Published in final edited form as:

Brain Res. 2009 January 9; 1247: 159–170. doi:10.1016/j.brainres.2008.10.022.

Reduced early hypoxic/ischemic brain damage is associated with increased GLT-1 levels in mice expressing mutant (P301L) human tau

Guanghong Liao¹, Miou Zhou², Simon Cheung¹, James Galeano¹, Nam Nguyen¹, Michel Baudry², and Xiaoning Bi¹

¹ Department of Basic Medical Sciences, COMP, Western University of Health Sciences, Pomona, CA 91766

² Neuroscience program, University of Southern California, Los Angeles, CA 90089

Abstract

Mutations in tau proteins are associated with a group of neurodegenerative diseases, termed tauopathies. To investigate whether over-expressing human tau with P301L mutation also affects stroke-induced brain damage, we performed hypoxia/ischemia (H/I) in young adult P301L tau transgenic mice. Surprisingly, brain infarct volume was significantly smaller in transgenic mice compared to wild-type mice 24 h after H/I induction. TUNEL staining also revealed less brain apoptosis in transgenic mice following H/I. H/I resulted in a significant increase in tau fragments generated by caspase activation and a marked decrease in tau phosphorylation at residue T231 in cortex of wild-type but not transgenic mice. Activation of calpain and caspase-3 following H/I was also reduced in transgenic compared to wild-type mice, as reflected by lower levels of the specific spectrin breakdown products generated by calpain or caspase-3. Finally, basal levels of the glial glutamate transporter, GLT-1, were higher in brains of transgenic as compared to wild-type mice. These results support the idea that enhanced levels of GLT-1 in transgenic mice are responsible for reducing H/I-induced brain damage by decreasing extracellular glutamate accumulation and subsequent calpain and caspase activation.

Keywords

calpain; caspase; glia; glutamate transporter; neurodegeneration; stroke

1. Introduction

Stroke and dementia are leading neurological disorders in the elderly population. Epidemiological studies have revealed a close correlation between ischemia and Alzheimer's disease- (AD) type dementia, as more than half of AD patients exhibit cerebrovascular pathology at autopsy, a coincidence that is more than by chance alone (Kalaria, 2000). Animal studies indicate that cerebral ischemia upregulates the expression of the amyloid precursor protein and enhances the production and accumulation of the amyloid β -peptide in adult rats

Send Proofs and Correspondence to: Dr. Xiaoning Bi, Dept. of Basic Medical Sciences, COMP, Western University of Health Sciences, Pomona, CA 91766-1854, Tel: 909-469-5487, Email: E-mail: xbi@westernu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Nihashi et al., 2001; Shi et al., 2000). Ischemic insults also affect tau proteins, which are the main components of neurofibrillary tangles, as indicated by a recent study showing that transient cerebral ischemia induces tau hyperphosphorylation in wildtype mice (Wen et al., 2004). However, whether predisposition conditions for AD or for dementia due to tauopathies affect stroke-induced brain damage has not been thoroughly investigated. Recently, a transgenic mouse model (hereafter referred to as P301L tau transgenic mice) overexpressing human tau with a mutation (P301L) found in frontotemporal dementia patients has been developed (Lewis et al., 2000). These mice exhibit neurofibrillary tangles in neurons of amygdala, hypothalamus, brainstem and spinal cord, and pre-tangles in hippocampus and cortex. This transgenic model thus provides a unique opportunity to investigate whether ischemia insults exacerbate tauopathies and whether overexpression of mutant tau affects ischemia-induced brain damage. This study represents the first attempt to answer these questions.

Numerous reports have indicated that spreading of neurological damage following ischemia may result from a process known as excitotoxicity, in which neurons die as a consequence of overstimulation of NMDA receptors due to excessive glutamate release (Choi, 1988; Nakanishi, 1992; Olney et al., 1986). Stimulation of NMDA receptors results in widespread increases in cytosolic calcium, which in turn activates cytotoxic cascades (Arundine and Tymianski, 2003; Choi, 1995), including the activation of calcium-dependent proteases, calpains (Czogalla and Sikorski, 2005), and apoptotic protease caspases (Love, 2003; Mattson, 2000). In addition to neuronal glutamate transporters, glial specific glutamate transporters, glutamate transporter 1 [GLT-1, also known as excitatory amino acid transporter 2 (EAAT2)] and glutamate-aspartate transporter (GLAST, also known as EAAT1) regulate extracellular glutamate levels under normal and pathological conditions (Rossi et al., 2007; Schousboe and Waagepetersen, 2006; Sheldon and Robinson, 2007; Yi and Hazell, 2006). Because excitotoxicity has been proposed to be a major contributor to hypoxia/ischemia-induced neuronal death, the present study also investigated the expression of GLT-1 and its potential relationship to hypoxia/ischemia-induced brain damage in tau transgenic mice.

2. Results

2.1. Decreased brain damage in P301L tau transgenic compared to wild-type mice 24 h after H/I induction

To induce H/I, 8 week-old P301L tau mice and age-matched wild-type B6D2F1 and C57BL/6 mice were subjected to a permanent occlusion of the right common carotid artery followed by 40 min of hypoxia. Neurological scores were evaluated 24 h after H/I induction; the percentage of mice with scores between 3 and 5 was not significantly different between P301L tau and wild-type mice (68% vs 85%, respectively). Brains from both transgenic and wild-type mice were collected 24 h after H/I induction and processed for Nissl staining; areas of damaged tissues (stained “pale” purple while healthy brain tissues stained “darker” purple, Fig. 1A) were quantified in 8 coronal sections using Image J software. H/I-induced brain damage was significantly smaller in transgenic mice in all 8 sections (Fig. 1B; $n=8$ for wild-type and 10 for transgenic mice; $p<0.01$ for each section). Differences in neuronal damage between the two types of mice were noticeably larger in anterior and posterior sections than in middle sections, suggesting that spreading of H/I-induced neuronal damage was reduced in transgenic mice. Total infarct volume in transgenic mice was about 37% of that in wild-type mice (25.8 mm^3 vs 69.4 mm^3 ; Fig. 1C). DNA damage and potential apoptotic cell death were also assessed by TUNEL staining; while numerous TUNEL-positive cells were detected in cortex and hippocampus of the H/I side from wild-type mice, fewer were observed in transgenic mice (Fig. 1D). H/I-induced neuronal damage was further analyzed by Fluoro-Jade B staining, which labels injured neurons (Schmued and Hopkins, 2000). In general, Fluoro-Jade B staining

labeled more neurons in both types of mice than TUNEL. However, microscopy examination showed that Fluoro-Jade B-positive areas were much smaller in transgenic than in wild-type mice (Fig. 1E). This initial impression was confirmed by a semi-quantitative analysis (Fig. 1F). In addition, *in vitro* experiments indicated that oxygen and glucose deprivation induced more severe damage in hippocampal slices cultured from wild-type than from transgenic mice (Fig. 2A); propidium iodide (PI) uptake was increased 4.4 fold in slices from wild-type mice as compared to 3.1 fold in slices from transgenic mice (Fig. 2B; $n=10-13$ sections; $p < 0.05$, Student's *t* test).

2.2. Reduced H/I-induced tau truncation in P301L tau transgenic mice

To investigate whether H/I alters tau biochemistry in P301L transgenic mice and whether tau modifications are associated with different responses to H/I in the two types of mice, tau phosphorylation and truncation were evaluated by immunoblotting and immunohistochemistry. Immunoblots were probed with the Tau5 antibody (which recognizes all tau isoforms), the AT8 antibody (which recognizes tau phosphorylated at Ser199/202) and the anti-Tau-p-Thr231 antibody. Levels of total tau proteins and phosphorylated tau isoforms recognized by AT8 antibody were higher in untreated tau P301L than in untreated wild-type mice (not shown), a result which is in agreement with the literature (Boekhoorn et al., 2006; Ramsden et al., 2005). Levels of tau proteins phosphorylated at Thr231 did not differ between the two genotypes of mice; however, H/I insults resulted in a marked decrease in this tau isoform in wild-type but not tau transgenic mice (Fig. 3A and C). Levels of tau proteins labeled with AT8 and Tau5 antibodies were not significantly altered by H/I (not shown). An antiserum, specifically recognizing fragments generated by caspase-mediated tau truncation (TauC), was used to further analyze caspase-mediated tau truncation. Basal levels of the fragments were higher in transgenic than in wild-type mice (Fig. 3B); while H/I induction induced a decrease in tau fragments in cortex of transgenic mice, it resulted in a significant increase in wild-type mice (Fig. 3B). H/I-induced increase in TauC in wild-type mice was confirmed by immunohistochemistry (Fig. 3D). A slight increase in TauC was also observed in the H/I “core” (e.g. striatum in Fig. 3D) in transgenic mice, but a decrease was found in the penumbra (e.g. cortex in Fig. 3D).

2.3. Reduced H/I-induced calpain and caspase-3 activation in P301L tau transgenic mice

As TUNEL staining revealed the existence of apoptosis and TauC immunoblotting indicated caspase-3 activation, especially in wild-type mice, the effects of H/I on both caspase-3 and calpain were investigated. Spectrin is a well known calpain and caspase substrate and increased levels of its specific breakdown products have been widely used as an indication of calpain or caspase activation (Siman and Noszek, 1988; Wang et al., 1998). Immunoblots labeled with anti-spectrin antibodies were therefore used to examine calpain and caspase activation. A significant increase in levels of the calpain-specific 145/150 kDa spectrin breakdown products was observed in cortex of wild-type but not transgenic mice 24 h after H/I (Fig. 4A, B). Levels of the 120 kDa spectrin breakdown product, which is generally considered to reflect caspase activation, were also increased 24 h after H/I in cortex of wild-type but not transgenic mice (Fig. 4A, C). Immunofluorescence using an antiserum that specifically recognizes calpain-mediated spectrin breakdown product showed that, following H/I, intense fluorescence was present in both cell bodies and neuropil in cortex of wild-type mice, while moderate levels of fluorescence were present only in a few scattered cell bodies in cortex of transgenic mice (Fig. 4D). Cells labeled with spectrin-breakdown antibodies were also labeled with anti-NeuN antibodies, suggesting that they were neurons (Fig. 4D).

Caspase-3 activation was further investigated by immunoblots probed with an antibody recognizing the cleaved/active caspase-3 isoforms with apparent molecular weights of 17 and 19 kDa. H/I induced a significant increase in both isoforms in cortex of wild-type but not

transgenic mice (Fig. 5; $p < 0.05$). Immunofluorescence with anti-cleaved caspase-3 antibody indicated that there were numerous immunopositive cells in damaged brain areas, including striatum, thalamus, and cortex in wild-type mice, while very few were found in transgenic mice. Double-labeling immunofluorescence indicated that caspase-3 immunoreactive cells were also labeled with anti-CD11b and anti-F4/80 (both are microglial markers) antibodies, but rarely with anti-NeuN (a neuronal marker) antibodies (not shown).

2.4. Increased brain GLT-1 levels in P301L tau transgenic mice

Results described above indicate decreased calpain and caspase activation and less excitotoxicity in transgenic than in wild-type mice. Excitotoxicity after H/I is in part due to excessive glutamate release that overcomes glutamate uptake capacity. Glutamate is normally rapidly taken up by glutamate transporters located on neurons or glia. Recent work has shown that expression of the glutamate transporters GLT-1 and GLAST in astrocytes is rapidly altered following H/I (Sheldon and Robinson, 2007). Some studies have suggested that a rapid decrease in levels of the glutamate transporters, GLT-1 and GLAST, following H/I may contribute to increased extracellular levels of glutamate (Chen et al., 2005; Rothstein et al., 1996; Torp et al., 1995), and thus to excitotoxicity. We therefore compared the levels of GLT-1 in control (untreated) and H/I-treated P301L tau and wild-type mice. Levels of GLT-1 in cortex of untreated P301L tau mice were 25% higher than in untreated wild-type mice ($n = 4$ for each group; $p < 0.05$; Fig. 6A&B). Immunohistochemistry for GLT-1 in hippocampus and cortex revealed the presence of densely labeled puncta (arrows in Fig. 6C; possibly astrocytic “feet”) surrounding the cell bodies and dendrites in P301L tau mice. Twenty-four h after H/I induction, GLT-1 levels were significantly increased in both transgenic and wild-type mice albeit a larger increase was apparent in transgenic than in wild-type animals (Fig. 6A&B).

To determine whether increased GLT-1 levels in P301L tau transgenic mice could result from gliosis induced by over-expression of mutant human tau, astrocytic expression of GFAP was evaluated. Immunoblotting analysis showed that GFAP levels in cortex of transgenic mice were in fact significantly lower than in wild-type mice (48% of wild-type; $n = 4$; $p < 0.05$), and were increased in both genotypes 24 h after H/I induction, with a larger increase in transgenic mice (Fig. 7A&C). Lower GFAP levels in transgenic than in wild-type mice were confirmed by immunohistochemical staining (Fig. 7C). Immunohistochemistry for GFAP in hippocampus showed fewer resident astrocytes in untreated P301L tau than in wild-type mice. Twenty-four hours after H/I induction, the number of GFAP-labeled positive cells increased in hippocampus of P301L tau mice. In both P301L tau mice and wild-type mice, GFAP-labeled cells exhibited enlarged cell bodies and shorter processes, indicating activated astrocytes (not shown). These results suggest that astrocytes in P301L tau transgenic mice have larger potential at buffering glutamate without exhibiting typical gliosis features.

3. Discussion

Results from the present study clearly indicate that 24 h after experimental H/I, P301L tau transgenic mice exhibited less brain damage than wild-type mice. At this time point, transgenic mice also exhibited less apoptosis, less tau truncation, and decreased activation of two proteases, calpain and caspase-3, which are generally associated with neurodegeneration. In addition, decreased brain damage in transgenic mice was associated with enhanced brain levels of the glial glutamate transporter GLT-1, which was located in puncta surrounding cell bodies and dendrites of cortical and hippocampal neurons, presumably reflecting astrocytic localization. GFAP levels were decreased in brains of transgenic mice, and immunohistochemistry confirmed the decrease in GFAP staining, thus indicating that increased GLT-1 levels in transgenic mice were not due to astrogliosis. The potential links between these events are discussed in the following sections.

3.1. Decreased calpain and caspase activation is associated with decreased brain damage in P301L transgenic mice

Severe H/I often leads to spreading of neurological damage beyond the initial necrotic core (i.e., the tissue directly supplied by the occluded vasculature) into the surrounding “penumbral” tissue (Zivin and Choi, 1991). Neuronal death in penumbral areas involves excitotoxicity, a consequence of overstimulation of NMDA receptors due to excessive glutamate release following ischemia (Choi, 1988; Nakanishi, 1992; Olney et al., 1986). Excessive stimulation of NMDA receptors results in widespread increases in cytosolic calcium, which in turn activates cytotoxic cascades (Arundine and Tymianski, 2003; Choi, 1995). One of the consequences of calcium influx consists in calpain activation. Although the mechanisms by which calpain induces neuronal death are not completely understood, recent studies indicate that this protease participates in excitotoxic cell death by cleaving the $\text{Na}^+\text{-Ca}^{++}$ exchanger (Bano et al., 2005; Hara and Snyder, 2007) and the metabotropic glutamate receptor mGluR1 α (Xu et al., 2007). Calpain also truncates a number of anti-apoptotic proteins, such as Bcl-2, Bcl-XL and Bid, which could account for the role of calpain in apoptosis.

Caspase activation is also critical for apoptotic cell death in ischemia-induced brain damage, especially in penumbra areas [for a recent review, see (Love, 2003; Mattson, 2000)]. Activation of caspase-3 was found in the boundary of the ischemic infarct within the first 24 h of ischemia (Didenko et al., 2002; Ferrer et al., 2003; Manabat et al., 2003). Caspase-3 knockout mice had smaller infarct volumes than wild-type mice (Le et al., 2002), while mice overexpressing human caspase-3 exhibited increased apoptosis and larger lesion volumes following cerebral ischemia (Kerr et al., 2004). Furthermore, intra-cerebroventricular administration of caspase inhibitors before ischemia reduced infarct size (Ma et al., 1998). Caspase-3 activation has also been associated with ischemic neuronal apoptosis in humans (Love et al., 2000a; Love et al., 2000b; Rosell et al., 2007).

Immunoblotting results of the present study indicated that both calpain and caspase-induced spectrin truncations were lower in transgenic compared to wild-type mice 24 h after H/I induction, indicating that calpain and caspase activation was reduced in transgenic compared to wild-type mice. Decreased calpain activation in tau transgenic mice was further confirmed by immunofluorescent staining using an antibody specific for spectrin breakdown products generated by calpain cleavage. Since calpain and caspase activation is critical for the spreading of H/I-induced brain damage, reduced activation of these proteases could account for decreased brain damage in transgenic mice. Whether H/I-induced cell death in wild-type mice is due to apoptosis or necrosis is not clear; the larger number of TUNEL-positive neurons in wild-type than in transgenic mice suggests a greater induction of apoptotic cell death in wild-type than in transgenic mice. However, the fact that Fluoro-Jade B labeled more neurons than TUNEL staining clearly indicates a large participation of non-apoptotic processes in H/I-induced brain damage.

3.2. Can increased astrocytic GLT-1 levels lead to decreased calpain and caspase activation in P301L transgenic mice?

The transgenic mouse model used in the present study was generated following the observations that mutations in tau gene (Mtap) caused frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), which demonstrated that tau dysfunction could directly result in neurodegeneration (Lewis et al., 2000). These mice exhibit motor impairment and develop neurofibrillary tangles in amygdala, brainstem, hypothalamus, spinal cord and other regions starting at 4.5 to 6.5 months of age; in contrast, in cortex and hippocampus only tau-immunoreactive pre-tangles are present (Lewis et al., 2000). Astrocytes and oligodendrocytes also show filamentous tau inclusion, albeit at a later age (Lin et al., 2003), which nonetheless indicates that over-expressing mutant human tau results in alterations

in glial properties. These mice also develop gliosis in brain areas exhibiting neurofibrillary tangles. In the present study, male transgenic mice were used at 2 months of age. It is thus unlikely that increased GLT-1 levels are due to gliosis, as no clear pathologies have been reported at this age, especially in cortex, hippocampus, and striatum, areas that are mostly affected by H/I. In addition, decreased GFAP levels in transgenic mice confirmed that changes in GLT-1 were not due to gliosis. Increases in GLT-1 in transgenic mice could result from either over-expression of tau proteins or from the prion promoter used to generate the transgenic mice. The prion promoter has been widely used for generating transgenic mice because it increases gene expression in both neurons and glia throughout the CNS (Borchelt et al., 1996; Zerbinatti et al., 2004; Jankowsky et al., 2001; Lesuisse et al., 2001; Gotz et al., 2001; Lewis et al., 2000). Since we are not aware of any report indicating that GLT-1 expression is enhanced in the majority of the transgenic mice generated with this promoter, the increased GLT-1 expression in astrocytes we observed is most likely due to over-expression of mutant human tau proteins. The mechanism linking tau over-expression with increased GLT-1 expression is not clear at present, but available evidence suggests that it might be a compensatory astrocytic reaction. Abnormal tau function resulting from mutations, hyperphosphorylations, or over-expression, affects its binding to microtubules, which in turn produces axonal transport impairment, especially anterograde transport of mitochondria (Mandelkow et al., 2003). Lack of mitochondria in synapses leads to reduced ATP levels (Thies and Mandelkow, 2007), which would not only affect membrane potential and ion transports but also reduce neuronal glutamate uptake. These events could promote elevated extracellular glutamate levels, therefore stimulating GLT-1 expression in astrocytes.

The present study indicated that the reduced brain damage observed 24 h after H/I induction in tau transgenic mice was associated with enhanced levels of GLT-1. Expression of the glutamate transporters GLT-1 and GLAST in astrocytes is rapidly altered following H/I (Sheldon and Robinson, 2007). Some studies have suggested that a rapid decrease in levels of the glutamate transporters GLT-1 and GLAST may contribute to H/I-elicited increase in extracellular glutamate levels (Chen et al., 2005; Rothstein et al., 1996; Torp et al., 1995), and thus to excitotoxicity. Antisense knockdown of GLT-1 in astrocytes but not of EAAC1 in neurons increases infarct volume and neuronal damage in cortex and striatum after transient focal cerebral ischemia in rats (Rao et al., 2001). A recent genetic study revealed that elimination of GLT-1 increases glutamate buildup during the first 5 min of severe global ischemia but decreases glutamate buildup during longer periods of ischemia (Mitani and Tanaka, 2003). The same study also showed that GLT-1 knockout leads to a more severe delayed cell death following a short period of ischemia, although it had no effect on cell death resulting from longer periods of ischemia. Pharmacological blockade of GLT-1 function results in elevated extracellular glutamate levels in the penumbral region of focal ischemia (Feustel et al., 2004). Preconditioning with a sub-lethal ischemic period has been shown to be neuroprotective against subsequent stroke (Davis and Patel, 2003). Interestingly, preconditioning is blocked dose-dependently by the GLT-1 inhibitor, dihydrokainate (Zhang et al., 2007), and by an antagonist of the anti-inflammatory peroxisome proliferator-activated receptor (PPAR), which regulates GLT-1 expression (Romera et al., 2007). Furthermore, in vitro studies showed that increase in GLT-1 expression produced by beta-lactam antibiotics or by an agonist of PPAR, rosiglitazone, is also neuroprotective (Romera et al., 2007).

Taken together, all these results strongly support our hypothesis that increased GLT-1 levels are likely responsible for the decrease in H/I-induced calpain/caspase activation and acute brain damage in P301L tau transgenic mice. However, we would like to emphasize that first, our study only addresses brain injury and biochemical changes 24 h after H/I; whether H/I leads to delayed severer neuropathology in the transgenic than wild-type mice remains to be determined. Secondly, only young transgenic mice without full-blown neurofibrillary tangles were used in the present study, and it is possible that old P301L tau mice react differently than

young animals. Considering the late-onset neuropathology in these mice and literature implicating H/I in Alzheimer's disease, it is tempting to speculate that H/I may result in severer damage in old transgenic mice. Nonetheless, our results further strengthen the notion that glial cells play important roles in controlling stroke outcome by taking up excessive glutamate levels, thereby decreasing subsequent calpain and caspase activation and ultimately decreasing spreading of neuronal damage. Considering that previous academic and pharmaceutical research focusing on neurons as the main target has had little success for developing novel stroke drugs, the present results suggest that glial cells should be considered as new therapeutic targets.

4. Experimental Procedures

4.1. Animals and Hypoxia/ischemia Induction

Eight to fourteen week-old male JNPL-3^{tau} transgenic mice (Lewis et al., 2000) and their wild-type controls (C57BL/6, and B6D2F1; all animals were purchased from Taconic) were used in the present study. Initial experiments indicated that H/I-induced brain damage in non-transgenic litter mates and C57BL/6 and B6D2F1 mice (the two strains used to generate and breed P301L mice, respectively) was not different; therefore, data from both strains were combined and referred to as wild-type. Hypoxia/ischemia (H/I) was induced as previously described (Adhami et al., 2006) following mouse anesthesia with pentobarbital (50 mg/kg). Briefly, the right common carotid artery was permanently occluded by transecting between two sutures. Hypoxia was then induced by applying 8% O₂ balanced with 92% N₂ through a gas mask and was maintained for 40 minutes under anesthesia. Mouse body temperature was maintained at 37 ± 0.5 °C with a temperature controller (model EW-89000-00; Cole Parmer, Vernon Hills, IL) connected to a rectal probe and a heating pad. After recovery from anesthesia, mice were returned to the animal care facility and inspected daily. The animal procedures were approved by the local Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

4.2. Neurological Deficit Score

After H/I induction, mouse behavior was measured based on a 6-point scale for neurological deficits according to a recent publication (Adhami et al., 2006): 0, no detectable neurological deficit; 1, ptosis of eyelid ipsilateral to the occluded common carotid artery side and/or failure to extend ipsilateral forepaw; 2, animal persistently walks in large circles toward the ipsilateral side; 3, animal persistently walks in small circles and/or rolls over repeatedly toward the ipsilateral side; 4, animal lies nearly motionless on the contralateral side; 5, animal dies after recovery from the anesthesia.

4.3. Measurement of brain damage

Twenty-four hour after H/I induction, mice were killed under deep anesthesia with pentobarbital (100 mg/kg) by perfusion with phosphate-buffered saline followed by 4% paraformaldehyde. For cryosectioning, brains were taken through graded sucrose solutions. Brains were then cut into serial coronal sections (25 µm) using a frozen-microtome and sections were kept at -20 °C in cryoprotectant until use. Eight sections corresponding to +1.98, +1.18, 0.50, 0.22, -1.06, -1.82, -2.46, and -3.28 mm to the Bregma point (Franklin and Paxinos, 1997) were mounted on slides and Nissl stain was performed with cresyl violet. Infarct area was measured and expressed as percent of the ipsilateral hemisphere area using a digital image analysis system (Image J, National Institutes of Health). Mean areas from different experimental groups (wild-type and P301L tau mice with sham surgery or H/I) were shown as mean ± SEM and were compared using one-way ANOVA with post-hoc analysis.

4.4. Fluoro-Jade B histochemistry

Fluoro-Jade B staining was performed following previously described procedures (Schmued and Hopkins, 2000). Briefly, coronal sections mounted on slides were incubated in 80% ethanol with 1% NaOH for 5 min, 70% ethanol for 2 min, and transferred to 0.06% KMnO₄ solution for 10 min. They were then rinsed in distilled water (dH₂O) for 2 min, and incubated in 0.004% Fluoro-Jade B working solution (Chemicon, Temecula, CA) in 0.1% acetic acid for 20 min. Sections were then rinsed in dH₂O 3 times, dried on a slide warmer, cleared with xylene, and coverslipped with DPX mounting medium (Sigma, St. Louis, MO). Images were obtained with a Nikon confocal microscope (Nikon TE 2000U with D-Eclipse C1 system). Semi-quantification was done by grading positively stained sections into four groups as previously described (Schiefer et al., 2002): Grade 0, no staining; grades 1 to 3 representing 1-30%, 30-60%, and >60% of the area stained positively for Fluoro-Jade B, respectively.

4.5. TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay

TUNEL was done with digoxigenin-11-dUTP following a previously described procedure (Adhami et al., 2006) with slight modifications. Twenty-five μ m thick sections mounted on slides were rinsed in 0.1 M phosphate buffer (PB), dehydrated through a series of graded ethanol, and defatted in xylene. After rehydration, sections were treated with 0.2% Triton X-100 and 0.25% acetic anhydride. Terminal deoxynucleotidyl transferase (TdT) reaction was then carried out at 37 °C according to the manufacturer's instructions (TdT kit from Roche Diagnostics, Indianapolis, IN). Binding of dUTP was revealed by using a sheep anti-Digoxigenin Fab fragment (1:1000, Roche Diagnostics, Indianapolis, IN), followed by a biotinylated anti-sheep antibody (1:400, Santa Cruz Technology) and then by a CyTM2-conjugated streptavidin antibody (1:1500, Jackson Immunoresearch Lab, PA). Finally, the slides were coverslipped with VectaShield mounting medium containing DAPI to stain nuclei (vector Laboratories Inc, Burlingame, CA), and images were taken using a Nikon confocal microscope.

4.6. Oxygen and Glucose Deprivation (OGD) in cultured hippocampal slices

“Hippocampal” slices containing hippocampus, entorhinal cortex, and portions of the adjacent neocortex were prepared from postnatal 7-9 day-old P301L tau and non-transgenic littermates mice, placed onto Millicell-CM membrane inserts (Millipore Corporation, Bedford, MA) in a six-well culture cluster plate (Corning Inc., Acton, MA) containing culture medium (1 ml/well) consisting of minimum essential medium, 30 mM dextrose, 30 mM HEPES, 5 mM Na₂HCO₃, 3 mM glutamine, 0.5 mM ascorbic acid, 2 mM CaCl₂, 2.5 mM MgSO₄, 1 mg/l insulin, and 20% horse serum (pH 7.4), and incubated at 36 °C with 5% CO₂ for 12 days with the medium being changed 3 times a week (Bi et al., 2001). For OGD treatment, hippocampal slices were incubated with OGD solution containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.5 MgSO₄, 2.5 CaCl₂ (pH 7.5) at 37 °C for 1 h in an anaerobic chamber saturated with nitrogen (Laake et al., 1999). Neuronal damage was assessed by propidium iodide (PI) uptake as previously described (Laake et al., 1999; Zhou and Baudry, 2006). PI (4.6 μ g/ml) was added to OGD solution and after 24 h incubation, slices were fixed overnight in 4% paraformaldehyde. Slices were then transferred to 0.1M PB with 20% sucrose for 24 h and sectioned at 20 μ m using a freezing microtome. PI uptake was visualized using a 5X objective with a microscope fitted with fluorescence detection, and images of PI-labeled slices were captured with a CCD camera; at this magnification, one image was sufficient to analyze an entire hippocampal slice. To obtain the best intensity of images and to avoid saturation, all acute hippocampal slices were exposed for 50 ms, and the camera gain was kept constant throughout each experiment. Fluorescence intensity was estimated by the following method: first, images were adjusted to gray levels and captured with Adobe Photoshop, with

the background of images in white and PI-stained structures in black; second, modified images were analyzed quantitatively by densitometry with Image J software.

4.7. Immunohistochemistry and double-immunofluorescence

Immunohistochemistry and double-immunofluorescence were carried out using free-floating sections as previously described (Liao et al., 2007). Immunohistochemistry was performed using the avidin-biotin horseradish peroxidase complex (ABC) method. Briefly, free-floating sections were first incubated in 10% normal horse serum (for monoclonal antibodies) or 3% normal goat serum (for polyclonal antibodies) diluted in PB with 0.1% Triton X-100 for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4 °C. Antibodies used were: mouse anti-GLT-1 (1:1,000, BD Biosciences, San Jose, CA), mouse anti-GFAP (1:10,000, Sigma, St. Louis, MO), and mouse anti-caspase-cleaved tau (TauC, 1:500, Chemicon, Temecula, CA). After 3 washes in PB, sections were incubated with corresponding biotinylated secondary antibodies (1:400; Vector Laboratories, Burlingame, CA) in 5% normal horse serum or 1.5% normal goat serum solution for 2-3 h, then in ABC diluted in PB for 45 min. Peroxidase reaction was carried out with 3,3'-diaminobenzidine tetrahydrochloride (0.05% in 50 mM Tris-HCl buffer, pH 7.4) as chromogen and 0.03% H₂O₂ as oxidant. Free-floating sections were mounted on pre-coated slides (SuperPlus, Fisher Scientific International Inc.) and air-dried. Sections were then dehydrated in graded ethanol and finally covered with Permount (Fisher). Images of immunostained sections from different brain regions were visualized using a Zeiss microscope (Axioskop 2 *mot plus*) and digitized with a Zeiss digital photo camera (AxioCam Hrc) and the program, Axiovision v 3.1 (Zeiss), was used to capture and save digitized images. Digitized images were then assembled in Photoshop (version 7).

Double-labeling immunofluorescence was done with sections first incubated with primary antibodies [rabbit anti-cleaved caspase-3 (1:500, Cell Signaling Technology) in combination with either rat anti-F4/80 (1:1000; Serotec) or rat anti-CD11b (1:500, Chemicon abcam.com) or mouse anti-NeuN (1:1000, Chemicon), in combination with rabbit antibodies specific for calpain-mediated spectrin breakdown products (1:20,000; a gift from Dr. Robert Siman, Department of Pharmacology, University of Pennsylvania, School of Medicine), then with corresponding secondary antibodies conjugated with Alexa Fluor® 488 or Alexa Fluor® 594; both secondary antibodies were purchased from Invitrogen (Carlsbad, CA). For tissues stained with cleaved-caspase 3 antibodies, Sudan black was used to minimize autofluorescence (Baschong et al., 2001). Briefly, after fluorescence labeling, tissues were mounted onto slides and dried for 30 minutes, then the slides were incubated in 0.025% Sudan Black B/70% ethanol solution (Fluka, St. Louis, MO) for 15 min. Slides were washed in PB, air dried, and then coverslipped with VectaShield mounting media. Images of double immunofluorescent labeled sections were acquired by using a Nikon confocal microscope.

4.8. Immunoblotting

Mice were killed by decapitation and different brain regions were dissected on ice and immediately frozen. Proteins were extracted with RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml Protease inhibitor cocktail, 1 mM Na₃VO₄, 1 mM NaF), and a total of 30-50 µg proteins were denatured by boiling for 5 min in sample buffer [2% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol and 0.1% bromophenol blue], and then separated by electrophoresis on SDS-polyacrylamide gels, after which proteins were transferred to PVDF membranes (GE healthcare, UK). PVDF membranes were incubated with primary antibodies for 12-16 h at 4 °C; immunoreactivity was visualized by using enhanced chemiluminescence (ECL Plus kit and reagents; Amersham Pharmacia Biotech, Piscataway, NJ). Antibodies used were: anti-cleaved caspase-3 (1:400, Cell Signaling Technology,

Danvers, MA), anti-spectrin (1:10,000, Santa Cruz technology, Santa Cruz, CA), anti-GLT-1 (1:1,000, BD Biosciences), anti-GFAP (1:7500, Sigma, St. Louis, MO), anti-pT231 tau (1:2,000, Biosource, Camarillo, CA), anti-TauC (1:500, Chemicon, Temecula, CA), anti-AT8 (1:500, Innogenetics, Gent, Belgium) and anti-Tau5 (1:5,000, Chemicon) antibodies. Anti-GAPDH (1:20,000, Chemicon, Temecula, CA) antibody was used as loading control in immunoblots for all the antibodies mentioned above. The optical density of antibody-labeled bands was analyzed using NIH Image J software. Mean densities from different experimental groups were shown as means $\pm$ SEM following normalization with GAPDH band intensity and were compared using one-way ANOVA with post-hoc analysis (tau transgenic mice with sham surgery or H/I and wild-type mice with sham surgery or H/I).

Acknowledgements

The work was supported by grants from NINDS (NS048423 to XB and NS048521 to MB) and by funds from Western University to X.B. Xiaoning Bi was also supported by funds from the Daljit and Elaine Sarkaria Chair.

Literature References

- Adhami F, Liao G, Morozov YM, Schloemer A, Schmithorst VJ, Lorenz JN, Dunn RS, Vorhees CV, Wills-Karp M, Degen JL, Davis RJ, Mizushima N, Rakic P, Dardzinski BJ, Holland SK, Sharp FR, Kuan CY. Cerebral ischemia-hypoxia induces intravascular coagulation and autophagy. *Am J Pathol* 2006;169:566–83. [PubMed: 16877357]
- Arundine M, Tymianski M. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* 2003;34:325–37. [PubMed: 12909079]
- Bano D, Young KW, Guerin CJ, Lefevre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P. Cleavage of the plasma membrane Na⁺/Ca²⁺ exchanger in excitotoxicity. *Cell* 2005;120:275–85. [PubMed: 15680332]
- Baschong W, Suetterlin R, Laeng RH. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). *J Histochem Cytochem* 2001;49:1565–72. [PubMed: 11724904]
- Bi X, Yong AP, Zhou J, Ribak CE, Lynch G. Rapid induction of intraneuronal neurofibrillary tangles in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A* 2001;98:8832–7. [PubMed: 11438710]
- Boekhoorn K, Terwel D, Biemans B, Borghgraef P, Wiegert O, Ramakers GJ, de Vos K, Krugers H, Tomiyama T, Mori H, Joels M, van Leuven F, Lucassen PJ. Improved long-term potentiation and memory in young tau-P301L transgenic mice before onset of hyperphosphorylation and tauopathy. *J Neurosci* 2006;26:3514–23. [PubMed: 16571759]
- Borchelt DR, Davis J, Fischer M, Lee MK, Slunt HH, Ratovitsky T, Regard J, Copeland NG, Jenkins NA, Sisodia SS, Price DL. A vector for expressing foreign genes in the brains and hearts of transgenic mice. *Genet Anal* 1996;13:159–63. [PubMed: 9117892]
- Chen JC, Hsu-Chou H, Lu JL, Chiang YC, Huang HM, Wang HL, Wu T, Liao JJ, Yeh TS. Down-regulation of the glial glutamate transporter GLT-1 in rat hippocampus and striatum and its modulation by a group III metabotropic glutamate receptor antagonist following transient global forebrain ischemia. *Neuropharmacology* 2005;49:703–14. [PubMed: 16019039]
- Choi DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 1988;11:465–9. [PubMed: 2469166]
- Choi DW. Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci* 1995;18:58–60. [PubMed: 7537408]
- Czogalla A, Sikorski AF. Spectrin and calpain: a ‘target’ and a ‘sniper’ in the pathology of neuronal cells. *Cell Mol Life Sci* 2005;62:1913–24. [PubMed: 15990959]
- Davis DP, Patel PM. Ischemic preconditioning in the brain. *Curr Opin Anaesthesiol* 2003;16:447–52. [PubMed: 17021495]
- Didenko VV, Ngo H, Minchew CL, Boudreaux DJ, Widmayer MA, Baskin DS. Caspase-3-dependent and -independent apoptosis in focal brain ischemia. *Mol Med* 2002;8:347–52. [PubMed: 12393932]

- Ferrer I, Friguls B, Dalfo E, Justicia C, Planas AM. Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat. *Neuropathol Appl Neurobiol* 2003;29:472–81. [PubMed: 14507339]
- Feustel PJ, Jin Y, Kimelberg HK. Volume-regulated anion channels are the predominant contributors to release of excitatory amino acids in the ischemic cortical penumbra. *Stroke* 2004;35:1164–8. [PubMed: 15017010]
- Franklin, KBJ.; Paxinos, G. The mouse brain in stereotaxic coordinates, Vol. Academic Press; San Diego: 1997.
- Gotz J, Tolnay M, Barmettler R, Chen F, Probst A, Nitsch RM. Oligodendroglial tau filament formation in transgenic mice expressing G272V tau. *Eur J Neurosci* 2001;13:2131–40. [PubMed: 11422454]
- Hara MR, Snyder SH. Cell signaling and neuronal death. *Annu Rev Pharmacol Toxicol* 2007;47:117–41. [PubMed: 16879082]
- Jankowsky JL, Slunt HH, Ratovitski T, Jenkins NA, Copeland NG, Borchelt DR. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol Eng* 2001;17:157–65. [PubMed: 11337275]
- Kalaria RN. The role of cerebral ischemia in Alzheimer's disease. *Neurobiol Aging* 2000;21:321–30. [PubMed: 10867217]
- Kerr LE, McGregor AL, Amet LE, Asada T, Spratt C, Allsopp TE, Harmar AJ, Shen S, Carlson G, Logan N, Kelly JS, Sharkey J. Mice overexpressing human caspase 3 appear phenotypically normal but exhibit increased apoptosis and larger lesion volumes in response to transient focal cerebral ischaemia. *Cell Death Differ* 2004;11:1102–11. [PubMed: 15153940]
- Laake JH, Haug FM, Wieloch T, Ottersen OP. A simple in vitro model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. *Brain Res Brain Res Protoc* 1999;4:173–84. [PubMed: 10446412]
- Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA, Moskowitz MA. Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. *Proc Natl Acad Sci U S A* 2002;99:15188–93. [PubMed: 12415117]
- Lesuisse C, Xu G, Anderson J, Wong M, Jankowsky J, Holtz G, Gonzalez V, Wong PC, Price DL, Tang F, Wagner S, Borchelt DR. Hyper-expression of human apolipoprotein E4 in astroglia and neurons does not enhance amyloid deposition in transgenic mice. *Hum Mol Genet* 2001;10:2525–37. [PubMed: 11709540]
- Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van Slegtenhorst M, Gwinn-Hardy K, Paul Murphy M, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M. Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat Genet* 2000;25:402–5. [PubMed: 10932182]
- Liao G, Yao Y, Liu J, Yu Z, Cheung S, Xie A, Liang X, Bi X. Cholesterol accumulation is associated with lysosomal dysfunction and autophagic stress in *Npc1* ^{-/-} mouse brain. *Am J Pathol* 2007;171:962–75. [PubMed: 17631520]
- Lin WL, Lewis J, Yen SH, Hutton M, Dickson DW. Filamentous tau in oligodendrocytes and astrocytes of transgenic mice expressing the human tau isoform with the P301L mutation. *Am J Pathol* 2003 Jan;162(1):213–8. [PubMed: 12507904]
- Love S, Barber R, Srinivasan A, Wilcock GK. Activation of caspase-3 in permanent and transient brain ischaemia in man. *Neuroreport* 2000a;11:2495–9. [PubMed: 10943710]
- Love S, Barber R, Wilcock GK. Neuronal death in brain infarcts in man. *Neuropathol Appl Neurobiol* 2000b;26:55–66. [PubMed: 10736067]
- Love S. Apoptosis and brain ischaemia. *Prog Neuropsychopharmacol Biol Psychiatry* 2003;27:267–82. [PubMed: 12657366]
- Ma J, Endres M, Moskowitz MA. Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice. *Br J Pharmacol* 1998;124:756–62. [PubMed: 9690868]
- Manabat C, Han BH, Wendland M, Derugin N, Fox CK, Choi J, Holtzman DM, Ferriero DM, Vexler ZS. Reperfusion differentially induces caspase-3 activation in ischemic core and penumbra after stroke in immature brain. *Stroke* 2003;34:207–13. [PubMed: 12511776]

- Mandelkow EM, Stamer K, Vogel R, Thies E, Mandelkow E. Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses. *Neurobiol Aging* 2003;24:1079–85. [PubMed: 14643379]
- Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol* 2000;1:120–9. [PubMed: 11253364]
- Mitani A, Tanaka K. Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. *J Neurosci* 2003;23:7176–82. [PubMed: 12904478]
- Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. *Science* 1992;258:597–603. [PubMed: 1329206]
- Nihashi T, Inao S, Kajita Y, Kawai T, Sugimoto T, Niwa M, Kabeya R, Hata N, Hayashi S, Yoshida J. Expression and distribution of beta amyloid precursor protein and beta amyloid peptide in reactive astrocytes after transient middle cerebral artery occlusion. *Acta Neurochir (Wien)* 2001;143:287–95. [PubMed: 11460917]
- Olney JW, Price MT, Fuller TA, Labruyere J, Samson L, Carpenter M, Mahan K. The anti-excitotoxic effects of certain anesthetics, analgesics and sedative-hypnotics. *Neurosci Lett* 1986;68:29–34. [PubMed: 3523314]
- Ramsden M, Kotilinek L, Forster C, Paulson J, McGowan E, SantaCruz K, Guimaraes A, Yue M, Lewis J, Carlson G, Hutton M, Ashe KH. Age-dependent neurofibrillary tangle formation, neuron loss, and memory impairment in a mouse model of human tauopathy (P301L). *J Neurosci* 2005;25:10637–47. [PubMed: 16291936]
- Rao VL, Dogan A, Todd KG, Bowen KK, Kim BT, Rothstein JD, Dempsey RJ. Antisense knockdown of the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. *J Neurosci* 2001;21:1876–83. [PubMed: 11245672]
- Romera C, Hurtado O, Mallolas J, Pereira MP, Morales JR, Romera A, Serena J, Vivancos J, Nombela F, Lorenzo P, Lizasoain I, Moro MA. Ischemic preconditioning reveals that GLT1/EAAT2 glutamate transporter is a novel PPARgamma target gene involved in neuroprotection. *J Cereb Blood Flow Metab* 2007;27:1327–38. [PubMed: 17213861]
- Rosell A, Cuadrado E, Alvarez-Sabin J, Hernandez-Guillamon M, Delgado P, Penalba A, Mendioroz M, Rovira A, Fernandez-Cadenas I, Ribo M, Molina CA, Montaner J. Caspase-3 is related to infarct growth after human ischemic stroke. *Neurosci Lett*. 2007
- Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. *Nat Neurosci* 2007;10:1377–86. [PubMed: 17965658]
- Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 1996;16:675–86. [PubMed: 8785064]
- Schiefer J, Alberty A, Dose T, Oliva S, Noth J, Kosinski CM. Huntington's disease transgenic mice are resistant to global cerebral ischemia. *Neurosci Lett* 2002;334:99–102. [PubMed: 12435481]
- Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res* 2000;874:123–30. [PubMed: 10960596]
- Schousboe A, Waagepetersen HS. Glial modulation of GABAergic and glutamatergic neurotransmission. *Curr Top Med Chem* 2006;6:929–34. [PubMed: 16787266]
- Sheldon AL, Robinson MB. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. *Neurochem Int* 2007;51:333–55. [PubMed: 17517448]
- Shi J, Yang SH, Stubley L, Day AL, Simpkins JW. Hypoperfusion induces overexpression of beta-amyloid precursor protein mRNA in a focal ischemic rodent model. *Brain Res* 2000;853:1–4. [PubMed: 10627301]
- Siman R, Noszek JC. Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. *Neuron* 1988;1:279–87. [PubMed: 2856162]
- Thies E, Mandelkow EM. Misrouting of tau in neurons causes degeneration of synapses that can be rescued by the kinase MARK2/Par-1. *J Neurosci* 2007;27:2896–907. [PubMed: 17360912]
- Torp R, Lekieffre D, Levy LM, Haug FM, Danbolt NC, Meldrum BS, Ottersen OP. Reduced postischemic expression of a glial glutamate transporter, GLT1, in the rat hippocampus. *Exp Brain Res* 1995;103:51–8. [PubMed: 7615037]

- Wang KK, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, Glantz SB, Morrow JS. Simultaneous degradation of alphaII- and betaII-spectrin by caspase 3 (CPP32) in apoptotic cells. *J Biol Chem* 1998;273:22490–7. [PubMed: 9712874]
- Wen Y, Yang S, Liu R, Brun-Zinkernagel AM, Koulen P, Simpkins JW. Transient cerebral ischemia induces aberrant neuronal cell cycle re-entry and Alzheimer's disease-like tauopathy in female rats. *J Biol Chem* 2004;279:22684–92. [PubMed: 14982935]
- Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M. Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. *Neuron* 2007;53:399–412. [PubMed: 17270736]
- Yi JH, Hazell AS. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochem Int* 2006;48:394–403. [PubMed: 16473439]
- Zerbinatti CV, Wozniak DF, Cirrito J, Cam JA, Osaka H, Bales KR, Zhuo M, Paul SM, Holtzman DM, Bu G. Increased soluble amyloid-beta peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. *Proc Natl Acad Sci U S A* 2004;101:1075–80. [PubMed: 14732699]
- Zhang M, Li WB, Geng JX, Li QJ, Sun XC, Xian XH, Qi J, Li SQ. The upregulation of glial glutamate transporter-1 participates in the induction of brain ischemic tolerance in rats. *J Cereb Blood Flow Metab* 2007;27:1352–68. [PubMed: 17228332]
- Zhou M, Baudry M. Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. *J Neurosci* 2006;26:2956–63. [PubMed: 16540573]
- Zivin JA, Choi DW. Stroke therapy. *Sci Am* 1991;265:56–63. [PubMed: 1679258]

Abbreviations

GFAP	glial fibrillary acidic protein
GLT-1	glial glutamate transporter 1 (also known as excitatory amino acid transporter 2 (EAAT2))
H/I	hypoxia/ischemia
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

Figure 1. Decreased brain damage in P301L tau mice after ischemia/hypoxia

A. Nissl-stained brain sections in wild-type (WT) and P301L tau transgenic mice (Tau) 24 h after ischemia/hypoxia. B. Comparison of infarct sizes in 8 selected sections showing that P301L tau mice have less brain damage in each section (n=8 for wild-type mice and 10 for P301L mice, $p < 0.01$ for each section). C. Total infarction volume of P301L and wild-type mice (** $p < 0.001$). D. TUNEL staining of apoptotic cells in cortex and CA1 region of wild-type and P301L mice. Scale bar = 20 μ m. E. Fluoro-Jade B staining in striatum, cortex, hippocampus and thalamus in wild-type and P301L mice. Scale bar = 50 μ m. F. semi-quantitative data for Fluoro-Jade B positive neurons (grade 0, no staining; grade 1, 1-30%; grade 2, 31-60%; grade 3, >60%. n=8 for wild-type and 10 for P301L mice, * $p < 0.05$, ** $p < 0.01$ compared to wild-type).

Figure 2. Oxygen-glucose deprivation -induced neuronal damage in cultured hippocampal slices
 A. Propidium iodide (PI) staining in cultured hippocampal slices of wild-type and P301L tau mice 24 h after OGD, indicating that hippocampal slices from the transgenic mice have less PI uptake than wild-type mice. B. Quantitative data for PI staining (* $p < 0.05$). CN, control; OGD, oxygen and glucose deprivation.

Figure 3. Tau truncation and phosphorylation in P301L and wild-type mice after ischemia/hypoxia
 A. Levels of pT231 were dramatically reduced in wild-type mice (n=6 for wild-type and 9 for P301L mice, *p < 0.05). B. Quantitative immunoblotting data for the levels of TauC (n=4 for wild-type and 9 for P301L mice, *p < 0.05). C. Representative images of immunoblots labeled with anti-pT231-tau or anti-TauC antibodies. D. TauC immunostaining in P301L tau and wild-type mice after ischemia/hypoxia. CN, control; H/I, hypoxia/ischemia.

Figure 4. Hypoxia/ischemia-induced calpain activation in P301L tau and wild-type mice

A. Representative images of immunoblots probed with anti-spectrin antibodies. B. Quantitative immunoblotting data for the 145/150 kDa spectrin break-down products (SBDP). C. Quantitative immunoblotting data for the 120 kDa SBDP (n=4, *p < 0.05, **p < 0.01 for both B and C). D. Immunostaining for calpain-cleaved SBDP and NeuN in cortex of wild-type and P301L tau mice (scale bar = 20 μ m).

Figure 5. Caspase-3 activation in wild-type and P301L tau mice after ischemia/hypoxia
 A. Representative images of immunoblots probed with antibodies against cleaved caspase-3.
 B&C. Quantitative data for the 19 kDa (B) and 17 kDa (C) isoforms of caspase-3 (* $p < 0.05$ compared to WT control).

Figure 6. Higher levels of glial glutamate transporter GLT-1 in P301L tau transgenic than in wild-type mice

A. Representative images of immunoblots probed with anti-GLT-1 or anti-GAPDH (used as loading controls). B. Quantitative immunoblotting data for GLT-1 (expressed as % of WT control; $n = 4$; * $p < 0.05$ compared to WT controls, # $p < 0.05$ compared to transgenic controls). CN, control; H/I, hypoxia/ischemia. C. Dense GLT-1 positive puncta (arrows) were enriched in cortex (CX) and hippocampus (CA1) of P301L tau transgenic mice without ischemia/hypoxia (scale bar = 10 μm).

Figure 7. Astrocyte activation in wild-type and P301L tau mice after hypoxia/ischemia
 A. Representative images of immunoblots labeled with antibodies against GFAP. B. Quantitative immunoblotting data for GFAP levels (n=4, *p < 0.05 compared to WT controls, #p < 0.05 compared to Tau controls.). C. Immunostaining with anti-GFAP antibodies in CA1 region of P301L tau and wild-type mice without hypoxia/ischemia (scale bar = 50 μ m).