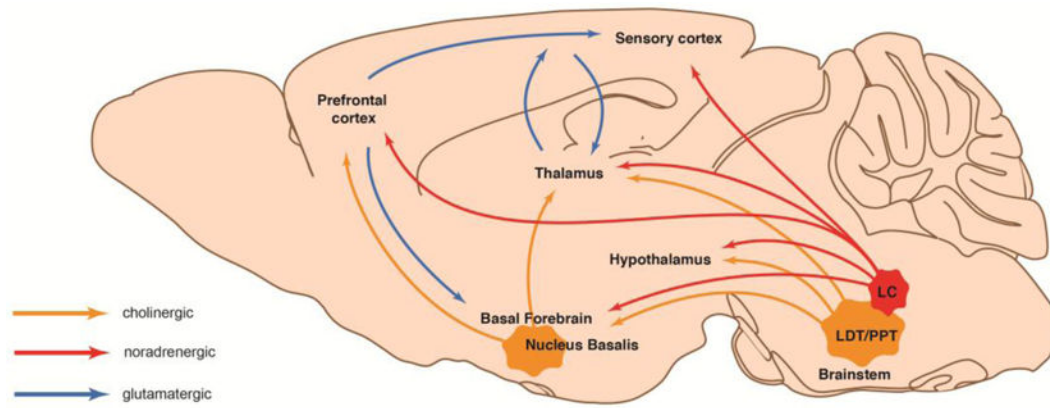


Fig. 2. Diagram displaying selected pathways involved in brain state changes
Sagittal view of a mouse brain showing glutamatergic (blue), noradrenergic (red), and cholinergic (orange) nuclei and projection pathways involved in shifts between resting and working activity states. LC: locus coeruleus; LDT: laterodorsal tegmental nucleus; PPT: pedunculopontine tegmental nucleus. Adapted from Lee and Dan, 2012 [8].

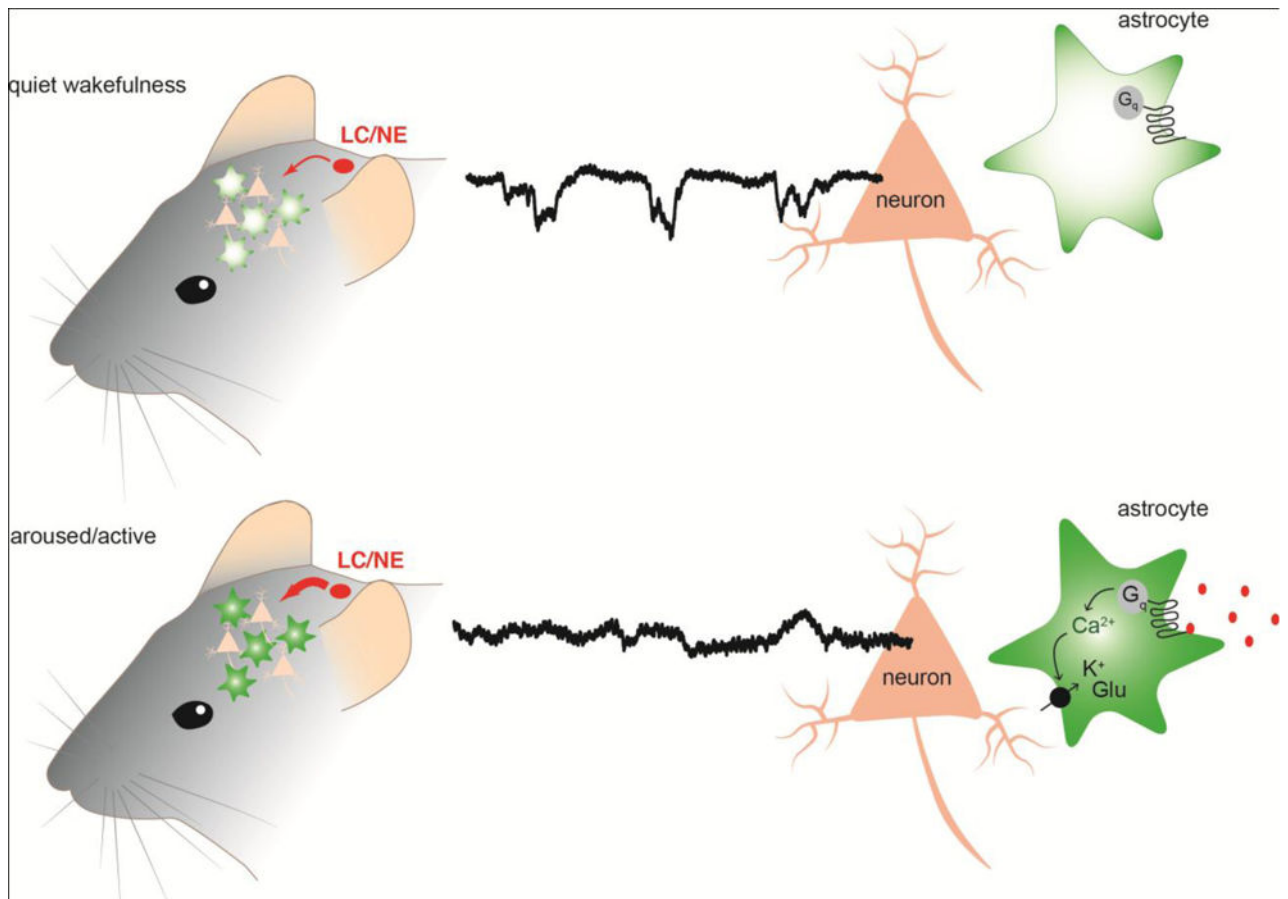


Fig. 3. Graphical summary of hypothesis

Top panel: during quiet wakefulness, the cortical noradrenergic and cholinergic pathways release low levels of neuromodulators, and astrocytes display transient asynchronous Ca^{2+} fluctuations within their processes. Bottom panel: during active brain states, there is increased cortical noradrenaline (NE) and acetylcholine (ACh) release. This activates G_q -coupled receptors located on astrocytes and leads to global astrocytic Ca^{2+} signaling. These Ca^{2+} level increases in astrocytes could lead to increased uptake of K^+ and glutamate (Glu) from the extracellular space into astrocytes thereby possibly regulating the excitability of neural circuits.