Accepted Date : 04-Apr-2013
Article type : Original Paper

Neuroprotective role of γ-enolase in microglia in a mouse model of Alzheimer's disease is regulated by cathepsin X

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Running title

Neuroprotective role of microglial γ-enolase

Keywords

γ-Enolase; cathepsin X; microglia; Alzheimer's disease; amyloid-β; neuroprotection;

Checklist

Listing of Figures:

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Summary

γ-Enolase is a neurotrophic-like factor promoting growth, differentiation, survival and regeneration of neurons. Its neurotrophic activity is regulated by cysteine protease cathepsin X which cleaves the C-terminal end of the molecule. We have investigated the expression and co-localization of γ-enolase and cathepsin X in brains of Tg2576 mice overexpressing amyloid precursor protein. *In situ* hybridization of γ-enolase and cathepsin X revealed that mRNAs for both enzymes were expressed abundantly around amyloid plaques. Immunostaining demonstrated that the C-terminally cleaved form of γ-enolase was present in the immediate plaque vicinity, whereas the intact form, exhibiting neurotrophic activity, was observed in microglia cells in close proximity to senile plaque. The upregulation of γ-enolase in microglial cells in response to amyloid-β peptide (Aβ) was confirmed in mouse microglial cell line EOC 13.31 and primary microglia and medium enriched with γ-enolase proved to be neuroprotective against Aβ toxicity, however, the effect was reversed by cathepsin X proteolytic activity. These results demonstrate an upregulation of γ-enolase in

microglia cells surrounding amyloid plaques in Tg2576 transgenic mice and demonstrate its neuroprotective role in amyloid-β-related neurodegeneration.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive impairment that is a consequence of extensive neuronal loss (Braak *et al*., 1999). The principal pathological feature of the disease is the extracellular deposition of the fibrillar amyloid-β and its compaction into senile plaques, which provokes complex cellular reaction involving the activation of adjacent microglia and astrocytes (Blasko *et al*., 2004).

Microglial activation is regarded as a protective event in the neuroregenerative process (Streit *et al*., 2002; Polazzi *et al*., 2009), since activated microglia are capable of phagocytosis of amyloid-β deposits (Koenigsknecht *et al.,* 2004; Fuhrmann 2010) and of releasing neurotrophic factors such as NGF, NT-3 and BDNF known to be neuroprotective (Nakajima *et al*., 2001; Schindowski *et al*., 2008). However, in several neuropathologies, where chronic inflammation is present, the inflammatory products derived from activated microglia may also promote neurodegeneration and contribute to neuronal loss (González-Scarano *et al*., 1999). In addition to inflammatory cytokines, activated microglia secretes lysosomal proteases, the cathepsins, including cathepsin X (Nakanishi 2003; Wendt *et al*., 2009). Cathepsin X expression and proteolytic activity were strongly upregulated in the mouse brain, in particular in glial cells and aged neurons, and its association with senile plaques was also observed (Wendt *et al.,* 2007). We showed that cathepsin X impairs neuronal survival and neuritogenesis; two processes needed for proper function of the nervous system by proteolytic removal of two C-terminal amino acids in γ-enolase (Obermajer *et al*., 2009).

Enolase is a multifunctional glycolytic enzyme expressed in diverse cells and tissues. While the αα isozyme (alpha-enolase, non-neuronal enolase) is expressed ubiquitously, including

macrophages, neurons and glial cells (Ueta *et al*., 2004), the αγ and γγ isozymes (gamma-enolase, neuron-specific enolase) are found only in neurons and neuroendocrine cells (Schmechel *et al*., 1987). Further, gamma-enolase is considered to be a neuronal marker (Herrmann *et al*., 2003) as well as a marker for morphological status in AD (Chaves et *al*., 2010) and possesses a neurotrophic activity (Hattori *et al*., 1995). It controls neuronal survival, differentiation and neurite regeneration by activating phosphatidylinositol 3-kinase (PI 3-k) and mitogen-activated protein kinase (MAPK) signal transduction pathways (Hafner *et al*., 2012), as do other neurotrophic factors, such as NGF (Kaplan & Miller, 1997). γ-Enolase neurotrophic activity is associated with the ability to be translocated towards the plasma membrane and with its intact C-terminal end, which is cleaved by cysteine protease cathepsin X (Obermajer *et al*., 2009; Hafner *et al*., 2010).

In the present study, we investigated the interplay between γ-enolase and cathepsin X under neuropathological conditions. Their expression and patterns of co-localization were characterized in a transgenic mouse model of Alzheimer's disease - aged Tg2576 mice that develop β-amyloid plaques in the brain (Hsiao *et al*., 1996) and was further confirmed *in vitro* by using microglial cell cultures treated with amyloid-β peptide (Aβ). Moreover, on a co-culture model of microglial medium of Aβ-stimulated microglia and neuronal PC12 cells intoxicated with Aβ, we evaluated the neuroprotective role of microglia-released γ-enolase on neuronal cell survival and neuritic regeneration.

Results

Plaque-associated overexpression of γ-enolase and cathepsin X in aged Tg2576 brains

To investigate the involvement of γ-enolase and cathepsin X in neurodegenerative disorders, we analyzed their expression pattern in aged Tg2576 transgenic mice containing the Swedish double mutation of the human amyloid precursor protein 695. These mice develop

several characteristics of hippocampal and cortical AD pathology, such as inflammatory changes with activated microglial cells surrounding β-amyloid plaques, bulb-like dystrophic neurites and reactive astrocytes (Hsiao *et al*., 1996, Alpár *et al*., 2006; Reese *et al*., 2008). Microscopic examination of consecutive brain cryo-sections, processed for *in situ* hybridization and emulsion autoradiography and counterstained with methylene blue, revealed upregulated γ-enolase mRNA (Fig. 1a) and cathepsin X mRNA (Fig. 1b), seen as clusters of silver grains associated with the plaques and also individual cells surrounding them. Counterstaining brain sections with thioflavine-S, which labels fibrillary β-amyloid plaques, further disclosed strong γ-enolase overexpression on or very close to senile plaques (Fig. 1c). This pattern of staining was evident for total γ-enolase (revealed by N-terminal recognizing antibody), whereas the upregulated intact-active form (revealed by C-terminal recognizing antibody that recognizes the intact C-terminal end of γ-enolase 11-fold stronger than a two-residue-shorter protein; *data not shown*) was more evident in close proximity to the sites of positive thioflavine-S staining (Fig. 1d).

Plaque-associated co-localization of γ-enolase with cathepsin X

The neurotrophic activity of γ-enolase in neuronal cells is regulated by proteolytic cleavage by cathepsin X (Obermajer *et al*., 2009). To evaluate the interaction of γ-enolase and cathepsin X *in vivo*, aged brains of Tg2576 mice were subjected to double immunofluorescence staining. Strong co-localization of cathepsin X with γ-enolase on, and at the edge of, the β-amyloid plaque was evident, but less in the surroundings of the plaque (Fig. 2a). In contrast, when using an antibody specific only for the intact-active form of γ-enolase, a weaker co-localization with cathepsin X was observed (Fig. 2b). Additionally, the co-localisation with a lysosomal marker LAMP1 was performed. Results show a significant co-localisation of cathepsin X and LAMP1 in vesicular structures in cells in the vicinity of

amyloid plaques, whereas co-localisation of γ-enolase and LAMP1 was not observed (Fig. S1a and S1b).

Plaque-associated microglial cells express γ-enolase and cathepsin X

To identify the cells expressing γ-enolase in the area of amyloid plaques, cells were immunostained for γ-enolase, the neuronal marker NeuN, the microglial marker OX-6 and the astrocyte marker GFAP (Fig. 3). In accord with γ-enolase being localized to neurons and neuroendocrine cells (Schmechel *et al.*, 1987), almost all cells expressing NeuN in plaque-free areas of the brain section were γ-enolase positive (Fig. 3b). Remarkably, no γ-enolase-positive neuronal cells were found to be localized in the vicinity of β-amyloid plaques (Fig. 3a). The majority of γ-enolase-positive cells in this area expressed the microglial marker OX-6 (Fig. 3c), while the microglial cells found in plaque-free areas of the transgenic brain did not show immunostaining for γ-enolase (Fig. 3d). Similarly, reactive astrocytes surrounding β-amyloid plaques did not display any γ-enolase-immunoreactivity, as demonstrated using GFAP (Fig. 3e). In brains of aged Tg2576 mice, cathepsin X-immunoreactivity was located preferentially in microglial cells, which showed a large number of cathepsin X dots surrounding senile plaque (Fig. 4a), and in some neuronal cells localized both close to and distal to the β-amyloid plaque (Fig. 4b). Only a few cathepsin X-immunoreactive astrocytes were observed (Fig. 4c). These observations are in line with a previous report demonstrating cathepsin X expression in APP/PS1 transgenic mouse brain, with a preference for glial cells, and association with the plaques (Wendt *et al.,* 2007). As a confirmation to these findings, we demonstrated the presence of γ-enolase in mouse microglia EOC 13.31 cells and its strong co-localization with cathepsin X in a perimembrane region (Fig. S2). Although γ-enolase is present predominantly in the cytoplasm, the co-staining of γ-enolase and cathepsin X with a vesicular marker LAMP1 in microglial cells revealed a vesicular co-localization of γ-enolase and cathepsin X, however, the co-localization of LAMP1 with cathepsin X was more prominent than it was with the γ-enolase (Fig. S3a).

Aβ induces γ-enolase production from microglia

The localization of microglial γ-enolase was further evaluated *in vitro* in microglial cell line EOC 13.31 exposed to fibrillar amyloid-β peptide (Aβ). In non-stimulated microglial cells, γ-enolase was localized mainly in the cytosol (Fig. 5a) whereas, after 24 h stimulation with $A\beta_{25-35}$, γ-enolase was highly concentrated at the plasma membrane (Fig. 5b). Flow cytometry analysis indicated that $A\beta_{25-35}$ treatment at 200 nM for 3 h significantly increased intact-active γ-enolase protein expression as compared to non-stimulated cells (Fig. 5c). In contrast, cells treated with $A\beta_{25-35}$ for 24 h did not display so highly increased protein expression (Fig. 5c). As shown by western blotting, both peptides amyloid beta fragment $A\beta_{25-35}$ and full-length $A\beta_{1-42}$ (200 nM) treatment significantly increased secretion of the intact-active form of γ-enolase from microglia cells after 3 h, whereas the level of total γ-enolase in the culture medium remained unchanged (Fig. 5d). It has been postulated that an alteration in the concentration of the stimulus for microglia activation could significantly affect the beneficial or harmful microglial function (Li *et al.*, 2007). We therefore studied the dose response of γ-enolase secretion to both Aβ peptides treatment after 3h. In the range from 50 nM to 500 nM, both $A\beta_{25-35}$ and $A\beta_{1-42}$ were effective in increasing intact-active γ-enolase secretion from microglial cells (Fig. 5e). However, no significant change in total γ-enolase was observed. At the highest concentration of Aβ (1 μM) the level of secreted intact-active γ-enolase was lower, presumably due to increased activity of cathepsin X. Specific inhibitor of cathepsin X AMS36 (10 μM) reversed the level of intact-active γ-enolase after exposure to Aβ (1 μM), whereas inhibitor of matrix metalloproteinases batimastat (10 μM) did not significantly affected the protein level of intact-active γ-enolase (*data not shown*). Additionally, the findings were substantiated in primary mouse microglia. A similar increase in secretion of the intact-active form of γ-enolase from microglia was observed in response to full-length $A\beta_{1-42}$, whereas the level of total γ-enolase remained unchanged (Fig. 5f). However, in primary microglia at 500 nM of $A\beta_{1-42}$, γ-enolase secretion in its intact-active form from microglia dropped to almost the same level as control. These results indicate that

microglia respond fairly quickly to Aβ stimulation, by upregulating and releasing the γ-enolase intact-active form.

γ-Enolase rescues neuronal cells from degeneration caused by Aβ

The effect of γ-enolase on the neurotoxic potential of Aβ was studied by assessing its ability to prevent Aβ-related death of neuronal cells. Neuronal differentiated PC12 cells were treated with Aβ, which showed a significant dose-dependent decrease in neuronal cell survival. However, exposure to γ-enolase peptide (γ-Eno), mimicking the active form of γ-enolase, 1 h prior to Aβ, resulted in almost complete preservation of cells, even at the highest Aβ concentration (Fig. 6a).

The neuroprotection conferred by microglial medium is mediated by up-regulated γ-enolase

Since direct intercellular interactions or microglial secreted factors influence neuronal cell survival (Figueiredo *et al.*, 2008), we investigated whether the γ-enolase released by microglia is a crucial factor determining Aβ induced neuronal cell death. For this purpose, microglial cells were treated with Aβ (200 nM), which increases the release of intact-active γ-enolase (Fig. 5d, e). The resulting microglial culture medium (MCM[Aβ]) was added to differentiated PC12 cells during their stimulation with Aβ and significant decrease of the Aβ neurotoxic effect was observed (Fig. 6b). This protection was reversed when MCM[Aβ] was either depleted in γ-enolase by immunoprecipitation (MCM[Aβ]+ID), or by addition of recombinant cathepsin X (MCM[Aβ]+rhCX) (Fig. 6b), where γ-enolase immunodepletion or cleavage, respectively, were confirmed by western blotting (*data not shown*). This result confirms the role of cathepsin X, which specifically cleaves the C-terminal dipeptide of γ-enolase, in diminishing γ-enolase neurotrophic activity. The protective effect of γ-enolase

was observed also in the test for overall neurite outgrowth (Hafner *et al*., 2012), as followed by the levels of GAP-43, a recognized marker for axon growth zones (Van Hooff *et al*., 1989). Differentiated PC12 cells treated by Aβ showed a significant reduction in GAP-43 expression (Fig. 6c, e). However, MCM[Aβ] preserved GAP-43 levels in Aβ-treated PC12 cells (Fig. 6c), but not when γ-enolase was depleted from medium (MCM[Aβ]+ID) or when was inactivated by recombinant cathepsin X (MCM[Aβ]+rhCX) (Fig. 6c). Moreover, immunofluorescence staining for GAP-43 also confirmed that microglial γ-enolase preserved GAP-43 expression in growth cones (Fig. 6g). Nevertheless, the pattern of GAP-43 expression in modified MCM[Aβ] (Fig. 6h, i) was comparable to that of PC12 cells treated with Aβ alone (Fig. 6e), again suggesting that γ-enolase released from microglia contributes to, but does not completely account for its neuroprotective action.

Discussion

γ-Enolase possesses a neurotrophic function that is abolished by the proteolytic activity of cathepsin X (Obermajer *et al*., 2009), however, the relevance of this regulatory process in neurodegenerative disorders remains elusive. In the present study, we have examined the interplay between γ-enolase and cathepsin X in a mouse model of Alzheimer's disease. Our results identify plaque-associated mRNA and immunostaining of γ-enolase and cathepsin X in senile plaque areas. The C-terminally cleaved form of γ-enolase associated with cathepsin X in the immediate plaque vicinity, whereas the intact-active form was observed in close proximity of senile plaque, with a preferential localization in microglia cells. Co-localization of γ-enolase and cathepsin X was also confirmed in microglia EOC 13.31 cells. Additionally, *in vitro* γ-enolase was significantly upregulated in response to Aβ in microglia cell culture, and its secreted intact-active form expressed a protective action on neuronal cells pretreated with Aβ peptide.

The Tg2576 transgenic mouse model used in our study mimics the pathological hallmarks of AD, including deposition of β-amyloid plaques (Apelt *et al*., 2001). The

upregulation of cathepsin X mRNA and the protein distribution around the senile plaques observed in our model are comparable to the results obtained in APP/PS1 transgenic mice and to observations on AD patients (Wendt *et al*., 2007). A similar plaque-associated deposition has been described for other cathepsins (Bernstein *et al*., 1990; Cataldo *et al*., 1990; Lemere *et al*., 1995), suggesting their role in inflammatory reactions associated with neurodegeneration. However, little research to date has focused on the expression and function of the cathepsin X target, γ-enolase, in AD, although enolase has been identified as one of the most consistently upregulated and oxidatively modified proteins in brain with AD pathology (Butterfield *et al.,* 2009). By *in situ* hybridization, we confirmed that γ-enolase is upregulated in the hippocampus and cortex regions in brains of transgenic Tg2576 mice and, moreover, its overexpression is associated with β-amyloid plaques. Simultaneous staining with thioflavine-S showed the presence of γ-enolase around senile plaque, with differential localization patterns of its intact-active and total forms. The latter, including the inactive, C-terminally cleaved form, was present mainly on the plaque, in contrast to the intact-active form that was detectable only in the vicinity of senile plaque. By double immunostaining we observed a strong co-localization of γ-enolase and cathepsin X around and on β-amyloid plaque. Nevertheless, when using a specific antibody to the intact-active form of γ-enolase, the co-localization with cathepsin X upon senile plaque was weaker. These observations are in line with our previous results showing that cathepsin X cleaves the C-terminal end of γ-enolase, abolishing in this way its neurotrophic activity (Obermajer *et al*., 2009). Therefore the expression of both proteins by cells surrounding β-amyloid plaques implies the possibility that cathepsin X regulates the neurotrophic activity of upregulated γ-enolase by its proteolytic activity.

γ-Enolase has been used for many years as a marker for neuronal loss (Herrmann *et al*., 2003), and is expected to be present in brain tissue in neuronal cells. However, we provide here the first evidence that this isoenzyme is also expressed in microglial cells surrounding the senile plaques. Most of the cells close to the plaque were doubly-immunostained, for microglia marker and for γ-enolase, whereas more peripheral microglia

cells were devoid of γ-enolase. These observations are in line with the plaque-associated microglial localization of other neurotrophic factors (Cummings *et al*., 1993; Burbach *et al*., 2004; Schindowski *et al*., 2008). Several studies link activated microglia with neuroprotection and it has been suggested that, in response to neural injury in AD, glial cells are up-regulated and consequently release neurotrophic factors such as NFG, BDNF, NT-3, and NT-4/5, which may locally modulate cell-cell interactions and exhibit a neuroprotective effects (Mallat *et al*., 1989; Lindsay *et al*., 1994; Elkabes *et al*., 1996; Nakajima *et al.,* 2001).

We have confirmed microglial expression of γ-enolase *in vitro*, by using a mouse microglia EOC 13.31 cell line and primary microgia derived from mouse brain. Like in brain tissue the co-localization of γ-enolase with cathepsin X was confirmed in a perimembrane region. More detailed triple fluorescence co-localization of γ-enolase, cathepsin X and LAMP1 revealed intravesicular co-localization of γ-enolase and cathepsin X. In non-stimulated microglial cells γ-enolase localization was mainly seen in the cytoplasm. However, in cells treated with fibrilar Aβ that exerts neurotoxical properties of the native full-length Aβ peptide (Zhang *et al.,* 2010), more peripheral localization pattern was observed. This suggests that γ-enolase is released from microglia as described for other neurotrophic factors. Indeed, Aβ significantly increased the secretion of intact-active γ-enolase from microglial cells soon after 3 h of treatment. Various factors produced in activated microglia have been reported to vary with the time of exposure to and concentration of the activation stimulus; lower concentrations of stimulus result in neuroprotection, and higher concentrations in neurotoxicity (Li *et al.,* 2007). Also, lower concentrations of Aβ have been found to respond with significant release of intact-active form of γ-enolase, whereas higher concentrations led to significant decrease of the intact-active form. The latter coincides with increased activity of cathepsin X at higher concentrations of Aβ that is capable of γ-enolase cleavage and its inactivation. Nevertheless, the specific inhibitor of cathepsin X reversed the decreased level of intact-active γ-enolase. Thus, the neuroprotective or neurotoxic effect of

activated microglia indeed correlates with the intensity of the stimulus and is reflected in the profile of the secreted factors.

γ-Enolase, released from microglia in an intact-active form, is capable of reducing Aβ mediated neuronal death of differentiated PC12 cells that are susceptible to Aβ stimulus (Lou *et al.*, 2011). Additionally, we have shown that the released microglial γ-enolase is an essential factor mediating the neuroprotective effect against Aβ toxicity on neuronal cells. We showed that neuronal cells become resistant to Aβ-induced cell death when cultured in the presence of microglial medium containing elevated levels of γ-enolase. This neuroprotection is lost when γ-enolase was depleted from the conditioned medium or when γ-enolase activity was reduced by cathepsin X cleavage. Besides protecting neuronal survival, γ-enolase also diminished neuritic degeneration caused by Aβ, as demonstrated by GAP-43 expression, a neuron-specific-protein associated with the formation of growth cones and axon growth (Van Hooff *et al.*, 1989). In brains with AD pathology, extensive neuritic dystrophy is accompanied by a dramatic remodelling of actin filaments (Heredia *et al.*, 2006; Braithwaite *et al.*, 2010). γ-Enolase is involved in cytoskeleton remodelling by activating PI 3-K and MAPK signalling pathways (Hafner *et al.*, 2012), therefore it could also have a beneficial effect on neuronal functions by promoting neurite outgrowth.

In conclusion, our study provides a new insight into the mechanism of cathepsin X/γ-enolase function related to AD. In AD brain we found that γ-enolase is upregulated around senile plaques in Tg2576 mouse brain and localized predominantly in microglia. *In vitro*, microglial cells, activated by Aβ peptide, synthesize and secrete γ-enolase into the medium, which is protective for neuronal cells when exposed to Aβ. Neuroprotection is a characteristic of intact-active γ-enolase and is abolished after proteolytic processing of the C terminus of the latter by cathepsin X. Our results propose intact-active γ-enolase as a candidate in neuroprotective strategies for treatment of AD.

Experimental procedures

Tg2576 mouse model of Alzheimer's disease

The transgenic mice used in this study contained the human amyloid precursor protein (APP) 695 with the double mutation (K670N, M671L) as developed and described previously by Hsiao et al. (Hisao *et al.*, 1995; 1996). The transgene is expressed in C57B6/SJL F1 mic, backcrossed to C57B6 breeders. Age-matched non-transgenic littermates served as controls. The transgenity was determined in 2-month-old animals in tail biopsy material by PCR (Hsiao et al., 1995). Four Tg2576 19-month-old mice were used for experiments.

Tissue preparation

For *in situ* hybridization the brains were removed, quickly frozen on dry ice and stored at -80°C in a freezer until cryostat sections could be cut. Before cutting, the brains were allowed to equilibrate at -20°C in a cryostat chamber. Coronal cryosections (10 µm) were cut through the striatum, hippocampus and substantia nigra and thaw mounted onto microscope glass slides coated with a 0.01% solution of (poly)L-lysine. The slides were then vacuum-packed and stored in a freezer at –20°C until being further processed. The sections were fixed in cold 4% phosphate-buffered paraformaldehyde, washed twice in 0.1 M phosphate-buffered saline (PBS) (pH 7.2-7.4) and dehydrated in 70% and 95% ethanol. For immunofluorescence or thioflavine-S staining, Tg2576 mice were transcardially perfused with cold saline followed by cold 4% paraformaldehyde in 0.1 M PBS (pH 7.2-7.4) (4°C). Thereafter, the brains were removed, fixed overnight in 20% sucrose in 4% paraformaldehyde, and submerged in 20% sucrose in PBS at 4ºC for 48 h. Coronal (20 µm) sections were cut using a freezing-stage microtome. Free-floating sections were stored at -20°C in a cryoprotectant solution for immunofluorescence processing.

In situ hybridization

The standard procedure described by Zivin *et al.* (1996) was used for *in situ* hybridization. Sections were incubated with 3' end ^{35}S-labelled oligodeoxyribonucleotide antisense probes (45 bases long) complementary to the mouse γ-enolase mRNA (ENO2; sequence 5'-TCC TGG TAG AGT GCC CCC

AGC TGG TCC CCA GTG ATG TAT CGG GAA-3') andmouse cathepsin X mRNA (CTSZ; sequence 5'-CCC AAA TGT GCA GGC ACT CTC GAT GGC AAG GTT GTA GCT GTC ACC-3'). GenBank accession numbers used to design the probes were as follows: mouse ENO2 NM013509 and mouse CTSZ NM022325.Hybridized sections were exposed to X-ray film (Scientific Imaging Film X-OmatTM AR, Kodak, Rochester, NY) for 2–3 weeks and developed using standard darkroom techniques.

Emulsion autoradiography

The slides from *in situ* hybridization were immersed in Amersham LM-1 nuclear emulsion (Amersham, Uppsala, Sweden) and exposed for 2 weeks at 4°C in the dark before being developed with Kodak GBX developer (Sigma-Aldrich, St. Louis) (1:1, vol/vol) for 5 min, then fixed in Kodak GBX fixer (Sigma-Aldrich; 1:3, vol/vol) for 10 min. After washing with water, the sections were dehydrated and counterstained with 0.2% methylene blue. Sections were mounted, coverslipped with DePeX and photographed under a light microscope (Olympus IX81) with a digital camera (Olympus DP71).

Immunofluorescence

Primary antibodies were used to correlate γ-enolase expression with cathepsin X and to identify the protein expressing cells. The following antibodies were used: mouse anti-γ-enolase C-term (1:20; Santa Cruz Biotechnology; for detecting the C-terminal end of γ-enolase, mouse anti- γ-enolase N-term (1:20; Santa Cruz Biotechnology; for detecting the N-terminal end of γ-enolase), goat anti-cathepsin X (1:50; R&D System), the neuronal marker, mouse NeuN (1:100; Chemicon), the microglia marker, mouse OX-6 (1:400; Abcam), the astrocyte marker, mouse anti-GFAP (1:400, Abcam), rabbit anti LAMP1 (1:400, Abcam). For thioflavine-S staining, free-floating sections of Tg2576 washed and stained as described by Schmidt *et al*. (1995). Double immunofluorescence staining was performed by pre-incubating the 20 µm sections for 20 min in a 1% solution of bovine serum albumin (BSA) in 0.1 M PBS containing 0.1% Triton X-100, followed by overnight incubation at 4°C with primary antibodies in blocking solution to be co-stained. Thereafter, sections were incubated with a secondary antibody cocktail consisting of AlexaFluor-555 donkey-anti-goat and AlexaFluor-488 rabbit-anti-mouse or AlexaFluor-488 donkey-anti-rabbit antibodies (Invitrogen) and were applied at a concentration 4 µg/ml in PBS containing 2% BSA and 0.02% Triton X-100 for 1 h. After washing the sections with PBS, the

Prolong Antifade kit (Molecular Probes) was used for mounting the sections on glass slides. Fluorescence microscopy was performed using a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen). Images were analyzed using Carl Zeiss LSM image software, version 3.0 and the colocalization areas are presented in fourth quadrant of the images.

Cell cultures

The mouse microglia EOC 13.31 cell line, derived from the brain of an apparently normal 10 day old mouse, was obtained from American Type Culture Collection (CRL-2468, Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with mouse macrophage colony stimulating factor 1 (R&D Systems, Minneapolis, M) at a concentration of 20 ng/ml, 10 % fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM L-glutamine (Sigma), 50 U/ml penicillin and 50 ug/ml streptomycin (Sigma) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. PC12 cell line was derived from a transplantable rat pheochromocytoma (ATCC; CRL-1721). These cells were maintained in DMEM supplemented with 5% FBS, 10% heat-inactivated horse serum, 2 mM L-glutamine and antibiotics under 37 °C in a humidified atmosphere containing 5% CO_2. PC12 were treated with nerve growth factor (NGF; 50 ng/ml) for 5 days, and allowed to differentiate into neuronal phenotype. Primary microglia cultures were derived from postnatal day 2 mouse brain (BALB/c) as described (Combs *et al.*, 1999). Purity of cultures was determined by flow cytometry analysis using an CD11b (BD) antibody as marker for microglial identification and was also observed under inverted microscope (Olympus IX 81) (Fig. S4a and S4b).

Aβ and γ-Eno treatments

Aβ peptides (Bachem, Torrance, CA), a 11 amino acids fragment ($A\beta_{25-35}$) and a full-lenght amyloid beta ($A\beta_{1-42}$), were dissolved in deionized distilled water at a concentration of 1 mM and incubated at 37 °C for 72 h to induce aggregation (Lou et al., 2011). This both peptides were added to microglia cultures and $A\beta_{25-35}$ to neuronal differentiated PC12 cells. Confluent microglia EOC 13.31 cells and primary mouse microglia were stimulated with different concentrations of $A\beta_{25-35}$ and$A\beta_{1-42}$ (50 nM,

200nM, 500 nM and 1 µM) in serum-free medium and maintained for 3 h, 6 h or 24 h. Aliquots of the medium for western blotting were pre-filtered on Microcon-YM-10 filters (Millipore Co., Billerica, MA). To assess neuronal survival by flow cytometry, neuronal differentiated PC12 cells were cultured in serum-free medium on a pre-coated collagen, type 1 derived from rat tail. After 24 h, cells were incubated with γ-enolase peptide (γ-Eno; AKYNQLMRIEEELGDEARFAGHNFRNPSVL, Biosynthesis, Lewisville, TX), a peptide mimicking the intact-active C-terminal end of γ-enolase protein, for 1 h. $A\beta_{25-35}$ peptide was then added at concentrations of 1, 10, 25 and 50 µM for an additional 24 h.

Conditioned medium transfer

To test the effect of γ-enolase secreted by activated microglia, microglia EOC 13.31 cells were stimulated with 200 nM $A\beta_{25-35}$ in serum-free medium. After 24 h, microglia conditioned medium (MCM[Aβ]) was collected, aliquoted and stored at - 20°C until used. To clarify the neuroprotective role of γ-enolase, some aliquots of MCM[Aβ] were treated with 1 ug/ml of recombinant cathepsin X for 1 h at 37 °C (MCM[Aβ]+rhCX). The enzyme activity was then destroyed by heating the media at 95 °C for 10 min. Other aliquots were immunodepleted for γ-enolase from MCM[Aβ] (MCM[Aβ]+ID). 600 µl samples of either control (PC12 culture medium; control medium) or MCM[Aβ] were incubated with 1 µg of γ-enolase antibody that recognizes the C-terminal end of the protein and 30 µl protein A-Sepharose at 4 °C for 16 h with constant rotation. The immunocomplex was removed by centrifugation at 300 *g* for 1 min at 4 °C and the supernatant collected and stored at -20 °C before use. To quantify and assess the effects of the resulting MCM[Aβ]'s on neuronal survival, differentiated PC12 cells were cultured in mixed serum-free medium and conditioned medium of Aβ-stimulated EOC 13.31 cells in a 2:1 ratio.

Assessment of neuronal survival

Cell survival was determined with propidium iodide (PI). Differentiated PC12 cells were treated as mentioned above and after 24 h labeled with PI (30 µM) for additional 30 min at 37 °C. Afterwards,

cells were analyzed for cytotoxicity with flow cytometry on FACS Calibur (BD Bioscience). The percentage viability was evaluated by FlowJo software (Ashland, OR).

Protein quantification by flow cytometry

Flow cytometry was used to analyze the protein expression of γ-Enolase in microglia EOC 13.31 cells and for expression of GAP-43 in differentiated neuronal PC12 cells. Cells were washed with ice-cold PBS and then fixed with 5 % formalin at room temperature for 15 min and further permeabilized with ice-cold methanol for 20 min at 4 °C. Nonspecific staining was blocked with 3 % BSA in PBS for 30 min. Next, cells were incubated with specific antibody to C-terminal end of γ-enolase (Santa Cruz Biotechnology) or GAP-43 (Abcam) in blocking buffer for 45 min at 4 °C. Cells were then incubated with Alexa-Fluor-488-conjugated antibodies for an additional 30 min at room temperature, protected from light. Finally, cells were washed twice in PBS and analyzed by flow cytometry (FACS Calibur) and protein expression was evaluated by FlowJo software. Alexa-Fluor-488-IgG antibody was used as an isotype control.

Western blot analysis

To analyze protein secretion of γ-enolase in microglial culture medium, protein determination and western blot analyses were performed as described (Hafner *et al.,* 2012). In western blot the following primary antibodies were applied: monoclonal mouse anti-C-terminal of γ-enolase, suitable for detection of its active form (1 : 500) (Santa Cruz Biotechnology) and monoclonal mouse anti-internal region of γ-enolase, suitable for detecting active and cleaved forms of γ-enolase (1 : 500) (Santa Cruz Biotechnology) and the following secondary HRP-coupled anti-mouse antibodies (1:5 000; Millipore. Blots were detected with the Super-Signal chemiluminescence substrate (Thermo Scientific, Rockford, IL). The band intensities were quantified using GeneTools software (Sygene, UK), and expressed as values relative to those of the controls.

Immunofluorescence staining in vitro

For γ-enolase localization and γ-enolase, cathepsin X and LAMP1 co-localization, microglial EOC 13.31 cells were cultured on glass coverslips (2 x 10^4/ml) and after 24 h cells were treated with 200 nM Aβ in serum-free medium. For GAP-43 localization, PC12 cells were differentiated with NGF for 5 days on glass coverslips precoated with collagen (20 µg/ml) and further treated as stated above. After treatment, the cells were fixed with 5 % formalin at room temperature for 30 min and permeabilized with 0.05 % Tween 20 for 10 min. Non-specific staining was blocked with 3 % BSA in PBS, pH 7.4 for 30 min. Cells were then incubated with mouse anti-γ-enolase (1:20; Santa Cruz Biotechnology), goat anti-cathepsin X (1:500, R&D System), rabbit anti-LAMP1 (1:400, Sigma) or with rabbit anti-GAP-43 (1:500, Abcam) antibodies in blocking buffer, for 2 h at room temperature. The cells were washed with PBS and further incubated with Alexa 488-labelled donkey anti-mouse, anti-goat or anti-rabbit, Alexa 555-labelled donkey anti-mouse or anti-rabbit and Alexa 4633-labelled donkey anti-goat secondary antibodies. After washing with PBS the Prolong Antifade kit was used for mounting coverslips on glass slides. Fluorescence microscopy for GAP-43 expression was performed using Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen) with Carl Zeiss LSM image software, version 3.0, and co-localization study of γ-enolase, cathepsin X and LAMP1 was performed using Carl Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen) with ZEN 2011 image software.

Statistical analysis

SPSS PC software (Release 13.0) was used for statistical analysis. Differences between groups were evaluated using the non-parametric Mann-Whitney test. P values < 0.05 were considered to be statistically significant.

Acknowledgments

The authors acknowledge Prof. Roger Pain for critical review of the manuscript. This study was supported by a grant from the Research Agency of the Republic of Slovenia (grants P4-0127 and J4-4123 to JK, P3-0171 to JS).

Author Contributions

A.H. designed and carried out immunofluorescence confocal microscopy and molecular biology experiments, contributed to *in situ* hybridization and wrote the manuscript draft. G.G. designed and performed *in situ* hybridization analysis and reviewed the manuscript. N.O. contributed to design of experiments and preparation of the manuscript. M.Z. contributed to design the experiments and reviewed the manuscript. R.S. provided Tg2576 mouse model of Alzheimer's disease. J.K. coordinated the research, contributed to the preparation of manuscript and supervised the study.

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Figure legends

Fig. 1 Expression of γ-enolase and cathepsin X in brain sections obtained from 19-month-old Tg2576 mice. *By situ* hybridization, γ-enolase (a) and cathepsin X (b) mRNAs are seen as clusters of silver grains (arrows). Representative images of consecutive brain sections, immunostained for both forms of γ-enolase, i.e. C-terminally cleaved form and intact-active form, respectively, and counterstained with thioflavine-S (c, d), demonstrating a strong γ-enolase overexpression on or very close to senile plaques (arrows) (c), whereas labelling of intact-active γ-enolase was seen mainly around senile plaques (arrows) (d). *Scale bars* 20 μm

Fig. 2 Differential association of γ-enolase with cathepsin X in 19-month-aged Tg2576 brain sections. Representative images of double immunofluorescence staining for cathepsin X with γ-enolase (a, b). Note strong co-localization of total γ-enolase and cathepsin X (arrows) on or very close to β-amyloid plaque (a), whereas there is a weak co-localization of the intact-active form of γ-enolase with cathepsin X (arrows) (b). *Scale bars* 20 μm

Fig. 3 Phenotype of γ-enolase-immunopositive cells surrounding amyloid-plaque. Double immunofluorescence staining for γ-enolase with NeuN (a), OX-6 (b) and GFAP (c) were performed on the cortex regions of 19-month-old transgenic Tg2576 mice. Confocal microscopy revealed γ-enolase-positive neuronal cells in areas distal from the amyloid plaque (arrows) (a, b), whereas the neuronal cells found around the plaque were negative for γ-enolase staining. Note that amyloid plaque was surrounded by γ-enolase-positive microglial cells (arrows) (c), while astroglial cells in the plaque periphery were γ-enolase-negative (e). In addition, γ-enolase-positive neuronal cells (b), but not microglial cells (d) were observed in plaque-free areas of brain. *Scale bars* 20 μm

Fig. 4 Phenotype of cathepsin X-immunopositive cells surrounding amyloid plaque. Double immunofluorescence staining for cathepsin X with OX-6 (a), NeuN (b) and GFAP (c) were performed on the cortex regions of 19-month-old transgenic Tg2576 mice. Expression of cathepsin X by activated microglia is restricted to close proximity to the plaque (a), whereas cathepsin X-positive neuronal cells are seen in the wider area of amyloid plaque (b). Note a single cathepsin X-positive astroglial cell in the plaque periphery (c). *Scale bars* 20 µm

Fig. 5 Aβ affects γ-enolase production from microglial cells. Localization of γ-enolase in microglial EOC 13.31 cells. In non-stimulated cells, γ-enolase is localized predominantly in the cytosol (arrow) (a), while Aβ treatment of microglial cells for 24 h showed dense localization of γ-enolase at the perimembrane region (arrow) (b). Expression pattern of γ-enolase in EOC 13.31 cells after $A\beta_{25-35}$ treatment was assessed by flow cytomery analysis (c). In a time-dependent manner, Aβ stimulation for 3 h significantly up-regulated the secretion of intact-active γ-enolase from microglial cells, but not of total γ-enolase (d). In a dose-dependent manner, secretion of intact-active γ-enolase was upregulated in the range from 50 nM to 500 nM of Aβ peptide. (e). The dose-dependent relation between Aβ concentration and γ-enolase secretion from primary microglia after 3 h of treatment (f). Protein levels of procathepsin X and cathepsin X in microglia EOC13.31 and primary microglia culture medium after exposure to $A\beta_{1-42}$ at indicated. Values are given as means ± SD (c-f); Graphs below western blot images indicate the amount of intact-active γ-enolase protein level relative to the respective control. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.05$.

Fig. 6 Involvement of γ-enolase in neuroprotection against Aβ-induced cytotoxicity. Pre-exposure of differentiated PC12 cells to γ-Eno peptide 1 h prior to treatment with Aβ preserved cell death (a) (* $P <$ 0.005). The presence of γ-enolase in microglial medium of Aβ-stimulated microglia (MCM[Aβ]) reduced Aβ-induced PC12 cell death, whereas medium, immunodepleted of γ-enolase protein (MCM [Aβ]+ID) or treated with recombinant cathepsin X (MCM [Aβ]+CatX), respectively, reversed this effect (b). Analysis of GAP-43 expression in PC12 cells 24 h after treatment with Aβ in presence or abence of microglial medium (c). A significant reduction in GAP-43 protein level was observed after Aβ treatment, whereas MCM[Aβ] preserved GAP-43 levels in Aβ-treated PC12 cells. Such effect was not observed in the presence of modified medium. Representative immunofluorescence images of PC12 cells treated as above and stained for GAP-43 (d-i). Aβ reduced GAP-43 expression at the tips of neurite (arrows) (e) in comparison to control (d). MCM[Aβ] preserved GAP-43 expression in growth cones (arrows) (g), whereas modified medium did not exert such an effect (arrows) (h, i). All values were normalized to control medium without Aβ and are given as means ± SD of three independent experiments (a-c). * $P < 0.005$, ** $P < 0.001$, *** $P < 0.01$, **** $P < 0.0001$. *Scale bars* 10 µm