Published in final edited form as:

Glia. 2016 February ; 64(2): 317–329. doi:10.1002/glia.22931.

Expression of familial Alzheimer disease presenilin 1 gene attenuates vesicle traffic and reduces peptide secretion in cultured astrocytes devoid of pathologic tissue environment

Matjaž Stenovec^{1,2,*}, Saša Trkov^{1,2,*}, Eva Lasi², Slavica Terzieva^{1,2,3}, Marko Kreft^{1,2,4}, José Julio Rodriguez Arellano³, Vladimir Parpura⁶, Alexei Verkhratsky^{1,2,3,5}, and Robert Zorec^{1,2,+}

¹Celica BIOMEDICAL, Tehnološki park 24, 1000 Ljubljana, Slovenia, EU

²Laboratory of Neuroendocrinology-Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia, EU

³Achucarro Center for Neuroscience, IKERBASQUE, Basque Foundation for Science, 48011 Bilbao, Spain and University of the Basque Country UPV/EHU and CIBERNED, Leioa, Spain, EU

⁴University of Ljubljana, Biotechnical Faculty, Department of Biology, CPAE, Večna pot 111, 1000 Ljubljana, Slovenia, EU

⁵Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK, EU

⁶Department of Neurobiology, Civitan International Research Center and Center for Glial Biology in Medicine, Evelyn F. McKnight Brain Institute, Atomic Force Microscopy & Nanotechnology Laboratories, 1719 6th Avenue South, CIRC 429, University of Alabama at Birmingham, Birmingham, AL 35294-0021, USA

Abstract

In the brain, astrocytes provide metabolic and trophic support to neurones. Failure in executing astroglial homeostatic functions may contribute to the initiation and propagation of diseases, including Alzheimer disease (AD), characterized by a progressive loss of neurones over years. Here, we examined whether astrocytes from a mice model of AD isolated in the pre-symptomatic phase of the disease exhibit alterations in vesicle traffic, vesicular peptide release and purinergic calcium signalling. In cultured astrocytes isolated from a newborn wild-type (wt) and 3xTg-AD mouse, secretory vesicles and acidic endosomes/lysosomes were labelled by transfection with plasmid encoding atrial natriuretic peptide tagged with mutant green fluorescent protein (ANP.emd) and by LysoTracker, respectively. The intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was monitored with Fluo-2 and visualized by confocal microscopy. In comparison with controls, spontaneous mobility of ANP- and LysoTracker-labelled vesicles was diminished in 3xTg-AD astrocytes; the track length (TL), maximal displacement (MD) and directionality index (DI) were

⁺**Correspondence:** Professor Robert Zorec, Laboratory of Neuroendocrinology-Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia. Tel.: +386 1 5437080, fax: +386 1 5437036, robert.zorec@mf.uni-lj.si.

^{*}Matjaž Stenovec and Saša Trkov contributed equally to this work.

Conflict of Interest

The authors declare no conflict of interest.

all reduced in peptidergic vesicles and in endosomes/lysosomes ($P<0.001$), as was the ATP-evoked attenuation of vesicle mobility. Similar impairment of peptidergic vesicle trafficking was observed in wt rat astrocytes transfected to express mutated presenilin 1 (PS1_{M146V}). The ATP-evoked ANP discharge from single vesicles was less efficient in 3xTg-AD and PS1_{M146V}-expressing astrocytes than in respective wt controls ($P<0.05$). Purinergic stimulation evoked biphasic and oscillatory $[Ca^{2+}]_i$ responses; the latter were less frequent ($P<0.001$) in 3xTg-AD astrocytes. Expression of PS1_{M146V} in astrocytes impairs vesicle dynamics and reduces evoked secretion of the signalling molecule ANP; both may contribute to the development of AD.

Keywords

vesicle mobility; regulated exocytosis; ANP.emd; LysoTracker; calcium signalling

Introduction

Alzheimer disease (AD) is a progressive, irreversible and ultimately fatal neurodegenerative disease, which leads to cognitive/memory impairment; AD arguably is the most common cause of dementia in the elderly. Histopathologic hallmarks of AD are represented by (i) focal extracellular deposits of fibrillar β -amyloid (A β), also known as senile or neuritic plaques and (ii) intraneuronal accumulation of neurofibrillary tangles composed of filamentous aggregates of hyper-phosphorylated Tau protein (Selkoe 2001). The degeneration and loss of synapses precedes neuronal death (DeKosky and Scheff 1990; Scheff et al. 1991) and may account for early cognitive impairment. It is believed that neurodegeneration occurs gradually and dementia represents the end stage of accumulating pathologic changes that may start to develop decade(s) before the onset of the earliest clinical symptoms (Jack et al. 2010). The current view is that neurodegeneration in AD reflects neurone-specific deficits. It may, however, begin as a failure in neuroglia, which are involved in the maintenance of brain homeostasis (cellular, micro-architectural, vascular, metabolic, neurotransmitter, ion) (Giaume et al. 2007; Heneka et al. 2010; Kano and Hashimoto 2009; Nedergaard et al. 2010; Terry 2000). The pathologic potential of neuroglia was first recognized by Alois Alzheimer himself, who found that glia populated senile plaques and were in close contact with damaged neurones (Alzheimer 1910; Strassnig and Ganguli 2005). In post-mortem AD human tissue, astroglial hypertrophy accompanied with increased levels of glial fibrillary acidic protein (GFAP) and S100 β , a calcium binding protein, are often observed (Beach and McGeer 1988; Griffin et al. 1989; Mrak and Griffin 2005; Nagele et al. 2004; Verkhratsky et al. 2014). In an animal model of familial AD, astroglial atrophy in addition to hypertrophy was detected in the hippocampus and prefrontal and entorhinal cortices (Kulijewicz-Nawrot et al. 2012; Olabarria et al. 2010; Olabarria et al. 2011; Yeh et al. 2011); it preceded the appearance of senile plaques and appeared first in the entorhinal cortex, the region earliest affected by AD pathology (Yeh et al. 2011). Astroglial atrophy suggests reduced synaptic coverage and failure to provide neuroprotective support, which may cause disruption in synaptic connectivity, imbalance in neurotransmitter homeostasis, and neuronal death through increased excitotoxicity (Hynd et al. 2004). The atrophic phenotype itself indicates altered functional architecture of astrocytes that is predominantly determined by intracellular vesicle trafficking, a fundamental biological

process required for cell-to-cell signalling. Astrocytes communicate with neighbouring cells in a slow time domain via the vesicular release of gliosignalling molecules (Guerk et al. 2012; Parpura and Zorec 2010). This communication relies on vesicle transport to provide a slow but continuous delivery of vesicular material, be that luminal- and/or membrane-laden cargo, towards the plasma membrane. Moreover, dysfunctional trafficking of endocytic vesicles, which otherwise retrieve plasmalemmal as well as extracellular contents, affects effective clearance of extracellular material engulfed by astrocytic endocytosis (Vardjan et al. 2012), also relevant in AD (Xiao et al. 2014).

Here, we aimed to provide experimental evidence for the early onset of cell-autonomous astrocyte dysfunction in the context of familial AD that affects cell capability for targeted delivery and regulated vesicular secretion. We performed these experiments in astroglial cells isolated from 3xTg-AD mouse model that mimics histopathology of AD (Oddo et al. 2003), with the exception of neuronal loss. In this animal model astrocytes express a single mutated gene, namely a mutant presenilin-1 (PS1_{M146V}), whereas neurones express in addition mutated amyloid precursor protein (APP) and Tau. Astroglial cells were maintained *in vitro* to prevent exposure to the pathological tissue environment associated with accumulation of A β , neuroinflammatory response or compromised blood brain barrier on longer term. In these astrocytes (cultured for ~3–4 weeks) we examined the spontaneous and ATP-stimulated mobility of peptidergic vesicles and endosomes/lysosomes, ATP-evoked peptide release and purinergic cytosolic Ca²⁺ signalling; as a control, we used a background strain referred henceforth as wild-type (wt). We further verified our findings in wt rat astrocytes transfected to express mutated presenilin 1 (PS1_{M146V}).

The results of this study show that vesicle traffic, secretory discharge of a peptide gliotransmitter and cytoplasmic Ca²⁺ homeostasis are all significantly impaired in astrocytes expressing a mutated AD-related gene, suggesting astroglia as a novel target for the development of pharmacologic manipulation in treating the early stages of AD.

Materials and methods

Animal model and cell culture

Primary astrocyte cultures were prepared from the cortices of 1–3-day-old 3xTg-AD and wt control mice as well as Wistar rats of either sex, in accordance with European and Slovenian legislation. The 3xTg-AD mouse harbours mutant genes for amyloid precursor protein (APP_{Swe}), presenilin 1 (PS1_{M146V}) and microtubule-associated protein Tau (Tau_{P301L}) (Oddo et al. 2003), and mimics the spatiotemporal progression of amyloid and Tau protein pathology as human AD. Genes for APP_{Swe} and Tau_{P301L} were co-microinjected into single-cell embryos from homozygous presenilin PS1_{M146V} knock-in mice. The background of the PS1 knock-in mouse was a hybrid 129/C57BL6. The wt control mice used were from the same strain and genetic background as the presenilin 1 (PS1_{M146V}) knock-in mice, but they harboured the endogenous wild-type mouse PS1 gene. All 3xTg-AD and wt littermates were from homozygous breeders. The animals were grouped by gender and genotype, housed under a controlled temperature and 12 h light/12 h dark cycles with access to food and water *ad libitum*. Cells obtained from neocortices of the animals, were grown in high-glucose Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 1 mM

sodium pyruvate, 2 mM L-glutamine, and 25 µg/ml penicillin/streptomycin at 37°C, in an atmosphere of 5% CO₂/95% air. Subconfluent cultures were shaken at 225 rotations per minute overnight with three subsequent medium changes. Before the experiments, the cells were trypsinized, sub-cultured onto poly-L-lysine-coated coverslips and maintained in culture medium at 37°C, in an atmosphere of 5% CO₂/95% air.

Solutions

Extracellular solution (ECS) consisted of (in mM): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 D-glucose, and 10 HEPES (4–2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.2 with NaOH. Unless stated otherwise, all chemicals were of the highest purity available (Sigma-Aldrich, Germany).

Cell transfection

To visualize individual secretory vesicles, cells were transfected with the plasmid-encoding atrial natriuretic peptide C-terminally tagged with emerald green fluorescent protein (ANP.emd; a gift from Dr. Ed Levitan, University of Pittsburgh, Pittsburgh, PA, USA; (Han et al. 1999; Krzan et al. 2003)) using Lipofectamine LTX Reagent (Life Technologies, USA). Briefly, DNA (1 µg/µl) was mixed with 1 µl of Plus Reagent, diluted in 50 µl of serum-free culture medium; 2 µl of Lipofectamine LTX Reagent was diluted in 50 µl of serum-free culture medium. Both solutions were mixed and incubated for 5 min at room temperature. Astrocytes were supplemented with 900 µl of serum-free culture medium and 100 µl of the lipofection mixture was pipetted onto the cells. The cultures were incubated for 3 h at 37°C in an atmosphere of 5% CO₂/95% air and then 30 µl of Ultrosor G (Life Technologies) was added. The medium was exchanged for fresh culture medium the next day. Transfected cells were observed after 48–72 h. To independently examine functional deficits arising from expression of the mutated presenilin 1 (PS1_{M146V}), cultured normal rat astrocytes were co-transfected with the plasmid encoding ANP.emd and the plasmid encoding PS1_{M146V} (kindly provided by Dr. Ilya Bezprozvanny, UT Southwestern Medical Center at Dallas, Dallas, TX, U.S.A; (Tu et al. 2006) while control cells were transfected with pANP.emd alone (1 µg/µl). Briefly, DNA plasmids encoding ANP.emd (0.5 µg/µl) and PS1_{M146V} (0.5 µg/µl) were mixed with 1 µl Plus Reagent, diluted in 50 µl serum-free DMEM; 2 µl of lipofectamine LTX was diluted in 50 µl serum-free DMEM. The subsequent steps were performed equally as described for cells transfected with the single plasmid (pANP.emd). In a subset of experiments, acidic endosomes/lysosomes were visualized in astrocytes by the addition of 200 nM of LysoTracker Red DND-99 (LyTR, Life Technologies) to the culture medium for 5 min at 37°C.

Immunocytochemistry

Non-transfected and transfected cultured astrocytes were washed (3 min) with phosphate buffered saline (PBS) and fixed in paraformaldehyde (4% in PBS) for 15 min, permeabilised with 0.1% Triton X-100 for 10 min and then washed four times with PBS at RT. The non-specific background staining was reduced by incubating cells in a blocking buffer with 10% (v/v) goat serum in PBS for 1 h at 37°C. The cells were then washed four times with PBS and incubated with primary mouse anti-glial fibrillary acidic protein (GFAP; 1:100; Sigma-Aldrich, USA) antibody diluted in 3% (w/v) bovine serum albumin (BSA) in PBS overnight

at 4°C. Afterwards, the cells were rinsed in PBS and stained with secondary anti-mouse antibodies conjugated to Alexa488 or Alexa546 (1:600; Life Technologies, USA) at 37°C for 45 min. At the end of the staining protocol, the cells were mounted onto glass slides using SlowFade Gold antifade agent (Life Technologies, USA). Single and double-fluorescent cells were observed with a confocal microscope (LSM 780, Zeiss, Germany) using a plan-apochromatic oil-immersion objective 63×/NA 1.4. Confocal images were obtained with a 488-nm argon laser and 561-nm diode-pumped solid-state laser excitation and the fluorescence emission bandpass filtered at 500–525 nm and 565–600 nm, respectively.

Analysis of vesicle mobility and cargo secretion

Astrocyte-loaded coverslips were mounted into the recording chamber and transferred to a confocal microscope (LSM 780; Zeiss) equipped with a plan-apochromatic oil-immersion objective 63×/NA 1.4. ANP.emd was excited by a 488-nm argon laser line and emission fluorescence was filtered with a bandpass filter 495–545 nm; LyTR was excited by a 561-nm DPSS laser line and emission fluorescence was filtered with a bandpass filter 575–640 nm. Time-lapse images were acquired every ~0.5 s for 1 min before and 4 min after stimulation with 100 μM ATP. The mobility of peptidergic (ANP.emd) vesicles and endosomes/lysosomes (LyTR) was analysed by ParticleTR software (Celica, Ljubljana, Slovenia) in exported tiff files (Potokar et al. 2005). Typically, ~50 randomly selected vesicles were tracked per cell and the track length (TL; the pathway that individual vesicles travelled) and maximal displacement (MD; the farthest translocation of a vesicle), the directionality index ($DI = MD/TL$), and the speed were determined; all parameters were estimated for 15-s epochs. In addition, we measured step lengths (defined as the distance that individual vesicles travelled within 485 ms, the interval corresponding to an acquisition rate of ~2Hz), and constructed the frequency distribution plots. Analysis of the vesicle mobility was performed before and 2–4 min after the onset of stimulation with 100 μM ATP. The mean (±SEM) TL and MD were determined in 4–8 stimulated and 12 non-stimulated cells.

The elementary events of astrocyte ANP.emd secretion (Krzan et al. 2003) were identified visually in time-lapse images. Vesicle fusion followed by ANP release appeared as a sudden decrease in vesicle fluorescence indicating complete discharge of the fluorescent cargo to the exterior (Stenovec et al. 2004). The time-resolved fluorescence changes (in arbitrary units, AU) at the place of secreting vesicles were obtained by the LSM 780 software (Zeiss) using a circular region of interest ($r=0.528 \mu\text{m}$, $S=0.876 \mu\text{m}^2$). The overall efficacy of ATP-evoked peptide secretion was estimated by counting all vesicles completely releasing the fluorescent cargo within 4 min after cell stimulation with 100 μM ATP. For statistical evaluation, the mean number of release events per cell within 4 min after ATP stimulation was compared between eight wt and seven 3xTg-AD mouse astrocytes, and eight wt and eight PS1^{M146V}-expressing rat astrocytes.

Calcium imaging

Astrocyte-loaded coverslips were incubated for 30 min at room temperature in culture medium supplemented with 1 μM Fluo-2-AM, a cell-permeant fluorescent calcium indicator

(Teflabs, TX, USA). Cells were then washed with ECS, incubated for 30 min to allow de-esterification of acetoxymethyl (AM) ester and mounted into the chamber on a confocal microscope (LSM 780, Zeiss) equipped with a Plan-Apochromat air objective 20×/NA 0.8. The Fluo-2 was excited by a 488-nm argon laser line and emission fluorescence was filtered with a 495–565 nm bandpass filter. Time-lapse images were acquired every second for 3 min before and 10 min after the bolus addition of ATP to reach a final concentration of 100 μ M. The changes in Fluo-2 fluorescence indicating increases in $[Ca^{2+}]_i$ were acquired in 115 wt and 150 3xTg-AD astrocytes using LSM780 software (Zeiss) within the regions of interest that encompassed individual cells. The time-resolved fluorescence changes were analysed by custom-written Matlab software (MathWorks, Natick, MA, USA) to obtain the peak calcium amplitude (F/F_0) and the surface under the curve (corresponding to the calcium amplitudes integrated over time ($F/F_0 \times t$)). The baseline fluorescence (F_0) was determined at the beginning of each recording as the average intensity (in AU) during the first 180 frames.

Statistical analysis

The parameters of vesicle mobility (TL, MD, DI and speed), the number of elementary secretory events and the parameters of calcium responses (peak and integrated increase) were expressed as means $\pm$ SEM. Statistical significance was determined with the Mann-Whitney *U* test using SigmaPlot 11.0 (Systat Software Inc., USA) and with ANCOVA using Matlab (MathWorks).

Results

Reduced spontaneous mobility of peptidergic vesicles in 3xTg-AD astrocytes

Mobility of peptidergic secretory vesicles loaded with ANP.emd (Fig. 1A,B) was imaged and the motion patterns were analysed in single cortical astrocytes maintained in a highly purified culture (Supp. Info. Fig. 1A,B). Vesicle tracks obtained in wt and 3xTg-AD astrocytes exhibited directional (elongated tracks) and non-directional (contorted tracks) mobility (for detailed definitions, see (Potokar et al. 2005)). However, in wt astrocytes, we usually observed more elongated vesicle tracks (Fig. 1C) than in 3xTg-AD astrocytes (Fig. 1D), indicating reduced directional vesicle mobility. In wt cells, the vesicle step lengths were bimodally distributed around the peak values of 0.07 μ m and 0.31 μ m (range 0–1.42 μ m; Fig. 1E). In 3xTg-AD cells, the step lengths were unimodally distributed around the peak value of 0.10 μ m (range 0–1.92 μ m; Fig. 1F). The frequency distribution plots were fitted with Gaussian (wt) and logarithmic Gaussian functions (3xTg-AD) (for details, see legend to Fig. 1E,F). The length of steps in 3xTg-AD astrocytes compared with wt astrocytes was substantially smaller. Approximately ~52% of steps (2629 of 5025) in wt astrocyte were short (<0.2 μ m) and ~48% (2396 of 5025) were long (>0.2 μ m), whereas in 3xTg-AD astrocyte ~72% of steps were short (3672 of 5072) and ~28% (1400 of 5072) were long ($P<0.001$, χ^2 test). To further quantify differences in spontaneous mobility of vesicles, four parameters (TL, MD, DI and vesicle speed) were determined in 15-s epochs. The mobility of 1668 vesicles in wt ($N=12$) and 1993 vesicles in 3xTg-AD cells ($N=12$) was analysed. The vesicle TL was significantly reduced by ~24% in 3xTg-AD cells ($N=12$, Fig. 1G; $P<0.001$). Similarly, the vesicle MD was significantly reduced by ~29% in 3xTg-AD

cells ($N=12$, Fig. 1H; $P<0.001$). The vesicle DI was less affected, but still significantly reduced by ~6% in 3xTg-AD cells ($N=12$, Fig. 1I, $P<0.001$). Finally, the vesicle speed was reduced by ~24% in 3xTg-AD cells ($N=12$, Fig. 1J; $P<0.001$). Overall, in comparison with wt astrocytes, the spontaneous mobility of peptidergic vesicles was substantially diminished in 3xTg-AD astrocytes.

ATP-evoked reduction in peptidergic vesicle mobility was larger in 3xTg-AD than in wt astrocytes

Since it was previously shown that peptidergic vesicle mobility is reduced in astrocytes stimulated with metabotropic agents (Potokar et al. 2008), we next examined how an increase in $[Ca^{2+}]_i$ affects vesicle mobility. Time-dependent changes in vesicle TL and MD were recorded 1 min before and 2 min after cell stimulation with 100 μ M ATP. The post-stimulation data were divided into two groups; data recorded during the first minute and during the second minute. A robust time-dependent decrease in TL and in MD was recorded after the application of ATP (Fig. 2A–D). In wt astrocytes, during the first post-stimulation minute, TL was reduced ($P<0.001$) by ~26% and by ~34% during the second minute of recording, whereas the MD was reduced ($P<0.001$) by ~34% during the first minute and by ~50% during the second minute after ATP application. In 3xTg-AD astrocytes, similar decrements in TL were observed, however those in MD appeared larger than in wt cells.

Next, we examined the effect of ATP stimulation on the directionality of vesicle movements by plotting the relationship between TL and MD and fitting a linear function [$MD=MD_0+a \times (TL)$] to the data. The slope (a) of the function corresponds to the directionality index (DI). Although both the vesicle TL and MD strongly decreased, only a minor decrease in the DI was observed in the post-stimulation data. During the second post-stimulation minute, the DI decreased, albeit not significantly ($P=0.142$), from 0.77 ± 0.01 to 0.73 ± 0.01 in wt cells; in contrast, in 3xTg-AD cells, the DI decreased modestly and significantly ($P<0.001$) from 0.79 ± 0.01 to 0.70 ± 0.01 (Fig. 2E,F).

ATP-evoked peptide secretion is strongly reduced in 3xTg-AD astrocytes

Subsequently, we examined whether the exocytotic $[Ca^{2+}]_i$ -dependent peptide discharge evoked by ATP stimulation is altered in 3xTg-AD astrocytes. The individual secretory events (Fig. 3A) were observed for 4 min after the application of ATP (100 μ M) to astrocytes. The normalised, time-dependent fluorescence plots of cargo discharge from individual vesicles, seen as a step-wise decrease in fluorescence signal to a level comparable with the background (auto)fluorescence, indicated a similar rate of release in both wt and 3xTg-AD astrocytes (Fig. 3B). A rapid and apparently complete discharge of ANP.emd, indicative of full-collapse vesicle fusion (Vardjan et al. 2009) was accomplished within 0.5 s, between the two successive image frames (Fig. 3B), as also observed in rat astrocytes (Trkov et al. 2012). The rate of peptide discharge from an individual vesicle was the same in wt and 3xTg-AD astrocytes. In contrast, the number of ATP-evoked elementary secretion events per cell and unit time was substantially diminished in 3xTg-AD astrocytes; it was 38 ± 6 in wt ($N=8$) versus 16 ± 6 in 3xTg-AD ($N=7$), respectively ($P<0.05$; Fig. 3C). Overall, the Ca^{2+} -dependent exocytotic secretion of ANP was reduced in 3xTg-AD astrocytes.

Reduced spontaneous mobility of peptidergic vesicles and ATP-evoked peptide discharge in normal rat astrocytes expressing PS1_{M146V}

To independently verify that functional deficits observed in 3xTg-AD mouse astrocytes depend on expression of the mutated presenilin 1 gene and not on particular rodent species, rat astrocytes were double-transfected with the plasmids encoding ANP.emd and mutated presenilin 1 (PS1_{M146V}); the respective controls were transfected with the pANP.emd alone. As before, the differences in the spontaneous mobility of 1405 peptidergic vesicles in wt ($N=12$; transfected with ANP.emd.) and 1416 vesicles in PS1_{M146V}-expressing astrocytes ($N=12$; transfected with ANP.emd and PS1_{M146V}) were analysed and compared in 15-s epochs. The vesicle TL was significantly reduced by ~23% in PS1_{M146V}-expressing cells ($N=12$, Fig. 4A; $P<0.001$). Similarly, the vesicle MD was significantly reduced by ~24% in PS1_{M146V}-expressing cells ($N=12$, Fig. 4B; $P<0.001$). The vesicle DI was less affected and was reduced by ~3% in PS1_{M146V}-expressing cells ($N=12$, Fig. 4C), while the vesicle speed was significantly reduced by ~23% in PS1_{M146V}-expressing cells ($N=12$, Fig. 4D; $P<0.001$). Overall, a similar and strong reduction in the spontaneous mobility of peptidergic vesicles was observed in both 3xTg-AD mouse (Fig. 1I–J) and PS1_{M146V}-expressing rat astrocytes (Fig. 4A–D) when compared to their respective wt controls. In addition, we also examined alterations in ATP-evoked peptide discharge in PS1_{M146V}-expressing astrocytes within 4 min after the application of ATP (100 μ M) to astrocytes. Again, the number of ATP-evoked elementary secretion events per cell and unit time was substantially diminished in PS1_{M146V}-expressing cells; it was 17 ± 2 in wt ($N=8$) versus 5 ± 1 in PS1_{M146V}-expressing cells ($N=8$), respectively ($P<0.001$; Fig. 4E). Thus, the Ca^{2+} -dependent exocytotic secretion of ANP was reduced in both 3xTg-AD mouse (Fig. 3C) and PS1_{M146V}-expressing rat astrocytes (Fig. 4E).

Spontaneous mobility of LysoTracker-labelled vesicles is diminished in 3xTg-AD astrocytes

Next we monitored the mobility of LysoTracker (LyTR)-labelled vesicles, which predominantly include endosomes/lysosomes with acidified lumen (Potokar et al. 2010). The spontaneous mobility of LyTR-labelled vesicles ($N=4296$) was analysed in wt ($N=12$) and 3xTg-AD cells ($N=12$). In comparison with wt cells, both the vesicle TL and MD were reduced significantly ($P<0.001$) by ~5% and ~7% in 3xTg-AD astrocytes (Fig. 5A,B). In 3xTg-AD astrocytes, the vesicle DI and speed were reduced ($P<0.001$) by ~2% and ~5%, respectively (Fig. 5C,D). Thus, the spontaneous mobility of LyTR-labelled vesicles was diminished in 3xTg-AD astrocytes; however, the reduction in mobility did not appear as strong as in secretory peptidergic vesicles (Fig. 1).

Stimulation decreased LyTR-labelled vesicle mobility was stronger in 3xTg-AD than wt astrocytes

As for peptidergic vesicles, ATP stimulation evoked a robust and time-dependent decrease in mobility (TL and MD) of LyTR-labelled vesicles. After stimulation, the TL decreased ($P<0.001$) by ~20% during the first minute and by ~22% during the second minute after stimulation in wt cells (Fig. 6A). In 3xTg-AD cells, the TL diminished ($P<0.001$) by ~11% during the first minute and by ~23% during the second minute after stimulation (Fig. 6B). In

wt cells, the initial reduction in MD (Fig. 6C) appeared more intense (by ~49% and by ~52%, during the first and second minute after stimulation, respectively) than in 3xTg-AD cells, in which the MD diminished by ~23% and ~53% during the first and second minute after stimulation, respectively (Fig. 6D). The reduction in the DI was more profound in wt than in 3xTg-AD astrocytes; in wt cells it reduced from 0.80 ± 0.01 to 0.38 ± 0.01 ($P < 0.001$) (Fig. 6E), whereas in 3xTg-AD it changed from 0.73 ± 0.01 to 0.49 ± 0.02 ($P < 0.001$) during the second minute after stimulation (Fig. 6F). Cell stimulation with ATP did not evoke detectable discharge of fluorescent dye, as was observed in peptidergic vesicles (Fig. 3), consistent with a previous report in rat astrocytes (Stenovec et al. 2011).

ATP-evoked biphasic and oscillatory increases in $[Ca^{2+}]_i$ in wt and 3xTg-AD astrocytes

We monitored ATP-mediated increases in $[Ca^{2+}]_i$ in astrocytes with Fluo-2 microfluorimetry (Fig. 7A, left). The application of ATP (100 μ M) increased indicator fluorescence, indicating an increase in $[Ca^{2+}]_i$ (Fig. 7A, middle and right). The ATP-induced increases in $[Ca^{2+}]_i$ lasted for minutes in wt and 3xTg-AD cells (Fig. 7B,C). Two apparent types of Ca^{2+} responses were observed: (i) biphasic increases in $[Ca^{2+}]_i$ (transient peak followed by a decline towards the baseline) (Fig. 7B,C, left) and (ii) oscillatory increases in $[Ca^{2+}]_i$ (Fig. 7B,C, right). To analyse these traces, we established criteria to assign the individual responses into either the first (i, biphasic) or the second (ii, oscillatory) category of Ca^{2+} responses.

We measured the Ca^{2+} peak amplitude (p , in AU) in the case of the biphasic response, and in the case of the oscillatory response we also measured the follow-up Ca^{2+} peak amplitudes (p_{osc} , in AU) and the number of individual increases in $[Ca^{2+}]_i$. A given increase in $[Ca^{2+}]_i$ was considered oscillatory if the difference in the fluorescence intensity between the successive minima and maxima was ≥ 2 AU, and if the adjacent fluorescence maxima were separated by ≥ 6 s. Then we calculated the ratio between the sum of follow-up Ca^{2+} peak values ($\sum p_{osc}/p$) and the first Ca^{2+} peak (F/F_0) ($\sum p_{osc}/p$). The relationship between $\sum p_{osc}/p$ and the number of oscillations (No. p_{osc}) was plotted for wt (Fig. 7D) and 3xTg-AD (Fig. 7E) astrocytes. If $\sum p_{osc}/p \geq 1$ and the number of Ca^{2+} oscillations > 3 , the Ca^{2+} response was considered to be an oscillatory response. Approximately 1/3 of responses in wt astrocytes (Fig. 7D) were oscillatory (39 of 115), whereas in 3xTg-AD astrocytes (Fig. 7E) these accounted only for ~1/10 (17 of 150) ($P < 0.001$, χ^2 test) of responses. Individual Ca^{2+} responses were quantified by measuring the peak amplitude (p) and the integrated increase (S , $F/F_0 \times s$) in $[Ca^{2+}]_i$ (Fig. 7B,C). In general, more free Ca^{2+} was mobilised during biphasic response than during oscillatory responses; therefore the responses were compared separately.

ATP evoked higher Ca^{2+} peaks in wt than in 3xTg-AD astrocytes during biphasic responses ($P < 0.05$; Fig. 8A), whereas it mobilized a similar cumulative amount of free Ca^{2+} (Fig. 8B) in the cytosol. In contrast, ATP evoked higher amplitude Ca^{2+} peaks in 3xTg-AD astrocytes than in wt astrocytes during oscillatory responses ($P < 0.05$; Fig. 8C), but mobilized a similar cumulative amount of free Ca^{2+} (Fig. 8D). Differences in ATP-induced changes in Ca^{2+} signalling may contribute to reduced peptide discharge from 3xTg-AD astrocytes.

Discussion

The main result of this study is that vesicle mobility and Ca^{2+} -dependent peptide secretion are reduced in astrocytes expressing AD-related mutant gene for presenilin 1 (PS1_{M146V}). The impaired vesicle mobility was observed in cultured astrocytes from neonatal 3xTg-AD mice as well as in rat astrocytes transfected with PS1_{M146V} gene. The spontaneous mobility of endosomes/lysosomes compared with peptidergic vesicles in 3xTg-AD astrocytes appears less affected (i.e. reduction in vesicle speed by ~24% in ANP and by ~2% in LyTR vesicles; $P < 0.001$). Disproportionally affected vesicle trafficking indicates imbalance in vesicle dynamics that may alter the cell surface signalling landscape and cell shape on a longer timescale. Our observations are consistent with earlier morphologic observations (Kulijewicz-Nawrot et al. 2012; Olabarria et al. 2010; Olabarria et al. 2011; Yeh et al. 2011) demonstrating a reduced surface area and volume of astroglial GFAP- and glutamine synthetase-positive astroglial profiles in the hippocampus, prefrontal and entorhinal cortex of 3xTg-AD mice, with astrocytes in the entorhinal cortex showing remarkable atrophy as early as in 1-month-old animals. A relevant question is how physiologic alterations observed *in vitro* in this study affect the performance of astrocytes *in vivo*.

Reduced vesicle dynamics contributes to the development of AD

Currently, it is unclear whether astrocytes in AD brain participate in the clearance and degradation of A β (Gunette 2003) or its production (Heneka et al. 2005; Rossner et al. 2005). Conceptually, imbalanced intracellular vesicle trafficking may affect proper distribution of proteins, including APP. In neurones, axonal aggregates of vesicles, organelles and kinesin have been reported in AD patients with either the Swedish or London mutations in APP (Stokin et al. 2005). Overexpressed APP blocked axons in fly and mouse models of disease (Gunawardena and Goldstein 2001) and the axonal swellings occurred well before the onset of symptoms or amyloid deposition (Stokin et al. 2005). Genetic reduction in kinesin light chain 1 expression also enhanced axonal accumulations of APP (Gunawardena and Goldstein 2001), indicating that altered APP transport may increase its exposure to γ -secretase to enhance pathogenic proteolysis (Gunawardena and Goldstein 2001; Stokin et al. 2005). Physiologically, A β clearance is facilitated by extracellular proteolysis, export across the blood–brain barrier, and cellular uptake. Astrocytes take up and degrade A β (Xiao et al. 2014), but diminished mobility of lysosomes, the major degrading organelles, wherein A β localizes after uptake (Basak et al. 2012), may insufficiently support effective degradation and thus contribute to the development of amyloid plaque pathogenesis in a longer time domain. Impaired intracellular transport following A β 42 clearance may lead to an increase in A β 42 accumulation that was additionally found to remodel astroglial metabolism; both increased (Allaman et al. 2010) and decreased (Schubert et al. 2009) glucose utilization were reported after prolonged (24 h) astrocyte exposure to A β .

Altered vesicle dynamics due to expression of the mutated presenilin 1

Trafficking of peptidergic vesicles (which in healthy cells move relatively fast) in 3xTg-AD cells decreased more (0.46 vs 0.35 $\mu\text{m/s}$; wt vs 3xTg-AD; Fig. 1) than trafficking of slower endosomes/lysosomes (0.24 vs 0.23 $\mu\text{m/s}$; wt vs 3xTg-AD; Fig. 5). The mechanistic reasons

for diminished mobility are unclear, yet they may include (i) altered three-dimensional structure of the cytoskeleton (Ebner et al. 1998; Yeh et al. 2011), in particular of microtubules, which act as tracks (Stenovec et al. 2014) for intracellular organelle transport by motor proteins (Ebner et al. 1998); (ii) altered cytosolic calcium homeostasis that takes place in AD (Abramov et al. 2004; Lim et al. 2014) (and in this study); (iii) altered mitochondrial ATP production (Ebner et al. 1998); or (iv) altered ATP utilization by molecular motors that propel vesicles along the cytoskeleton (Soldati and Schliwa 2006). The microtubule cytoskeleton plays an important role in intracellular traffic (Ebner et al., 1998). The decreased instantaneous speed in fast-moving peptidergic vesicles (compare Fig. 1E,F) may either indicate that vesicles were arrested more frequently along the cytoskeleton or were less effectively dragged by the motor proteins during processive (for up to several micrometers without dissociating from their track) motor “walking” along the microtubules. In accordance with the abovementioned possibilities, four times more pauses (no vesicle displacement during 485 ms) were observed in 3xTg-AD astrocytes (4%) than in wt astrocytes (1%).

PS1_{M146V} mutation or loss of PS1 function were reported to increase activation of glycogen synthase kinase 3 β (GSK3 β), which arguably increases the phosphorylation of kinesin, thereby leading to reduced binding of microtubules associated motor proteins to the membrane-bound cargo (Gunawardena et al. 2013; Pigino et al. 2003). *In vivo* GSK3 phosphorylates kinesin light chains that causes the release of kinesin-1 from membranous organelles, leading to a reduction in kinesin-1 driven motility (Morfini et al. 2002). In cultured mouse neurones the relative levels of GSK3 β activity were increased in the presence of mutated PS1 (PS1_{M146V}). Concomitant with an increased GSK3 β activity, relative levels of kinesin light chains phosphorylation were increased, and the amount of kinesin-1 bound to membranous organelles was reduced. Consistent with a deficit in kinesin-1-mediated fast axonal transport, densities of synaptophysin- and syntaxin-1-containing vesicles and mitochondria were reduced in neuritic processes of knock-in PS1_{M146V} hippocampal neurones. It appears thus that mutated PS1 compromises neuronal function by affecting GSK-3 activity and kinesin-1-based motility at sites of vesicle delivery and membrane insertion (Pigino et al. 2003). Moreover, in *Drosophila* larvae neurones reduced expression of presenilin with ~50% amino acid sequence identity to human PS1 increased both anterograde and retrograde velocities of APP-YFP-positive vesicles that required functional kinesin and dynein motors (Gunawardena et al. 2013). In hepatocytes, phosphorylation of cytoplasmic dynein contributed to changes in dynein ATPase activity and reduced ability of dynein to tether membranous organelles to microtubules (Runneger et al. 1999). Reduced dynein activity resulted in a marked reduction of dynein ATPase activity, essential to generate force from the hydrolysis of ATP to transport cargo along microtubules. By collectively considering these results, it appears that altered vesicle trafficking in 3xTg-AD mouse and PS1_{M146V}-expressing rat astrocytes originates from mutant PS1 (characteristic for the early-onset familial AD), which may alter microtubules associated motor protein activity by their phosphorylation via GSK3 β (Wang et al. 2013).

The ATP-evoked reduction in the directional mobility of peptidergic vesicles was larger in 3xTg-AD astrocytes. Incidentally, the same stimulus exerted a stronger reduction in vesicle

directionality in endosomes/lysosomes than in peptidergic vesicles, while the directionality of peptidergic vesicles was less affected in ATP-stimulated wt astrocytes. This may suggest that the structural integrity of the trafficking substrate, the cytoskeleton that assists in guiding directional vesicle movements (Soldati and Schliwa 2006), remained largely intact after ATP-induced increase in $[Ca^{2+}]_i$. Distinct sensitivity of mobile vesicles to agonist-evoked increase in $[Ca^{2+}]_i$ was also obvious (compare the reduction in DI after ATP stimulation in peptidergic vesicles and in endosomes/lysosomes). The latter comparison may indicate that endosomes/lysosomes transiently disconnected from the cytoskeleton tracks and were temporarily displaced in the cytosol by Brownian motion. Thus, the mobility of distinct vesicle types (secretory and endocytic) is differentially regulated by physiologic agents that increase $[Ca^{2+}]_i$.

Altered calcium homeostasis in 3xTg-AD astrocytes

In addition to putative changes in microtubules associated motor protein activity, impaired Ca^{2+} homeostasis may account for changes in vesicle mobility in 3xTg-AD astrocytes at rest (Fig. 1,5) and after neurochemical stimulation (Fig. 2,6). Diminished mobility of peptidergic vesicles and of dextran-laden late endosomes/lysosomes, positive for the major histocompatibility complex class II molecules, correlates well with increased astrocytic $[Ca^{2+}]_i$ (Stenovec et al. 2014; Vardjan et al. 2012). The proportion of astrocytes responding with oscillatory Ca^{2+} responses to ATP was substantially smaller in 3xTg-AD astrocytes ($\sim 1/10$) than in wt astrocytes ($\sim 1/3$). Our observation contrasts with the findings of Johnston et al. (2006), who reported a similar fraction of oscillating Ca^{2+} responses ($\sim 1/10$) in PS_{L286V} and in wild-type PS1 expressing astrocytes, but observed more Ca^{2+} oscillations in PS_{L286V}-expressing astrocytes after the application of a lower ATP concentration (1 μ M). Collectively, both studies indicate that PS1 mutations may introduce aberrations in agonist-evoked intracellular Ca^{2+} mobilization in astrocytes that potentially contribute to the pathogenesis of familial AD (FAD). The seemingly disparate findings may be a result not only of the use of different ATP concentrations but also of expression of PS1_{M146V} in our study as opposed to PS1_{L286V} in the other study. In our experiments, a smaller astrocyte population responded to the agonist challenge with Ca^{2+} oscillations. The remaining population generating larger calcium responses is more likely to spread the excitation via Ca^{2+} waves (Mattson and Chan 2003). The aberrant astrocyte Ca^{2+} waves were observed *in vivo* in a mouse model of AD expressing APP_{Swe} and PS1_{E9} (Kuchibhotla et al. 2009). In this animal, astrocyte network exhibited elevations in resting calcium and increased network-level functional activity due to senile plaque deposition that affected calcium homeostasis in astrocytes; no evidence for altered astrocytic calcium activity was found before development of senile plaques at age 3 to 3.5 months. FAD PS mutations could influence intra- and intercellular communication pathways in astrocytes, including APP processing and A β clearance, known to be affected by aberrant Ca^{2+} homeostasis (Mattson 2004). A small disturbance in signalling pathways could thus result in significant cumulative effects over time and determine the development of AD pathology. Aberrant Ca^{2+} signalling with subsequent glutamate toxicity has also been implicated in AD (Mattson 2004; Mattson and Chan 2003), and Ca^{2+} may regulate the interaction of PS1 with cytoskeletal proteins, affecting the processing of APP to A β (Tezapsidis et al. 2003). Furthermore, an increased concentration of mislocalised phosphorylated Tau isolated from human post-mortem AD

brain samples and aggregated into paired helical filaments (Wischik et al. 1988), can affect the transport of mitochondria or the components of the endoplasmic reticulum to the peripheral cell regions and lead to a decrease in glucose metabolism and ATP synthesis in these compartments, causing energy deficits or a loss of Ca^{2+} homeostasis (Mattson and Guo 1997). Thus, future work linking the function of mutated presenilins with mitochondrial energy supply in astrocytes might also give us further clues in the understanding of AD.

In summary, experiments presented in this report revealed, for the first time, that expression of mutated presenilin 1 (PS1_{M146V}) in astrocytes alters vesicle dynamics and reduces evoked secretion of the signalling molecule ANP; both may contribute to the development of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Slovenian Research Agency grants #P3 310, J3 3632, J3 4051, J3-4146, J3 6790. V.P.'s work is supported by the National Institutes of Health (HD078678). We thank Ms. Maja Ruper i and Mi o Boži for precious technical support.

References

- Abramov AY, Canevari L, Duchen MR. Calcium signals induced by amyloid beta peptide and their consequences in neurons and astrocytes in culture. *Biochim Biophys Acta*. 2004; 1742(1–3):81–87. [PubMed: 15590058]
- Allaman I, Gavillet M, Bélanger M, Laroche T, Viertl D, Lashuel HA, Magistretti PJ. Amyloid-beta aggregates cause alterations of astrocytic metabolic phenotype: impact on neuronal viability. *J Neurosci*. 2010; 30(9):3326–3338. [PubMed: 20203192]
- Alzheimer, A. Beiträge zur Kenntnis der pathologischen Neuroglia und ihrer Beziehungen zu den Abbauvorgängen im Nervengewebe. In: Franz Nissl, AA., editor. *Histologische und histopathologische Arbeiten über die Grosshirnrinde mit besonderer Berücksichtigung der pathologischen Anatomie der Geisteskrankheiten*. Jena: G Fischer; 1910. p. 401-562.
- Basak JM, Verghese PB, Yoon H, Kim J, Holtzman DM. Low-density lipoprotein receptor represents an apolipoprotein E-independent pathway of A β uptake and degradation by astrocytes. *J Biol Chem*. 2012; 287(17):13959–13971. [PubMed: 22383525]
- Beach TG, McGeer EG. Lamina-specific arrangement of astrocytic gliosis and senile plaques in Alzheimer's disease visual cortex. *Brain Res*. 1988; 463(2):357–361. [PubMed: 3196922]
- DeKosky ST, Scheff SW. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann Neurol*. 1990; 27(5):457–464. [PubMed: 2360787]
- Ebneth A, Godemann R, Stamer K, Illenberger S, Trinczek B, Mandelkow E. Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *J Cell Biol*. 1998; 143(3):777–794. [PubMed: 9813097]
- Giaume C, Kirchhoff F, Matute C, Reichenbach A, Verkhratsky A. Glia: the fulcrum of brain diseases. *Cell Death Differ*. 2007; 14(7):1324–1335. [PubMed: 17431421]
- Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, White CL 3rd, Araoz C. Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc Natl Acad Sci U S A*. 1989; 86(19):7611–7615. [PubMed: 2529544]
- Gu ek A, Vardjan N, Zorec R. Exocytosis in Astrocytes: Transmitter Release and Membrane Signal Regulation. *Neurochem Res*. 2012

- Guénette SY. Mechanisms of Abeta clearance and catabolism. *Neuromolecular Med.* 2003; 4(3):147–160. [PubMed: 14716023]
- Gunawardena S, Goldstein LS. Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*. *Neuron.* 2001; 32(3):389–401. [PubMed: 11709151]
- Gunawardena S, Yang G, Goldstein LS. Presenilin controls kinesin-1 and dynein function during APP-vesicle transport in vivo. *Hum Mol Genet.* 2013; 22(19):3828–3843. [PubMed: 23710041]
- Han W, Li D, Stout AK, Takimoto K, Levitan ES. Ca²⁺-induced deprotonation of peptide hormones inside secretory vesicles in preparation for release. *J Neurosci.* 1999; 19(3):900–905. [PubMed: 9920653]
- Heneka MT, Rodriguez JJ, Verkhratsky A. Neuroglia in neurodegeneration. *Brain Res Rev.* 2010; 63(1–2):189–211. [PubMed: 19944719]
- Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, Van Leuven F. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. *J Neuroinflammation.* 2005; 2:22. [PubMed: 16212664]
- Hynd MR, Scott HL, Dodd PR. Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. *Neurochem Int.* 2004; 45(5):583–595. [PubMed: 15234100]
- Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol.* 2010; 9(1):119–128. [PubMed: 20083042]
- Johnston JM, Burnett P, Thomas AP, Tezapsidis N. Calcium oscillations in type-1 astrocytes, the effect of a presenilin 1 (PS1) mutation. *Neurosci Lett.* 2006; 395(2):159–164. [PubMed: 16300890]
- Kano M, Hashimoto K. Synapse elimination in the central nervous system. *Curr Opin Neurobiol.* 2009; 19(2):154–161. [PubMed: 19481442]
- Krzan M, Stenovec M, Kreft M, Pangrsic T, Grilc S, Haydon PG, Zorec R. Calcium-dependent exocytosis of atrial natriuretic peptide from astrocytes. *J Neurosci.* 2003; 23(5):1580–1583. [PubMed: 12629160]
- Kuchibhotla KV, Lattarulo CR, Hyman BT, Bacskai BJ. Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. *Science.* 2009; 323(5918):1211–1215. [PubMed: 19251629]
- Kulijewicz-Nawrot M, Verkhratsky A, Chvátal A, Syková E, Rodríguez JJ. Astrocytic cytoskeletal atrophy in the medial prefrontal cortex of a triple transgenic mouse model of Alzheimer's disease. *J Anat.* 2012; 221(3):252–262. [PubMed: 22738374]
- Lim D, Ronco V, Grolla AA, Verkhratsky A, Genazzani AA. Glial calcium signalling in Alzheimer's disease. *Rev Physiol Biochem Pharmacol.* 2014; 167:45–65. [PubMed: 24935225]
- Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature.* 2004; 430(7000):631–639. [PubMed: 15295589]
- Mattson MP, Chan SL. Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium.* 2003; 34(4–5):385–397. [PubMed: 12909083]
- Mattson MP, Guo Q. Cell and molecular neurobiology of presenilins: a role for the endoplasmic reticulum in the pathogenesis of Alzheimer's disease? *J Neurosci Res.* 1997; 50(4):505–513. [PubMed: 9404712]
- Morfini G, Szebenyi G, Elluru R, Ratner N, Brady ST. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *EMBO J.* 2002; 21(3):281–293. [PubMed: 11823421]
- Mrak RE, Griffin WS. Glia and their cytokines in progression of neurodegeneration. *Neurobiol Aging.* 2005; 26(3):349–354. [PubMed: 15639313]
- Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol Aging.* 2004; 25(5):663–674. [PubMed: 15172746]
- Nedergaard M, Rodriguez JJ, Verkhratsky A. Glial calcium and diseases of the nervous system. *Cell Calcium.* 2010; 47(2):140–149. [PubMed: 20045186]

- Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron*. 2003; 39(3):409–421. [PubMed: 12895417]
- Olabarria M, Noristani HN, Verkhratsky A, Rodríguez JJ. Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia*. 2010; 58(7):831–838. [PubMed: 20140958]
- Olabarria M, Noristani HN, Verkhratsky A, Rodríguez JJ. Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: mechanism for deficient glutamatergic transmission? *Mol Neurodegener*. 2011; 6:55. [PubMed: 21801442]
- Parpura V, Zorec R. Gliotransmission: Exocytotic release from astrocytes. *Brain Res Rev*. 2010; 63(1–2):83–92. [PubMed: 19948188]
- Pigino G, Morfini G, Pelsman A, Mattson MP, Brady ST, Busciglio J. Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. *J Neurosci*. 2003; 23(11):4499–4508. [PubMed: 12805290]
- Potokar M, Kreft M, Pangrsic T, Zorec R. Vesicle mobility studied in cultured astrocytes. *Biochem Biophys Res Commun*. 2005; 329(2):678–683. [PubMed: 15737639]
- Potokar M, Stenovec M, Gabrijel M, Li L, Kreft M, Grilc S, Pekny M, Zorec R. Intermediate filaments attenuate stimulation-dependent mobility of endosomes/lysosomes in astrocytes. *Glia*. 2010; 58(10):1208–1219. [PubMed: 20544856]
- Potokar M, Stenovec M, Kreft M, Kreft ME, Zorec R. Stimulation inhibits the mobility of recycling peptidergic vesicles in astrocytes. *Glia*. 2008; 56(2):135–144. [PubMed: 17990309]
- Rossner S, Lange-Dohna C, Zeitschel U, Perez-Polo JR. Alzheimer's disease beta-secretase BACE1 is not a neuron-specific enzyme. *J Neurochem*. 2005; 92(2):226–234. [PubMed: 15663471]
- Runnagar MT, Wei X, Hamm-Alvarez SF. Increased protein phosphorylation of cytoplasmic dynein results in impaired motor function. *Biochem J*. 1999; 342(Pt 1):1–6. [PubMed: 10432292]
- Scheff SW, Scott SA, DeKosky ST. Quantitation of synaptic density in the septal nuclei of young and aged Fischer 344 rats. *Neurobiol Aging*. 1991; 12(1):3–12. [PubMed: 2002880]
- Schubert D, Soucek T, Blouw B. The induction of HIF-1 reduces astrocyte activation by amyloid beta peptide. *Eur J Neurosci*. 2009; 29(7):1323–1334. [PubMed: 19519624]
- Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*. 2001; 81(2):741–766. [PubMed: 11274343]
- Soldati T, Schliwa M. Powering membrane traffic in endocytosis and recycling. *Nat Rev Mol Cell Biol*. 2006; 7(12):897–908. [PubMed: 17139330]
- Stenovec M, Kreft M, Poberaj I, Betz WJ, Zorec R. Slow spontaneous secretion from single large dense-core vesicles monitored in neuroendocrine cells. *FASEB J*. 2004; 18(11):1270–1272. [PubMed: 15180959]
- Stenovec M, Milošević M, Petrušić V, Potokar M, Stević Z, Prebil M, Kreft M, Trkov S, Andjuz PR, Zorec R. Amyotrophic lateral sclerosis immunoglobulins G enhance the mobility of Lysotracker-labelled vesicles in cultured rat astrocytes. *Acta Physiol (Oxf)*. 2011; 203(4):457–471. [PubMed: 21726417]
- Stenovec M, Trkov S, Kreft M, Zorec R. Alterations of calcium homeostasis in cultured rat astrocytes evoked by bioactive sphingolipids. *Acta Physiol (Oxf)*. 2014; 212(1):49–61. [PubMed: 24825022]
- Stokin GB, Lillo C, Falzone TL, Brusch RG, Rockenstein E, Mount SL, Raman R, Davies P, Masliah E, Williams DS, et al. Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease. *Science*. 2005; 307(5713):1282–1288. [PubMed: 15731448]
- Strassnig M, Ganguli M. About a peculiar disease of the cerebral cortex: Alzheimer's original case revisited. *Psychiatry (Edmont)*. 2005; 2(9):30–33. [PubMed: 21120106]
- Terry RD. Cell death or synaptic loss in Alzheimer disease. *J Neuropathol Exp Neurol*. 2000; 59(12):1118–1119. [PubMed: 11138931]
- Tezapsidis N, Merz PA, Merz G, Hong H. Microtubular interactions of presenilin direct kinesin of Abeta peptide and its precursors. *FASEB J*. 2003; 17(10):1322–1324. [PubMed: 12738804]

- Trkov S, Stenovec M, Kreft M, Potokar M, Parpura V, Davletov B, Zorec R. Fingolimod--a sphingosine-like molecule inhibits vesicle mobility and secretion in astrocytes. *Glia*. 2012; 60(9): 1406–1416. [PubMed: 22639011]
- Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee SF, Hao YH, Serneels L, De Strooper B, Yu G, Bezprozvanny I. Presenilins form ER Ca²⁺ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. *Cell*. 2006; 126(5):981–993. [PubMed: 16959576]
- Vardjan N, Gabrijel M, Potokar M, Svajger U, Kreft M, Jeras M, de Pablo Y, Faiz M, Pekny M, Zorec R. IFN- γ -induced increase in the mobility of MHC class II compartments in astrocytes depends on intermediate filaments. *J Neuroinflammation*. 2012; 9:144. [PubMed: 22734718]
- Vardjan N, Stenovec M, Jorgacevski J, Kreft M, Grilc S, Zorec R. The fusion pore and vesicle cargo discharge modulation. *Ann N Y Acad Sci*. 2009; 1152:135–144. [PubMed: 19161384]
- Verkhatsky A, Marutle A, Rodríguez-Arellano JJ, Nordberg A. Glial Asthenia and Functional Paralysis: A New Perspective on Neurodegeneration and Alzheimer's Disease. *Neuroscientist*. 2014
- Wang HM, Zhang T, Li Q, Huang JK, Chen RF, Sun XJ. Inhibition of glycogen synthase kinase-3 β by lithium chloride suppresses 6-hydroxydopamine-induced inflammatory response in primary cultured astrocytes. *Neurochem Int*. 2013; 63(5):345–353. [PubMed: 23871716]
- Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA. Structural characterization of the core of the paired helical filament of Alzheimer disease. *Proc Natl Acad Sci U S A*. 1988; 85(13):4884–4888. [PubMed: 2455299]
- Xiao Q, Yan P, Ma X, Liu H, Perez R, Zhu A, Gonzales E, Burchett JM, Schuler DR, Cirrito JR, et al. Enhancing astrocytic lysosome biogenesis facilitates A β clearance and attenuates amyloid plaque pathogenesis. *J Neurosci*. 2014; 34(29):9607–9620. [PubMed: 25031402]
- Yeh CY, Vadhvana B, Verkhatsky A, Rodríguez JJ. Early astrocytic atrophy in the entorhinal cortex of a triple transgenic animal model of Alzheimer's disease. *ASN Neuro*. 2011; 3(5):271–279. [PubMed: 22103264]

Main Points

1. Mobility of peptidergic secretory vesicles and endo-/lysosomes is diminished in 3xTg-AD astrocytes.
2. ATP-evoked decrease in vesicle mobility is larger in 3xTg-AD astrocytes.
3. ATP-evoked peptide discharge is reduced in 3xTg-AD astrocytes.

FIGURE 1.

Attenuated spontaneous mobility of peptidergic vesicles in 3xTg-AD astrocytes. (A) Live cultured wt astrocyte under DIC optics and (B) the confocal image of the same cell expressing ANP.emd stored in individual vesicles observed as bright fluorescent puncta; scale bars, 10 μm . (C) Vesicle tracks ($N=50$) obtained in a 15-s epoch of imaging representative control (wt) and (D) 3xTg-AD astrocytes expressing ANP.emd, respectively. Note less elongated vesicle tracks in the 3xTg-AD astrocyte. (E, F) Frequency histogram of the step length in spontaneously moving vesicles in wt ($N=5025$, E) and 3xTg-AD ($N=5072$,

F) astrocytes. The data were fitted with the function $f=a \times \exp(-0.5 \times (x/x_0)/b)^2/x$, where $a=17.88 \pm 0.00$, $b=0.07 \pm 0.00 \mu\text{m}^{-0.5}$, $x_0=0.07 \pm 0.00 \mu\text{m}$ (black curve) and $a=6.53 \pm 0.13$, $b=0.19 \pm 0.01 \mu\text{m}^{-0.5}$, $x_0=0.31 \pm 0.01 \mu\text{m}$ (grey curve) in wt astrocyte, and with the function $f=a \times \exp(-0.5 \times (\ln x/x_0)/b)^2/x$, where $a=1.96 \pm 0.04$, $b=0.92 \pm 0.02 \mu\text{m}^{-0.5}$, $x_0=0.10 \pm 0.00 \mu\text{m}$ (black curve) in 3xTg-AD astrocyte. The vertical dashed line indicates the step length of $0.2 \mu\text{m}$ obtained close to the intersection of distributions (black and grey curve) in wt astrocytes to discriminate small ($<0.2 \mu\text{m}$) from large ($>0.2 \mu\text{m}$) steps. Note the higher proportion (%) of smaller steps lengths in the 3xTg-AD astrocyte indicated by the absence of the second mode distribution seen in wt astrocytes. **(G)** Track length (TL), **(H)** maximal displacement (MD), **(I)** directionality index (DI) and **(J)** speed of ANP-loaded vesicles in wt and 3xTg-AD astrocytes. Note substantially diminished TL, MD, DI and vesicle speed in 3xTg-AD astrocytes. The numbers above the top of the bars (mean $\pm$ SEM) indicate the number of vesicles analysed; the numbers at the bottom of the bars indicate the number of cells analysed.

FIGURE 2.

ATP-evoked reduction in vesicle mobility is larger in 3xTg-AD than in wt astrocytes. (**A–D**) Mobility of peptidergic vesicles (**A, B**, TL; **C, D**, MD; mean±SEM) in control (wt) and 3xTg-AD astrocytes before (–), and within the first (1') and the second (2') minute after stimulation with 100 μM ATP. Note the stronger reduction in vesicle mobility in 3xTg-AD cells. The numbers at the top indicate the number of vesicles and the numbers at the bottom of the bars indicate the number of cells analysed. *** $P < 0.001$ vs resting (non-stimulated) vesicle mobility (Mann-Whitney U test). (**E, F**) The plots displaying the relationship

between MD and TL (directionality index, DI) in ANP vesicles in wt ($N=8$) and 3xTg-AD ($N=7$) astrocytes before stimulation (**E**, **F**, white circles) and during the second minute (**E**, **F** black circles) after stimulation with 100 μ M ATP. A linear function (black lines) of the form $[MD=MD_0+a \times (TL)]$ was fitted to the data; the slopes ($a \pm SEM$) correspond to the DI. This parameter (displayed in the top right corners) diminished significantly ($P < 0.001$; ANCOVA) within the second minute after ATP stimulation in 3xTg-AD astrocytes but not in wt astrocytes.

FIGURE 3.

Reduction of evoked peptide secretion in 3xTg-AD astrocytes. **(A)** Confocal images of individual peptidergic vesicles in wt (top) and 3xTg-AD (bottom) astrocytes discharging fluorescent ANP.emd cargo during cell stimulation with 100 μ M ATP. The rapid decrease in fluorescence (~ 0.5 s) indicates rapid discharge of the vesicle cargo. Time marks in the top right corners indicate the time related to the arbitrary starting point (0 s). Scale bar, 0.5 μ m. **(B)** Normalized time-dependent changes in individual vesicle fluorescence (from **A**). The horizontal dotted lines indicate the minimum and maximum fluorescence levels. The vertical

dashed lines indicate the period where the vesicle fluorescence was measured from the images displayed in **A**. The addition of ATP (100 μ M) is indicated by the horizontal rectangle. **(C)** The number of ATP-evoked secretory events indicative of full-collapse vesicle fusion is strongly decreased in 3xTg-AD astrocytes. * P <0.05 vs wt (Mann-Whitney U test).

FIGURE 4.

Attenuated spontaneous mobility of peptidergic vesicles and reduced ATP-evoked peptide secretion from PS1_{M146V}-expressing rat astrocytes. (A) TL, (B) maximal MD, (C) DI and (D) speed of ANP-loaded vesicles in wt and PS1_{M146V} rat astrocytes. Note diminished TL, MD, and vesicle speed in PS1_{M146V}-expressing astrocytes. The numbers above the top of the bars (mean±SEM) indicate the number of vesicles analysed; the numbers at the bottom of the bars indicate the number of cells analysed. (E) The ATP-evoked secretory events were

substantially decreased in PS1_{M146V}-expressing astrocytes. *** $P < 0.05$ vs wt (Mann-Whitney U test).

FIGURE 5.

Attenuated spontaneous mobility of LysoTracker-labelled vesicles in 3xTg-AD astrocytes. (A) Track length (TL), (B) maximal displacement (MD), (C) directionality index (DI) and (D) speed of LyTR-labelled vesicles (indicating acid endosomes/lysosomes) in wt and 3xTg-AD astrocytes. Note diminished TL, MD, DI and vesicle speed in 3xTg-AD astrocytes. The numbers above the top of the bars (mean $\pm$ SEM) indicate the number of vesicles analysed; the numbers at the bottom of the bars indicate the number of cells analysed. *** P <0.001 vs wt (Mann-Whitney U test).

FIGURE 6.

ATP-evoked reduction in LysoTracker-labelled vesicle mobility is larger in wt than in 3xTg AD astrocytes. (A–D) Mobility of LyTR-labelled vesicles (A, B, TL; C, D, MD; mean $\pm$ SEM) in control (wt) and in 3xTg-AD astrocytes before (–), and within the first (1') and the second (2') minute after stimulation with 100 μ M ATP. Note a faster reduction in vesicle mobility in wt cells. The numbers above the top of the bars indicate the number of vesicles and the numbers at the bottom of the bars indicate the number of cells analysed. *** $P < 0.001$ vs resting (non-stimulated) vesicle mobility (Mann-Whitney U test). (E, F) The plots

displaying the relationship between MD and TL (directionality index, DI) in LyTR vesicles in wt ($N=4$) and 3xTg-AD ($N=4$) astrocytes before stimulation (**E**, **F**, white circles) and during the second minute (**E**, **F**, black circles) after stimulation with 100 μ M ATP. A linear function (black lines) of the form $[MD=MD_0+a \times (TL)]$ was fitted to the data, the slopes ($a \pm SEM$) correspond to the DI (displayed in the top right corners). This parameter decreased significantly ($P<0.001$; ANCOVA) within the second minute after ATP stimulation in wt and in 3xTg-AD astrocytes.

FIGURE 7.

Reduced proportion of ATP-evoked oscillatory calcium responses in 3xTg-AD astrocytes. (A) Confocal images of astrocytes containing the fluorescent Ca^{2+} indicator Fluo-2. The images display astrocytes before (0 s, left) and after stimulation with $100 \mu\text{M}$ ATP (30 and 180 s; middle and right, respectively). ATP evoked strong increases in intracellular calcium activity as indicated by the pseudocoloured intensity scale (right, 0–255 intensity levels). Scale bars, $50 \mu\text{m}$. ATP (white rectangle) evoked two types of calcium responses in wt (B) and 3xTg-AD (C) astrocytes: (i) biphasic transients and (ii) oscillatory calcium responses.

The peak (p , mean $\pm$ SEM) and the time-integrated $[Ca^{2+}]_i$ (S) evoked by 100 μ M ATP. The horizontal dotted line indicates the baseline fluorescence level (F_0). The downward (black) and upward (white) arrowheads indicate successive minima and maxima (p_{osc}), respectively, in $[Ca^{2+}]_i$ during oscillatory responses. (**D, E**) Plots displaying the relationship between the ratio of the sum of follow-up peak calcium amplitudes – oscillations (Sum p_{osc}) and the first calcium peak amplitude (p), and the number of oscillations within 4 min (No. $p_{osc}/4$ min) in calcium responses evoked by 100 μ M ATP in wt (**D**) and 3xTg-AD (**E**) astrocytes. The non-oscillatory (i) calcium responses are confined to the grey shaded areas delineated with dashed lines, while the white zone of the plots show the oscillatory (ii) responses (the details are described in the Results section). The relative proportion (%) of non-oscillatory (i, left) and oscillatory (ii, right) responses in wt ($N=115$) and 3xTg-AD ($N=150$) astrocytes are displayed at the top of the plots. Note that ATP evoked three times more oscillatory responses in wt than in 3xTg-AD astrocytes.

FIGURE 8.

Altered peak calcium responses evoked by purinergic receptor stimulation in 3xTg-AD astrocytes. (**A**, **C**) The peak (p) in ATP-evoked biphasic calcium transients (**A**) was smaller in 3xTg-AD astrocytes, while the peak (p) in oscillatory calcium responses (**C**) was larger in 3xTg-AD astrocytes. (**B**, **D**) ATP mobilized a similar cumulative amount of free Ca^{2+} during biphasic (**B**) and oscillatory calcium responses (**D**) in both wt and 3xTg-AD astrocytes as indicated by the integrated calcium activity (S). The numbers at the bottom of the bars indicate the number of cells analysed. * $P < 0.05$ vs wt (Mann-Whitney U test).