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Trafficking of astrocytic vesicles in hippocampal slices

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Abstract

The increasingly appreciated role of astrocytes in neurophysiology dictates a thorough understanding of the mechanisms underlying the communication between astrocytes and neurons. In particular, the uptake and release of signaling substances into/from astrocytes is considered as crucial. The release of different gliotransmitters involves regulated exocytosis, consisting of the fusion between the vesicle and the plasma membranes. After fusion with the plasma membrane vesicles may be retrieved into the cytoplasm and may continue to recycle. To study the mobility implicated in the retrieval of secretory vesicles, these structures have been previously efficiently and specifically labeled in cultured astrocytes, by exposing live cells to primary and secondary antibodies. Since the vesicle labeling and the vesicle mobility properties may be an artifact of cell culture conditions, we here asked whether the retrieving exocytotic vesicles can be labeled in brain tissue slices and whether their mobility differs to that observed in cell cultures. We labeled astrocytic vesicles and recorded their mobility with two-photon microscopy in hippocampal slices from transgenic mice with fluorescently tagged astrocytes (GFP mice) and in wild-type mice with astrocytes labeled by Fluo4 fluorescence indicator. Glutamatergic vesicles and peptidergic granules were labeled by the anti-vesicular glutamate transporter 1 (vGlut1) and anti-atrial natriuretic peptide (ANP) antibodies, respectively. We report that the vesicle mobility parameters (velocity, maximal displacement and track length) recorded in astrocytes from tissue slices are similar to those reported previously in cultured astrocytes.

Keywords

Astrocyte; Brain Slice; Glia; Cytoskeleton; Trafficking

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Introduction

Astrocytes are one of the most intensively studied types of brain cells in recent years [1–4]. They appear to be actively implicated in a number of processes important for functioning of the nervous system [2,3,5], therefore it is of key importance to understand the mechanisms that underlie the communication between astrocytes and neighboring cells, especially neurons. Astrocytes take up and release a number of substances which affect neuronal physiology and neurotransmission. The release of gliotransmitters from astrocytes may occur through several mechanisms, all of which are under intense investigation. One of the confirmed ways of substance discharge from astrocytes is regulated exocytosis [6–11]. In exocytosis, involving the merging of the vesicle and the plasma membrane, a fusion pore is formed that connects the vesicle lumen with the extracellular space. This allows labeling of vesicles which recycle back to the cytoplasm [12], and specific labeling and mobility characterization of recycling vesicles was recently reported in cultured live rat astrocytes [12,13]. Whether vesicles in intact tissue exhibit similar properties to cultured cells, is unclear. Therefore, we here labeled recycling vesicles in astrocytes in hippocampal tissue slices, since brain tissue slices represent a preparation which is physiologically closer to that occurring *in vivo*, i.e. it preserves cell-to-cell contacts and tissue architecture as present in the brain.

The results show that the mobility of specifically labeled recycling glutamatergic vesicles and peptidergic granules (immuno positive for vGlut1 or ANP, respectively) in astrocytes from tissue slices, determined by two-photon microscopy, exhibit similar properties to those in cultured astrocytes [12,13].

Materials and Methods

Brain slices

The images were acquired in the Phil Haydon's Laboratory at Department of Neuroscience, University of Pennsylvania. All procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Institutional Animal Care. Cortical–hippocampal slices (300–340 μm) were obtained from wild-type mice (C57BL/6J) at postnatal days 10–13 and from one month old GFP mice [14]. The mice were anesthetized with halothane inhalation (1–2 drops of halothane in an anesthetization chamber, 10cm $\times$ 10 cm $\times$ 12 cm). After cervical dislocation the brain was removed and put in an ice-cold artificial cerebrospinal fluid (aCSF) solution containing (in mM): 124 NaCl, 3.1 KCl, 1.25 $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 26 NaHCO_3 , 10 D-glucose, 2 MgCl_2 , 1 CaCl_2 at pH 7.4 (with O_2 95%, CO_2 5%). After removal of the cerebellum, the brain was glued and horizontal slices (300 μm) were cut using a vibratome (VT1000S; Leica, Mannheim, Germany). Prior to experiments, slices were incubated in the aCSF at 35° C for at least 1 h. Slices from wild-type mice were bulk loaded for 1.5 h at room temperature in aCSF containing Fluo4 (12.5 $\mu\text{g}/\text{ml}$, Invitrogen, Carlsbad, CA) and pluronic acid (1 $\mu\text{l}/\text{ml}$ of 20% DMSO solution, Invitrogen, Carlsbad, California, USA) saturated with 95% O_2 and 5% CO_2 to label astrocytes [15]. Experiments were performed in sets up to 5 slices. In some of experiments, following Fluo4 loading, slices were transferred to aCSF containing sulforhodamine 101 (SR101, 25 μM , Invitrogen, Carlsbad, California, USA) for 10 min, to selectively label astrocytes [16]. Fluo4 and SR101 co-labeled slices were imaged with A1 confocal microscope (Nikon, Tokyo, Japan).

Vesicle labeling and imaging

Vesicles were labeled with primary and secondary antibodies; primary antibodies used were Rabbit polyclonal to vGlut1 (1:2000, Synaptic Systems, Goettingen, Germany) and Rabbit polyclonal to atrial natriuretic peptide (ANP) (1:600, Abcam, Cambridge, UK). Secondary

antibodies were against rabbit IgG conjugated to the fluorescent dye Alexa Fluor 546 (1:600; Invitrogen, Carlsbad, California, USA). Anti-ANP antibody recognizes ANP in the vesicle lumen and anti-vGlut1 antibody recognizes epitopes on the cytoplasmic, transmembrane and luminal loop of the vGlut1 transporter [17].

Slices were briefly rinsed in normal aCSF which contained 124 NaCl, 3.1 KCl, 1.25 $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, 26 NaHCO_3 , 10 D-glucose, 1 MgCl_2 , 2 CaCl_2 at pH 7.4 (with O_2 95%, CO_2 5%). Vesicles were then labeled by the following procedure: slices were washed in 3% bovine serum albumin (BSA) in aCSF and sequentially incubated with primary and secondary antibodies diluted in 3% BSA in aCSF at RT (20 min with each antibody). Between incubations with different antibodies slices were rinsed three times in aCSF (2 min). Following the labeling, slices were rinsed two times in aCSF (2 min) and transferred into recording chamber for imaging. During labeling, rinsing and imaging slices were continuously kept in an aerated aCSF (O_2 95%, CO_2 5%).

Labeled slices were put into recording chamber. Fluorescently labeled vesicles in brain slices were observed with a two-photon microscope by using an Ultima scanhead (Prairie Technologies, Middleton, WI, USA) attached to an Olympus BX51WI microscope equipped with a $\times 60$ water-immersion objective. Excitation for Alexa Fluor 546 was provided at 840 nm, and emission was detected by the external photomultiplier tube (607/45 nm). Time series images were recorded in 2 s intervals.

Analysis

Vesicle mobility was analyzed by ParticleTR software (Celica Biomedical Center, Ljubljana, Slovenia). The parameters of vesicle mobility were determined as described [18]. Briefly, the current time (time from the beginning of tracking for a single vesicle), step length (displacement of a vesicle in the time interval of 2 s), track length (the total length of the analyzed vesicle pathway), directionality index (the ratio between the maximal displacement and the track length), velocity and maximal displacement of vesicles were estimated as described previously [13,18,19]. In recordings where the same particle was present in all analyzed images and in which the tracks of particles do not intercept, tracking and analysis was fully automatic. When the tracks of particles intercepted, the tracking was stopped. Vesicle and granule mobility was analyzed in up to 13 cells from wild-type mice and in up to 12 cells from GFP mice (for ANP and vGlut1 vesicles, respectively). The analysis of the vesicle mobility was performed for epochs of 30 s. Statistical significance was determined with the two-tailed *t* test. Values are expressed as mean $\pm$ s.e.m. Values were considered significantly different when $p < 0.001$.

To determine the extent of Fluo4 and SR101 co-labeling inside astrocytes we counted cells in the green channel (Fluo4), the red channel (SR100) and the merged channel (cells simultaneously labeled with Fluo4 and SR101). The extent of co-labeling is expressed as mean $\pm$ s.e.m.

Results and Discussion

While most of the primary physiological phenomena are studied in cultured single astrocytes, brain slice preparations are being widely used to study the function of astrocytes, mainly to exclude the possibility that phenomena described in cultured cells are not due to an artifact of culturing conditions. Therefore, we here considered whether retrieving vesicles can be stained in acute brain slices, similar to what was shown in primary astrocyte cultures [12,13]. Astrocytes in slices from wild type (WT) mice were identified by pre-labeling with Fluo4 dye, which was observed as green fluorescence (Figure 1C), consistent with previous reports [15, 16]. The SR101 dye which labels astrocytes [16] was highly colocalized with Fluo4 labeled cells ($94 \pm 1\%$, n (Fluo4 labeled cells) = 222). Astrocytes in slices from GFP mice were identified

via the specific expression of the GFP protein in these cells. Therefore astrocytes were easily determined in tissue slices under the microscope. Vesicles and granules were observed in cell soma and major processes of labelled astrocytes. In different sets of experiments we incubated slices from wild-type and GFP mice with antibodies either against the granule cargo protein: atrial natriuretic peptide (ANP) or against a protein integrated into the vesicle membrane: vesicular glutamate transporter 1 (vGlut1), a hallmark for glutamatergic vesicles. Both ANP and glutamate have been described as gliotransmitters [1,20].

The recording of vesicle/granule mobility with two-photon microscopy was performed 44 μm deep from the tissue slice surface, in indicating that the antibodies penetrated to the level of the focal plane through the extracellular space before they have been taken up by astrocytes or, alternatively, they were taken up by astrocyte processes at the slice surface and were transported into the astrocyte along the cytoskeleton. The role of the cytoskeleton in astrocytes was previously shown to play a crucial role in micrometer distances transport of secretory vesicles of cultured cells [12].

According to the afore-described protocol (Materials and Methods, Vesicle labeling) primary antibodies were applied first (Figure 1A2). After the uptake of secondary antibodies, presumably in another vesicle recycling process, (Figure 1A3) double labeled vesicles which have had their lumen exposed to the extracellular milieu resealed and trafficked back to the cytoplasm (Figure 1A4). Vesicles in astrocytes from CA1 region of the hippocampus (Figure 1A1) were then observed and recorded with a two-photon microscope. Vesicles were seen as fluorescence puncta (Figure 1B), which were not observed in slices exposed only to secondary antibodies, thus the labeling is unlikely unspecific. The fluorescent puncta exhibited two types of mobility, non-directional and directional as described previously [18,19]. The first mode of mobility was characterized by less directed, shorter movements, whereas the directional mobility was characterized by longer, almost straight-lined movements, as seen by recorded tracks (Figure 2 and Figure 3).

Trajectories of analyzed ANP-granules are shown in Figure 2A,B for wild-type and GFP mice, respectively. Mobility parameters, maximal displacement and track length were calculated as described [18] and exhibit a linear relationship, which is similar in both types of slices (Figure 2C,D). Slopes of the lines fitted to the data represent correlation between the maximal displacement and the track length and describe directionality of vesicle pathway. Directionality can be described with values from near 0 (low directionality, highly random movement) to 1 (linear movement). Slopes (a) of the lines fitted to the data were 0.245 ± 0.011 in wild-type and 0.324 ± 0.022 in GFP mice. The average velocity of granules in slices from wild-type mice was $0.037 \pm 0.001 \mu\text{m/s}$ ($n = 485$) and $0.045 \pm 0.001 \mu\text{m/s}$ ($n = 205$) from GFP mice. This is similar to what was reported for recycling ANP-granules in rat primary astrocyte cultures ($0.06 \pm 0.001 \mu\text{m/s}$; [12]), but one order of magnitude slower from the velocity of pre-fusion proANP-Emd labeled granules [18,19], which average velocity was $0.4 \mu\text{m/s}$. The vGlut1-vesicles were analyzed similarly and trajectories from wild-type and GFP mice are shown in Figure 3A,B. The correlation between maximal displacement and track length is presented in Figure 3C,D. Slopes (a) of the lines fitted to the data were 0.436 ± 0.024 in wild-type and 0.194 ± 0.015 in GFP mice. The vGlut1-vesicles were slightly slower than ANP-granules; their average velocity was $0.028 \pm 0.001 \mu\text{m/s}$ ($n = 202$; wild-type mice) and $0.026 \pm 0.001 \mu\text{m/s}$ ($n = 249$; GFP mice). Velocities of recycling vGlut1 vesicles in slices are also slightly slower from recycling vGlut1 vesicles from rat primary astrocyte cultures ($0.05 \mu\text{m/sec}$; [13]). Both, ANP granules and vGlut1 vesicles were substantially slower from vesicles in mammalian neurons, where fast transport velocities of the vesicles are in the range $0.8\text{--}3.5 \mu\text{m/s}$ [21]. Further mobility parameters analyzed are the following: The average maximal displacement for ANP-granules was $0.311 \pm 0.011 \mu\text{m}$ and $0.387 \pm 0.019 \mu\text{m}$ for wild-type mice and GFP mice, respectively. The average maximal displacement for vGlut1-vesicles was $0.440 \pm 0.020 \mu\text{m}$ (wild-type),

significantly different from $0.318 \pm 0.011 \mu\text{m}$ in slices from GFP mice ($*p < 0.001$; Figure 4). The average TL for ANP-granules was 1.104 ± 0.025 (wild-type mice), significantly different from $1.362 \pm 0.040 \mu\text{m}$ in GFP mice ($*p < 0.001$; Figure 4). TL for vGlut1-vesicles was $0.841 \pm 0.024 \mu\text{m}$ (wild-type mice) and $0.812 \pm 0.035 \mu\text{m}$ (GFP mice; Figure 4). TL were slightly lower for vGlut1 vesicles, similarly as it was observed in primary astrocyte cultures (Figure 4; [12,13]). The small differences in maximal displacement (MD) of vGlut1 vesicles and in track length (TL) of ANP granules between wild-type and GFP mice may emerge from GFP interference with trafficking vesicles or alternatively from possible relatively unselective labeling of astrocytes by Fluo4, a calcium-binding molecule, in the wild-type slices (Figure 4). Considering our recent studies in cultured cells, where we have shown that the application of agents to cells to increase cytosolic calcium, reduces the mobility of ANP-labelled granules [12], and that this leads to an enhanced mobility of vGlut1-positive vesicles [13], it is possible that the slightly lower mobility of ANP-granules in WT vs. GFP slices (Figure 2) is due to a Fluo4-mediated increase in basal calcium. Moreover, if Fluo4 treatment increases basal cytosolic calcium, one would also expect to see an increased mobility of vGlut1-positive vesicles in WT. vs GFP slices, which we have recorded (Figure 3).

These results show that the experimental procedures previously used in cultured astrocytes to study the mobility of retrieving secretory vesicles are appropriate to be used in tissue slices. Moreover, it is unlikely that the properties measured in cultured cells are an artifact, since similar properties were observed in tissue slices.

Conclusions

This study shows that the single vesicle mobility of ANP-granules and vGlut1 vesicles can be studied in brain tissue slices and that the mobility parameters match those obtained in cell cultures previously. Therefore, vesicle dynamics results obtained in cell cultures are not merely an artifact.

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Abbreviations

ANP	atrial natriuretic peptide
vGlut1	vesicular glutamate transporter 1
MD	maximal displacement
TL	track length

Figure 1. Vesicles/granules were successfully immunolabeled in acute hippocampal slices

Labeled vesicles/granules in astrocytes were imaged in the CA1 region of a hippocampus (A1). The procedure of vesicle/granule labeling with antibodies is presented in panels A2–A4. After the fusion pore opening primary antibodies from the extracellular solution bind to vesicle/granule proteins of recycling vesicles (A2). Secondary antibodies bind to primary antibodies in one of the following vesicle/granule cycles (A3). Vesicles/granules labeled with both antibodies (A4) can be imaged, subsequently. Successfully labeled granules with anti-ANP primary antibodies and anti-rabbit Alexa Fluor secondary antibodies were observed under the microscope as fluorescent dots in cells from WT (B1) and GFP mice (B2).

plasma membrane, ○ - vesicle, ⊗ - vesicle luminal protein, ▲ - primary antibody, ▼ - secondary antibody. Astrocytes were labeled in hippocampal slice (C1) (left, bar 20 μm) with Fluo4 (C2, green). To confirm identity of Fluo4 labeled astrocytes secondary labeling with SR101 was performed (C3, red). The staining revealed co-labeling with both dyes (C4, Merge, bar 200 μm).

Figure 2. Mobility of ANP-labeled granules in astrocytes from wild-type and GFP mice

Encircled are labeled ANP-granules which were subjected to the mobility analysis; granules' trajectories are shown in astrocytes from wild-type (WT) and transgenic (GFP) mice (A and B, respectively). Bars: 5 μm. Granules were labeled with primary antibodies against ANP (1:600, rabbit, Abcam) and with secondary antibodies Alexa Fluor 546 Goat anti-Rabbit IgG (1:600, Molecular Probes). Graphs C and D represent the relationship between the track length (TL) and the maximal displacement (MD) for WT and GFP mice. n = number of analyzed vesicles. A linear function with the form: $[MD = MD_0 + a \cdot (TL)]$ was fitted to the data. The slopes (a) of the lines fitted were 0.2451 ± 0.011 (WT) and 0.3238 ± 0.022 (GFP), significantly different ($p < 0.05$). Dashed line denotes maximal displacement of 1 μm. 12 cells from wild-type astrocytes were analyzed (A,C) and 7 cells from GFP astrocytes (B,D).

Figure 3. Mobility of vGlut1-labeled vesicles in astrocytes from WT and GFP mice

Encircled are labeled and analyzed vGlut1-vesicles; their trajectories are shown in panels (A, B) for WT and GFP mice, respectively. Bars: 5 μm. Vesicles were labeled with primary antibodies against vGlut1 (1:2000, rabbit, Synaptic Systems) and with secondary antibodies Alexa Fluor 546 Goat anti-Rabbit IgG (1:600, Molecular Probes). Scatter plots in panels C and D show the relationship between track length (TL) and maximal displacement (MD) for wild type (WT) and transgenic (GFP) mice. n = number of analyzed vesicles. A linear function with the form: $[MD = MD_0 + a \cdot (TL)]$ was fitted to the data. The slopes (a) of the lines fitted were 0.436 ± 0.024 (WT) and 0.194 ± 0.015 (GFP), significantly different ($p < 0.05$). Dashed line denotes maximal displacement of 1 μm. 13 cells from wild-type astrocytes were analyzed (A,C) and 12 cells from GFP astrocytes (B,D).

Figure 4. Mobility parameters of ANP-granules and vGlut1-vesicles

Graph A) The average values for maximal displacement (MD). MD values for ANP-granules were $0.321 \pm 0.008 \mu\text{m}$ and $0.387 \pm 0.019 \mu\text{m}$ for WT and GFP mice, respectively. The average MD for vGlut1 vesicles in WT mice was $0.440 \pm 0.020 \mu\text{m}$, significantly different from $0.311 \pm 0.011 \mu\text{m}$ in GFP mice ($*p < 0.001$).

Graph B) The average track length (TL). TL for ANP-granules in WT mice was $1.104 \pm 0.025 \mu\text{m}$ and TL in GFP mice was significantly different ($1.362 \pm 0.040 \mu\text{m}$, $*p < 0.001$). In vGlut1-vesicles TL was $0.841 \pm 0.024 \mu\text{m}$ and $0.812 \pm 0.035 \mu\text{m}$, for WT and GFP mice, respectively. Numbers adjacent to columns represent number of analyzed vesicles.