Histol Histopathol (2011) 26: 277-284

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Physiopathologic dynamics of vesicle traffic in astrocytes

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Summary. The view of how astrocytes, a type of glial cells, contribute to the functioning of the central nervous system (CNS) has changed greatly in the last decade. Although glial cells outnumber neurons in the mammalian brain, it was considered for over a century that they played a subservient role to neurons. This view changed. Functions thought to be exclusively present in neurons, i.e. excitability mediated release of chemical messengers, has also been demonstrated in astrocytes. In this process, following an increase in cytosolic calcium activity, membrane bound vesicles, storing chemical messengers (gliotransmitters), fuse with the plasma membrane, a process known as exocytosis, permitting the exit of vesicle cargo into the extracellular space. Vesicles are delivered to and are removed from the site of exocytosis by an amazingly complex set of processes that we have only started to learn about recently. In this paper we review vesicle traffic, which is subject to physiological regulation and may be changed under pathological conditions.

Key words: Astrocyte, Vesicles, Mobility, Atrial Natriuretic Peptide, Glutamate

Introduction

During the last decade a significantly new view of how glial cells function in the central nervous system (CNS) has emerged. For over a century the paradigm was held that these brain cells, consisting of astroglia, oligodendroglia and microglia, are subservient to neurons.

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Astrocyte, the most abundant glial cell type in the brain, is now known to be an important player in integrating functions of brain cells, especially neurons. Astrocytes provide metabolic support to neurons (Nedergaard et al., 2003), integrate neuronal transmission (Haydon, 2001), regulate synaptogenesis (Stevens, 2008), brain microcirculation (Anderson and Nedergaard, 2003; Zonta et al., 2003; Gordon et al., 2007) and are implicated in pathologic immune response (Dong and Benveniste, 2001; De Keyser et al., 2003). Several gliotranmitters have been described in astrocytes (Martin, 1992; Parpura et al., 1994) along with their mode of release. One of the increasingly recognized types of gliotransmitter release is Ca^{2+}-dependent exocytosis (Parpura and Zorec, 2010; Parpura et al., 2010), which occurs in response to neurotransmitter release from neurons (Porter and McCarthy, 1996; Pasti et al., 1997; Kang et al., 1998; Araque et al., 2002). Until now it was recognized that Ca^{2+}-dependent exocytosis mediates glutamate release (Bezzi et al., 2004), D-serine release (Martineau et al., 2006, 2008), release of adenosine 5'-triphosphate (ATP, Coco et al., 2003; Pangršič et al., 2007) and peptides, such as atrial natriuretic peptide (Kržan et al., 2003), brain-derived neurotrophic factor (BDNF, Jean et al., 2008) and neuropeptide Y (NPY, Ramamoorthy and Whim, 2008). Gliotransmitters are released into the extracellular space from vesicles and transported to the cell surface where they get fused with the plasma membrane. Transport of astrocytic vesicles is guided to the plasma membrane by cytoskeleton (Potokar et al., 2005, 2007).

Cytoskeleton in astrocytes

In general, membrane bound vesicles in the cytoplasm of eukaryotic cells are transported along the cytoskeleton. This transport is modulated by the dynamics of cytoskeleton filaments, different types of

molecular motor proteins, and is controlled by a pleiad of regulatory and signaling molecules (Chang and Goldman, 2004). Besides ubiquitous microtubules and actin filaments, astrocytes also express specific intermediate filaments (IF), which form a third part of the cytoskeleton in astrocytes. IFs are differently expressed in various developmental and physiological stages of astrocytes, and consist of glial fibrillary acidic protein (GFAP), vimentin (Fig. 1), nestin and synemin (Pekny et al., 1999; Pekny and Pekna, 2004; Jing et al., 2007). GFAP and vimentin form IF cytoskeleton in nonreactive astrocytes in the adult CNS (Pekny and Pekna, 2004). In contrast, in reactive astrocytes under pathological conditions, along with increased expression of GFAP and vimentin, nestin and synemin filaments are also formed (Eliasson et al., 1999; Jing et al., 2007). Unlike actin filaments and microtubules, intermediate filaments are devoid of enzymatic activity and are the least understood part of the cytoskeleton (Eliasson et al., 1999; Chang and Goldman 2004; Pekny and Pekna, 2004). The cytoskeleton is a highly dynamic structure which is altered in astrocytes by different physiologic stimuli and by various pathologies (Potokar et al., 2008). Several key observations of cytoskeleton-dependent vesicle traffic in astrocytes have been published in recent years and are reviewed in the present paper.

Properties of vesicle traffic along the secretory pathway

The characteristics of single vesicle traffic in astrocytes were first described in primary cultured rat astrocytes (Potokar et al., 2005). To fluorescently label single vesicles the fusion protein between ANP and emerald green fluorescent protein (proANP-Emd, Han et al., 1999) was used. Parameters to describe vesicle mobility, such as speed, step length, track length, maximal displacement, and mean square displacement, were calculated (Qian et al., 1991; Saxton, 1993; Wacker et al., 1997; Oheim and Stühmer, 2000; Potokar et al., 2005). Vesicles containing recombinant peptide proANP-Emd revealed distinct types of vesicle mobility (directional and non-directional, Fig. 2), similar to other cell types (Burke et al., 1997; Tvarusko et al., 1999; Duncan et al., 2003; Hill et al., 2004). The directional mode of vesicle mobility (described for maximal displacements above 1 μm, Fig. 2B) was observed as tracks close to a straight-line appearance, frequently interrupted by switches in speed and direction. Maximal displacement is a measure for the net translocation of a vesicle (Wacker et al., 1997, Fig. 2A) and is used for the determination of vesicle directionality (Fig. 2B). The average speed of directional proANP-Emd vesicles (0.5±0.01 μm/s) was comparable to the directional movement of vesicles in neurons (Grafstein and Forman, 1980). Only a subpopulation of proANP-Emd vesicles (35%) exhibited directional mobility (Potokar et al., 2005). This is similar to records of synaptobrevin2-EGFP (Syb2-EGFP) tagged vesicles in astrocytes, where a subpopulation of vesicles (25%) is highly mobile and fuses with the plasma membrane upon stimuli that trigger elevations in concentration of free calcium ions

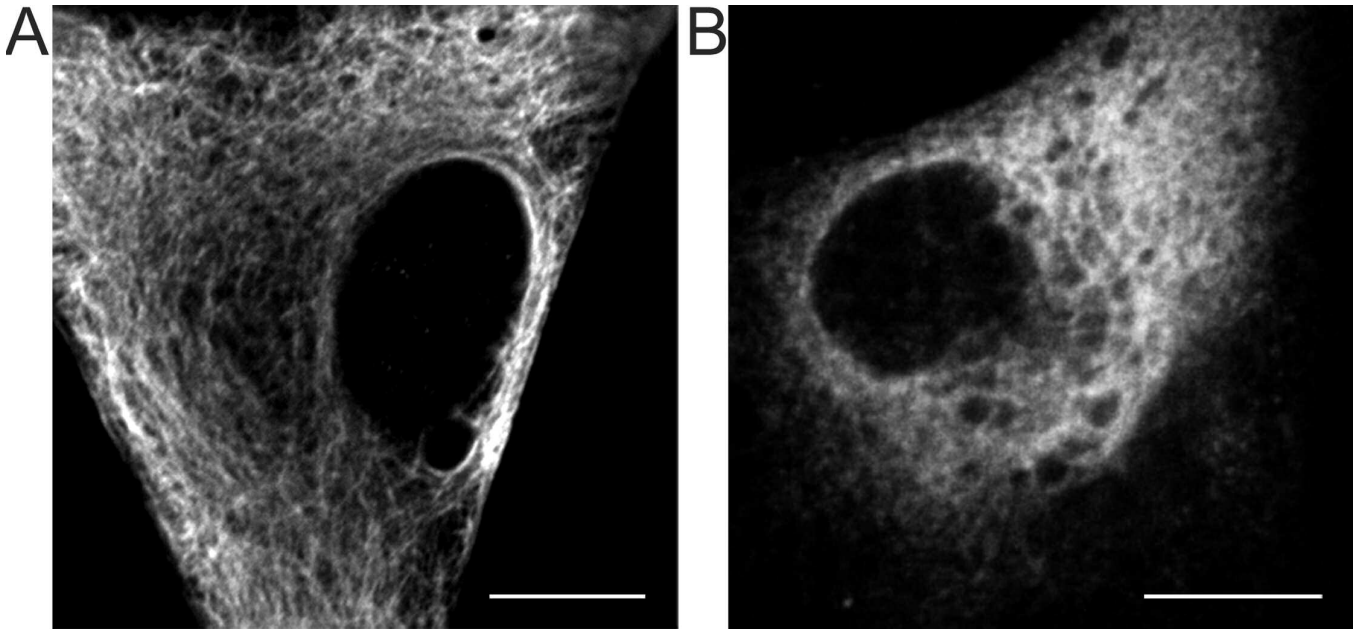

Fig. 1. Intermediate filaments in rat astrocytes. Astrocytes in the adult CNS express two forms of intermediate filaments, as shown in rat astrocytes: vimentin **(A)** and glial acidic fibrilary protein (GFAP, **B**). Filaments were labeled with rabbit primary antibodies against vimentin (1:200, Abcam) or GFAP (1:700, Chemicon) and goat secondary antibodies against rabbit IgG (1:600, Molecular Probes). Bars: 10 μm.

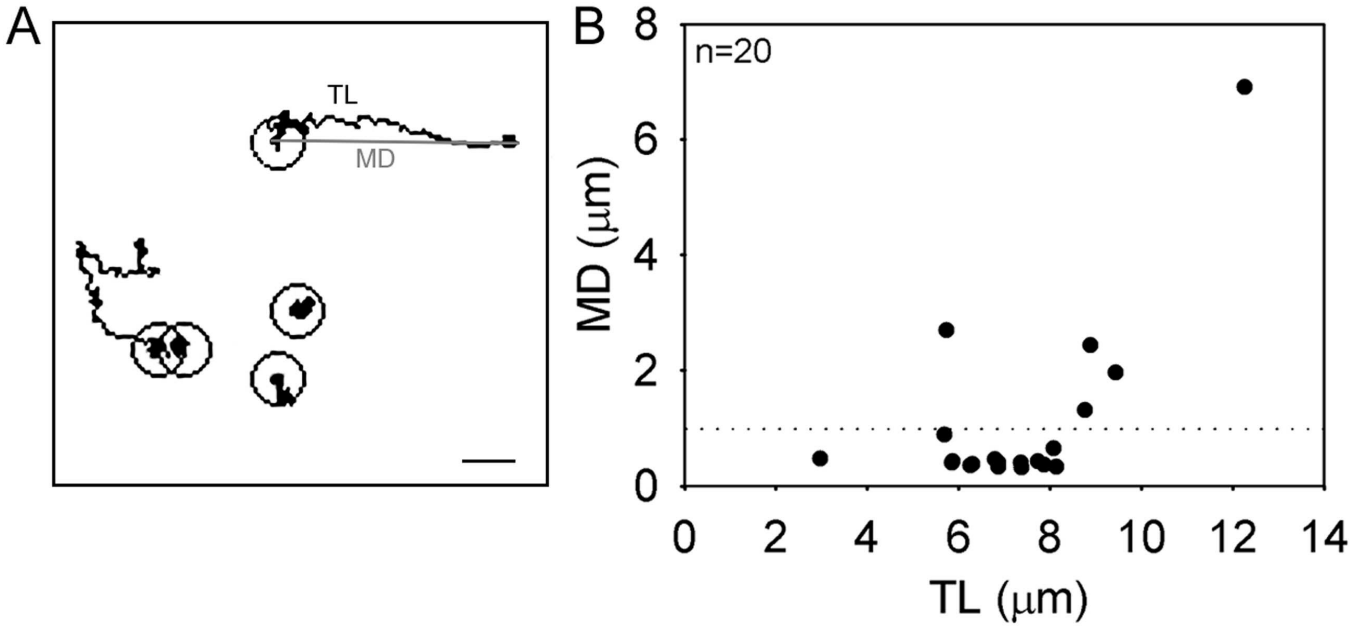

Fig. 2. Directional and non-directional mobility of vesicles. Vesicle trajectories (pathways, tracks) of five vesicles in 2 min recording time. **A.** Encircled are vesicle positions at the beginning of mobility analysis. Vesicles tend to move in a directional manner (black extended trajectories) or in a non-directional manner during the recording time (black clumped trajectories). Maximal displacement (MD, gray) is the distance between the two most distant points in vesicle trajectory. Bar: 2.5 μm. Ratio between MD and trajectory length (TL) represents vesicle directionality. **B.** MD and TL are calculated for 30 s periods, n = number of analyzed periods. Peptidergic ANP-Emd vesicles with MD lower that 1 μm (dotted line) are described as non-directional in astrocytes.

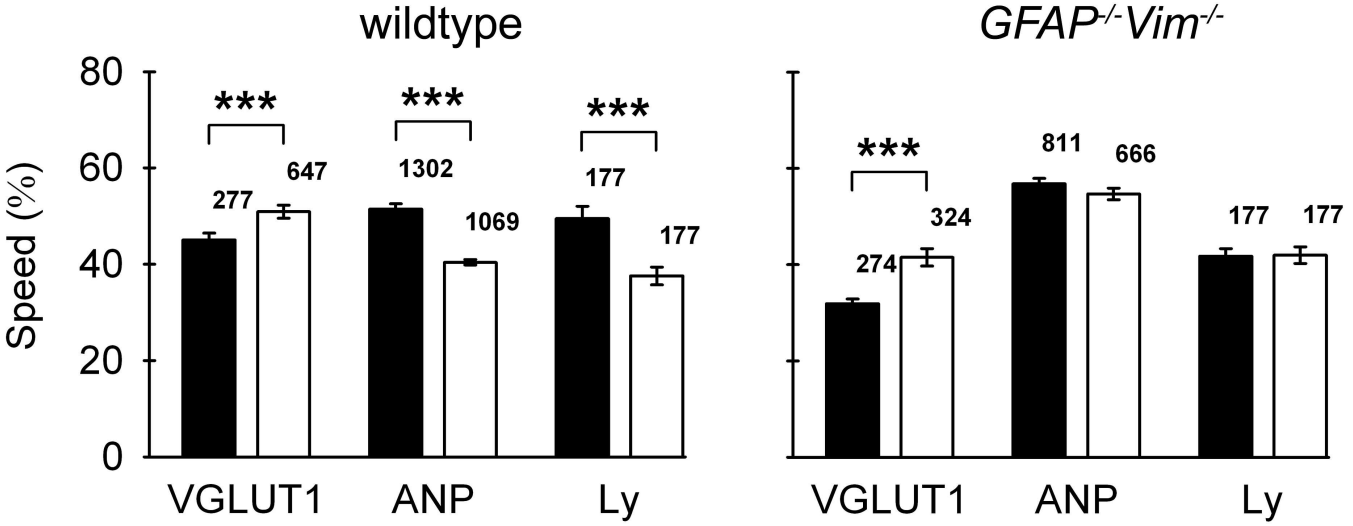

Fig. 3. Stimulation-dependent vesicle speed modulation is affected by intermediate filaments. Relative change (%, in reference to 95 percentile) of speed values in VGLUT1-vesicels, ANP-vesicles and Lysotracker-labelled vesicles in mouse astrocytes. Spontaneous (non-stimulated, black bars) and ionomycin-stimulated (to increase cytosolic calcium, white bars) mobility of VGLUT1, ANP vesicles and the mobility of Lysotracker-labeled vesicles (Ly) in cells with IFs (wildtype), and in mutated cells devoid of intermediate filaments (IFs; *GFAP$^{-/-}$Vim$^{-/-}$*). Note the differential effects of stimulation on the mobility of stained organelles. In general, the mobility of VGLUT1-vesicles increased following cell stimulation. In contrast, the mobility of ANP vesicles and Lysotracker-stained organelles diminished following stimulation in wildtype cells; in *GFAP$^{-/-}$Vim$^{-/-}$* cells the mobility either diminished slightly or was not affected by stimulation. Bars indicate relative average speed (±SEM in %) of stained organelles. The numbers above the bars indicate the number of analyzed vesicles. ***P<0.01 vs control (non-stimulated). Figure was modified from Potokar et al., 2010. Reprinted with permission of John Wiley & Sons, Inc..

in the cytoplasm $[Ca^{2+}]_i$ (Crippa et al, 2006). The disappearance of proANP-Emd vesicles after triggered increase in $[Ca^{2+}]_i$ was also observed, although without changes in speed measured 1 min after triggering a rise in $[Ca^{2+}]_i$ (Kržan et al., 2003; Potokar et al., 2007). Interestingly, the average directional speed of proANP-Emd and Syb2-EGFP vesicles is very similar, 0.50±0.01 μm/s and 0.65±0.04 μm/s, respectively.

The directional mobility of proANP-Emd vesicles was further explored to reveal a role of the astrocyte cytoskeleton in vesicle traffic. Fluorescently tagged proANP-Emd vesicles were shown to be transported with high speed along microtubules, but other types of the cytoskeleton (actin and intermediate filaments) were also shown to be importantly involved in their traffic (Potokar et al., 2007). For example, after de-polymerization of actin filaments, vesicle mobility was severely impaired, indicating the role of actin filaments in their traffic. Similar observations were discovered in ARPE-19 human retina cells, where vesicles remained trapped in a mesh of depolymerized actin filaments and remained mobile by free diffusion (Aschenbrenner et al., 2004). Actin filaments seem to play different roles in specific types of cells. In contrast to observations in astrocytes and retinal pigment epithelial cells, in PC12 neuroendocrine cells actin filaments trap vesicles and constrain their access to microtubule tracks (Rudolf et al., 2001). Novel discoveries were also described for the role of intermediate filaments in astrocytes; in addition to primarily maintaining cell shape (Chang and Goldman, 2004) they affect vesicle traffic of secretory vesicles as well (Potokar et al., 2007). In astrocytes lacking IFs *(GFAP$^{-/-}$Vim$^{-/-}$*, Eliasson et al., 1999) the fraction of vesicles with directional mobility was lower than in wild type astrocytes (Potokar et al., 2007). These data are in agreement with the hypothesis that IFs have a role in vesicle traffic (Styers et al., 2005). These data are in favor of the hypothesis that intermediate filaments are required for long-range directional vesicle mobility, by acting as a three-dimensional lattice (Potokar et al., 2007).

Traffic properties of glutamatergic and peptidergic recycling vesicles and endocytotic vesicles

After exocytosis, the process where fusion of the vesicle membrane and plasma membrane occurs, the fusion pore may be resealed and the vesicle is retrieved into the cytoplasm (Jahn and Sudhof, 1999; Valtorta et al., 2001; Taraska et al., 2003). Not only in small synaptic-like vesicles containing amino acids, but the same process has also been proposed for larger peptidergic vesicles (secretory granules) and was referred to as granule cavity recapture or granule recapture; similar to the "kiss-and-run" exocytosis of synaptic vesicles (Taraska et al., 2003). In three studies the traffic of peptidergic and glutamatergic recycling vesicles in astrocytes has been extensively studied. Both types of vesicles were fluorescently labeled by antibodies, which were taken up into the vesicle lumen when fused vesicles captured extracellularly applied antibodies. Such vesicles were termed recycling or retrieving vesicles (Stenovec et al., 2007; Potokar et al., 2008). There are two possible ways of how this labeling was obtained; first, vesicles that were labeled have had their lumen exposed to the extracellular milieu via the fusion pore several times before they were retrieved back into the cytoplasm. Or second, multiple cycles of fusions and retrievals of vesicles may also explain the staining of vesicles by primary antibodies first and subsequently by secondary antibodies. This vesicle labeling is not unique to peptidergic vesicles, since a similar method was used to stain synaptic vesicles by Sara et al. (2005), where vesicles were labeled first with primary antibodies against synaptotagmin I and subsequently with secondary antibodies.

In rat, retrieving peptidergic vesicles containing ANP (ANP-vesicles) and retrieving glutamatergic VGLUT1-vesicles are acidic and a third of them are cytochemically positive for synaptotagmin IV (SytIV), a protein proposed to be essential for regulated exocytosis in astrocytes (Zhang et al., 2004; Stenovec et al., 2007; Potokar et al., 2008). These vesicles are small, between 45 and 50 nm in diameter, as shown by immunogold electron microscopy studies (Stenovec et al., 2007; Potokar et al., 2008). The traffic of retrieving vesicles has different properties from peptidergic vesicles in the post-synthetic secretory pathway, where vesicles travel to the plasma membrane. They are one order of magnitude slower than post-synthetic proANP-Emd vesicles (ANP-vesicles: 0.06±0.001 μm/s, VGLUT1 vesicles: 0.05±0.001 μm/s). These velocities are similar to the measured velocity of ATP-containing vesicles (Pangršič et al., 2007; Potokar et al., 2008).

The role of the cytoskeleton in retrieving vesicle traffic was determined by disintegration of microtubules, actin filaments and vimentin intermediate filaments. In all cases significant reductions in vesicle track length, maximal displacement and speed of ANP-retrieving and VGLUT1-retrieving vesicles were observed (Stenovec et al., 2007; Potokar et al., 2008). This is consistent with results where perturbations of actin filaments were shown to impair the retrieval of synaptic vesicles to the plasma membrane (Shupliakov et al., 2002) and with the impaired traffic of G-protein-coupled receptor CB1R in astrocytes when the dynamics of actin filaments or microtubules was perturbed (Osborne et al., 2009). A similar result was obtained on peptidergic vesicles in pituitary cells with depolymerized actin cytoskeleton (Chowdhury et al., 2002). Along with actin filaments, the vesicle recycling process was shown to require an interaction of endocytotic organelles with tubulin cytoskeleton (Gruenberg et al., 1989; Apodaca, 2001; Hoepfner al, 2005). Recent data also revealed a role of intermediate filaments in retrieving vesicle mobility in astrocytes (Gillard et al., 1998; Stenovec et al., 2007;

Potokar et al., 2008). In pathologic CNS conditions, such as neurotrauma, brain ischemia or neurodegenerative diseases, astrocytes become reactive (Eddleston and Mucke, 1993; Pekny and Nilsson, 2005), hypertrophic and up-regulate a number of proteins; a hallmark of this reaction is the up-regulation of intermediate filament (IF) proteins, GFAP, vimentin, nestin and synemin (Pekny et al., 1999; Pekny and Pekna, 2004; Jing et al., 2007). Increased expression of intermediate filament proteins appears to have a specific effect on vesicle traffic and consequently on the delivery of vesicle cargo to the plasma membrane (Potokar et al., 2007), transporter recycling (Stenovec et al., 2008) and recycling of signalling molecules implicated in the cell–cell communication (Fiúza and Arias, 2007). Moreover, the newest findings show that astrocyte IFs differentially affect the stimulation-dependent mobility of recycling vesicles and endosomes (Potokar et al., 2010). Stimuli that increase $[Ca^{2+}]_i$ enhance the mobility of VGLUT1-positive glutamatergic vesicles, and inhibit the mobility of ANP-positive peptidergic vesicles in wild type astrocytes and both effects seem to be attenuated by the absence of IFs. On the other hand, the mobility of endosomes/lysosomes labelled by Lysotracker dye in stimulated conditions was severely reduced in the absence of IFs (see Fig. 4 in Potokar et al., 2010). These vesicles were slow in comparison to the pre-fusion peptidergic vesicles and exhibited mobilities of recycling vesicles. Their mobility was however increased by stimulation in the absence of IFs (Potokar et al., 2010).

Additionally, *in vivo* imaging of recycling vesicles was recently reported in brain slices, a preparation which is physiologically closer to that occurring *in vivo*. Here, cell-to-cell contacts are preserved and tissue architecture is closer to the one present in the brain (Potokar et al., 2009). The mobility of specifically labeled recycling glutamatergic and peptidergic vesicles (immuno positive for VGLUT1 or ANP, respectively) exhibited similar mobilities to those in cultured astrocytes (Stenovec et al., 2007; Potokar et al., 2008).

An increase in $[Ca^{2+}]_i$ differently affects vesicle mobility of distinct recycling vesicles

Neurotransmitters trigger an increase in $[Ca^{2+}]_i$ in astrocytes through metabotropic and ionotropic receptors (Araque et al., 1999; Alvarez-Maubecin et al., 2000; Haydon, 2001; Halassa and Haydon, 2010), which are over-stimulated in pathophysiologic conditions (Koizumi, 2010).

Experimentally, a $[Ca^{2+}]_i$ rise in astrocytes is predominantly triggered by different stimulations: ATP (Bennett et al., 2005; North and Verkhratsky, 2006; Pangršič et al., 2006), glutamate (Cornell-Bell et al., 1990; Dani et al., 1992; Porter and McCarthy, 1996) and ionomycin (Kržan et al., 2003; Porras et al., 2004; Oguri et al., 2006; Pangršič et al., 2006). It has been reported that an increase in $[Ca^{2+}]_i$ does not cause a significant difference in the mean velocity of proANP-Emd labeled vesicles (0.29 ± 0.01 μm/s before stimulation and 0.27 ± 0.01 μm/s after stimulation) (Ng et al., 2002; Potokar et al., 2007). In contrast, the triggered increase in $[Ca^{2+}]_i$ strongly reduced the mobility of recycling ANP-vesicles (Potokar et al., 2008); as was observed for ATP-vesicles in astrocytes (Pangršič et al., 2007) and endocytotic vesicles in wild type astrocytes (Potokar et al., 2010). Their velocity decreased and directional mobility was lost (Pangršič et al., 2007; Potokar et al., 2008). At elevated $[Ca^{2+}]_i$ all ATP-vesicles were significantly slower and it appears that they are kept within specific cell domains, close to the plasma membrane for a prolonged period of time (Pangršič et al., 2007; Pryaznikov and Khiroug, 2008). $[Ca^{2+}]_i$ may affect vesicle traffic through remodeling the cytoskeleton by actin associated proteins (Steinmetz et al., 1997; Potokar et al., 2008). However, a specific role of $[Ca^{2+}]_i$ in vesicle traffic is yet to be determined. The task to elucidate the role of calcium in vesicle traffic seem to be even more demanding, since it appears that different vesicle types are distinctly regulated by Ca^{2+}. For example, peptidergic proANP-Emd labeled vesicles in nerve terminals respond to a $[Ca^{2+}]_i$ rise with mobilization of apparently restrained vesicles (Shakiryanova et al., 2005). This is the opposite of what was found for proANP-Emd vesicles in astrocytes (Potokar et al., 2007) and similar to VGLUT1 vesicles, which accelerated their mobility in increased $[Ca^{2+}+]_i$ (Stenovec et al., 2007; Potokar et al., 2008).

Conclusions and future perspectives

Novel potential implications of astrocytes in brain functioning urge us to thoroughly understand underlying mechanisms and their communication with neighbouring neurons, endothelial and other types of glial cells. One of the key aspects in this quest is to understand the delivery of molecules in membrane-bound vesicles to the plasma membrane and their retrieval from the plasma membrane following their lumen exposure to the extracellular milieu. Recent studies of single vesicle traffic in astrocytes revealed the properties of glutamatergic and peptidergic vesicle mobility and their specific responses to extracellular stimuli. These responses appear to be specific for a particular type of vesicle and detailed mechanisms, together with the consequences on astrocyte physiology, remain to be unraveled by further studies. In future it will be important to identify molecules specific for regulation of trafficking of specific vesicle types and how vesicles interact with the cytoskeleton. It appears that all vesicles in astrocytes do not respond to altered physiological and pathological conditions in the same pattern, therefore this finding may represent a new cellular paradigm of how astrocytes contribute to the phenotype of brain function in physiological and pathological conditions.

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Accepted October 8, 2010