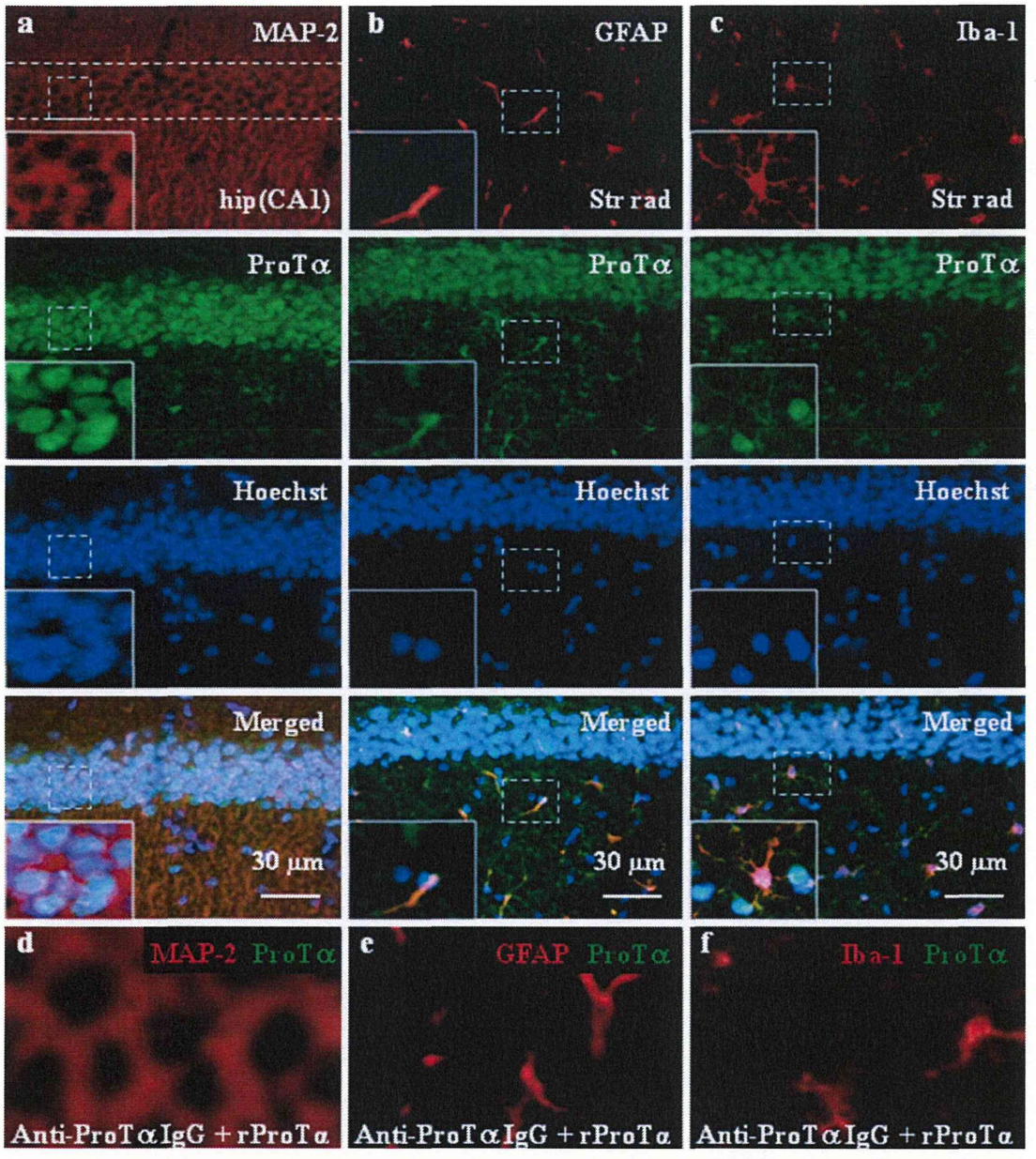

Fig. 2 Cell type-specific sub-cellular localization of ProTα. **a–c** Triple immunofluorescence staining was performed using brain coronal sections. Immunostaining data indicate that ProTα is localized in nucleus in the CA1 pyramidal cell layer neurons indicated by dot lines in the hippocampus (**a** MAP-2, *red*; ProTα, *green*; Hoechst, *blue*). ProTα is localized both in cytosolic space of processes and cell body in astrocytes (**b** GFAP, *red*; ProTα, *green*; Hoechst, *blue*) and microglia (**c** Iba-1, *red*; ProTα, *green*; Hoechst, *blue*) in the stratum radiatum of hippocampus. *Insets* in **a–c** indicate the high-magnification view of ProTα localization noted by *squares*. For antibody characterization, anti-ProTα IgG was pretreated with recombinant ProTα (rProTα) for 30 min, and subsequent double immunostaining of brain coronal sections was performed. Addition of recombinant ProTα in the antibody completely abolished the ProTα signal in MAP-2-positive neurons (**d**), GFAP-positive astrocytes (**e**), and Iba-1-positive microglia (**f**). *Scale bars* **a–c** 30 μm

plexus is mainly involved in the production of cerebrospinal fluid (CSF) and in a variety of neurological disorders (Wolburg and Paulus 2009; Wrede et al. 2009). Recently, habenula has attracted a great deal of attention for its prominent role in the regulation of dopamine and serotonin systems in terms of depression, anxiety, sleep mode, and extrapyramidal motor functions (Lecourtier et al. 2006; Hikosaka 2010). As depression is associated with cell death (Arantes-Goncalves and Coelho 2006; McKernan et al. 2009) and ProTα is a potent neuroprotective protein (Ueda et al. 2007), the role of ProTα in the habenula may be an intriguing subject in the phenotypic analysis of

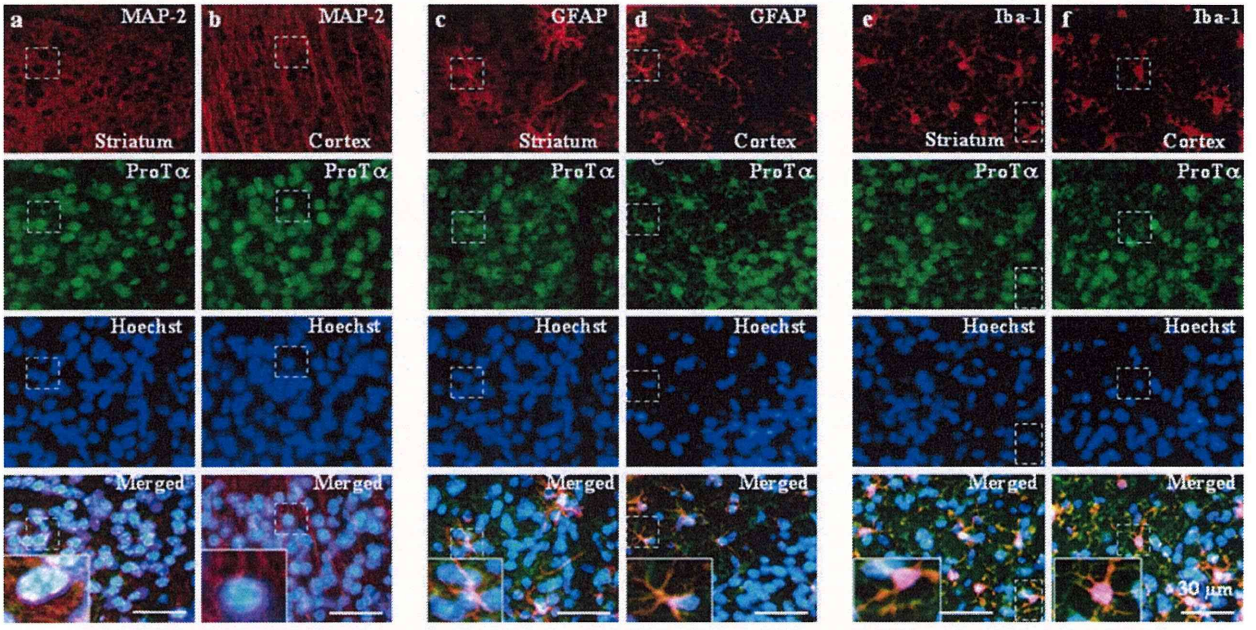

Fig. 3 Cell type-specific sub-cellular localization of ProTα in the striatum and cortex. Triple fluorescence staining of brain coronal indicates that ProTα was localized in nucleus in striatal (**a** MAP-2, *red*; ProTα, *green*; Hoechst, *blue*) and cortical neurons (**b**). ProTα is localized both in cytosolic space of processes and cell body in astrocytes in the striatum (**c** GFAP, *red*; ProTα, *green*; Hoechst, *blue*) and cortex (**d**). The expression of ProTα in the striatal (**e** Iba-1, *red*; ProTα, *green*; Hoechst, *blue*) and cortical microglia (**f**). *Insets* in **a**–**f** indicate the high-magnification view of ProTα localization noted by squares. *Scale bars* 30 μm

ProTα-related transgenic mice. Most interestingly, stronger ProTα signals that were found in neurons in Purkinje cell layer of cerebellum play an important role in movement, posture, and cognitive functions (Jorntell and Hansel 2006; Iscru et al. 2009). In this study we successfully demonstrated the cell type-specific sub-cellular localization of ProTα in adult brain. Detailed immunohistochemical findings revealed that ProTα was strictly confined in the nuclei of neuronal cells, whereas the expression was observed in the cytosolic space of astroglial and microglial processes and cell body in the adult brain. Several investigations demonstrated that ProTα binds to partner histone H1 in the nuclei and helps in DNA packaging (Papamarcaki and Tsolas 1994; Diaz-Jullien et al. 1996; George and Brown 2010), participates in chromatin remodeling (Gomez-Marquez and Rodriguez 1998), and regulates gene transcription (Trumbore and Berger 2000; karetsou et al. 2002). It is evident that ProTα regulates transcriptional activity of the estrogen receptor by sequestering repressor from the estrogen receptor complex (Martini et al. 2000). Therefore, it is suggested that nuclear ProTα may play roles in epigenetic regulation in neurons. In addition to such nuclear functions, ProTα has various putative functions in the cytosol space (Jiang et al. 2003; Karapetian et al. 2005). There is a challenging investigation about ProTα-mediated inhibition of translation by regulating the phosphorylation of eukaryotic elongation factor 2 during mitotic cell division (Vega et al. 1998; Enkemann et al. 1999). ProTα protects cells from oxidative stress through the dissociation of the intranuclear Nrf2–Keap1 complex and facilitates expression of oxidative stress-protecting genes (Karapetian et al. 2005). It has also been hypothesized that ProTα prevents cells from apoptosis through the inhibition of apoptosome formation (Jiang et al. 2003; Letsas and Frangou-Lazaridis 2006). The cytosol expression of ProTα in glial cells may be related to the fact that glial cells have more resistance to cell death stress than neurons.

Recent in vitro study has postulated that ProTα is localized in the nuclei of primary culture of rat embryonic astrocytes (Matsunaga and Ueda 2010). This finding revealed a discrepancy from the present study, in which ProTα is found in both cytosol and nucleus of astrocytes. There are several reports hypothesizing that the fragmentation of ProTα was mediated by caspase-3 enzyme at C-terminal side located within the spacer region bipartite nuclear localization signal (NLS) in ProTα (Rubtsov et al. 1997; Enkemann et al. 2000; Matsunaga and Ueda 2010). It has recently been described that active caspase-3 is present in the nuclei of astrocytes in normal brain (Duran-Vilaregut et al. 2010). Based on these findings, we attempted to see effects of ZVAD-fmk, a caspase-3 inhibitor, and found that nuclear localization of ProTα was significantly increased at 3–24 h in astrocytes after i.c.v administration. Therefore, it is supposed that the C-terminal part possessing NLS of

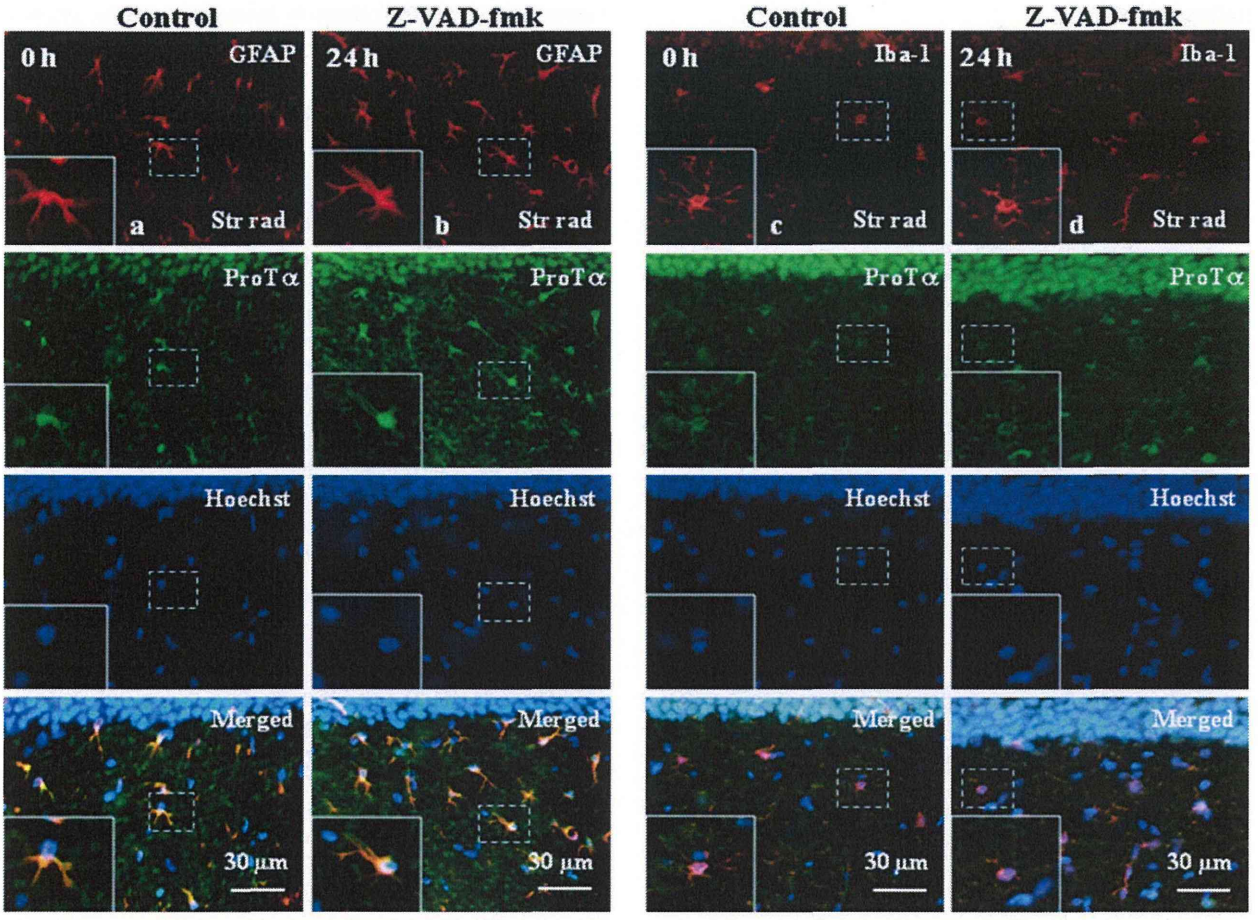

Fig. 4 Redistribution of ProTα by caspase-3 inhibitor treatment in astrocytes. ZVAD-fmk, a caspase-3 inhibitor, was injected intracerebroventricularly (i.c.v.) in mice brain, and ProTα immunostaining was performed at 24 h after treatment. Nuclear intensity of ProTα was significantly increased in astrocytes (**b** GFAP, *red*; ProTα, *green*; Hoechst, *blue*) in the stratum radiatum of hippocampus at 24 h after injection compared to the control (**a**), but there was no change in ProTα signals in microglia (**d** Iba-1, *red*; ProTα, *green*; Hoechst *blue*), compared to the control (**c**). *Insets* in **a–d** indicate the high-magnification view of ProTα localization denoted by *squares*. *Scale bars* 30 μm

ProTα may be cleaved by active caspase-3 in adult astrocytes of normal brain. Similarly, ProTα expression was also observed in both cytosol and nucleus in microglia. However, the expression pattern of ProTα in microglia was unchanged in the ZVAD-fmk-treated brain, though detailed mechanisms underlying this discrepancy in subcellular localization among neurons, astrocytes, and microglia would become the subject of future research. Taken together, the present findings encourage us to investigate the possible intracellular roles of cytosolic ProTα in astrocytes and microglia.

In conclusion, our study demonstrates that ProTα is expressed ubiquitously throughout the adult brain. There are very strong signals observed in some regions, especially in the neurogenesis-related zones in brain. The pattern of ProTα localization in neurons was completely different from that in astrocytes or microglia in the adult brain. Our demonstration would contribute to further understanding of the physiological and pathophysiological roles of ProTα in the brain.

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ORIGINAL ARTICLE

Neuron-specific non-classical release of prothymosin alpha: a novel neuroprotective damage-associated molecular patterns

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Abstract

Prothymosin alpha (ProTα), a nuclear protein devoid of signal sequence, has been shown to possess a number of cellular functions including cell survival. Most recently, we demonstrated that ProTα is localized in the nuclei of neurons, while it is found in both nuclei and cytoplasm in the astrocytes and microglia of adult brain. However, the cell type-specific non-classical release of ProTα under cerebral ischemia is yet unknown. In this study, we report that ProTα is non-classically released along with S100A13 from neurons in the hippocampus, striatum and somatosensory cortex at 3 h after cerebral ischemia, but amlexanox (an anti-allergic compound) reversibly blocks this neuronal ProTα release. We found that none of ProTα is released from astrocytes and microglia under ischemic stress. Indeed, ProTα intensity is increased gradually in astrocytes and microglia through 24 h after the cerebral ischemia. Interestingly, Z-Val-Ala-Asp fluoromethyl ketone, a caspase 3 inhibitor, pre-treatment induces ProTα release from astrocytes in the ischemic brain, but this release is reversibly blocked by amlexanox. However, Z-Val-Ala-Asp fluoromethyl ketone as well as amlexanox has no effect on ProTα distribution in microglia upon cerebral ischemia. Taken together, these results suggest that only neurons have machineries to release ProTα upon cerebral ischemic stress *in vivo*.

Keywords: apoptosis, cerebral ischemia, necrosis, neuroprotective DAMPs, non-classical release, S100A13.

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The ischemia in the central nervous system is a complex pathophysiological condition, in which neuronal necrosis in the ischemic core causes progressive secretion of cytotoxic mediators, which in turns further cause extended neuronal death (Danton and Dietrich 2003; Swanson *et al.* 2004; Ueda 2009; Niizuma *et al.* 2010; Zhao and Rampe 2010). A wide variety of intracellular molecules termed as damage-associated molecular patterns (DAMPs) are secreted into the extracellular environment upon necrotic/ischemic stress and play key roles in such deterioration of cellular damages (Rubartelli and Lotze 2007; Kono and Rock 2008; Chen and Nunez 2010; Schmidt and Tuder 2010; Zitvogel *et al.* 2010; Pisetsky 2011). Among these molecules, high mobility group box-1 (HMGB-1) is a representative DAMPs protein, which is extracellularly released from the nuclei of neurons upon ischemic damages (Lotze and Tracey 2005; Liu *et al.* 2007; Muhammad *et al.* 2008; Qiu *et al.* 2008; Sims *et al.* 2010; Yang *et al.* 2010; Zhang *et al.* 2011). However, there is also a case that neuroprotective molecule, such as prothymosin alpha (ProTα), is released into the extracellular milieu upon ischemic/necrotic stress in culture experiments (Ueda and Fujita 2004; Fujita and Ueda 2007; Ueda *et al.* 2007, 2010; Fujita *et al.* 2009; Ueda 2009). In this sense, ProTα may be called as a new member of cytoprotective DAMPs molecules.

ProTα is a nuclear protein and functionally implicated with cellular proliferation and survival (Pineiro *et al.* 2000; Jiang

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Abbreviations used: Amx, amlexanox; CA, cornu ammonis; DAMPs, damage-associated molecular patterns; HMGB-1, high mobility group box-1; Hip, hippocampus; i.c.v., intracerebroventricular; GFAP glial fibrillary acidic protein, intracerebroventricular; MAP-2, Microtubule-associated protein; PBS, phosphate-buffered saline; ProTα, prothymosin alpha; Str rad, stratum radiatum; tMCAO, transient middle cerebral artery occlusion; Z-VAD-fmk, Z-Val-Ala-Asp fluoromethyl ketone.

et al. 2003; Gomez-Marquez 2007; Ueda 2009), chromatin remodeling (Gomez-Marquez and Rodriguez 1998), DNA packaging (Diaz-Jullien *et al.* 1996; George and Brown 2010), and regulation of transcription (Martini *et al.* 2000; Karetsou *et al.* 2002). In addition to this, extracellular roles of ProTα have also been reported (Baxevanis *et al.* 1992; Garbin *et al.* 1997; Mosoian *et al.* 2006). There is an exciting report about the involvement of Toll-like receptor-4 in ProTα-induced immunoprotection against virus (Mosoian *et al.* 2010). The recent *in vitro* investigations described that ProTα is localized in nuclei of both cultured cortical neurons and embryonic astrocytes, and that is extracellularly released from these cells upon ischemic stress (Matsunaga and Ueda 2010). The mode of ischemia-induced non-classical release of ProTα was characterized in the experiments using C6 astroglioma cells *in vitro* (Matsunaga and Ueda 2010). This study explained that ProTα is first diffused from the nucleus to cytosol, and in turn immediately co-released to the extracellular space with S100A13 (a Ca^{2+}-binding cargo protein) and this release is reversibly blocked by amlexanox, an anti-allergic drug.

Most recently, we demonstrated that ProTα is strictly localized in the nucleus of adult brain neurons, whereas it is expressed both in the cell body and cytosolic space of processes in the astrocytes and microglia, an indication of big difference between *in vitro* and *in vivo* studies in terms of ProTα localization in astrocytes (Matsunaga and Ueda 2010; Halder and Ueda 2012). Interestingly, nuclear ProTα intensity was drastically increased in astrocytes by diminishing cytosolic contents, but not in microglia after the pre-treatment with Z-Val-Ala-Asp fluoromethyl ketone (Z-VAD-fmk), a caspase 3 inhibitor (Halder and Ueda 2012). The existence of caspase 3 activity in astrocytes in the adult brain as well as the caspase 3-mediated ProTα fragmentation *in vitro* has been reported previously (Enkemann *et al.* 2000; Evatafieva *et al.* 2003; Duran-Vilaregut *et al.* 2010; Matsunaga and Ueda 2010). Taken together, these studies suggested that caspase 3 controls the distribution of astroglial ProTα in the brain. However, the ischemia-induced ProTα release from brain is still under investigation. In the present study, we firstly attempted to see the cell type-specific non-classical release of ProTα as well as the effect of amlexanox on ProTα distribution in brain after cerebral ischemia.

Materials and methods

Middle cerebral artery occlusion mouse model

The transient middle cerebral artery occlusion (tMCAO) model was induced following the method as described previously (Egashira *et al.* 2004). Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and body temperature was monitored and maintained at 37°C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8-0 in size monofilament nylon surgical suture (Natsume Co. Ltd., Tokyo, Japan) coated with silicon (Xantopren; Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 1 h tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. As the silicon-coated nylon suture also plugs the branch from middle cerebral artery to supply blood to hippocampus in mice, due to small brain size, the ischemia-induced brain damages are also observed in the hippocampus. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21; Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100; Advance Co.) inserted into the left striatum (anterior: 20.5 mm; lateral: 1.8 mm from bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

Drug treatment

Amlexanox (kindly provided by Takeda Pharmaceutical Company Ltd, Osaka, Japan) was dissolved in 0.05 N NaOH in phosphate buffered saline [K^+ free phosphate-buffered saline (PBS), pH 7.4], adjusted pH 7.6 by 0.1 M H_3PO_4, and finally diluted in PBS. Using Hamilton syringe, amlexanox was injected intracerebroventricularly (i.c.v.) at a dose of 10 μg/5 μL in the brain 30 min before ischemia. Vehicle was treated with equal volume of solution containing 0.05 N NaOH and 0.1 M H_3PO_4 in PBS 30 min before ischemia in a similar manner. However, Z-VAD-fmk was purchased from Sigma-Aldrich, St Louis, MO, USA and dissolved in dimethyl sulfoxide and finally diluted in artificial CSF. Z-VAD-fmk was delivered i.c.v. at a dose of 1 μg/5 μL in the brain 30 min before ischemia (1 h tMCAO). Following similar way, vehicle was treated with equal volume of artificial CSF in the brain 30 min before cerebral ischemia.

Cell counting

Measurements of ProTα- and S100A13-positive cells in the brain were done using the BZ Image Measurement software. Briefly, cell counts were carried out in bright field images following the protocol as reported previously (Matsumoto *et al.* 2006). The number of ProTα- and S100A13-positive neurons, astrocytes and microglia in the somatosensory cortex of brain were stereologically counted (bregma 0.62 to −2.06) in the square fields (approximately 250 μm × 250 μm) of vehicle-treated ($n = 3$) and amlexanox-treated ($n = 3$) ischemic brain and were normalized to those obtained identically in the control brain ($n = 4$). However, to determine the ProTα- and S100A13-positive cells, we carried out the counts using specific cell markers with clearly visible nuclei. The quantification was expressed as average percentage of the total number of cell type-specific ProTα- and S100A13-positive cells in the 4–7 brain sections per mouse.

Statistical analysis

All results are shown as means ± SEM. Two independent groups were compared using the Student's *t*-test. Multiple groups were compared using Dunnett's multiple comparison test after a one-factor ANOVA. $P < 0.01$ was considered significant.

Other methods

Animals, tissue preparations, antigen retrieval microwave technique and proteinase K treatment, and immunohistochemical analysis are available as Appendix S1.

Results

Cerebral ischemia-induced rapid depletion of ProTα from hippocampal neurons

Following cerebral ischemia (1 h tMCAO) in mice, we examined time-dependent changes in ProTα expression in the ipsilateral hippocampus throughout 1–24 h. The 3,3′-diaminobenzidine tetrahydrochloride immunostaining data revealed that ProTα depletion in ipsilateral CA1 pyramidal neurons starts as early as 1 h after the cerebral ischemia (Fig. 1b), completes at 3 h (Fig. 1c) and followed by a gradual recovery of ProTα signals through 24 h (Fig. 1–d–f). However, there were some cells showing intense ProTα-immunoreactivity in the ipsilateral stratum radiatum of hippocampus at 3 h (Fig. 1c). The ProTα signals in various cells in the stratum radiatum gradually increased as time goes thereafter (Fig. 1–d–f). However, there was no significant change in ProTα reactivity in the respective contralateral hippocampus of ischemic brain (Fig. 1–a–f). Similar results of ProTα depletion and recovery were also observed in the regions of striatum (Fig. 2–a–f) and somatosensory cortex (Fig. 2–g–l).

Ischemia-induced depletion of neuronal ProTα

To identify the cell type specificity for ProTα release after cerebral ischemia (1 h tMCAO) and reperfusion, coronal brain sections were co-stained with anti-ProTα IgG and antibody against Microtubule-associated protein (MAP-2), a cytoplasmic neuronal marker. Our double fluorescence immunohistochemical data explained that ProTα immunoreactivity is strictly localized in nuclei of MAP-2-positive CA1 pyramidal neurons of hippocampus in the control mice (Fig. 3a). In confocal microscopy observation, ProTα signals were also found in nuclei in the MAP-2-positive CA1 pyramidal neurons of control hippocampus (Fig. 3g). As early as 3 h after the cerebral ischemia and reperfusion, ProTα signals in CA1 pyramidal neurons were completely lost, whereas the signals were significantly enhanced in some non-neuronal cells in the stratum radiatum of hippocampus (Fig. 3b). ProTα in the nuclei of pyramidal neurons was recovered largely to the control levels, and the signals were also localized in the nuclei at 24 h (Fig. 3c). Like control, there was also no change in nuclear ProTα levels observed in the contralateral side of brain (data are not shown). Similar patterns of ProTα release at 3 h and recovery at 24 h were observed in MAP-2-positive neurons in the striatum (Fig. 3i) and somatosensory cortex (Fig. 3j and k) of ischemic brain.

Amlexanox-reversible blockade of neuronal ProTα release

To understand the phenomenon whether amlexanox blocks ischemia-induced non-classical release of ProTα *in vivo*, mice was treated with amlexanox (10 μg/5 μL; i.c.v.) 30 min before cerebral ischemia (1 h tMCAO). Our immunohistochemical results revealed that ProTα signals are completely lost in ipsilateral MAP-2-positive CA1 pyramidal neuronal cells in the hippocampus of PBS-pre–treated

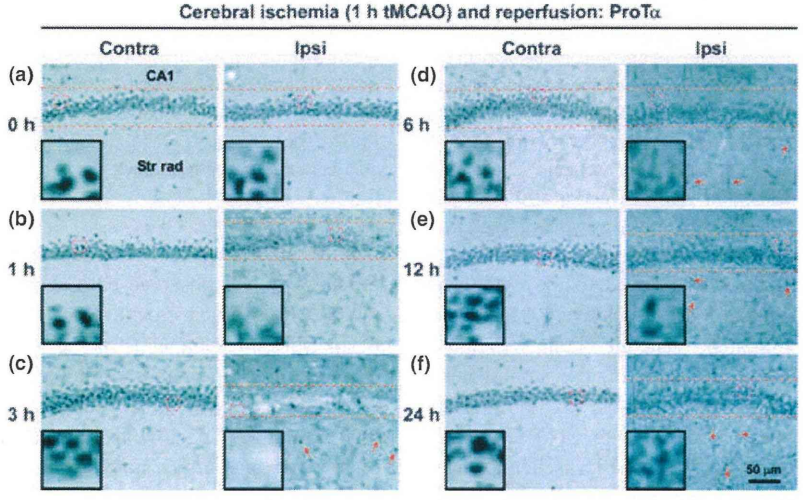

Fig. 1 Depletion of ProTα in the CA1 pyramidal cell layer of hippocampus under cerebral ischemia. (a–f) Immunostaining of ProTα in adult mice brain after ischemic stress. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) immunostaining data of coronal brain sections indicate that ProTα is partially released at 1 h (b) followed by complete release at 3 h (c) in the ipsilateral CA1 pyramidal cell layer of hippocampus after ischemic stress (1 h tMCAO). Some cells show intense ProTα reactivity at 3 h in the stratum radiatum of hippocampus noted by arrow points (c). (d–f) ProTα level is recovered gradually in the ipsilateral CA1 pyramidal cell layer at the later time points that starts from 6 h (d) continuing 12 h (e) and 24 h (f) after ischemic stress. ProTα intensity is also gradually increased in the ipsilateral stratum radiatum of hippocampus through 24 h after ischemic stress noted by arrows (d–f). There is no change in ProTα staining at 0 h (a) as well as in the respective contralateral sides of hippocampus after ischemia. Insets in panels (a–f) indicate the higher magnification view of ProTα intensity noted by red squares.

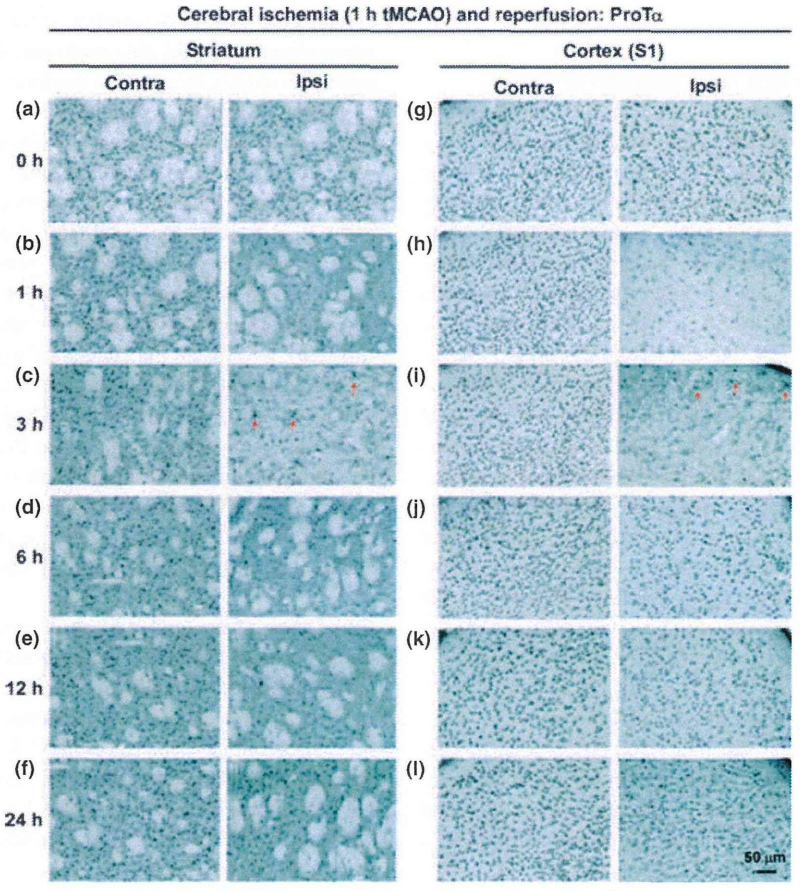

Fig. 2 ProTα is released from striatum and somatosensory cortex of ischemic brain. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) immunostaining of coronal brain sections is performed using antibody against ProTα. (a–f) ProTα signal is partially lost at 1 h (b), followed by complete lost at 3 h (c) and recovery gradually at 6 h (d), 12 h (e) and 24 h (f) in the ipsilateral striatum after ischemic stress (1 h tMCAO) in adult mice, retaining the normal staining at 0 h (a) and also in the respective contralateral sides. (g–l) DAB immunostaining data show the time–course ProTα expression in the ipsilateral as well as contralateral somatosensory cortex of brain from 0 to 24 h after ischemic stress. (c, i) Some non-neuronal-like cells show intense ProTα reactivity at 3 h in the ipsilateral striatum (c) and somatosensory cortex (i) noted by arrows.

(vehicle) brain at 3 h after ischemia and reperfusion (Fig. 3d). However, ProTα was diffused to the cytoplasm from the nucleus in ipsilateral MAP-2-positive CA1 neurons in the amlexanox-pre–treated ischemic brain (Fig. 3e), preserving normal nuclear staining in the contralateral hippocampus (Fig. 3f). In confocal microscopy observation, we also found that ProTα is diffused to MAP-2-positive neuronal cytoplasm from nucleus at 3 h after ischemic stress in the amlexanox-pre–treated brain (Fig. 3h). Similar results were also observed in MAP-2-positive neuronal cells of ipsilateral striatum (Fig. 3i) and somatosensory cortex (Fig. 3j and k) in the ischemic brain.

Caspase 3 inhibition causes ProTα release from astrocytes

To investigate the clue whether ProTα is released from astrocytes *in vivo*, coronal brain sections were co-stained with anti-ProTα IgG and antibody against an astroglial marker, glial fibrillary acidic protein (GFAP). ProTα immunoreactivity was observed both in nucleus and cytoplasm in the GFAP-positive astrocytes located in the stratum radiatum of control hippocampus (Fig. 4a and g). In the presence of ischemic stress in brain, the findings clarified that the ProTα immunoreactivity is still observed with higher intensity in ipsilateral GFAP-positive astrocytes in the stratum radiatum of hippocampus at 3 h after cerebral ischemia and reperfusion (Fig. 4b), compared with the control brain (Fig. 4a). Indeed, the ProTα signals were increased gradually in astrocytes through 24 h (Fig. 4c). Similar results were also observed in astrocytes in the ipsilateral striatum (Fig. 4h) and somatosensory cortex (Fig. 4i and j) after ischemic stress in brain. Most recently, we demonstrated that ProTα is distributed in both cell body and cytosolic space of processes in adult astrocytes of mouse brain, and that ProTα signal in the astroglial nuclei is drastically increased by diminishing

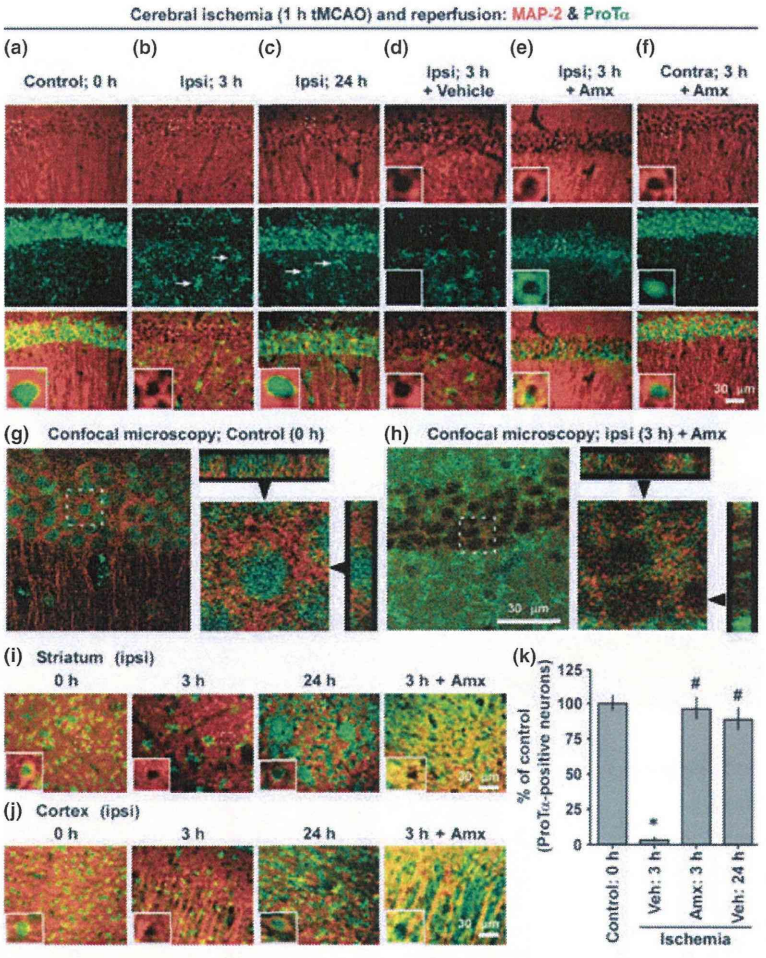

Fig. 3 Ischemia-induced ProTα depletion in neurons is blocked by amlexanox. Amlexanox (Amx) is injected (10 μg/5 μL; i.c.v.) in the mice brain 30 min before cerebral ischemia (1 h tMCAO). (a–j) Coronal brain sections are co-stained with antibodies against ProTα and MAP-2. (a, b) Double fluorescence immunostaining shows that ProTα signal is completely lost in MAP-2-positive neurons in the ipsilateral CA1 pyramidal cell layer of hippocampus (MAP-2, red; ProTα, green) at 3 h after cerebral ischemia (1 h tMCAO), compared with the nuclear ProTα staining in the MAP-2-positive neurons of control brain. (b) Some non-neuronal cells shows higher ProTα immunoreactivity in the ipsilateral stratum radiatum of hippocampus indicated at 3 h by arrow points. (c) ProTα is recovered in nuclei in the ipsilateral CA1 pyramidal neuronal cells at 24 h, but some non-neuronal cells shows the higher ProTα intensity in the stratum radiatum noted by arrows. (d) ProTα staining is completely lost in the ipsilateral MAP2-positive CA1 pyramidal neurons of PBS-pre–treated (vehicle) ischemic mice at 3 h. (e) Following Amx pre-treatment in ischemic mice brain, ProTα release is blocked in the ipsilateral MAP-2-positive CA1 pyramidal neurons and consequently translocated in the neuronal cytoplasmic spaces at 3 h after stress, compared with the contralateral side (f). (g, h) Conforcal microscopy observation indicates ProTα signals in the MAP-2-positive CA1 pyramidal neurons of hippocampus. A higher magnification view is indicated as doted square in (g) and (j), respectively. Arrowheads indicate the 3D imaged line (thickness: 10 μm), as shown in upper (*x*-axis) and right panels (*y*-axis). (i, j) Double fluorescence immunostaining shows that ProTα signal is completely lost in MAP-2-positive neurons in the ipsilateral striatum and somatosensory cortex at 3 h after 1 h tMCAO, compared with the normal nuclear ProTα staining in the control. ProTα signal is recovered in nuclei in the ipsilateral striatum and somatosensory cortex at 24 h after ischemia. Amx injection 30 min before ischemia inhibits the release of neuronal ProTα in the ipsilateral striatum and somatosensory cortex at 3 h after ischemic stress. Insets indicate the higher magnification view of ProTα localization in CA1 pyramidal neurons noted by dotted squares. (k) Quantitative analysis of ProTα -positive neurons in the somatosensory cortex. Data represent the means ± SEM ($^{*,\#}p < 0.01$, vs. the control: 0 h and the Amx: 3 h, respectively).

cytosolic levels when pre-treatment with Z-VAD-fmk, a caspase 3 inhibitor (Halder and Ueda 2012). In the present study, we confirmed that ProTα is also localized in both cell body and processes in immature astrocytes in the neonatal mice brain including stratum radiatum of hippocampus (Fig. 4k). Our *in vivo* experiments revealed that ProTα

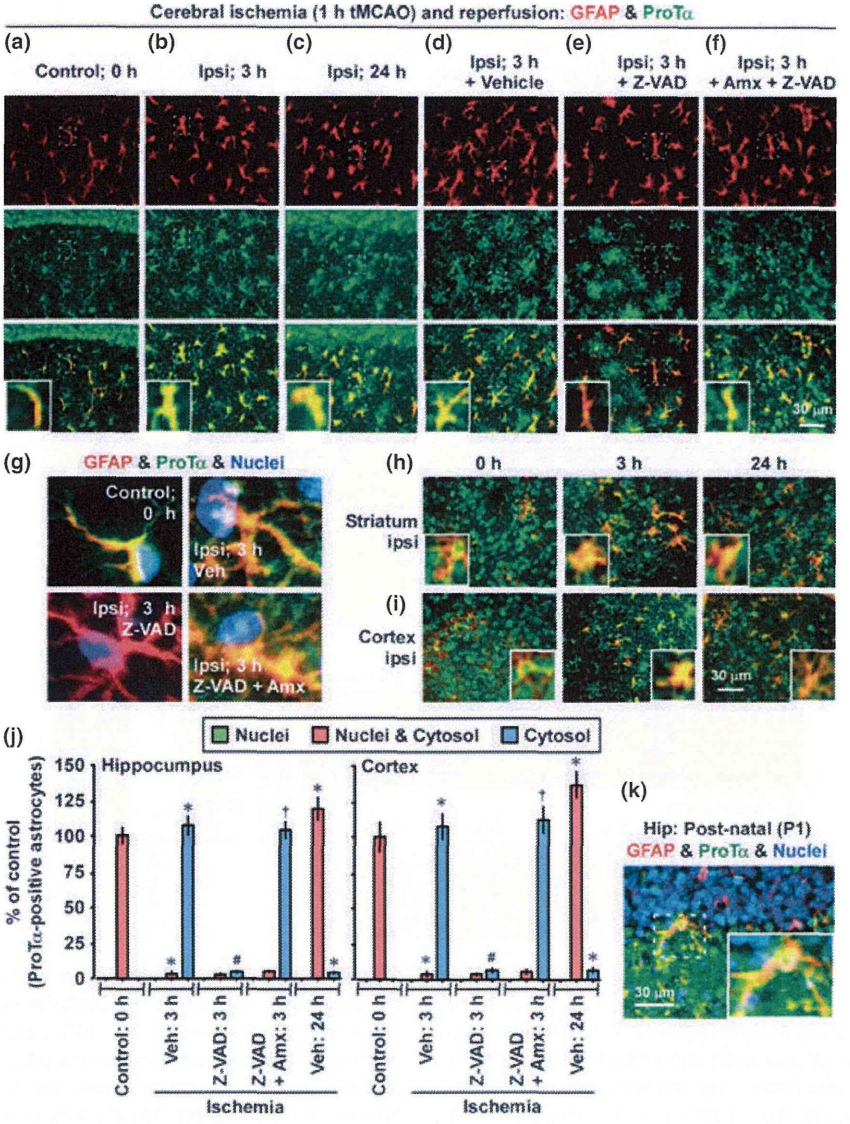

Fig. 4 Initiation of ProTα release from astrocytes by caspase 3 inhibition. Z-VAD-fmk (Z-VAD), a caspase 3 inhibitor (1 μg/5 μL) as well as Amx (1 μg/5 μL) is injected (i.c.v.) in the brain 30 min before cerebral ischemia (1 h tMCAO). Coronal brain sections are co-stained with antibodies against ProTα and GFAP. (a) Immunohistochemical analysis shows the expression of ProTα in GFAP-positive astrocytes in the stratum radiatum of hippocampus in control brain (GFAP, red; ProTα, green). (b) Higher ProTα signal is found in GFAP-positive astrocytes in the ipsilateral stratum radiatum at 3 h after ischemia. (c) The signal is gradually increased in astrocytes through 24 h after ischemia. (d) Intense ProTα signal is observed in GFAP-positive astrocytes in the ipsilateral stratum radiatum at 3 h after ischemia in the in PBS-pre-treated (vehicle) mice. (e) Following Z-VAD-fmk pre-treatment and ischemic stress, ProTα signal is significantly decreased in GFAP-positive astrocytes in the ipsilateral stratum radiatum at 3 h. (f) Z-VAD-fmk-induced ProTα release is blocked from GFAP-positive astrocytes in the ipsilateral stratum radiatum at 3 h after ischemia in Amx pre-treated brain. (g) Higher magnification views of ProTα in astrocytes in the control brain (upper left panel), the vehicle pre-treated ischemic brain (upper right panel), the Z-VAD-fmk pre-treated ischemic brain (lower left panel), and Amx + Z-VAD-fmk pre-treated ischemic brain (lower right panel) at 3 h after stress. (h, i) ProTα is not released from astrocytes in the striatum and somatosensory cortex after cerebral ischemia. ProTα signal is found with higher intensity in GFAP-positive astrocytes in the ipsilateral striatum and somatosensory cortex at 3 h after ischemic stress, compared with the ProTα signals in the control brain. ProTα intensity is gradually increased in GFAP-positive astrocytes in the ipsilateral striatum and somatosensory cortex through 24 h after ischemic stress. (j) Quantitative analysis of ProTα localization of astrocytes in the stratum radiatum of hippocampus (left panel) and somatosensory cortex (right panel). Data represent the means ± SEM. ($^{*,\#,\dagger}P < 0.01$, vs. the control: 0 h, the Veh: 3 h, and the Z-VAD: 3 h, respectively). (k) ProTα is localized both in cell body and processes in the astrocytes of post-natal (P1) mice brain (GFAP, red; ProTα, green; Nuclei, blue). Insets indicate the higher magnification view of ProTα expression in astrocytes noted by dotted squares.