Published in final edited form as:

Pharmacology. 2014 ; 93(0): 24–31. doi:10.1159/000357383.

Short- and Long-Term Treatment of Mouse Cortical Primary Astrocytes with β -Amyloid Differentially Regulates the mRNA Expression of L-Type Calcium Channels

Nina Daschil^a, Stefanie Geisler^b, Gerald J. Obermair^b, and Christian Humpel^a

^aDepartment of Psychiatry and Psychotherapy, University Clinic for General and Social Psychiatry, Innsbruck, Austria

^bDivision of Physiology, Innsbruck Medical University, Innsbruck, Austria

Abstract

Background—It is well established that reactive astrocytes express L-type calcium channels (LTCC), but their functional role is completely unknown. We have recently shown that reactive astrocytes highly express the $\text{Ca}_v1.2$ α_1 -subunit around β -amyloid (A β) plaques in an Alzheimer mouse model. The aim of the present study was to explore whether A β peptides may regulate the mRNA expression of all LTCC subunits in primary mouse astrocytes in culture.

Methods—Confluent primary astrocytes were incubated with 10 $\mu\text{g/ml}$ of human or murine A β or the toxic fragment A β_{25-35} for 3 days or for 3 weeks. The LTCC subunits were determined by quantitative RT-PCR.

Results—Our data show that murine A β_{42} slightly but significantly increased $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ expression when incubated for 3 days. This acute treatment with murine A β enhanced β_2 and β_3 mRNA levels but decreased $\alpha_2\delta$ -2 mRNA expression. When astrocytes were incubated for 3 weeks, the levels of $\text{Ca}_v1.2$ α_1 were significantly decreased by the murine A β and the toxic fragment. As a control, the protein kinase C- ϵ activator DCP-LA displayed a decrease in $\text{Ca}_v2.1$ expression.

Conclusion—In conclusion, our data show that A β can differentially regulate LTCC expression in primary mouse astrocytes depending on incubation time.

Keywords

L-type calcium channel; Astrocytes; Reverse transcription-polymerase chain reaction; mRNA; β -Amyloid

Introduction

Alzheimer's disease (AD) is the most common progressive neurodegenerative disorder in elderly people. The extracellular deposition of β -amyloid ($A\beta$) plaques, intraneuronal tau pathology, synaptic loss, cholinergic neuronal cell death and inflammatory processes are the major neuropathological hallmarks of AD. A possible role for Ca^{2+} dysregulation in the aging brain and in AD has been postulated [1, 2]. L-type calcium channels (LTCC) are assumed to be involved in Ca^{2+} homeostasis [3]. LTCC consist of a pore-forming α_1 -subunit ($Ca_v1.1$, 1.2, 1.3 or 1.4), which plays a role in voltage sensing and serves as a drug-binding site. Auxiliary β - and $\alpha_2\delta$ -subunits are responsible for membrane trafficking and the modulation of channel properties [4, 5].

Besides their occurrence in neurons, LTCC are also expressed by astrocytes [6–8]. After brain injury, glial cells become reactive and highly express glial fibrillary acidic protein (GFAP) [9]. In contrast, neuron-glial antigen 2 glia also reacts to degenerative stimuli but does not express GFAP [10]. It is well established that brain injury leads to an upregulation of $Ca_v1.2$ α_1 -subunits in reactive GFAP-positive astrocytes [11, 12]. The expression of $Ca_v1.2$ and $Ca_v1.3$ channels in primary astrocytes is well established [8, 13–15], but their function is not completely understood. We have recently shown that $Ca_v1.2$ α_1 -subunits are highly expressed in reactive astrocytes surrounding $A\beta$ plaques in a transgenic amyloid precursor protein-overexpressing mouse model [12, 16]. In addition, we have recently reported that reactive astrocytes around plaques do not coexpress the auxiliary β_4 -subunit [16]. The role and function of these calcium channels in astrocytes are not known. In order to investigate the role of astroglial LTCC channels, it would be necessary to explore the cellular regulation of all LTCC subunits in the astroglia. Unfortunately, no specific and immunohistochemically selective antibodies are available for most of the LTCC subunits.

The principal question arises whether astroglial $Ca_v1.2$ α_1 -subunit expression is caused by the AD pathology (including inflammation, oxidative stress or Ca^{2+} dysregulation) or whether it is caused directly by plaque generation induced by the $A\beta$ cascade. In the latter case, the question arises whether the plaques per se (containing aggregated $A\beta$ peptides) or the soluble $A\beta$ peptides alone may induce astroglial $Ca_v1.2$ α_1 -subunit expression. And third, it is possible that $Ca_v1.2$ α_1 -subunit expression is a consequence of the activation and reaction of the astrocytes rather than independent of plaques. In order to study these questions, we cultured primary astrocytes from the murine cortex to explore whether exogenous $A\beta$ peptides directly regulate expression of the LTCC channel. We show that $A\beta$ peptides moderately enhance LTCC expression after short incubation but decrease the expression after longer incubation.

Material and Methods

Animals

Male and female wild-type B6129SF2/J mice were purchased from the Jackson Laboratory (Bar Harbor, Minn., USA). All mice were housed according to standard animal care protocols, fed ad libitum with regular animal diet and maintained in a pathogen-free

environment. All experiments were approved by the Austrian Ministry of Science and Research and conformed to the Austrian guidelines on animal welfare and experimentation.

Primary Astrocyte Cell Culture

Primary astrocytes were cultured as previously reported, with slight modifications [17, 18]. Postnatal-day 4 mice were rapidly sacrificed. The cortices were dissected and pooled in 1.5 ml of pre-warmed sterile 0.25% trypsin solution. With a scalpel, the tissue was cut into small pieces. After an incubation time of 15 min at 37°C, the trypsin was discarded. To obtain single cells, the remaining tissue was passed through a 70-µm cell strainer (BD Falcon; Becton Dickinson, Franklin Lakes, N.J., USA) into a tube containing 1 ml of horse serum (16050-122; Gibco, USA) and 500 µl of DNase I (10 mg/ml; Roche Applied Sciences, Vienna, Austria). After 5 min of centrifugation (1,500 rpm), the pellet was resuspended in 10 ml of glial medium (5% horse serum, 0.5% FCS in 100 ml OptiMEM; Gibco). The cells were centrifuged again for 5 min at 1,500 rpm. The pellet was then resuspended in 2 ml of glial medium and transferred onto collagen-coated 6-well plates (150 µl of cells/well). The medium was changed 24 h after preparation and the cells were cultured for 2 weeks to become confluent. To remove residual microglia, the cells were shaken (180 rpm, 37°C) overnight and then again cultured in fresh medium. Astrocytes adhere to the surface, while other cell types peel off with the supernatant. We obtained a high astroglial purity with <0.1% oligodendrocytes or microglia.

Treatment with Exogenous Stimuli

Primary confluent astrocytes were incubated for either 3 days or 3 weeks by various treatments to induce cell activation: human Aβ₄₂ (10 µg/ml; Calbiochem, San Diego, Calif., USA); murine Aβ₄₂ (10 µg/ml; Calbiochem); the toxic Aβ₂₅₋₃₅ fragment (10 µg/ml; A4559; Sigma, St. Louis, Mo., USA); protein kinase C (PKC)-ε activator (3 µg/ml; DCP-LA, D5318; Sigma); and cAMP analog (12 µg/ml; Cpt-cAMP, C3912; Sigma). For some experiments, Aβ₂₅₋₃₅ treatments were performed at an acidic pH of 6.7 or with 45 µmol/l H₂O₂ in order to enhance astrogliosis. The 3-week treatments were conducted with 1% horse serum in glial medium.

Immunohistochemistry

Immunohistochemistry was performed as described [16]. Astrocytes were fixed in cold 4% paraformaldehyde/10 mmol/l PBS (4°C, 30 min). After fixation, the cells were washed with PBS and then incubated with 0.1% Triton X-100/PBS (T-PBS) for 30 min at room temperature, shaking. Subsequently, the astrocytes were pretreated for 20 min with 5% methanol/1% H₂O₂/PBS, and after thorough rinsing, the cells were blocked with 20% horse serum/0.2% BSA/T-PBS. Finally, they were incubated for 2–3 days at 4°C with a primary antibody diluted in 0.2% BSA/T-PBS. The primary antibodies used for this study were: rabbit anti-Ca_v1.2 α₁-subunit (1:2,000; Sigma C1603); chicken anti-GFAP (1:2,000; Millipore AB5541); and rabbit polyclonal to glutamine synthetase (1:2,000; GluS; Abcam). After incubation, the cells were washed in PBS and incubated with biotinylated secondary antibodies: α-rabbit (1:200; Vector V1011) or α-chicken (1:200; Vector K0722). After 1 h of incubation, the astrocytes were washed with PBS and incubated with an avidin-biotin complex solution (Vectastain Elite ABC reagent; Vector Laboratories) for another 1 h. After

washing with 50 mmol/l Tris-buffered saline, the signal was detected by using 0.5 mg/ml 3,3'-diaminobenzidine including 0.003% H₂O₂ as a substrate in Tris-buffered saline for 3–8 min. The reaction was terminated by adding Tris-buffered saline. The stained cells were rinsed in 10 mmol/l PBS, at a pH of 7.4. Control experiments for all antibodies were conducted by omitting the primary antibody. Staining was visualized with an Olympus BX61 fluorescence microscope and pictures were captured with Openlab software connected to an Apple computer. For colocalization studies, fluorescence immunohistochemistry was performed similarly as for chromogenic staining but without methanol pretreatment. After incubation with the primary antibody, the cells were washed in PBS and incubated with fluorescent Alexa 488 secondary antibodies (Invitrogen Life Technologies, Vienna, Austria). Additionally, DAPI staining was performed. After 1 h of incubation, the astrocytes were washed and ready for analysis.

RNA Isolation and Quantitative TaqMan-PCR

Quantitative RT-PCR was performed as recently described [16]. Astrocytes were rinsed quickly with PBS to remove the medium. Subsequently, by the addition of 600 µl of Buffer RLT (RNeasy Mini Kit; Qiagen, Hilden, Germany), cells were harvested with a cell scraper. Three wells from a 6-well dish were pooled. The astrocytes were disrupted by using QIAshredder columns, and total RNA was extracted utilizing the RNeasy Mini Kit. RNA concentrations were determined photometrically using BioPhotometer 6131 (Eppendorf, Hamburg, Germany). Reverse transcription was performed on 1 µg of RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) and random hexamer primers (Promega, Madison, Wisc., USA). The RT mix was incubated for 60 min at 37°C. The relative abundance of different Ca_v subunit transcripts was assessed by TaqMan quantitative PCR (qRT-PCR) using a standard curve method based on PCR products of known concentration in combination with normalization using the most stable control genes, as previously described [19]. TaqMan gene expression assays specific for all high-voltage-activated Ca²⁺ channel subunits (α₁, β and α_{2δ}), designed to span exon-exon boundaries, were purchased from Applied Biosystems (Foster City, Calif., USA). The following assays (Applied Biosystems) were used [name (gene symbol), assay ID]: Ca_v1.1 (Cacna1s), Mm00489257_m1; Ca_v1.2 (Cacna1c), Mm00437953_m1; Ca_v1.3 (Cacna1d), Mm01209919_m1; Ca_v1.4 (Cacna1f), Mm00490443_m1; Ca_v2.1 (Cacna1a), Mm00432190_m1; Ca_v2.2 (Cacna1b), Mm00432226_m1; Ca_v2.3 (Cacna1e), Mm00494444_m1; β₁ (Cacnb1), Mm00518940_m1; β₂ (Cacnb2), Mm00659092_m1; β₃ (Cacnb3), Mm00432233_m1; β₄ (Cacnb4), Mm00521623_m1; α_{2δ}-1 (Cacna2d1), Mm00486607_m1; α_{2δ}-2 (Cacna2d2), Mm00457825_m1; α_{2δ}-3 (Cacna2d3), Mm00486613_m1; and α_{2δ}-4 (Cacna2d4), Mm01190105_m1. The endogenous control genes (Applied Biosystems) included were [name (gene symbol), assay ID]: γ-cytoplasmic actin (Actb), Mm00607939_s1; β₂-microglobulin (B2m), Mm00437762_m1; glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Mm99999915_g1; hypoxanthine phosphoribosyltransferase 1 (Hprt1), Mm00446968_m1; succinate dehydrogenase complex, subunit A (Sdha), Mm01352363_m1; tata box-binding protein (Tbp), Mm00446973_m1; and transferrin receptor (Tfrc), Mm00441941_m1. qRT-PCR (50 cycles) was performed in duplicates, using 20 ng total RNA equivalents of cDNA and the specific TaqMan gene expression assay for each 20-µl reaction in TaqMan Universal PCR Master Mix (Applied

Biosystems). Analyses were performed using the 7500 Fast System (Applied Biosystems). The C_t values for each Ca_v gene expression assay were recorded for each individual preparation. To allow a direct comparison between expression levels in different tissues, we normalized all experiments to *Tbp*, *Hprt1* and *Actb*, which were determined to be the most stably expressed reference genes across all preparations and time points [20]. Subsequently, normalized molecule numbers were calculated for each Ca_v subunit from their respective standard curve [19].

Data Analysis and Statistics

The data were organized and analyzed using MS Excel and KaleidaGraph statistical software. Statistical significance was determined by one-way ANOVA followed by the Fisher protected least significant difference (PLSD) post hoc test, and $p < 0.05$ represents significance.

Results

Astroglial Cultures

Immunohistochemistry for GFAP revealed a moderate staining of confluent astrocytes, while only occasionally, large reactive strong GFAP astrocytes were visible (fig. 1a, c). Immunostaining for glutamate synthetase showed that the whole confluent layer was strongly positive (fig. 1b). The reactive GFAP-positive astrocytes were also seen by fluorescent immunostaining over DAPI-positive nuclei (fig. 1e, f). No expression of $Ca_v1.2$ α_1 -subunit-like immunoreactivity was seen in these astroglial cultures under all tested conditions (fig. 1d).

$Ca_v1.2$ α_1 -Subunit mRNA Expression after Three Days of Incubation

Neither the PKC- ϵ activator DCP-LA nor the cAMP agonist affected mRNA expression of α_1 -subunits (fig. 2). Human $A\beta_{42}$ had no effect on mRNA expression, while murine $A\beta_{42}$ slightly but significantly enhanced $Ca_v1.2$ and $Ca_v1.3$ α_1 -subunits (fig. 2). The toxic $A\beta_{25-35}$ fragment had no effect on expression of any tested α_1 -subunits (fig. 2). However, $Ca_v1.3$ was significantly decreased when costimulated with the toxic $A\beta_{25-35}$ fragment and H_2O_2 (fig. 2).

Expression of Auxiliary Subunits after Three Days

In order to test expression of all auxiliary subunits in the primary astrocytes, we focused on murine $A\beta_{42}$, which had a significant effect on $Ca_v1.2$ subunit expression. Among all auxiliary subunits tested, only β_2 and β_3 were significantly enhanced, while the mRNA for $\alpha_2\delta-2$ was significantly downregulated by murine $A\beta_{42}$ (fig. 3).

α_1 -Subunit mRNA Expression after Three Weeks of Incubation

A 3-week incubation of astroglia showed a significant increase for the mRNA of $Ca_v1.2$ α_1 and β_3 and $\alpha_2\delta-2$ compared with a 3-day control treatment (fig. 3). Surprisingly, a chronic treatment for 3 weeks with murine $A\beta_{42}$ and the toxic fragment $A\beta_{25-35}$ significantly decreased $Ca_v1.2$ α_1 -subunit expression, but not $Ca_v2.1$ and $Ca_v1.3$. The PKC- ϵ activator

DCP-LA significantly decreased $\text{Ca}_v2.1$, but not $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, subunit expression in primary murine astroglia (fig. 4).

Discussion

In the present study we found that A β peptides differentially regulate LTCC mRNA expression when incubated for a short (3 days) or long period (3 weeks).

Astroglial Cultures

It is assumed that astrocytes may contribute to several CNS pathologies, and reactive astrogliosis points to damaged tissue, which can easily be detected by GFAP staining, a reliable marker for activated astrocytes [9]. In addition, glutamine synthetase is another astroglia-specific staining. The primary astroglial culture model is well established in our research group [17, 18], displaying a confluent layer with some GFAP-positive reactive astroglia, and <1% of other cells. In order to culture astroglia for a longer time period, the cells are cultured with low serum to prevent overgrowth of the cells and subsequent cell death. In order to enhance astrogliosis, in some experiments the cells were incubated at a low pH (6.7) or together with hydrogen peroxide.

Effects of PKC- ϵ Activator and cAMP Agonist on LTCC Subunits

It has been reported that PKC- ϵ overexpression increases the mRNA levels of $\text{Ca}_v2.1$ and $\text{Ca}_v1.2$ α_1 -subunits in primary astrocytes [13]. In addition, a novel PKC- ϵ activator, DCP-LA, reduced A β levels [21], providing an important link to our A β experiments. Thus, as a control, we incubated our primary astrocytes with DCP-LA [21]. In fact, our data only showed a slight upregulation of the $\text{Ca}_v1.2$ subunit treated for 3 days with DCP-LA, while after 3 weeks, $\text{Ca}_v2.1$ was significantly downregulated. As another control, the effect of cAMP was tested, because it is well known that LTCC activity is regulated by cAMP production and subsequent activation of protein kinase A, involving β -adrenergic receptors [22, 23]. Further, increased cAMP levels in cultured astrocytes can convert flat polygonal astrocytes into process-bearing stellate astrocytes [24]. In order to increase intracellular cAMP, we used the highly membrane-permeable agonist cpt-cAMP. However, our data show that enhanced cAMP did not affect LTCC expression in primary astrocytes at all time points.

Effects of Acute (Three-Day) A β Treatment on α_1 -Subunits

We have recently shown that the $\text{Ca}_v1.2$ α_1 -subunit is highly expressed in GFAP-positive astrocytes surrounding A β plaques in an AD mouse model [16]. However, in this in vivo model, we cannot distinguish whether the peptides alone or the plaque per se may activate the calcium channels in these reactive astrocytes. Therefore, we aimed to investigate the mRNA expression levels of all LTCC subunits in primary astrocytes of postnatal mice. In the present study, we used 3 different A β peptides: human A β_{42} , murine A β_{42} and a toxic A β_{25-35} amino acid fragment. We tested human and murine peptides because 3 amino acids are different between these two species [25] and it may be possible that human A β does not act on primary murine astrocytes. A β_{25-35} was used because this small fragment is the main toxic component of the A β peptide. Previous work has shown that A β_{25-35} induces

cytoskeletal reorganization in cultured astrocytes [26] or revealed neurotrophic and neurotoxic effects on hippocampal neurons [27]. It was also suggested that this A β _{25–35} peptide destabilizes calcium homeostasis and renders human cortical neurons more vulnerable to excitotoxicity [28]. All peptides were used at a very high concentration of 10 μ g/ml because it has been reported that primary astrocytes are markedly affected under these conditions [29, 30]. Our present data show that the addition of murine A β ₄₂ for 3 days slightly but significantly upregulated LTCC mRNA expression but not the protein of Ca v 1.2 and Ca v 1.3 in primary astrocytes. Thus, we conclude that an acute, brief exposure to A β does not markedly influence LTCC mRNA expression in cultured astrocytes.

Effects on Auxiliary Subunits and Potentiation of Astrogliosis

In order to test whether the A β peptide activates auxiliary subunits in vitro (independent of the α_1 -subunit), we performed a qRT-PCR analysis of all subunits. We could identify that β_2 and β_3 were upregulated but $\alpha_2\delta$ -2 was downregulated after incubation with murine A β ₄₂ for 3 days. This differential expression profile is difficult to explain, and still the signaling process in primary astrocytes is unknown. More experiments using electrophysiology with the patch clamp technique are required to further investigate this issue. In order to enhance the reactivity of primary astrocytes, we incubated the cells at a low pH (acidosis, pH 6.7) or treated the cells with a high concentration of hydrogen peroxide together with the toxic A β _{25–35} fragment. Indeed, chronic hypoxia dramatically increased the current density of membrane channel proteins in HEK 293 cells [31]. It has also been reported that A β induces cell death by generation of H₂O₂ [32]. However, costimulation with acidosis did not markedly affect mRNA expression of the LTCC subunits, while H₂O₂ led only to a slight decrease in expression, suggesting that, again, another more robust mechanism may account for the calcium channel activation.

Long-Time Exposure of Primary Astrocytes with A β

Thus, we hypothesized that in order to enhance LTCC expression, primary astrocytes must be exposed to A β peptides for a longer time period. In addition, the higher A β concentration and longer incubation over 3 weeks at 37°C would also likely induce aggregation of A β peptides and provide more plaque-promoting conditions. In order to decrease dramatic proliferation, the primary astrocytes were cultured under low-serum conditions together with the A β peptides for 3 weeks. Unexpectedly, we observed that the Ca v 1.2 mRNA were significantly downregulated and again no Ca v 1.2 α_1 protein expression was seen. This suggests that neither soluble nor aggregated A β peptides alone are sufficient to induce a dramatic upregulation of the calcium channel in vitro. Thus, we conclude that more potent exogenous stimuli are necessary to activate calcium channels in reactive astrocytes in vitro, such as inflammation, oxidative stress and/or large A β plaques, or even an exposure for months. In fact, we have also shown in vivo that calcium channels are only upregulated at a very late stage (>11 months) in mice.

Conclusions

In conclusion, our data show that A β peptides differentially regulate LTCC mRNA expression in cultured primary cortical astrocytes when incubated for short and long periods.

It seems likely that A β peptides alone are not sufficient to activate astroglial calcium channels. More robust long-lasting effects are necessary to activate Ca²⁺ expression in GFAP-positive astrocytes.

Acknowledgements

We are thankful to Ursula Kirzenberger-Winkler for excellent technical assistance. This study was supported by the 'Sonderforschungsbereich SFB F4405-B19 and F4406-B19' of the Austrian Science Fund.

References

1. Landfield PW. 'Increased calcium-current' hypothesis of brain aging. *Neurobiol Aging*. 1987; 8:346–347. [PubMed: 3627350]
2. Khachaturian ZS. Hypothesis on the regulation of cytosol calcium concentration and the aging brain. *Neurobiol Aging*. 1987; 8:345–346. [PubMed: 3627349]
3. Thibault O, Gant JC, Landfield PW. Expansion of the calcium hypothesis of brain aging in Alzheimer's disease: minding the store. *Aging Cell*. 2007; 6:307–317. [PubMed: 17465978]
4. Catterall WA. Structure and regulation of voltage-gated Ca²⁺ channels. *Annu Rev Cell Dev Biol*. 2000; 16:521–555. [PubMed: 11031246]
5. Obermair, GJ.; Flucher, BE. Neuronal functions of auxiliary calcium channel subunits. In: Stephens, G.; Mochida, S., editors. *Modulation of Presynaptic Calcium Channels*. Springer Science; Dordrecht: 2013. p. 29-59.
6. MacVicar BA. Voltage-dependent calcium channels in glial cells. *Science*. 1984; 226:1345–1347. [PubMed: 6095454]
7. Latour I, Hamid J, Beedle AM, Zamponi GW, MacVicar BA. Expression of voltage-gated Ca²⁺ channel subtypes in cultured astrocytes. *Glia*. 2003; 41:347–353. [PubMed: 12555202]
8. D'Ascenzo M, Vairano M, Andreassi C, Navarra P, Azzena GB, Grassi C. Electrophysiological and molecular evidence of L-(Ca_v1), N-(Ca_v2.2), and R-(Ca_v2.3) type Ca²⁺ channels in rat cortical astrocytes. *Glia*. 2004; 45:354–363. [PubMed: 14966867]
9. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol*. 2010; 119:7–35. [PubMed: 20012068]
10. Nishiyama A, Yang Z, Butt A. Astrocytes and NG2-glia: what's in a name? *J Anat*. 2005; 207:687–693. [PubMed: 16367796]
11. Westenbroek RE, Bausch SB, Lin RCS, Franck JE, Noebels JL, Catterall WA. Upregulation of L-type Ca²⁺ channels in reactive astrocytes after brain injury, hypomyelination, and ischemia. *J Neurosci*. 1998; 18:2321–2334. [PubMed: 9502793]
12. Willis M, Kaufmann WA, Wietzorrek G, Hutter-Paier B, Moosmang S, Humpel C, Hofmann F, Windisch M, Knaus HG, Marksteiner J. L-type calcium channel Ca_v1.2 in transgenic mice overexpressing human A β PP751 with the London (V717I) and Swedish (K670M/N671L) mutations. *J Alzheimers Dis*. 2010; 20:1167–1180. [PubMed: 20413896]
13. Burgos M, Pastor MD, González JC, Martínez-Galan JR, Vaquero CF, Fradejas N, Benavides A, Hernández-Guijo JM, Tranque P, Calvo S. PKC ϵ upregulates voltage-dependent calcium channels in cultured astrocytes. *Glia*. 2007; 55:1437–1448. [PubMed: 17676593]
14. Yan E, Li B, Gu L, Hertz L, Peng L. Mechanisms for L-channel-mediated increase in [Ca²⁺]_i and its reduction by anti-bipolar drugs in cultured astrocytes combined with its mRNA expression in freshly isolated cells support the importance of astrocytic L-channels. *Cell Calcium*. 2013; 54:335–342. [PubMed: 24079970]
15. MacVicar BA, Hochman D, Delay MJ, Weiss S. Modulation of intracellular Ca⁺⁺ in cultured astrocytes by influx through voltage-activated Ca⁺⁺ channels. *Glia*. 1991; 4:448–455. [PubMed: 1718862]
16. Daschil N, Obermair GJ, Flucher BE, Stefanova N, Hutter-Paier B, Windisch M, Humpel C, Marksteiner J. Ca_v1.2 calcium channel expression in reactive astrocytes is associated with the

- formation of amyloid- β plaques in an Alzheimer's disease mouse model. *J Alzheimers Dis.* 2013; 37:439–451. [PubMed: 23948887]
17. Moser KV, Humpel C. Blood-derived serum albumin contributes to neurodegeneration via astroglial stress fiber formation. *Pharmacology.* 2007; 80:286–292. [PubMed: 17671402]
 18. Zassler Z, Blasig I, Humpel C. Protein delivery of caspase-3 induces cell death in malignant C6 glioma, primary astrocytes and immortalized and primary brain capillary endothelial cells. *J Neurooncol.* 2005; 71:127–134. [PubMed: 15690127]
 19. Schlick B, Flucher BE, Obermair GJ. Voltage-activated calcium channel expression profiles in mouse brain and cultured hippocampal neurons. *Neuroscience.* 2010; 167:786–798. [PubMed: 20188150]
 20. Vandesompele J, de Preter K, Pattyn F, Poppe B, van Roy N, de Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002; 3:RESEARCH0034. [PubMed: 12184808]
 21. Nelson TJ, Cui C, Luo Y, Alkon DL. Reduction of β -amyloid levels by novel protein kinase C activators. *J Biol Chem.* 2009; 284:34514–34521. [PubMed: 19850930]
 22. Harvey RD, Hell JW. $\text{Ca}_v1.2$ signaling complexes in the heart. *J Mol Cell Cardiol.* 2013; 58:143–152. [PubMed: 23266596]
 23. Hall DD, Davare MA, Shi M, Allen ML, Weisenhaus M, McKnight GS, Hell JW. Critical role of cAMP-dependent protein kinase anchoring to the L-type calcium channel $\text{Ca}_v1.2$ via A-kinase anchor protein 150 in neurons. *Biochemistry.* 2007; 46:1635–1646. [PubMed: 17279627]
 24. Won CL, Oh YS. cAMP-induced stellation in primary astrocyte cultures with regional heterogeneity. *Brain Res.* 2000; 887:250–258. [PubMed: 11134613]
 25. Yamada T, Sasaki H, Furuya H, Miyata T, Goto I, Sakaki Y. Complementary DNA for the mouse homolog of the human amyloid beta protein precursor. *Biochem Biophys Res Commun.* 1987; 149:665–671. [PubMed: 3322280]
 26. Salinero O, Moreno-Flores MT, Ceballos ML, Wandosell F. β -Amyloid peptide induced cytoskeletal reorganization in cultured astrocytes. *J Neurosci Res.* 1997; 47:216–223. [PubMed: 9008152]
 27. Yankner BA, Duffy LK, Kirschner DA. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science.* 1990; 250:279–282. [PubMed: 2218531]
 28. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. β -Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci.* 1992; 12:376–389. [PubMed: 1346802]
 29. Pentreath VW, Mead C. Responses of cultured astrocytes, C6 glioma and 1321NI astrocytoma cells to amyloid β -peptide fragments. *Nonlinearity Biol Toxicol Med.* 2004; 2:45–63. [PubMed: 19330108]
 30. Abramov AY, Canevari L, Duchon MR. Calcium signals induced by amyloid β peptide and their consequences in neurons and astrocytes in culture. *Biochim Biophys Acta.* 2004; 1742:81–87. [PubMed: 15590058]
 31. Scragg JL, Fearon IM, Boyle JP, Ball SG, Varadi G, Peers C. Alzheimer's amyloid peptides mediate hypoxic up-regulation of L-type Ca^{2+} channels. *FASEB J.* 2005; 19:150–152. [PubMed: 15494446]
 32. Brera B, Serrano A, de Ceballos ML. β -Amyloid peptides are cytotoxic to astrocytes in culture: a role for oxidative stress. *Neurobiol Dis.* 2000; 7:395–405. [PubMed: 10964610]

Fig. 1. Immunohistochemical characterization of primary murine astrocytes. GFAP staining revealed a moderate confluent layer of astrocytes, and some few highly positive reactive astrocytes (**a**, **c**). The staining for glutamine synthetase (GluS) showed a strong staining of astroglia all over the whole confluent layer (**b**). Nuclear DAPI (**e**, **f**) showed a confluent layer of cells. No immunostaining was evident for the $\text{Ca}_v1.2 \alpha_1$ channel (**d**). Scale bar = 20 μm (**a**, **c–f**), 10 μm (**b**).

Fig. 2. qRT-PCR expression of Cav2.1, Cav1.2 and Cav1.3 in primary astrocytes after 3 days of incubation. Confluent astroglia was incubated without (Control) or with the PKC-ε activator DCP-LA or the cAMP agonist (cpt-cAMP) or with human or murine Aβ₄₂ or the toxic fragment Aβ₂₅₋₃₅ for 3 days. The toxic fragment was also coincubated under acidic conditions (pH 6.7) or with H₂O₂. After 3 days, cells of three 6-well dishes were scraped and pooled. After that, these cells were further processed for qRT-PCR. Values are given as means ± SEM normalized transcript level. Statistical analysis was performed by one-way ANOVA with a subsequent Fisher PLSD post hoc test. * p < 0.05.

Fig. 3.

qRT-PCR expression of Cav1.2 and all auxiliary subunits (β₁₋₄ and α₂δ-1 to -4) in primary astrocytes. Confluent control (Control) astroglia was incubated without any exogenous stimulus for 3 days or 3 weeks. Furthermore, cells were treated with murine Aβ₄₂ for 3 days. At the end of the experiment, cells of three 6-well dishes were scraped and pooled and further processed for RTPCR. Values are given as means ± SEM normalized transcript level. Statistical analysis was performed by one-way ANOVA with a subsequent Fisher PLSD post hoc test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 4. qRT-PCR expression of Cav2.1, Cav1.2 and Cav1.3 in primary astrocytes after 3 weeks of incubation. Confluent astroglia was incubated without (Control) or with the PKC- ϵ activator DCP-LA or with human or murine A β or the toxic fragment A β ₂₅₋₃₅. After 3 weeks of incubation with reduced serum (1%), the cells of three 6-well dishes were scraped and pooled and further processed for qRT-PCR. Values are given as means $\pm$ SEM normalized transcript level. Statistical analysis was performed by one-way ANOVA with a subsequent Fisher PLSD post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.