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Abstract: Mitochondria are increasingly recognized for playing important roles in regulating the evolving metabolic state of mammalian cells. This is particularly true for nerve cells, as dysregulation of mitochondrial dynamics are invariably associated with a number of neuropathies. Accumulating evidence now reveals that changes in mitochondrial dynamics and structure may play equally important roles also in the cell biology of astroglial cells. Astroglial cells display a significant heterogeneity in their morphology and specialized functions across the different brain regions, however besides fundamental differences they seem to share a surprisingly complex meshwork of mitochondria, which is highly suggestive of tightly regulated mechanisms that contribute to maintain this unique architecture. Here, we summarize recent work performed in astrocytes in situ indicating that this may indeed be the case, with astrocytic mitochondrial networks shown to experience rapid dynamic changes in response to defined external cues. Although the mechanisms underlying this degree of mitochondrial re-shaping are far from being understood, recent data suggest that they may contribute to demarcate astrocyte territories undergoing key signalling and metabolic functions.

Spatiotemporal control of mitochondrial network dynamics in astroglial cells

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Highlights

- Astrocytes display highly complex mitochondrial architectures *in vivo*
- Mitochondrial trafficking and dynamics in astrocytes are regulated
- Mitochondria critically contribute to astrocyte Ca^{2+} handling
- Astrocyte reacting to injury undergo marked changes in mitochondrial dynamics

Abstract

Mitochondria are increasingly recognized for playing important roles in regulating the evolving metabolic state of mammalian cells. This is particularly true for nerve cells, as dysregulation of mitochondrial dynamics are invariably associated with a number of neuropathies. Accumulating evidence now reveals that changes in mitochondrial dynamics and structure may play equally important roles also in the cell biology of astroglial cells. Astroglial cells display a significant heterogeneity in their morphology and specialized functions across the different brain regions, however besides fundamental differences they seem to share a surprisingly complex meshwork of mitochondria, which is highly suggestive of tightly regulated mechanisms that contribute to maintain this unique architecture. Here, we summarize recent work performed in astrocytes *in situ* indicating that this may indeed be the case, with astrocytic mitochondrial networks shown to experience rapid dynamic changes in response to defined external cues. Although the mechanisms underlying this degree of mitochondrial re-shaping are far from being understood, recent data suggest that they may contribute to demarcate astrocyte territories undergoing key signalling and metabolic functions.

TEXT

Astrocytes are an abundant cell population that fulfils a range of functions, from regulating neurovascular coupling to local refinement in synapse structure and function[1,2,3]. The multifunctional role of these cells is mirrored morphologically by a highly complex yet well-delineated arrangement of branches and branchlets. On the one hand, these exceedingly thin processes provide a dynamic coverage of neuronal synaptic contacts, thereby regulating their formation, maturation and ultimately maintenance[1]. On the other, elaborated perivascular processes almost completely sheath the brain microvasculature through highly specialized terminals called end-feet, proposed to co-regulate vascular tone as well as blood-brain-barrier function[4,5]. Accumulating evidence indicates that most of the specialized signaling functions that perisynaptic and perivascular astrocytic processes fulfill are invariably mediated by local, regionalized changes in second messengers (e.g. Ca2+) associated with the secretion of transmitters and other metabolites[3,6,7], thereby suggesting the existence of well-organized compartments that timely regulate the availability of these molecules in concert with, e.g. neuronal activity or vascular/interstitial flow. One organelle likely involved in this regulation is the mitochondrial network, which can effectively couple alternating periods of high/low respiration with local changes in ions availability and metabolite supply[8,9,10,11], thereby potentially coordinating important intracellular signaling events in astrocytes. Interestingly, from an energy metabolism point of view it is well established that astrocytes have prevalent glycolytic and glycogenic rates whereas the precise extent of ongoing oxidative phosphorylation (OXPHOS) *in vivo* has remained a matter of debate for long time. By sorting astrocytes from whole mouse brains, several recent studies have now started to provide evidence that, alongside with a high expression of glycolytic enzymes, these cells are also enriched in several enzymes of the tricarboxylic acid (TCA) cycle[12,13], thus not only supporting the notion that astrocytes have a prominent oxidative capacity but also emphasizing their ability to flexibly modify their metabolic profile according to substrate availability and/or energy demand[10,14]. This degree of metabolic “plasticity” may also reflect the peculiar capability

of astrocytes to adapt to extreme conditions of metabolic challenge, as for instance during ischemia, injury and inflammation. This has been exemplified, for example, in cultured astrocytes that preferentially increase their rate of glycolysis at the expenses of mitochondrial respiration to maintain ATP production when directly exposed to pro-inflammatory stimuli[15,16,17]. Intriguingly, this seemingly unique ability to rapidly adapt their cellular energy state appears to reflect the pronounced changes in morphology, gene and marker expression that mark the acquisition of astrocytic reactive states during disease[18]. Thus, although the precise cellular and sub-cellular events underlying the progression through these evolving states of reactivity are only partly understood, changes in mitochondrial structure and function are becoming important hallmarks of the astrocytic response to brain insults. Here we present some of the most recent observations revealing the peculiar nature of the mitochondrial network in astrocytes, and discuss how dynamic changes in its architecture may contribute to regulate fundamental functions in astrocytes both under resting conditions and in response to injury.

Unique architecture of the astrocytic mitochondrial network

Owing to a very complex tridimensional morphology, which in most astrocytes is characterized by a number of major processes giving rise to thousands of ramified branchlets and leaflets, a detailed analysis of the mitochondrial network architecture in these cells *in situ* has been elusive. Only during the last decade electron microscopy studies have started to ascertain that astrocytes, and astroglial cells in general, are surprisingly enriched in mitochondria[12,19,20,21]. These organelles have been found invading most of the peripheral processes, including fine branchlets and terminal endfeet, where highly heterogeneous organelle morphologies were observed, presumably suggesting the capacity of the mitochondrial network to accommodate a certain degree of structural remodeling to match the spatial constrains of perineuronal and perivascular processes. In particular, within endfeet processes large bundles of both small and elongated, branched and unbranched mitochondria were described in close apposition to the perivascular membrane[19]. At the

perisynaptic level, however, the physical size of some of the finest astrocytic processes (so-called PAPs, tens of nanometers wide) appears to essentially prevent the entry of cellular organelles, yet mitochondria are often observed in the immediate apposition to these fine terminals[11,22,23,24]. Similar observations suggesting the existence of a highly complex mitochondrial network in protoplasmic astrocytes *in vivo* were independently provided by few recent studies utilizing high resolution light microscopy and genetically-encoded fluorescent reporters (mito-GFP or similar, either delivered by a virus-based approach[16] or expressed by inducible reporter mice[25,26]), to specifically illuminate astrocytic mitochondria. These works describe the existence of a densely packed network of highly interconnected mitochondria occupying a substantial volume of individual astrocytes, particularly main processes and branches, but also reaching fine protrusions and endfeet[16,26]. The morphology of these organelles in astrocytes was also confirmed to be rather heterogeneous, yet while a dense meshwork of elongated mitochondria was found in the soma and within main branches, thinner and shorter mitochondria mostly populated peripheral processes, including perineuronal protrusions. With slight variability, this peculiar distribution appears to be maintained across the different layers of the cortex (Figure 1A), yet analysis of mitochondrial network architecture in astroglia of other brain regions including striatum, hippocampus and cerebellum disclosed a certain degree of differences (Figure 1B), on account of essentially distinct astrocytic morphologies. These features therefore indicate that fundamentally distinct sub-populations of astrocytes across the brain may display an equally different architecture of their mitochondrial network, suggesting that these organelles may efficiently adapt to unique morphological constrains imposed by region-specific astrocyte geometries. It is also tempting to speculate that some of the anatomical differences observed in the diverse brain areas may reflect a broader reorganization of astrocytic mitochondrial networks owing to metabolic adaptations to activity patterns of, e.g., specific neuronal circuits or even subtypes of nearby synapses. Interestingly, the observed heterogeneity in astrocytic mitochondrial networks may mirror the increasingly recognized morphological and functional diversity of these cells, which is likely

determined by a combination of both intrinsically encoded factors and external cues provided by the surrounding microenvironment[27].

Mitochondrial trafficking and dynamics in astrocytes *in situ* are regulated

The complex and seemingly heterogeneous structure of the mitochondrial network in astrocytes is highly suggestive of prominent ongoing trafficking and dynamics of mitochondria. In mammals, experiments conducted predominately in neurons revealed that specific adaptor proteins (e.g., Miro and TRAKs) sit right at the core of the mitochondrial trafficking machinery, and mediate the reversible binding of mitochondria to proper motor proteins (kinesin and dynein)[28]. While the exact stoichiometry of these core elements are still debated, at least some of the mechanisms linking mitochondrial transport to synapse function have been proposed (e.g. via Ca^{2+}-sensing domains of the adaptor proteins, see below), as well as their implications in several neurological disorders[29]. On the other side, the proteins regulating mitochondrial dynamics (namely, fusion and fission) and how their dysfunction may lead to secondary alterations in mitochondrial structure and respiratory capacity, are a matter of equally intense investigation due to their direct association to human diseases. Indeed, pathogenic mutations in some of these proteins have been directly linked with the onset of certain classes of hereditary neuropathies, e.g. Charcot-Marie-Tooth disease type 2A[30] and dominant optic atrophy[31,32], as well as to abnormal brain development[33]. The chief mediators of mitochondrial dynamics include several dynamin-related GTP-binding proteins located on the outer and inner mitochondrial membranes, where they promote the catalytic reactions leading to fusion or division of mitochondria[34,35]. Fusion of the outer membrane is governed by mitofusin 1 and 2, while the protein optic atrophy 1 (OPA1) and in particular the balanced proteolytic processing of its several splice variants into long and short isoforms mediates inner mitochondrial fusion/fission dynamics[36]. In contrast, mitochondrial fission at the outer mitochondrial membrane is promoted by the dynamin-related protein 1 (Drp1), a cytosolic protein whose re-localization to/from the membrane is regulated by its phosphorylation state on distinct

serine residues, as well as by other post-transcriptional modifications (i.e., ubiquitylation, S-nitrosylation and SUMOylation)[37]. While the recruitment and subsequent assembly of Drp1 at the precise sites of mitochondrial fission is facilitated by other membrane adaptor proteins (e.g., mitochondrial fission factor, MFF, and mitochondrial fission protein, FIS1)[34,37], recent evidence suggests that the choice of these fission sites may be pre-determined by a local, dynamic rearrangement of nearby ER tubules who first mark and then physically constrict the mitochondrial membrane to allow Drp1 assembly[38]. Maintenance of an efficient mitochondrial network thus seems to be regulated by a complex choreography of membrane contact sites, fission and fusion events, whereby manipulating the expression of any of their regulatory proteins easily destabilizes this delicate balance by producing either a highly fragmented or hyper-tubulated network. Evidence *in vitro* indicates that astrocytes display substantial rates of mitochondrial fusion[39,40] in line with their overtly dynamic and motile network (Figure 2A-B). Further evidence also indicates that astrocytes respond to at least some of the aforementioned gene expression manipulations of dynamin-related proteins by rapidly altering their mitochondrial network morphology[16,41], although the precise consequences of these manipulations in astroglial cells have not been tested systematically yet. Nonetheless, it is reasonably safe to assume that, in glial cells as in other cell types, mitochondrial dynamics may be of key relevance for preserving quality control mechanisms and the intermixing of mitochondrial content (e.g., mtDNA and proteins), and thus facilitate changes in respiratory capacity to meet the evolving cellular demands[35]. It is also important to consider that as for many other cell types, most of these studies have assessed the extent and regulation of astrocytic mitochondrial dynamics in culture, where the phenotypic characteristics of these otherwise highly differentiated cells become significantly altered in terms of morphology and gene expression. For instance, astrocytes in culture lose their tri-dimensional polarized morphology by acquiring a planar geometry, also reflecting the lack of perisynaptic and –vascular processes. Moreover, astrocytes in culture are proliferative, in marked contrast to post-weaning/adult brain tissue, and their gene expression profile is more similar to a de-differentiated progenitor- or

reactive-like state[42]. Interestingly, the advent of accessible confocal and 2-photon microscopy techniques alongside with gene manipulation technology allowed starting to address how astrocyte mitochondrial dynamics and trafficking may be regulated *in situ* in mouse brain explants. Recent imaging work in brain slices following expression of mitochondrially-targeted fluorophores (GFP or similar) revealed that also in these settings, astrocytes possess a certain degree of mitochondrial movement (quantified as displacement), albeit at a level consistently lower than previously observed in culture. For instance, fluorescent labelling of the mitochondrial network via biolistic transfection[43] or viral transduction[44] of astrocytes in cultured organotypic brain slices revealed a predominant proportion of stationary organelles over the course of several minutes of imaging, with only 20-30% of them displaying active movement. Along the same vein, analysis of individual mitoGFP-expressing astrocytes in freshly cut, acute brain slices shows that the extent of mitochondrial displacement remains rather stable at a value of about 20% over the course of up to 2 hours of imaging (Figure 2C-G). While the measurements in these experiments cannot fully discriminate between net trafficking vs. fission/fusion or other remodeling dynamics of mitochondria, they provide a first estimate of their rate of overall temporal "displacement" in living cells within their native tissue. Interestingly, by focusing on peripheral astrocytic processes where isolated mitochondria could be better discerned from the rest of the network, recent works have begun to more specifically investigate the relative trafficking of these organelles in astrocytes, utilizing kymograph analysis similar to what has been previously done for neuronal axonal processes[45]. These studies show that astrocytic mitochondrial trafficking is consistently affected by slice manipulation of neuronal activity, with blockers (e.g., TTX) increasing whereas enhancers (glutamate or electric stimulation) reducing mitochondrial motility[43,44]. While this sort of activity-dependent repositioning of astrocytic mitochondria appeared to correlate with the spatial distribution of synapses of nearby neurons in slices, it is noteworthy to mention that also mitochondrial fusion dynamics - as evaluated with a photo-activatable mitochondrial probe - were sensitive to glutamate application, who produced a decrease in the basal rate of fusion[44].

Therefore, changes in both trafficking and fusion dynamics of mitochondria in cultured brain tissue explants may be modulated by the application of neurotransmitters, suggesting that astrocytes may reorganize their mitochondrial network in register with changes in network excitability. Whether this local reorganization of the mitochondrial network may in turn (directly or indirectly) modulate synaptic efficacy, for instance by contributing to local Ca^{2+} homeostasis in perisynaptic processes or by regulating the availability of transmitters, is unknown. Thus, future studies will have to address the question whether manipulations specifically perturbing either mitochondrial repositioning or fusion dynamics in astrocytes may produce any measurable effects on nearby synapses and, more broadly, on circuit functioning in a physiological context.

Mutual regulation of mitochondrial and Ca^{2+} dynamics in astrocytes

On account of their highly ramified morphology as well as expression of a wide repertoire of membrane receptors, transporters and channels, astrocytes are thought of regulating locally tissue homeostasis, including ions concentration and neuro/glio-transmitter uptake and release[3]. At the core of this capability of sensing and signaling regionalized changes in the activity of, e.g., nearby synapses is a very unique spatiotemporal regulation of Ca^{2+} events[46,47]. In fact, unlike neurons, astrocytes are electrically silent, however they display a surprisingly intense activity in Ca^{2+} dynamics, posing the question which intracellular stores may contribute to precisely shape these complex patterns of Ca^{2+} signaling events. Interestingly, Ca^{2+} transients in astrocytes seem to occur spontaneously, but they can also be triggered by specific stimulation with neurotransmitters and, *in vivo*, by sensory stimuli and locomotor activity[48,49]. Accumulating evidence from *ex vivo* and *in vivo* high-resolution confocal and 2-photon imaging studies suggests that astrocyte display a remarkable variety of Ca^{2+} dynamics, which may help explaining how Ca^{2+} signaling events in these cells can differentially transduce a diversity of stimuli and generate, in turn, specific effects at synapses and perivascular endfeet[47,50]. For instance, a large fraction of the Ca^{2+} events an astrocyte experiences during a typical imaging session of several minutes is essentially

characterized by transients extremely localized (usually spanning few μm), asynchronous in nature and of somewhat variable duration (from very fast to longer-lasting "expanding" transients), in contrast to less frequent but pronounced raises occurring in the cell soma[51]. On the one side, the temporal scale of these localized, fast events can reconcile the presumptive role of perisynaptic astrocytic processes in modulating synaptic function[3]. On the other, the identification of these functionally independent micro- (few μm in size) and expanding Ca^{2+} domains (also referred as to "waves", usually covering an area larger than microdomains) within astrocytic processes, together with their varied kinetics, has led to the hypothesis that their occurrence may facilitate the temporal "integration" of extracellular signals[47], thus strongly suggesting that astrocytes might effectively decode and thus respond to specific patterns of neuronal activity[3]. It is therefore not surprising that substantial effort has been made during the last few years in trying to understand what mechanisms regulate Ca^{2+} homeostasis and microscopic compartmentalization of signaling events in astrocytes[52,53,54,55,56]. Interestingly, the first obvious candidate and major intracellular pathway of Ca^{2+} homeostasis, i.e. the inositol triphosphate (IP3)-dependent release from the ER stores, appears to contribute mainly to large somatic Ca^{2+} events, having only partial effects on the generation and frequency of fluctuations occurring in the astrocytes' processes[54,57]. By contrast, an appreciable amount of local Ca^{2+} events, as detected by utilizing membrane-targeted genetically-encoded calcium indicators, rather relies upon distinct sources, and amongst these, mitochondria have recently emerged as key candidates. Indeed, in a recent study performed in astrocytes *in situ*, mitochondria were not only often found sitting in very close proximity to recorded microdomains but Ca^{2+} efflux in particular through the mitochondrial permeability transition pore (mPTP), a non-selective channel located at the inner mitochondrial membrane, was shown to directly supply these local signaling events[26]. As the authors of this study suggest, these observations may highlight the presumable capability of astrocytic mitochondria to dynamically couple periods of high respiration with the simultaneous generation of discrete Ca^{2+} signaling domains[26]. Indeed, Ca^{2+} efflux through periodic opening of the mPTP has been postulated as a mechanism capable of

ensuring the dissipation of excessive intra-mitochondrial Ca^{2+} accumulation (thus preventing mitochondrial overload and damage) in regions of the cells anyhow subjected to elevated Ca^{2+} fluxes (i.e., IP3R- or transmembrane-mediated)[58]. In this case, transient mitochondrial Ca^{2+} efflux may concurrently contribute to amplify spatially-restricted signaling events. It is established that mitochondrial Ca^{2+} uptake occurs via the low-affinity Ca^{2+} uniporter (MCU), whose opening is modulated by several accessory and regulatory proteins[8]. Interestingly, a number of studies performed in different cell types suggest that under physiological conditions, mitochondrial Ca^{2+} uptake occurs at spatially restricted intracellular locations subjected to particularly elevated Ca^{2+} concentrations[8], as those generated in close proximity (~30-100 nm)[59] to IP3 receptors on the ER (i.e., so-called mitochondria-ER tethering domains)[60], or to Ca^{2+} channels on the plasma membrane. Mitochondrial Ca^{2+} accumulation through MCU complex is then counteracted by the activity of the Na^{+} and H^{+}/Ca^{2+} antiporters located on the inner mitochondrial membrane or possibly even through transient mPTP opening, which altogether modulate the extent and spread of cytosolic Ca^{2+} transients[58,61]. To some extent, this has been also assessed in astrocytes in culture, for instance utilizing mitochondrially-targeted genetic indicators to monitor Ca^{2+} influx/efflux from these organelles[62,63]. The fact that mitochondria have been now directly suggested as key regulators of the "de novo" generation of fast Ca^{2+} events in astrocyte processes is therefore surprising, as not only the exceptionally rapid kinetics of these events would appear in contradiction with the somewhat much slower Ca^{2+} efflux from mitochondria[63], but to take effect this mechanism would also benefit from a long-lasting Ca^{2+} storage capacity in astrocytic mitochondria presumably independent from other surrounding Ca^{2+} sources. In support of this newly-proposed mechanism, experiments conducted following depletion of ER stores via thapsigargin or the use of IP3R2 knock-out astrocytes (in which IP3-dependent Ca^{2+} release from the ER is prevented) revealed that the persisting amount of fast Ca^{2+} events detected was sensitive to pharmacological inhibition of mitochondrial Ca^{2+} efflux[26]. However, experiments conducted in wild-type astrocytes of acute slices perfused with Ca^{2+} free media also demonstrated that these fast events are

primarily dependent on extracellular Ca^{2+} influx[57], suggesting the possibility that rather than generating Ca^{2+} microdomains, mitochondria may contribute to confine their magnitude and spread in proximity of the plasma membrane. Conceivably, in order to efficiently and rapidly influence localized changes in astrocytic Ca^{2+} homeostasis, this mitochondrial-dependent mechanism would require the existence of a widespread mitochondrial meshwork invading the most peripheral processes, which is indeed the type of structure observed in astrocytes (see also paragraph above). Yet, the presumable dynamic nature of the mitochondrial network in astrocytes, together with the well-described spatial constrains of the Ca^{2+} microdomains, would necessitate of mechanisms guiding the precise locations where mitochondria are most needed to exert these functions, and thus an efficient machinery regulating fusion-fission, membrane tethering as well as repositioning of mitochondria would be beneficial to let these organelles not only access but also demarcate sites of higher metabolic demand[64]. Interestingly, work performed *ex vivo* started to address, at least in part, what these mechanisms might be. Not entirely unexpected, motor adaptor proteins such as Miro1 and 2 have been proposed to drive also in astrocytes, as in neurons[45,65], the repositioning of mitochondria in a Ca^{2+}-dependent manner along the cells' processes. Miro1 and 2 are atypical Rho-GTPase proteins equipped with two Ca^{2+}-sensing EF-hands and a C-terminal transmembrane domain that mediates their localization to the mitochondrial outer membrane. Through conformational changes induced by Ca^{2+} binding, these proteins can reversibly associate with kinesin and dynein motors to promote mitochondrial trafficking along microtubules. Although the exact mechanisms regulating these conformational changes are still debated[29], experiments conducted in astrocytes in organotypic slices show that Miro proteins regulate the movement of mitochondria both under basal conditions and following induced stimulation in a Ca^{2+}-dependent manner[44,66]. While mitochondria thus become immobilized at sites of higher Ca^{2+} concentrations, disruption of Miro activity in astrocytes not only impairs the "activity-dependent" motility of their mitochondria, but also seems to influence the occurrence of cytosolic Ca^{2+} signaling domains[44,66]. If proven in a more physiological context, i.e. in preparations *in vivo*, the

fact that Miro proteins may regulate in a calcium-dependent manner mitochondria positioning in astrocyte processes poses the interesting question which primary and secondary mechanisms may mutually orchestrate Ca^{2+} homeostasis at spots of intense activity. Yet, the complex structure of the astrocytic mitochondrial network *in vivo* might suggest that the actual extent of net mitochondrial trafficking might not be as pronounced as observed in preparations *ex vivo* or *in vitro*. Mature protoplasmic astrocytes are indeed densely packed with mitochondria, with only very few of the optically resolved, peripheral fine processes left unoccupied[16,26]. What is more, mitochondria are continuously exposed to an intense variety of Ca^{2+} events throughout the astrocytes' volume, even in basal conditions, as mentioned above. It therefore seems unlikely that these organelles may undergo extensive or very frequent movements along astrocytic processes, in contrast to the much longer axonal processes where overt trafficking has been documented[67], thus suggesting that Miro proteins may preferentially contribute to co-regulate Ca^{2+} buffering capacity independent of their role in mitochondrial transport[68], at least in fully differentiated, adult astrocytes. On the contrary, during early post-natal stages when astrocytes are still maturing and may migrate or proliferate to populate their final locations in the brain[69], or even in a setting of injury in which astrocytes enter a state of reactivity marked by pronounced metabolic changes[42], Miro proteins may effectively play a significant role in regulating the positioning of mitochondria to match the evolving spatial metabolic needs of the cell. It is also not clear to which extent mitochondrial fusion-fission dynamics contribute to regulate, in astrocytes *in situ*, homeostatic changes in Ca^{2+} buffering at cellular microdomains. Growing evidence in astrocyte cultures suggests that this might be the case, as changes in mitochondrial dynamics and remodeling have been linked to Ca^{2+} handling, although these studies point to a primary dependence upon mitochondrial membrane potential and ROS production as the leading events involved in homeostatic Ca^{2+} changes[70,71,72,73,74]. Nonetheless, given the proposed role of at least one protein, i.e. Mfn2, in regulating the extent of mitochondria-ER functional tethering besides mitochondrial fusion in cells[75], it is tempting to speculate a direct involvement of mitochondrial dynamics in maintaining

the heterogeneity in Ca^{2+} signaling domains observed in astrocyte processes[47]. With the concurrent optimization of advanced genetically-encoded indicators and imaging technologies[76,77], future studies will therefore be needed not only to better clarify the actual role of Ca^{2+}-sensing, mitochondrial trafficking proteins in astrocytes *in vivo*, but also the precise mutual regulation of Ca^{2+} homeostasis/signaling and mitochondrial dynamics in these highly differentiated cells.

Mitochondrial dynamics and quality control in astrocytes reacting to injury

Over the course of the last years, astrocytes have gradually but progressively emerged as a group of cells invariably involved in virtually most brain diseases. While current evidence indicates that astrocytes do exert various and important functions in the healthy brain, presumably owning to their increasingly recognized cellular heterogeneity[27], to date we still know surprisingly little about the sub-cellular changes these cells experience when facing challenging and harmful conditions. Astrocytes are well recognized for their capability to markedly up-regulate classic markers of "gliosis" in response to tissue damage, degeneration and inflammation, however they have been also recently re-evaluated in terms of structural and functional changes, including gene expression profile, depending on the type of injury and anatomical location[18]. For instance, several lines of evidence now indicate that astrocytes can significantly adapt their metabolic state to distinct insults, thus revealing a previously unknown layer of complexity in the processes regulating the acquisition of reactive states in these cells[42,78]. While this form of plasticity is likely to involve multiple intracellular pathways and organelles, recent data suggest that the evolving state of reactive astrocytes is mirrored by a corresponding alterations of their mitochondrial network, the central player in energy metabolism and metabolic signalling. For instance, overall changes in mitochondrial markers and ultrastructure have been commonly described in several brain diseases or following traumatic injury/stroke in rodent models as well as human post-mortem material[79,80,81,82,83,84]. Also, marked changes in mitochondrial membrane potential, redox

state and Ca^{2+} influx have been linked in astrocytes, as in neurons, to several types of injury and excitotoxicity[80,85] via mechanisms likely involving the opening of the mPTP, which as mentioned earlier may lead to rapid dissipation of membrane potential and uncoupling of OXPHOS. Interestingly, accumulating evidence suggests that these are also common events occurring during brain trauma and ischemia, as well as in several neurodegenerative disorders, yet most of these data originate from *in vitro* or *post-mortem* studies, thus lacking sufficient molecular understanding to separate cause from consequence in the disease, in particular in the case of astrocytes. Likewise, neuropathologies that accompany proper mitochondrial diseases[86] often involve gliosis in conjunction with neuronal loss and axon degeneration, however whether alterations in astrocytic mitochondria significantly contribute to regulate the onset or progressive accumulation of irreversible tissue damage is unclear. While some studies have thus begun to utilize more sophisticated mouse models to assess the actual possible contribution of astrocytic mitochondrial function/dysfunction in the pathogenesis of brain diseases[87,88,89], the precise mechanisms underlying mitochondrial structural and functional changes during astrocyte reactivity *in vivo* are still poorly understood. One of the reasons for this lack of knowledge appears to be the paucity of methodologies to properly dissect the actual changes in the astrocytic mitochondrial network with sufficient spatiotemporal resolution, and assign them specific roles in influencing the metabolic state of reactive cells. While this task may conceivably require the development and optimization of new genetic tools[90], some promising results in this respect have nevertheless been provided by few recent studies. For instance, we now know that the mitochondrial network in protoplasmic astrocytes responding to cortical injury rapidly undergoes a morphological remodelling which follows the temporal progression of classic traits of reactivity (e.g., GFAP expression levels). In particular, the pro-inflammatory microenvironment resulting from the initial injury insult was shown to trigger a marked but localized reorganization of the otherwise tubular mitochondrial network in astrocytes, by promoting fragmentation and the generation of smaller mitochondria, independent of visible changes in cell viability[16]. These morphological alterations, which are

mediated by the recruitment onto astrocytic mitochondria of the pro-fission protein Drp1, are reminiscent of what has been described in cell culture[91] or in neurons after stroke[92], yet in astrocytes this phenomenon appears to large degree reversible in nature, reflecting the onset of quality-control (and presumably other) mechanisms that may contribute to limit excessive mitochondrial damage[16]. This would be in line with the notion that mitochondrial dynamics in cells contribute to maintain overall mitochondrial functionality, on the one side by promoting mitochondrial content mixing via fusion while on the other favouring segregation and elimination of irreversibly damaged organelles via fission[93]. Thus, the fact that astrocytes respond *in vivo* to stressful insults by dynamically reorganizing their mitochondrial network strongly suggest that this degree of remodelling may play a pivotal role in regulating energetic and metabolic challenges at the site of injury, although the underlying operating mechanisms remain to be addressed. Interestingly, forced astrocytic expression of a mutant isoform of the mitochondrial enzyme superoxide dismutase 1 ($SOD1^{G93A}$), which has been linked to amyotrophic lateral sclerosis and induces neurotoxic phenotypes in astrocytes[94,95,96], causes mitochondrial oxidative stress with ensuing dysregulation of Ca^{2+} homeostasis[26]. Whether this manipulation may mimic some of the features occurring in injury-induced reactive astrocytes is not known, however it reveals a plausible mechanism how damaged mitochondria may aggravate glial cells' reactivity and, possibly, neurotoxicity[88]. While the cell-autonomous implications of these changes in mitochondrial structure and function for astroglial cell reactivity are still poorly understood, and will likely be the focus of intense future investigation, recent works already begun to put forward the hypothesis that astrocytes may be involved in the intercellular transfer of mitochondria with neurons *in vivo*, particularly in a setting of injury. For example, it was proposed that during stroke astrocytes could transfer sill-functional mitochondria to nearby neurons via vesicular exocytosis in order to sustain neuronal metabolic activity and recovery[97], a phenomenon which is reminiscent of what has been previously described in injured lung cells or tumor models[98]. Likewise, reverse vesicular transfer of mitochondria from retinal ganglion axons to astrocytes was also shown as a mechanism of

transcellular mitochondrial recycling[99]. While these observations shed new light on the possible mitochondrial-dependent quality control mechanisms underlying neuroglia cross-talk, they also raise important points of criticism, some of which call for further work in order to corroborate these results[100]. For example, astrocyte-to-neuron transfer of mitochondria has been primarily detected in experiments conducted in cell cultures and utilizing soluble fluorescent dyes[97], which altogether may prevent a rigorous assessment of the specificity of this mechanism, especially in subsequent functional experiments conducted *in vivo*. Also, no real mechanisms were provided in these studies as to how mitochondria might be tagged and separated from the existing network for vesicular loading and transfer. Are fission-fusion dynamics and trafficking at specific perineuronal processes involved in the segregation of these mitochondria, somewhat similar to what has been proposed for cell-autonomous recycling of dysfunctional mitochondria via, e.g., autophagy-mediated mechanisms[93]? And are such processes specific for mitochondria or also take place for other organelles? Future studies will be needed to address these points and clarify what the mechanisms are that regulate astrocyte non- and cell-autonomous trafficking as well as disposal of mitochondria *in vivo*, with particular attention to how this relates to the metabolic cellular state in a setting of disease, where the functional contribution of reactive astrocytes is increasingly recognized[101,102].

Conclusions

Despite growing evidence for an important role of astrocytes in modulating synaptic and vascular functions, still little is known about how these cells may efficiently couple their response to external stimuli with local intracellular changes in their signalling and metabolic state. Our knowledge about the molecular processes underlying these changes is still rudimentary, yet recent progress in imaging techniques and genetic tools allowed to start unveiling a previously unappreciated complexity in the players regulating the type and compartmentalization of the metabolic events occurring throughout the astrocytes' territories. The particularly elaborated and dynamic structure

of the astrocytic mitochondrial network directly implicates mitochondria as potential co-regulators in most of the signalling pathways observed to date in astrocytes. Nonetheless, a systematic analysis of the actual contribution of mitochondria to astrocyte functions *in vivo* is still missing. Revealing the mechanisms underlying mitochondrial remodelling in astrocytes, as well as the interplay between mitochondria and other astrocytic organelles, may enable us to better dissect the evolving metabolic state of these cells while reacting to injury and disease, and thus significantly broaden our understanding of the events regulating tissue damage and repair.

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Conflict of interest

No potential conflicts of interest were disclosed.

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Figure legends

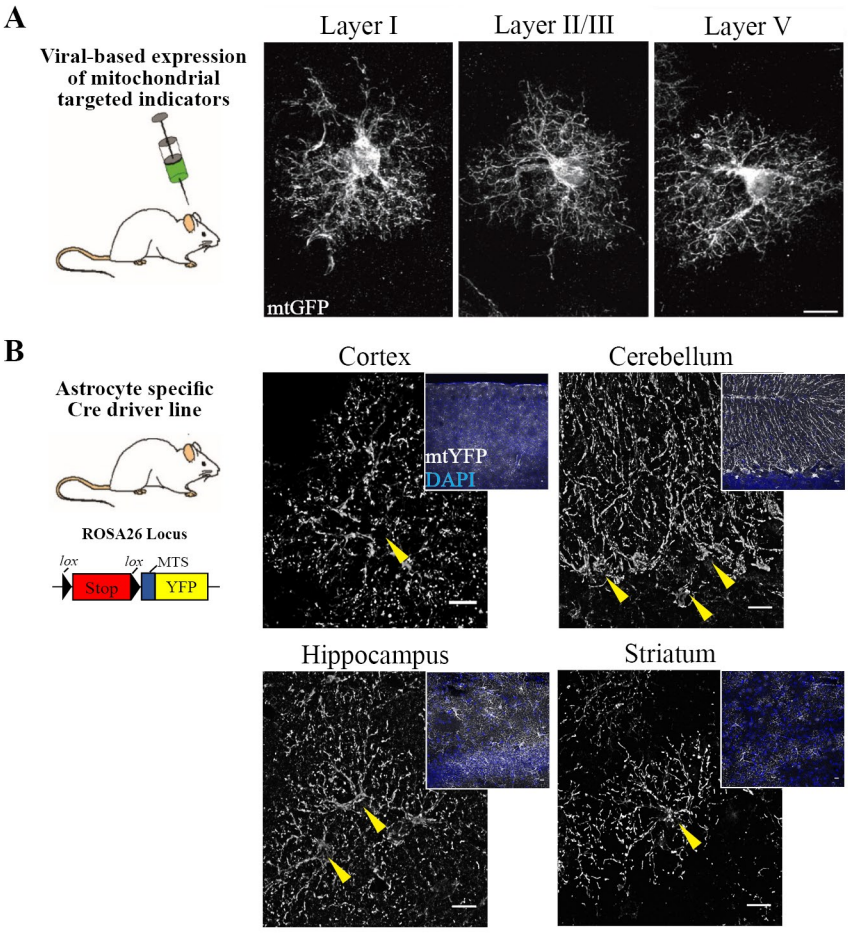
Figure 1: Mitochondrial network architecture in astroglial cells *in vivo*

(A) Schematic illustration of a viral-based approach to express mitochondrial-targeted GFP (mtGFP) in astrocytes. On the right, representative confocal pictures show the elaborated mitochondrial network of reconstructed mtGFP-expressing astrocytes across different layers of the neocortex. Bars 10 µm. **(B)** Utilization of an inducible reporter mouse to express mito-YFP (mtYFP) in the ROSA26 locus[25] specifically in astrocytes. Right images depict representative high-resolution acquisitions of mtYFP-expressing astroglia in different brain regions (see insets) including cortex, cerebellum (Bergmann glia), hippocampus and striatum. The yellow arrowheads point to astroglia cell bodies. Bars 10 µm.

Figure 2: Assessment of mitochondrial motility in astrocytes *in vitro* and *ex vivo*

(A) Time-lapse imaging of astrocytes in culture expressing mtGFP. Images show individual mitochondria tracked over the course of 1 minute and their relative classification according to their motility[16]. **(B)** Color-coded tracks of selected mitochondria are shown. Scale bar: 5 µm. **(C)** Time-lapse sequence of an mtGFP-expressing astrocyte *ex vivo* following viral transduction *in vivo*. Images show the distribution of astrocytic mitochondria during 30 minutes of imaging. Bar 20 µm. **(D)** Superimposition of the frames shown in C. Mitochondria which had moved between frames appear in single colors. Bar 20 µm. **(E and F)** High magnifications of the two areas boxed in D depicting mitochondria stable over time **(E)** compared to a slowly-moving mitochondrion **(F)**. Bars 10 µm. **(G)** Assessment of the overall mitochondrial displacement in four individual astrocytes expressing mtGFP and examined every 10 minutes during 2 hours of imaging.

Figure 1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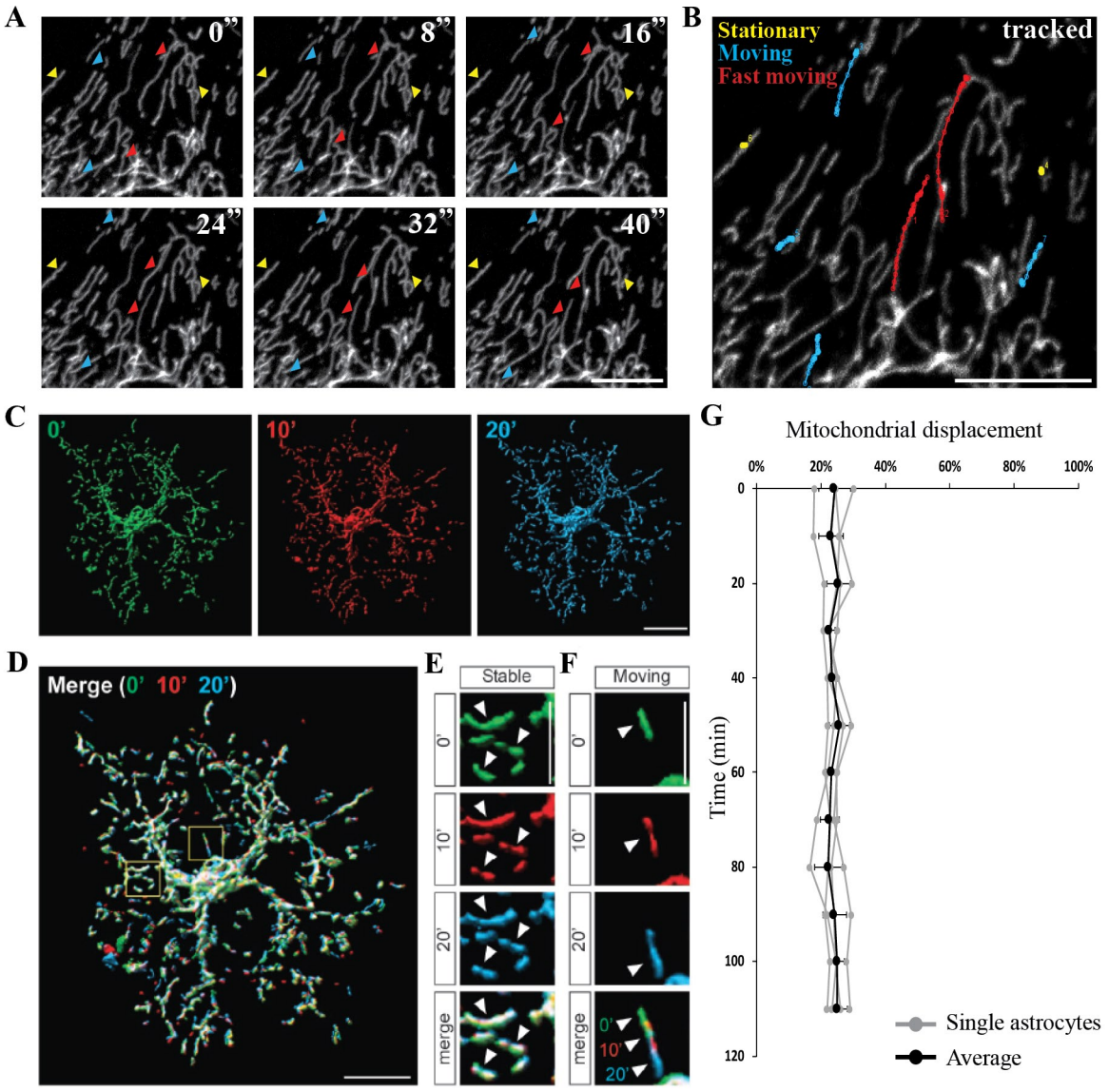
Cerebellum
mtYFP
DAPI
Hippocampus
Striatum

Figure 2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Average

***Conflict of Interest**