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Ischemic Tolerance in an *In Vivo* Model of Glutamate "Preconditioning"

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Abstract

Ischemia initiates a complicated biochemical cascade of events that triggers neuronal death. In this study, we focused on glutamate –mediated neuronal tolerance to ischemia-reperfusion. We employed an animal model of life-long excess release of glutamate, the glutamate dehydrogenase 1 transgenic (Tg) mouse, as a model of *in vivo* "glutamate preconditioning". Nine- and 22-month old Tg and wild type (wt) mice were subjected to 90 min of middle cerebral artery occlusion followed by 24 hr reperfusion. The Tg mice suffered significantly reduced infarction and edema volume, compared with their wt counterparts. We further analyzed proteasomal activity, level of ubiquitin immunostaining, and MAP2A expression to understand the mechanism of neuroprotection observed in the Tg Mice. We found that in the absence of ischemia, the Tg mice exhibited higher activity of the 20S and 26S proteasomes while there were no significant differences in the level of hippocampal ubiquitin immunostaining between wt and Tg mice. A surprising observation was that of a significant increase in MAP2A expression in neurons of the Tg hippocampus following ischemia-reperfusion, compared with that in wt hippocampus. The results suggest that increased proteasome activity and MAP2A synthesis and transport might account for the effectiveness of glutamate preconditioning against ischemia-reperfusion.

Keywords

stroke; ubiquitin; MAP2; aging; hippocampus

INTRODUCTION

Stroke results in a rapid cessation of blood flow that compromises energy metabolism in affected brain tissues and leads to large increases in glutamate release (Benveniste *et al.*, 1984). Glutamate is ubiquitously distributed in the brain, is present in concentrations that are higher than those of any other amino acid (Fonnum, 1984), and functions as the principal excitatory neurotransmitter in the central nervous system (CNS). Previous studies have demonstrated that blocking ionotropic glutamate receptors significantly reduces ischemic damage (Sims & Muyderman, 2010). This is attributed to the triggering by the released

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glutamate of an intracellular excitotoxic cascade involving an excessive accumulation of calcium (Ca^{2+}) and activation of downstream pathways that cause cell death. Therefore, impeding the development of excitotoxicity in animal experimental stroke has been a frequently targeted mechanism in the development of neuroprotective stroke treatments (O'Collins *et al.*, 2006; Minnerup *et al.*, 2012). The effect of over 20 anti-excitatory drugs has been evaluated in over 270 preclinical studies, yet none of them have been shown to be effective in the treatment of stroke in humans (O'Collins *et al.*, 2006).

There are increased efforts to identify potential strategies to protect neurons following ischemic stroke. A current focus is on understanding the protective mechanisms that induce tolerance following ischemic preconditioning. Current knowledge suggests that preconditioning with a brief period of ischemia is an effective approach to decrease neuronal death after a more severe ischemic episode (Kirino, 2002). The same concept can also be applied to excitotoxicity where preconditioning with a mild glutamate-induced stress can promote a tolerant state that reduces the injury caused by a subsequent, more severe glutamate exposure. The effect of glutamate preconditioning in the development of resistance to a subsequent ischemic insult has been assessed previously, although such preconditioning was produced in *in vitro*, not *in vivo*, preparations through the addition of exogenous glutamate or glutamate analogs, such as receptor agonists or antagonists. For example, the death of cortical neurons in primary culture following *in vitro* ischemia is prevented by pre-exposure of the cells to *N*-methyl-D-aspartate (NMDA), a selective glutamate NMDA receptor agonist (Lin *et al.*, 2008). In other studies, the ischemic preconditioning of primary hippocampal neurons was disrupted following exposure to the NMDA receptor inhibitor MK-801 (Mabuchi *et al.*, 2001), and the ischemic preconditioning of co-cultures of neurons and glial cells was suppressed following exposure to the NMDA receptor inhibitor 3-((D)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (Grabb & Choi, 1999). Both of these studies provided indirect evidence of glutamate involvement in the process of ischemic preconditioning. However, there are no studies that have examined the role of endogenous, moderate excess synaptic glutamate release and subsequent receptor activation *in vivo* on ischemia-induced neuronal damage.

In the present study, our main objective was to determine whether moderate excess synaptic release of endogenous glutamate throughout the lifespan of an organism, could lead to increased ischemic tolerance, i.e., a preconditioning-like state, and thus protect neurons from ischemia in an *in vivo* model. We used transgenic (Tg) mice that overexpress the gene for glutamate dehydrogenase 1 (*Glud1*) only in neurons, not in glial cells, of the CNS. These Tg mice are characterized by an increase in GLUD1 enzyme levels and activity (Bao et al. 2009; Hascup et al. 2011), relatively modest, yet significant, increases in brain glutamate concentrations (approximately 10–15% above wild-type littermates) throughout the lifespan (Bao et al. 2009; Choi et al. 2014), and a moderate increase (30–40% higher levels than normal) in depolarization-induced synaptic glutamate release (Bao et al. 2009; Michaelis et al. 2011). Unlike other models of excessive accumulation of extracellular glutamate (from 150% to approximately 30-fold increases in glutamate levels) because of null mutations of glial glutamate transporters or of regulators of such transporters (Rothstein et al. 1996; Tanaka et al. 1997; Zeng et al. 2007), the *Glud1* Tg mice do not suffer from massive

neuronal damage in the brain or an early death. Thus, the *Glud1* Tg mice offer a potentially good model for probing the effect of moderately increased, pulsatile, chronic release of endogenous glutamate by neurons on the development of resistance to an ischemic episode in adult and aged mice.

The hypothesis that we are testing is that chronic exposure of neurons to moderate levels of synaptically released glutamate would activate protective pathways that reduce damage after a stroke. Both gene ontology and pathway analyses of whole genome expression differences between *Glud1* Tg and wt mice have identified many genes that are related to glutamate and intracellular signaling as being over-expressed in the Tg mice (Wang et al. 2010; Wang et al. 2014). Some of these genes might lead to the development of resistance to ischemia. Our results demonstrated that the *Glud1* Tg mice were more resistant to ischemia/reperfusion, compared with wt mice. In order to ascertain the potential molecular mechanisms for the observed resistance to ischemia in the *Glud1* Tg mice, we explored the effect of glutamate hyperactivity on the proteolytic activity of the 20S and 26S proteasomes, and of glutamate hyperactivity and ischemia on the levels of ubiquitinated proteins. Proteasomal activity is decreased and ubiquitinated proteins accumulate following middle cerebral artery occlusion (MCAO) (Alves-Rodrigues et al. 1998; Keller et al. 2000a; Keller et al. 2000b), while intact proteasome activity is related to rapid ischemic tolerance (Meller et al. 2008). We hypothesized that in the *Glud1* Tg mice, the proteasome activity might be elevated and less affected by ischemia, thus providing relative neuroprotection from an ischemic episode. This hypothesis was based in part on the observed increases in the genomic expression of ubiquitin proteasome system (UPS) enzymes in *Glud1* Tg compared with wt mice (Wang et al. 2010; Wang et al. 2014).

To assess the susceptibility of neurons to the injurious effects of ischemia in Tg and wt mice, we examined the immune-labeling of neuronal cell bodies and dendrites by antibodies to microtubule-associated protein-2A (MAP2A). Acute glutamate- or NMDA-induced neuronal damage manifests as dendrite varicosities in neurons labeled with anti-MAP2A antibodies (Hoskison et al. 2007; Ikegaya et al. 2001). We previously showed that labeling with anti-MAP2A antibodies shows discontinuities in dendrites of 9-month old *Glud1* Tg mice but without evidence of varicosities (Bao et al. 2009).

MATERIALS AND METHODS

Generation of *Glud1* Tg mice

All experiments were conducted with the approval of the University of Kansas Institutional Animal Care and Use Committee. The *Glud1* Tg mice were generated as described (Bao *et al.*, 2009). Briefly, linearized DNA containing the cDNA of mouse GLUD1 was injected into fertilized C57BL6/SJL hybrid mouse oocytes. The cDNA was placed under the control of the Nse (neuron-specific enolase) promoter (Bao *et al.*, 2009). The animals used in the present study were male, 9-month (mo)-old Tg and wt. This age was selected as this is the age when CNS transcriptomic and structural differences between the Tg and wt are most significant (Bao *et al.*, 2009; Wang *et al.*, 2010; Michaelis *et al.*, 2011; Wang *et al.*, 2014). In studies designed to determine the effects of aging on ischemia-induced brain damage, 22-mo old mice were used.

In vivo ischemia model

Reversible occlusion of the middle cerebral artery was induced using a well-established protocol (Clark *et al.*, 1997). The mice were anesthetized with 3% Isoflurane and oxygen and maintained with 1.5% Isoflurane, or to desired anesthetic effect, throughout the procedure. Buprenorphine was used as the analgesic and was injected pre-operatively at 0.05 mg/kg. Male mice were subjected to MCAO followed by a 24 hour (hr) period of reperfusion. At the whole-brain level, TTC (2,3,5-triphenyltetrazolium chloride monohydrate) staining was used to assess brain damage (Ito *et al.*, 1997). Brain edema volume (V_{edema}) was also measured from the coronal sections that were stained by TTC by determining the volumes of both the ipsilateral (affected) hemisphere (V_{Ipsi}) and the contralateral hemisphere (V_{contra}) and using the equation: $V_{edema} = V_{contra} - V_{Ipsi}$ (Yan *et al.*, 2011).

Measurement of proteasomal activity

Brain tissues from 9-mo old wt and Tg mice were homogenized and 20 μg of the cell homogenate proteins were incubated with proteasome activity assay buffer in which their ability to cleave the fluorogenic peptide substrate, Succinyl-LeuLeuValTyr-7-amino-4-methly-coumarin (Suc-LLVY-AMC) was determined (Figueiredo-Pereira *et al.*, 1994). The assay buffer used in the measurement of the 26S proteasome function consisted of 50 mM Tris (pH 7.4), 5mM $MgCl_2$, 2 mM DTT, 2 mM ATP and Suc-LLVY-AMC (80 μM in 1% DMSO, Sigma-Aldrich). The buffer employed for the determination of 20S proteasome function contained 20 mM HEPES (pH 7.8), 0.5 mM EDTA, 0.03% SDS and 80 μM Suc-LLVY-AMC. The hydrolysis of Suc-LLVY-AMC into AMC was detected using a fluorescence plate reader at ex 380 nm and em 440 nm.

Immunohistochemistry

Immunostaining and statistical analyses were performed using methods similar to those described previously (Bao *et al.*, 2009) but with minor modifications in the initial steps of preparation of brain tissue following MCAO. Specifically, before fixation of the hemisected brain tissue (ipsilateral *vs*. contralateral hemispheres), 2 mm coronal sections were obtained as described above for the assessment of the effects of MCAO on brain at the macroscopic level using TTC staining. For the confocal microscopy studies, the 2 mm coronal sections were fixed and permeabilized and then subjected to rapid freezing and cryo-microtome sectioning into ~25 μm thick sections. The cryotome sections were incubated overnight in buffer containing a combination of the primary antibodies against ubiquitin (polyclonal; rabbit; 1:250; Cell Signaling) and against MAP2A (monoclonal; mouse; 1:250; Millipore). The sections were subsequently rinsed in PBS and incubated with fluorescent dye-labeled secondary antibodies (Alexa 568 goat anti-rabbit and Alexa 488 goat anti-mouse) at 37 °C for 2 hrs. Cortical and hippocampal regions were examined by confocal microscopy on a Leica SPE2 laser confocal microscope. Pixel density counts were determined using the Leica Application Suite software. The results reported are from the hippocampus as neuronal structure was frequently better preserved than in the cerebral cortex, albeit still showing extensive damage. The averaged pixel densities from each sub-layer of each region of the hippocampus, i.e., from the *stratum oriens* (SO), *stratum pyramidale* (SP) and *stratum*

radiatum (SR) of the CA1 and CA3 regions, from 3 pairs of animals, were statistically analyzed and graphed.

Statistical analysis

Student's *t*-test and one-way or three-way ANOVA were used to determine statistically significant differences employing SigmaPlot 12.5. The level of significance was set at $P \leq 0.05$.

RESULTS

Lower brain infarct and edema volume in *Glud1* Tg *vs*. wt mouse brain following MCAO

Nine-mo old male wt and Tg mice were subjected to MCAO followed by a 24 hr period of reperfusion. Using TTC staining, we assessed the extent of brain damage. The reduction of TTC by dehydrogenases in living cells leads to the formation of water-insoluble red formazan crystals, which outlines the area and volume of surviving tissue. This method can be used to determine percent infarct size (Ito *et al.*, 1997). The results revealed that the *Glud1* Tg mice had a 30% decrease in infarct volume compared to their wt cohorts (Figure 1B). Brain edema volume was measured using 2 mm coronal sections by determining the difference between the volumes of both the ipsilateral hemisphere and the contralateral hemisphere. As shown in Figure 1C, the *Glud1* Tg mice had a significantly lower edema volume following MCAO than the wt mice. Together, these results support our hypothesis that chronic exposure to moderate levels of glutamate released at synapses can protect against an ischemic insult.

Increased basal levels of proteasomal activity in the brain of *Glud1* Tg *vs.* wt mice

Next, we attempted to define molecular components of the adaptive mechanisms that allowed the *Glud1* Tg mice to be more resistant to ischemia. The ubiquitin-proteasome system (UPS) plays an essential role in ischemic tolerance (Meller *et al.*, 2008; Meller, 2009). To identify whether differences in the UPS between Tg and wt mice might account for the observed differential sensitivity to ischemia in these two types of mice, we measured the proteasomal activities in brain tissue of the *Glud1* Tg and wt cohorts. Brain tissue from 9-mo old wt and Tg mice that had not been subjected to MCAO were homogenized, and proteasomal function was assessed. An ATP-free assay buffer was used to differentiate 20S proteasomal function from the 26S proteasomal function. We found that basal proteasomal activities of both the 26S and 20S proteasome (i.e., in the absence of any prior treatment) were significantly elevated in the *Glud1* Tg mice compared with those in wt mice (Figure 2).

Ubiquitin labeling in hippocampus following MCAO in Tg and wt mice

The differentially higher activity of the 26S and 20S proteasomes in *Glud1* Tg *vs* wt mouse brain fit with the previously observed elevated gene expression of UPS enzymes in the Tg mouse hippocampus (Wang et al. 2010). Increases in UPS mRNA levels and, as shown in the present study in the proteasome activity in *Glud1* Tg mice, might represent adaptive responses to increases in the levels of damaged proteins brought about by glutamate hyper-stimulation of neurons in these mice. Overall increases in the activity of the UPS might account for the delayed appearance of significant accumulation of ubiquitin protein

aggregates in brain neurons of *Glud1* mice that is observed around the age of 16–20 months (Bao *et al.*, 2009). However, ischemia-reperfusion, we reasoned, may cause an increased burden in terms of damaged proteins in both wt and Tg mice (Alves-Rodrigues et al. 1998; Keller et al. 2000b). The effect of ischemia-reperfusion on ubiquitinated protein accumulation and proteasome degradation might be expected to be greater in the Tg than the wt mouse but these mice might have adapted to the increased burden of ubiquitinated proteins. We examined the levels of immunolabeled ubiquitinated proteins in brain sections from *Glud1* Tg and wt mice, both under baseline conditions and following cerebral artery occlusion. In contrast to our expectation, there were no significant differences in overall immune labeling of ubiquitin or ubiquitinated proteins in neurons of either the CA1 or the CA3 region of the hippocampus of Tg and wt mice subjected to ischemia (one-way ANOVA, Holm-Sidak *post-hoc* pairwise comparisons, $P>0.05$ for all comparisons; data not shown). The same was true also for the levels of immune reactivity under baseline conditions. The lack of differential labeling between wt and Tg mice matched the observations reported previously for 9 month old *Glud1* Tg and wt hippocampus (Bao et al. 2009).

Increased MAP2A labeling in the hippocampus of the *Glud1* Tg mice following MCAO

MAP2 is a microtubule-associated protein that is critical for the maintenance of normal cytoskeletal architecture in neurons and for the overall function of neurons (Yan *et al.*, 2010). MAP2 is localized in cell bodies and dendrites and is a useful marker of surviving neurons and a probe of dendrite structure (Bao et al. 2009; Hoskison et al. 2007; Ikegaya et al. 2001). In a previous study of 9-mo old *Glud1* Tg mice, it was noted that the hyperglutamatergic state in the brain of the Tg animals led to a diminution of MAP2A labeling by the anti-MAP2A antibodies, while the structure of dendrites remained relatively well preserved (Bao et al. 2009). These changes were attributed to a possible disruption of either MAP2A mRNA transport to or suppression of protein synthesis within the dendrites (Rehbein et al. 2000). In the present study, under baseline conditions, we observed a significant reduction in overall MAP2A labeling in both cell bodies and dendrites, and in both CA1 and CA3 regions of the hippocampus of the *Glud1* Tg as compared with that of the wt mice (Fig. 3, 4). Remarkably, following the induction of ischemia-reperfusion, the labeling of MAP2A in neuronal cell bodies and dendrites of the Tg mice was markedly enhanced above that observed at baseline, whereas the labeling of the hippocampus neurons in wt mice was markedly reduced in both the ipsilateral and contralateral CA1 and CA3 hippocampal regions (Fig. 5, 6).

Lower brain infarct and edema volume in aged *Glud1* Tg mice *vs* wt mice

Focal ischemia leads to increases in infarct volume in aged animals (Davis *et al.*, 1995; Sutherland *et al.*, 1996), and the effectiveness of preconditioning in inducing protection against severe ischemia has been reported to be decreasing with advancing age (He *et al.*, 2005). To determine whether the relative ischemia tolerance observed in our model of glutamate preconditioning, i.e., the *Glud1* mice, was altered by aging, we determined brain damage (infarct and edema volumes) in 22-mo old Tg and wt mice after MCAO and reperfusion. Figure 7 shows that the *Glud1* Tg mice at 22 mos of age were protected in terms of decreases in infarct and edema volumes, compared to their age-matched wt

counterparts ($P < 0.005$). Statistical analyses showed that there were no significant differences between the 22-mo old and the 8-mo old mice of the same genotype.

DISCUSSION

The key observation made in the present study was that either adult or aged *Glud1* Tg mice exhibited significantly greater resistance to ischemia-reperfusion than the Wt mice of the same age. The mechanisms for such tolerance to the effects of ischemia-reperfusion are still unknown. The first mechanism considered in the present study was that of a proteasome-mediated resistance to ischemia-reperfusion.

It is known that excess glutamate can induce the formation of reactive oxygen species (ROS) (Armstead et al., 1989; Duchen, 2000; Kahlert et al., 2005; Parfenova et al., 2006) and that ROS can alter proteasome activities (Ullrich et al., 1999; Ding et al., 2003; Grune et al., 2004; Aiken et al., 2011). The proteasome proteolytic pathways represent the main mechanisms responsible for the degradation of damaged or unwanted proteins. These pathways play an important role in maintaining normal cellular homeostasis, as null mutants for the proteasome subunits result in lethal phenotypes (Heinemeyer et al., 1991; Orlowski, 1999). Protein unfolding and aggregation are also dominant pathogenic events in vulnerable ischemic neurons (Ge et al., 2007). A study by Liu et al. (2005) showed that ischemic preconditioning alleviated protein aggregation in neurons. Whether this reduction is due to a decrease in overall cell damage or the result of an enhanced ability by neurons tolerant to ischemia in eliminating the irreparably damaged proteins has yet to be elucidated. Therefore, we tested the hypothesis that chronic exposure of neurons to increased levels of synaptic glutamate release, i.e., glutamate preconditioning, might lead to an increase in proteasome activity. We observed significant increases in both the 20S and 26S baseline proteasome activities in *Glud1* mouse brains as compared with those of wt mice. Ubiquitination is a key step in the degradation process of many proteins. Contrary to our hypothesis that elevated proteasomal activity would lead to decreases in ubiquitinated proteins following an episode of ischemia-reperfusion, we did not observed any difference in the level of ubiquitin immunoreactivity following ischemia-reperfusion in hippocampal neurons of the Tg, compared with those of the Wt mice. In summary, the increase of the proteasome activities but not of ubiquitination suggests that the proteasomal degradation pathways are involved in the neuroprotective effect of chronic glutamate synaptic release.

The molecular dynamics that lead to increases in proteasome activity in the *Glud1* mice are not known. Following an episode of stroke, the loss of ATP impairs the activity of the 26S proteasome (Keller et al., 2000). The 26S proteasome is an ATP-dependent protease composed of a core proteinase, the 20S proteasome, and two PA700 regulatory particles (the 19S complex) on both ends (Coux et al., 1996). These subunits associate and dissociate in an ATP-dependent manner, thus conditions of decreased ATP levels would bring about the dissociation of the subunits (Tanahashi et al., 2000). Following transient forebrain ischemia, the ATP-dependent re-association of the 20S catalytic and PA700 regulatory subunits to form the 26S proteasome is severely impaired in the hippocampus (Asai et al., 2002). The increased baseline proteasome activity in *Glud1* mouse brains might offer relative resistance to the ischemia-induced loss of ATP. However, the present studies did not link the

protective effect of chronic glutamate synaptic release in brain to the activation of proteasome degradation of proteins.

Besides the differential activities of the proteasomes in the Tg *vs.* wt brains, the opposite responses of neurons to ischemia-reperfusion in terms of the MAP2A levels in Tg and wt hippocampus, was one of the most remarkable differential characteristics observed. MAPs are key regulators of neuronal morphogenesis (Popa-Wagner *et al.*, 1999). The so-called "late MAPs", MAP2A and MAP2B, have a profound effect on the organization of cellular microtubules (Lewis *et al.*, 1989), provide structural stabilization during process outgrowth (Chen *et al.*, 1992), and assist in the maintenance of proper synaptic circuitry in the mature brain (Marsden *et al.*, 1996; Popa-Wagner *et al.*, 1999). MAP2A is preferentially associated with dendritic processes (Binder *et al.*, 1986). Stress conditions can alter MAP2 levels in the brain cortex and hippocampus (Yan *et al.*, 2010), and MAP2 localization and protein levels are disrupted in response to the stress induced by high extracellular glutamate levels (Arias *et al.*, 1997). Cerebral ischemia reduces MAP2 immunoreactivity in the hippocampus and cortex of both neonatal (Malinak & Silverstein, 1996) and adult rats (Kitagawa *et al.*, 1989; Dawson & Hallenbeck, 1996). The most significant loss of MAP2 following ischemia is in the CA1 region in both rats (Inuzuka et al., 1990) and gerbils (Yoshimi et al., 1991). The susceptibility of MAP2 to ischemia and excitotoxicity has been attributed to the elevation of intracellular Ca^{2+} concentrations that lead to rapid MAP2 proteolysis by calcium-activated proteinases (Kitagawa *et al.*, 1989). However, some have reported that MAP2 levels in the penumbra surrounding the core of the ischemic lesion, as well as in surviving neurons at the core of ischemic tissue, are increased rather than decreased (Li et al. 1998). The same is true for MAP2 levels following episodes of severe epileptic seizures in rats (Jalava et al. 2007). Thus, under conditions of increased glutamate release to the extracellular environment of neurons, i.e., ischemic penumbra and post-ictal state, certain neuronal populations respond to the stress by increasing the intracellular levels of MAP2 proteins.

In our studies, we observed that following MCAO, MAP2A was significantly increased in the CA1 region of the *Glud1* Tg mice compared to the wt littermates. This striking increase in MAP2 immunolabeling is more likely a result of increased protein levels and not an increase in the number of dendritic processes. This suggests that unlike the wt mice, the *Glud1* Tg mice may have a preserved ability to synthesize some of the components required for structural repair and maintenance (Popa-Wagner *et al.*, 1999). Since some MAP2 is synthesized in the cell body and then transported to dendrites (Okabe & Hirokawa, 1989), while a substantial amount of MAP2A is synthesized in dendrites from mRNA that has been transported to dendritic sites (Rehbein et al. 2000), it would appear that the *Glud1* Tg mice have improved protein and mRNA transport processes that lead to enhanced recovery following cerebral ischemia. In more recent studies, we have observed significant increases in the levels of proteins involved in the transport of membrane bound organelles in neurons, the kinesins, in mRNA levels for these proteins, and in overall axoplasmic transport in Tg *vs.* wt neurons (P. Lee et al., submitted for publication). Therefore, the possibility that neurons from the hyperglutamatergic mice might recover more readily the transport of particle-bound mRNA and local synthesis of MAP2A than neurons from wt mice seems possible but has to be examined in greater detail in future studies.

Previous studies have reported that the degree of preconditioning-induced protection is significantly diminished in aged rats (Fenton *et al.*, 2000; He *et al.*, 2005). In humans, transient ischemic attacks (TIA) were found to be neuroprotective against ischemic strokes. However, the protective mechanisms of TIAs were not present in elderly patients over the age of 65 (Della Morte *et al.*, 2008). To determine whether the tolerance induced by glutamate preconditioning was preserved in aged mice, we evaluated brain infarct size in 22-mo old *Glud1* Tg mice after MCAO and reperfusion. We showed that glutamate preconditioning was at least as, if not more, effective in the aged (22-mo) compared with the young adult (9-mo) mice. This was consistent with the results of another study in which gerbils preconditioned with 1.5 min of ischemia before a subsequent 5-min occlusion of both carotid arteries (global ischemia), exhibited differential levels of resistance to ischemia (Dowden & Corbett, 1999). The young animals had 53–67% protection of CA1 neurons while the older animals exhibited approximately 75% protection (Dowden & Corbett, 1999). The authors attributed this difference to the decrease in density and sensitivity of NMDA receptors that occur in the aged rodent brain (Gonzales *et al.*, 1991).

In conclusion, the phenomenon of ischemic preconditioning in the brain is well documented. In this report, for the first time, we demonstrate that *in vivo* increased synaptic release of glutamate can induce ischemic tolerance and maintain cell viability in young adult mice that were subjected to cerebral artery occlusion. This protection was preserved in the aged 22-mo old mice. The increase in MAP2 levels following ischemia-reperfusion in the resistant *Glud1* Tg mice, may suggest an enhancement in protein synthesis and protein and mRNA transport to distal processes in these mice. Increases in MAP2A synthesis and transport under conditions of neuronal stress may be an important component of the glutamate preconditioning process that leads to ischemic tolerance and, potentially, to tolerance to other stressful stimuli.

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ABBREVIATIONS

AMC	7-amino-4-methly-coumarin
ATP	adenosine triphosphate
CA	carbonic anhydrase
CNS	central nervous system
EAAT-2	excitatory amino acid transporter-2
Glud1	glutamate dehydrogenase gene
MAP	microtubule-associated proteins
MCAO	middle cerebral artery occlusion

mo	month
NMDA	N-methyl-D-aspartate
nse	neuron-specific enolase
ROS	reactive oxygen species
rt-PA	recombinant tissue plasminogen activator
SO	*stratum oriens*
SP	*stratum pyramidale*
SR	*stratum radiatum*
Tg	transgenic
TIA	transient ischemic attack
TTC	2,3,5-triphenyltetrazolium chloride monohydrate
UBE	ubiquitin-conjugating enzymes
UPS	ubiquitin proteasome system
wt	wild type

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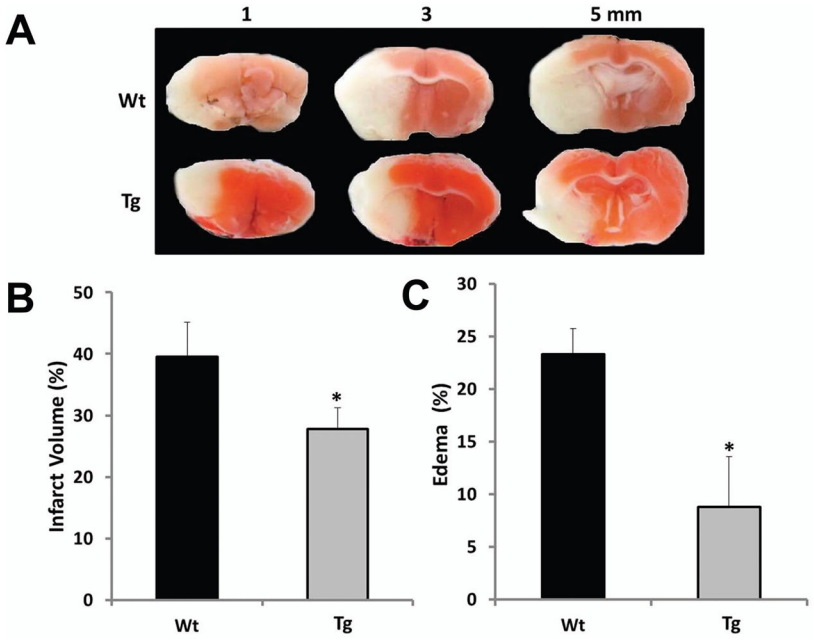

Figure 1. Effect of MCAO on brain tissue damage in Wt *vs. Glud1* Tg mice

Brain damage was determined by TTC staining after mice were subjected to 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections proceeding from frontal to caudal. Sections were taken from the 1 mm position of the frontal pole and proceeded in 2 mm intervals to 5 mm. (B) Quantification of infarct volume was determined in TTC stained sections. (C) Quantification of brain edema volume estimated from TTC stained sections (n=5). Data presented as means ± SEM. *$p < 0.05$ Tg *vs*. wt mice.

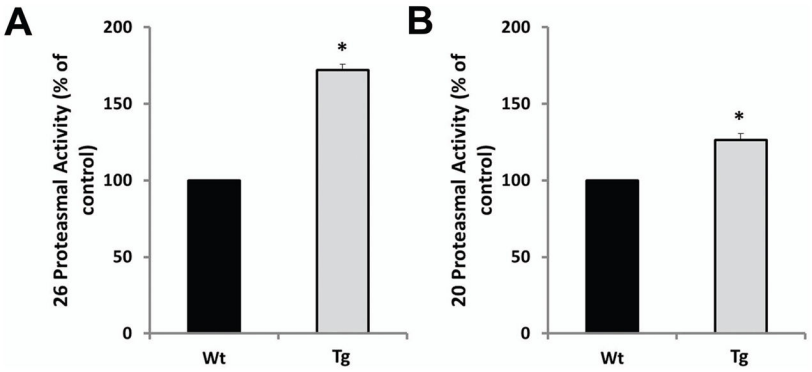

Figure 2. Increased brain proteasomal activity in the *Glud1* Tg mice

Activities of the (A) 26S and the (B) 20S proteasome were assayed by determining their ability to cleave Suc-LLVY-AMC (n=3). Data presented as means ±SEM. *p<0.05 Tg *vs*. wt mice.

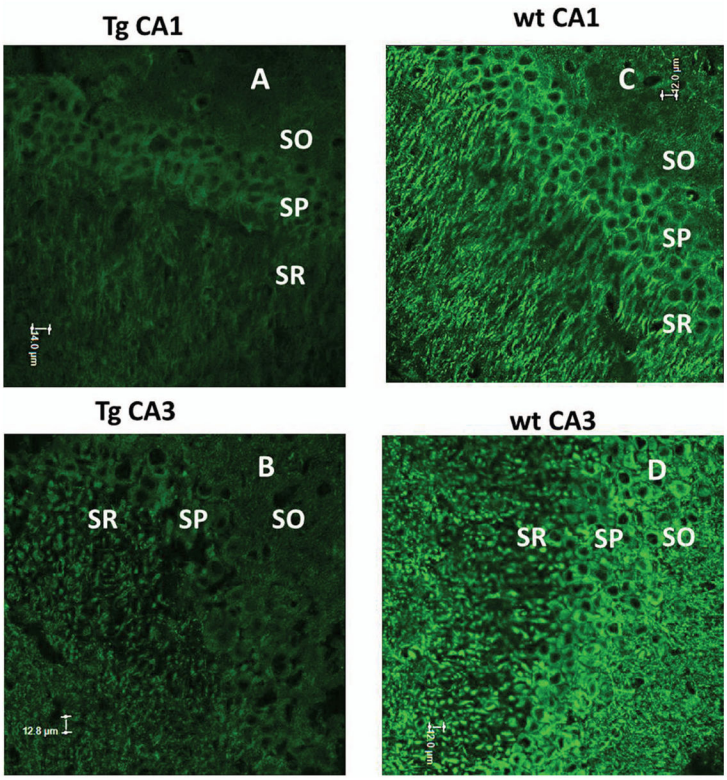

Figure 3. Immune-labeling of MAP2A in the hippocampus of *Glud1* Tg and wt mice under baseline conditions

Representative immunofluorescent images showing labeling of MAP2A in the CA1 and CA3 regions of the hippocampus in a pair of 9-mo old *Glud1* Tg and wt mice that were not subjected to *in vivo* ischemia-reperfusion. All images were obtained using identical laser intensity and fluorescence amplification. SO, *stratum oriens*; SP, *stratum pyramidale*; SR *stratum radiatum*. Scale bar =12 μm.

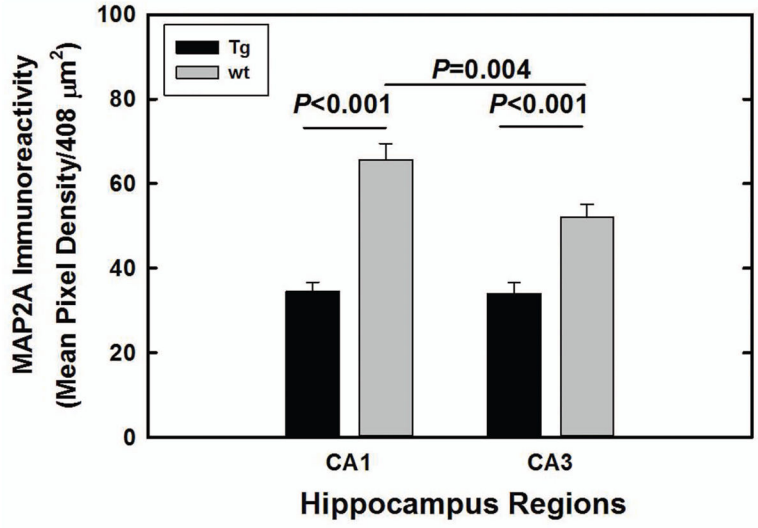

Figure 4. Quantification of MAP2A immunoreactivity in CA1 and CA3 regions of the hippocampus of Tg and wt mice under baseline conditions

The mean pixel densities (±SEM) of MAP2A immune labeling in the CA1 and CA3 regions represent the average of measurements obtained from multiple subfields from the three layers of each region of the hippocampus of 3 Tg and 3 wt mice. The data were analyzed by one-way ANOVA with *post hoc* analysis of pairwise comparisons (Holm-Sidak) and the significant differences between measurements are indicated.

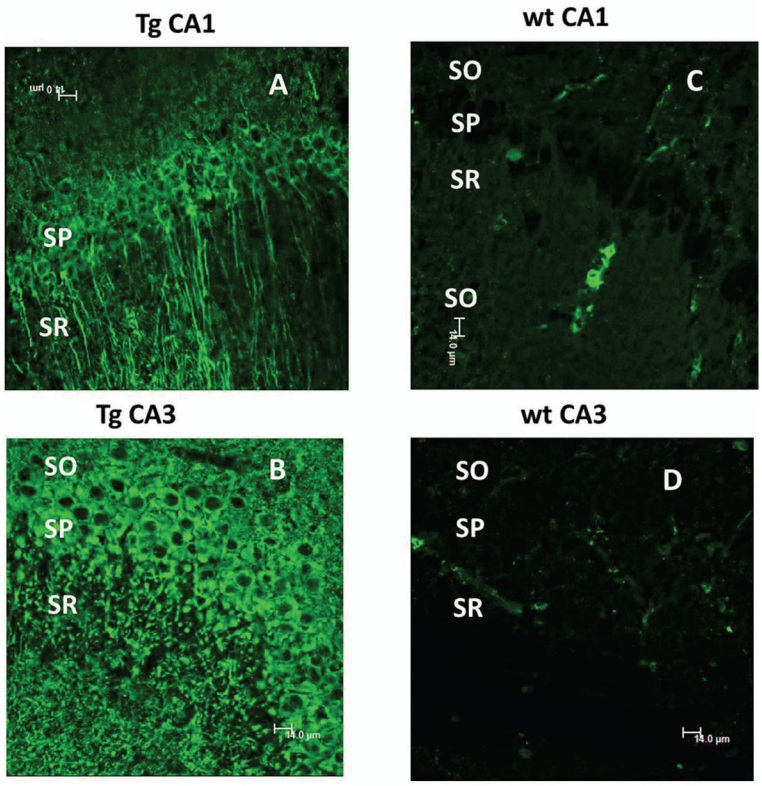

Fig. 5. Immune labeling of MAP2A in the hippocampus of *Glud1* Tg and wt mice ipsilateral to the side of MCAO

Representative immunofluorescent images showing labeling of MAP2A in the ipsilateral CA1 and CA3 regions of the hippocampus in a pair of 9-mo old *Glud1* Tg and wt mice that were subjected to *in vivo* ischemia-reperfusion. All images were obtained using identical laser intensity and fluorescence amplification. SO, SP, and SR, same as in figure 3. Scale bar =14 μm.

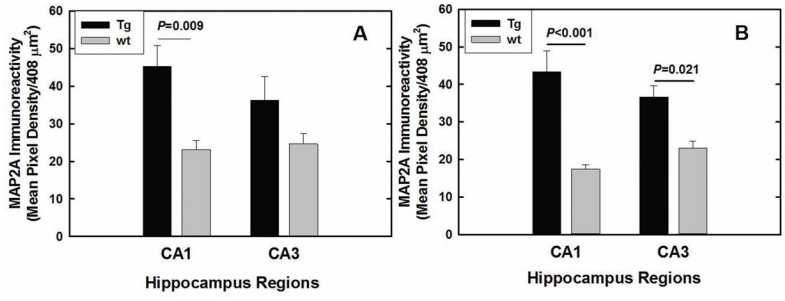

Figure 6. Quantification of MAP2A immunoreactivity in CA1 and CA3 regions of the hippocampus on the ipsilateral (A) and contralateral (B) sides to MCAO in Tg and wt mice

The mean pixel densities (±SEM) of MAP2A immune labeling in the CA1 and CA3 regions represent the average of measurements obtained from multiple subfields from the three layers of each region of the hippocampus, ipsilateral (A) or contralateral (B) to MCAO of 3 Tg and 3 wt mice. The data were analyzed by one-way ANOVA with *post hoc* analysis of pairwise comparisons (Holm-Sidak) and the significant differences between measurements are indicated.

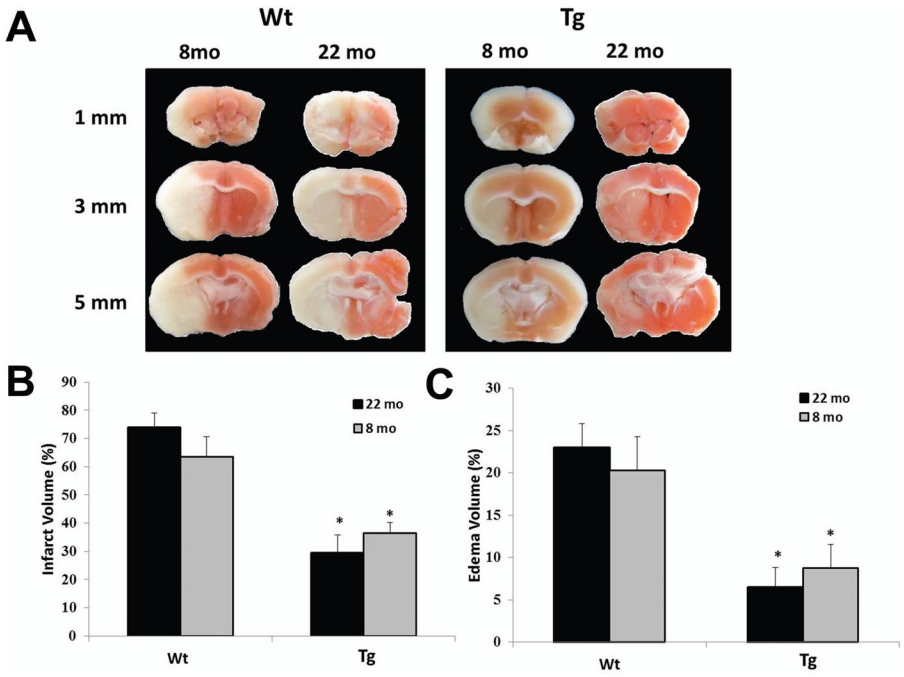

Figure 7. Effect of MCAO on brain tissue damage in 22 month old Wt and *Glud1* Tg mice

Brain damage was determined by TTC staining in 8-mo and 22-mo old Wt and *Glud1* Tg mice after 90 min ischemia followed by 24 hr reperfusion. (A) Representative TTC staining of brain coronal sections proceeding from frontal to caudal. Sections were taken from the 1 mM position of the frontal pole and proceeded in 2 mm intervals to 5 mm. (B) Quantification of infarct volume determined by TTC stained sections. (C) Quantification of brain edema volume estimated from TTC stained sections (n=3). Data presented as means ± SEM. *$p < 0.05$ Tg *vs*. wt mice.