Increasing O-GlcNAcylation is neuroprotective in young and aged brains after ischemic stroke

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ABSTRACT

Spliced X-box binding protein-1 (XBP1s) together with the hexosamine biosynthetic pathway (HBP) and O-GlcNAcylation forms the XBP1s/HBP/O-GlcNAc axis. Our previous studies have provided evidence that activation of this axis is neuroprotective after ischemic stroke and critically, ischemia-induced O-GlcNAcylation is impaired in the aged brain. However, the XBP1s' neuroprotective role and its link to O-GlcNAcylation in stroke, as well as the therapeutic potential of targeting this axis, have not been well established. Moreover, the mechanisms underlying this age-related impairment of O-GlcNAcylation induction after brain ischemia remain completely unknown. In this study, using transient ischemic stroke models, we first demonstrated that neuron-specific overexpression of Xbp1s improved outcome, and pharmacologically boosting O-GlcNAcylation with thiamet-G reversed worse outcome observed in neuron-specific Xbp1 knockout mice. We further showed that thiamet-G treatment improved long-term functional recovery in both young and aged animals after transient ischemic stroke. Mechanistically, using an analytic approach developed here, we discovered that availability of UDP-GlcNAc was compromised in the aged brain, which may constitute a novel mechanism responsible for the impaired O-GlcNAcylation activation in the aged brain after ischemia. Finally, based on this new mechanistic finding, we evaluated and confirmed the therapeutic effects of glucosamine treatment in young and aged animals using both transient and permanent stroke models. Our data together support that increasing O-GlcNAcylation is a promising strategy in stroke therapy.

1. Introduction

Activation of cellular pro-survival pathways is central to the brain’s resilience to stress conditions. However, the protective potency of these pathways declines with age (Lopez-Otin et al., 2013; Wang and Yang, 2019; Yang and Paschen, 2017). This decline critically contributes to worse recovery of neurologic functions in the aged brain after stroke (Wang and Yang, 2019; Yang and Paschen, 2017). Therefore, it is conceivable that a promising strategy to improve stroke outcome in elderly patients is to boost these pro-survival pathways, such as the unfolded protein response (UPR) (Wang and Yang, 2019; Yang and Paschen, 2017; Jiang et al., 2017; Yang and Paschen, 2016).

The main function of the UPR is to preserve and restore endoplasmic reticulum (ER) homeostasis. The UPR is activated primarily under pathologic conditions when unfolded/misfolded proteins accumulate in the ER and cause ER stress (Yang and Paschen, 2016). The most conserved UPR branch is mediated by the ER stress sensor inositol-requiring enzyme-1 (IRE1)(Chen and Brandizzi, 2013). Once activated, IRE1 is converted to an active endonuclease that splices X-box binding protein-1 (Xbp1) mRNA, which triggers a frame-shift of the coding region, leading to protein expression of XBP1s (Chen and Brandizzi, 2013). XBP1s is a transcriptional factor that regulates expression of many ER stress-related genes (Lee et al., 2003). Notably, XBP1s can up-regulate expression of major hexosamine biosynthetic pathway (HBP) enzymes including glutamine:fructose-6-phosphate aminotransferase (GFAT), the rate-limiting enzyme of the HBP (Jiang et al., 2017;
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Animal experiments were approved by the Duke University Animal Care (Jiang et al., 2017). Further, we reported that disrupting this axis in previous data indicate that this axis is activated after ischemic stroke (Table S1). outcome experiments is summarized in Supplementary Fig. S2 and formation for the experimental design and animals used in all stroke infarct volume (short-term) or neurologic score (long-term), which was used to determine the group size for each experiment based on our previous studies or pilot experiments. All stroke outcome data have been subjected to Shapiro-Wilk tests to assess normality. Most of the data were normally distributed and thus were analyzed with parametric tests, but when data did not exhibit a normal/Gaussian distribution, non-parametric tests were applied. Statistical analysis was assessed by unpaired Student’s t-test or Mann-Whitney U test. To compare more than 2 groups, one-way ANOVA with post hoc Holm-Sidak correction for multiple comparisons was performed. Data are presented as mean ± SD or the median (neurologic score). Univariate scatterplots were used to present all stroke outcome data. The level of significance was set at p < 0.05.

3. Results

Acute stroke outcome was improved in mice with neuron-specific overexpression of Xbp1s in the brain.

Ischemic stroke activates the IRE1/XBP1 pathway (Jiang et al., 2017; Yu et al., 2017). We previously reported that neuron-specific deletion of Xbp1 in Xbp1-cKO mice worsens stroke outcome, providing the first indirect evidence suggesting that stroke-activated XBP1 pathway is neuroprotective (Jiang et al., 2017). In the current study, we aimed to directly test whether boosting the activation of the IRE1/XBP1 pathway, which can be achieved by overexpressing the effector XBP1s of this pathway, protects the brain from stroke. Thus, we generated transgenic XBP1s-TG mice in which expression of functional XBP1s was controlled spatially by the neuron-specific Camk2a promoter and temporally by the Tet-off system. To ensure identical baseline conditions, both control and XBP1s-TG mice were maintained with drinking water containing doxycycline until experiments. Seven days before surgery, mice were switched to regular drinking water to activate expression of the Xbp1s transgene in the XBP1s-TG mice. Mice then underwent 45 min filament MCAO. On day 1 after stroke, XBP1s-TG vs. control mice exhibited significantly better neurologic scores and smaller infarct volumes (Fig. 1). These data, together with the worse stroke outcome previously observed in Xbp1-cKO mice (Jiang et al., 2017), clearly establish that...
activation of the XBP1 pathway is an endogenous protective response during the acute ischemic stroke phase.

Worse acute stroke outcome in Xbp1-cKO mice was reversed after pharmacologically boosting O-GlcNAcylation with thiamet-G.

One potential mechanism that underpins the neuroprotective effects of the XBP1 pathway in stroke is O-GlcNAcylation activation via the XBP1s/HBP/O-GlcNAc axis (Supplementary Fig. S1). This notion is based on our findings: stroke activates both the XBP1 pathway and O-GlcNAcylation; post-stroke activation of O-GlcNAcylation is severely impaired when Xbp1 is deleted in neurons; and pharmacologically boosting O-GlcNAcylation or overexpressing Xbp1s (Fig. 1) improves stroke outcome (Jiang et al., 2017). However, one missing link in this reasoning is that we still do not know whether increasing O-GlcNAcylation can rescue the worse stroke outcome observed in Xbp1-cKO mice (Jiang et al., 2017). To address this gap, Xbp1-cKO mice were treated with vehicle or thiamet-G, and 16 h later, were subjected to 30 min filament MCAO. On day 3 post stroke, functional deficits and infarct volumes were evaluated. Indeed, compared to vehicle-treated Xbp1-cKO mice, thiamet-G-treated Xbp1-cKO mice exhibited significant improvement in neurologic scoring, the adhesive tape removal test, and the pole test (Fig. 2A–C). Moreover, stroke infarct volumes were significantly reduced in the thiamet-G group (Fig. 2D). These data together support our proposed mechanism that the beneficial effects of the XBP1 pathway in stroke critically involve an increase in O-GlcNAcylation by activating the XBP1s/HBP/O-GlcNAc axis.

Long-term beneficial effects were observed in young and aged animals treated with thiamet-G after stroke.

Having clarified the significance of the XBP1s/HBP/O-GlcNAc axis in acute stroke outcome, we set out to determine whether activating this axis, more specifically increasing O-GlcNAcylation, during the acute/subacute phase after stroke confers beneficial effects on long-term neurologic outcome, a readout of high clinical relevance. Thus, we subjected young C57BL/6 mice to 30 min filament MCAO. Thiamet-G or vehicle was dosed at 1 h after reperfusion and then daily for a week. Four weeks later, mice were evaluated for neurologic deficits. Although this experiment suffered a high mortality rate (Supplementary Table S1), we were still able to find that thiamet-G-treated mice performed significantly better on the tape removal test, pole test, and neurologic scoring (Supplementary Fig. S3). Motivated by this promising finding, we decided to perform another long-term outcome experiment with a consideration of age and animal species, using our transcranial MCAO model, which produces moderate brain damage and exhibits an excellent long-term survival rate. Taking into account many physiological differences between two species mouse and rat, and the STAIR recommendations (Fisher et al., 2009), we set out to verify the neuroprotective effect of thiamet-G treatment in aged rats. In this experiment, we subjected aged rats to 1 h of transcranial MCAO followed by 4-week evaluation. Thiamet-G or vehicle was dosed 1 h after reperfusion and then daily for 3 days. Compared to vehicle-treated aged rats, thiamet-G-treated rats performed significantly better on the tape removal test (Fig. 3A) and exhibited better neurologic scores (Fig. 3B). Collectively, the results demonstrated that boosting O-GlcNAcylation conferred long-term beneficial effects in young and aged animals after ischemic stroke.

Aged brains showed reduced UDP-GlcNAc availability under physiologic and ischemic conditions, compared to young brains. The data above, together with our previous study (Jiang et al., 2017), demonstrate that pharmacologically increasing O-GlcNAcylation improves both short- and long-term stroke outcome in aged animals. Notably, we also showed that ischemia-activated induction of O-GlcNAcylation is impaired in the aged brain (Jiang et al., 2017; Shen et al., 2018; Liu et al., 2016). Therefore, in order to better understand
the therapeutic potential of targeting O-GlcNAcylation in brain ischemia/stroke, it is important to clarify the mechanisms that underpin this critical impairment. We could envision 3 scenarios that may account for this impairment: 1) an aging-related decline in ability to activate the XBP1 pathway upon ischemic stress, 2) an aging-related decline in the activity of the XBP1s/HBP/O-GlcNAc signal transduction, and 3) an aging-related impairment in the final conjugation steps of O-GlcNAcylation. However, the first scenario is unlikely because our data indicated that the extent of the increase in Xbp1s mRNA after brain ischemia is similar between young and aged brains (Liu et al., 2016).

To examine the second scenario, we had to ensure that the expression levels of XBP1s are similar in young and aged brains in order to compare their O-GlcNAcylation levels. To this end, a viral approach was used. We first constructed an AAV-XBP1s vector with GFP as indicator (Fig. 4A). To facilitate quantification of downstream O-GlcNAcylation levels by Western blot, hippocampus, a well-defined brain structure that can be reproducibly transduced, was chosen for viral injection. High-titer AAV-XBP1s viral particles were stereotaxically injected into the hippocampus regions of young and aged mouse brains. The immunofluorescence staining data confirmed hippocampal neuronal transduction with strong expression of Xbp1s transgene (Fig. 4B). Consistent with that the XBP1s/HBP/O-GlcNAc axis functions in the brain (Jiang et al., 2017), overexpression of Xbp1s significantly increased GFAT1 and O-GlcNAcylation levels (Fig. 4C). However, this increase was similar between the groups, suggesting that in the healthy brain, the XBP1s/HBP/O-GlcNAc signaling transduction is not impaired with age.

Lastly, the final O-GlcNAcylation step is tightly controlled by the availability of UDP-GlcNAc (Yang and Qian, 2017). Therefore, we speculated that UDP-GlcNAc production may be impaired in the aged brain. To test this, a LC-MS/MS-based method of UDP-GlcNAc measurement was developed. Representative LC-MS/MS chromatograms and calibration curve plot are shown in Fig. 5. To evaluate this method, we first analyzed brain samples from Xbp1c-KO and XBP1s-TG mice. As expected, compared to wild-type mice, UDP-GlcNAc levels were reduced by 27.3% ($p < 0.001$) in Xbp1-cKO mouse brains but increased by 26.3% ($p < 0.001$) in XBP1s-TG mouse brains (Fig. 6A). This result not only validated our method, but also provided the direct evidence of the coupling between the XBP1 pathway and the HBP (Wang et al., 2014). More interestingly, aged brains had about 89.0% ($p = 0.004$) of UDP-GlcNAc as in young brains, and after brain ischemia, these levels declined even further in aged brains (Fig. 6). Of note, in this experiment, we used a global brain ischemia model, because compared to focal ischemic stroke, more homogeneously affected ischemic brain tissue samples can be collected for analysis. Together, these findings provided the first evidence that reduced availability of UDP-GlcNAc may underpin impaired activation of O-GlcNAcylation in the aged brain after ischemia.

**Glucosamine is a promising neuroprotectant in stroke therapy.**

Our UDP-GlcNAc data above implicated that increasing the UDP-GlcNAc amount could be a direct, effective approach to correct the cause of impairment in post-ischemic O-GlcNAcylation activation in the aged brain. To test this approach, glucosamine is an excellent candidate because glucosamine can be transported into the cells via the glucose transporter system and then be phosphorylated to glucosamine-6-phosphate, which enters the HBP flux and subsequently increases the levels of UDP-GlcNAc (Supplementary Fig. S1)(Gu et al., 2017). Moreover, glucosamine is naturally synthesized in the human body and compelling evidence supports its safety in humans. Thus, glucosamine may represent an immediate translational opportunity here. To support this appealing possibility, we further evaluated the therapeutic potential of glucosamine in stroke. Here, we tested glucosamine in young and aged mice using both transient and permanent stroke models. In the permanent stroke experiment, mice were subjected to photothermoblastic stroke, and 30 min later, the first dose of glucosamine or vehicle was administered (Fig. 7). Compared to vehicle treatment, glucosamine significantly improved performance of mice in 2 sensitive behavioral tests on day 3 after stroke: cylinder test and foot fault test (Fig. 7B,C). Infarct volumes appeared to be smaller in treated mice, but this comparison did not reach statistical significance (Fig. 7D). Next, we evaluated glucosamine in aged mice after transient MCAO. One dose of glucosamine treatment at 30 min after reperfusion significantly improved functional performance of mice in pole test and neurologic scoring on day 3 after stroke (Fig. 8A,B). Moreover, mice treated with glucosamine exhibited significantly smaller infarct volumes, compared to vehicle-treated mice (Fig. 8C). Thus, our data, together with published evidence from other 2 independent groups (Gu et al., 2017; Hwang et al., 2010), strongly support that glucosamine is a promising neuroprotectant in stroke therapy.

**4. Discussion**

The XBP1s/HBP/O-GlcNAc axis has emerged as a pivotal pro-survival pathway under ischemic conditions, including brain ischemia (Jiang et al., 2017; Wang et al., 2014; Shen et al., 2018; Liu et al., 2016; Gu et al., 2017). Building on previous work, we here further provided critical missing evidence to support that the XBP1 pathway is coupled with O-GlcNAcylation to provide acute protection in stroke. Our data then showed that intervention with thiamet-G to increase O-GlcNAcylation in the brain can improve long-term functional outcome not only in young mice, but also in aged rats after stroke. Notably, we discovered...
that compared to young brains, there was reduced UDP-GlcNAc availability in aged brains, which constitutes a potentially key mechanism responsible for impaired O-GlcNAcylation activation in the aged brain after ischemia (Jiang et al., 2017; Shen et al., 2018; Liu et al., 2016). Finally, our data support that glucosamine is a potent neuroprotectant in stroke. Taken together, increasing O-GlcNAcylation is a promising therapeutic strategy for stroke therapy.

Many lines of evidence indicate that O-GlcNAcylation is a pro-survival pathway. For example, O-GlcNAcylation is activated in cells exposed to a variety of stress conditions including glucose deprivation, ER stress, and oxidative stress. Under these stress conditions, increasing O-GlcNAcylation levels promotes cell survival, while decreasing its levels adversely affects cell survival (Wang et al., 2014; Zachara et al., 2004; Zou et al., 2012; Jones et al., 2008). Thus, activation of O-GlcNAcylation appears to be an endogenous defense response in the cell. Further, mounting evidence demonstrates that increasing O-GlcNAcylation protects organs under ischemic stress (Wang et al., 2014; Liu et al., 2007; Champattanachai et al., 2007). For example, acutely increasing O-GlcNAcylation by treating the heart with an OGA inhibitor, either before ischemia or during reperfusion, improves functional recovery (Liu et al., 2007; Champattanachai et al., 2007). We have demonstrated that O-GlcNAcylation levels in the brain are increased after ischemia, which is partly dependent on the XBP1s/HBP/O-GlcNAc axis, and this increase is neuroprotective in stroke (Jiang et al., 2017). Here, we provided further supportive evidence by showing that compared to control mice, Xbp1s-TG mice had a better stroke outcome, and that thiamet-G treatment reversed the worse stroke outcome observed in Xbp1-cKO mice.

It remains to be determined how increased global O-GlcNAcylation offers neuroprotection in stroke. Since many proteins are believed to be targeted by O-GlcNAc modification, the protective effect is conceivably the net outcome of O-GlcNAcylated proteins regulated by brain ischemia. Although systematic profiling of protein O-GlcNAcylation in post-ischemic brain has not been performed yet, previous studies on functional analysis of O-GlcNAcylation of individual proteins may help us to understand the neuroprotective role of O-GlcNAcylation in stroke.

Fig. 4. A similar increase in O-GlcNAcylation was observed in young and aged mouse brains overexpressing Xbp1s. (A) Scheme of the AAV-XBP1s vector. The vector mainly consists of inverted terminal repeats (ITR) at both ends, the EF1α promoter, HA-tagged XBP1s linked with fluorescent protein GFP using the T2A sequence, and a polyA (pA) sequence. (B–C) AAV8-XBP1s was stereotaxically injected into one hippocampus in each mouse. Mice were then subjected to a 3-week recovery before analysis. (B) Verification of viral Xbp1s gene delivery to the hippocampus in the brain. Coronal sections (−2.0 mm AP from bregma) of the fixed brain were used for immunostaining with XBP1s. Images confirmed expression of viral XBP1s (purple), the co-localization of XBP1s staining with GFP signals (native; green), and an increase in O-GlcNAc signal (red) in transduced cells. (C) Viral Xbp1s expression-induced O-GlcNAcylation in the hippocampus. Hippocampus samples were collected from both contralateral (as controls) and virus-injected sides (AAV) for Western blot analysis. Intensities were measured and normalized to β-actin. The mean values from 4 control (2 young and 2 aged) samples were set to 1.0. Data are presented as mean ± SD (n = 3–4/group). *, p < 0.05, compared to control; ns, not significant.
Data indicate that isoflurane induces VDAC O-GlcNAcylation, which target, voltage-dependent anion channel (VDAC), is critically involved in stroke (Hirose et al., 2011). Interestingly, tau and eNOS are O-GlcNAcylation targets, and increasing their O-GlcNAcylation inhibits the mPTP opening and confers protection to ischemia-reperfusion stress (Cui et al., 2009). Another O-GlcNAcylation (He et al., 2020), and it has been shown that eNOS knockout mice have worse stroke outcome (Chatham et al., 2020). For example, glucose deprivation increases O-GlcNAcylation and activity of endothelial nitric oxide synthase (eNOS) (He et al., 2020), and it has been shown that eNOS knockout mice have worse stroke outcome (Chatham et al., 2020). Another O-GlcNAcylation target, voltage-dependent anion channel (VDAC), is critically involved in the mitochondrial permeability transition pore (mPTP) formation. Data indicate that isoflurane induces VDAC O-GlcNAcylation, which inhibits the mPTP opening and confers protection to ischemia-reperfusion stress (Hirose et al., 2011). Interestingly, tau and α-synuclein are O-GlcNAcylation targets, and increasing their O-GlcNAcylation prevents formation of aggregates (Yuzwa et al., 2012; Marotta et al., 2012), suggesting a role of O-GlcNAcylation in maintaining cellular protein homeostasis. Clearly, identifying the O-GlcNAcylated proteome regulated by stroke will provide in-depth insights on the molecular mechanisms underlying the beneficial effects observed here. In this regard, further research may benefit from recent technical advances on glycoproteomics (Xu et al., 2020; Xiao et al., 2018).

One notable technical contribution of this work is the development and validation of a LC-MS/MS-based method for directly measuring UDP-GlcNAc in the brain tissue. The novelty of this method lies in the use of Trizol reagent to prepare samples for downstream analysis. At first, our attempts to adopt a published method (Lopez-Gutierrez et al., 2017) did not result in optimal chromatographic separation, likely due to insufficient pre-cleaning of the complex brain tissue matrix. Inspired by a one-step protocol to generate all RNA, DNA, and protein using Trizol reagent, we postulated that this reagent may be capable of enriching UDP-GlcNAc while eliminating most of other highly present components such as DNA, proteins and lipids. In line with this thinking, the brain samples from the aqueous supernatant exhibited a strong and clear signal of UDP-GlcNAc. Since XBP1s is expected to increase UDP-GlcNAc production by upregulating HBP enzymes (Jiang et al., 2017), we would assume that overexpression of Xbp1s in XBP1s-TG mice increases the levels of UDP-GlcNAc, while deletion of Xbp1 in Xbp1-cKO mice decreases its levels in the brain. Indeed, the LC-MS/MS data agreed with our expectation, thus supporting the validity of the analytical method. This new assay is expected to be a valuable tool for dissecting the mechanisms responsible for changes in O-GlcNAc modification observed in brain disorders, such as neurodegenerative diseases (Ma et al., 2017).

Using this method, we discovered that compared to the young brain, the aged brain had lower baseline levels of UDP-GlcNAc, and that after ischemia, UDP-GlcNAc levels decreased further in the aged brain. Of note, the result that UDP-GlcNAc levels were not significantly increased in the ischemic brain of young mice seemed inconsistent to our finding of post-ischemic activation of the XBP1s/HBP/O-GlcNAc axis in the brain. Although the exact reason for this is currently unknown, one possible explanation is that the increase in O-GlcNAcylation after ischemia uses almost all the newly-produced substrate UDP-GlcNAc, which results in overall unchanged levels of UDP-GlcNAc. This explanation may also be applied to our findings in aged mice where UDP-GlcNAc levels were decreased after ischemia. After ischemia, due to impaired production of the substrate, the brain uses existing UDP-GlcNAc to maintain baseline or for a modest increase in O-GlcNAcylation may also be applied to our findings in aged mice where UDP-GlcNAc levels were decreased after ischemia. After ischemia, due to impaired production of the substrate, the brain uses existing UDP-GlcNAc to maintain baseline or for a modest increase in O-GlcNAcylation.

Clarification of this critical question warrants further research. Our finding that there is reduced availability of UDP-GlcNAc in the aged brain provided a novel mechanistic insight for worse stroke outcome in aged animals. Although the mechanism underlying this reduction warrants further research, the finding implicates that increasing UDP-GlcNAc could be a promising neuroprotective approach in stroke. Based on this, we further tested glucosamine treatment in stroke, and indeed observed its beneficial effects in young and aged mice after transient and permanent stroke. In 2010, Hwang et al. provided the

Fig. 5. Development of a new LC-MS/MS method for measurement of UDP-GlcNAc in brain tissue. A) Representative chromatograms of UDP-GlcNAc (analyte) and GDP-Glc (internal standard) in a mouse brain sample. B) Chromatograms of UDP-GlcNAc at lower limit of quantification (LLOQ = 10.2 ng/ml), demonstrating sufficient signal-to-noise ratio (S/N > 10). C) Calibration curve used for quantification of brain samples in the study. A series of diluted UDP-GlcNAc was analyzed alongside study samples as a single LC/MS/MS analytical run. Evident is a linear signal-vs-conc. relationship ($r^2 > 0.999$). Accuracy at LLOQ was > 99%.

Fig. 6. Quantification of UDP-GlcNAc in the brain. Young (2-3-month old) and aged (20-22-month old) were subjected to global brain ischemia or sham operation. After 3 h reperfusion, the whole brains were collected for LC-MS/MS analysis. Xbp1-cKO (cKO; 2-3-month old) and XBP1s-TG (TG; 2-3-month old) mice were used to validate the method. (A) Comparison between control mice. (B) Comparison between sham (S) and ischemia (I) samples. Mean values of the young/sham group were set to 100%. Data are presented as mean ± SD (n = 5). *, p < 0.05; **, p < 0.01, ***, p < 0.001.
first evidence that glucosamine exerts acute neuroprotective effects in a rat model of transient ischemic stroke (Hwang et al., 2010). Either pre-treatment at 1 h before or post-treatment at 30 min after tMCAO with glucosamine significantly reduced infarct volumes. Later, another study showed that glucosamine treatment increases O-GlcNAcylation in the brain and improves long-term (24 days after stroke) recovery of neurologic function after transient MCAO in young mice (Gu et al., 2017). Here, we provided additional evidence to illustrate the neuroprotective effects of glucosamine in a permanent stroke model, and more importantly in aged stroke mice. Collectively, glucosamine as a neuroprotectant in ischemic stroke has been demonstrated in both short- and long-term outcome, both young and aged animals, and both mice and rats. Therefore, it would be appealing to further examine its therapeutic potential in a pre-clinical setting using higher-order species (e.g., nonhuman primates) following the STAIR recommendations (Fisher et al., 2009; Savitz et al., 2019), because this may represent an immediate translational opportunity in stroke therapy, given that glucosamine has been widely used in humans for decades (Anderson et al., 2005). It would be also informative for future studies to analyze current databases to investigate whether there is evidence showing a correlation between glucosamine intake and human stroke outcome. Notably, one clinical study suggests that habitual use of glucosamine is associated with a significantly lower risk of stroke (Ma et al., 2019).

Limitations in the current study are noted. To examine the XBP1s/HBP/O-GlcNAc signaling in the aged brain, we used a viral approach and our data suggested that this signaling transduction was not impaired in the aged brain. However, this interpretation needs to be regarded cautiously because exogenous XBP1s was highly expressed by the viral vectors. Such levels of overexpression may make it difficult to detect modest defects of this signaling pathway in the aged brain. Also, to

Fig. 7. Acute outcome was improved in young mice treated with glucosamine after photothrombotic stroke. Mice were subjected to photothrombotic stroke and 30 min later, the first dose of vehicle or glucosamine (GlcN) was administered. Behavioral tests were performed on day 1 and day 3 for the open field test (A), and on day 3 for both the cylinder test (B), and foot fault test (C). Finally, mouse brains were used for measurement of infarct volumes (D). Data are presented as mean ± SD (n = 6-7/group). *, p < 0.05; ns, not significant.

Fig. 8. Acute outcome was improved in aged mice treated with glucosamine after transient stroke. Aged mice were subjected to 30 min MCAO and 30 min later, mice were dosed with vehicle or glucosamine (GlcN). Pole test (A) and neurologic scoring (B) were performed on day 3 after stroke. After behavioral tests, infarct volumes (C) were measured. Horizontal bars represent median value of neurologic scores. Data are presented as mean ± SD (n = 7-8/group). *, p < 0.05.
measure UDP-GlcNAc in the ischemic brain, we used a transient global brain ischemia model rather than a stroke model in order to minimize the effects of variations in infarct on UDP-GlcNAc measurements. Finally, the current work was focused on one downstream process (ie, O-GlcNAcylation) of the XBP1 pathway because we have shown previously that several HBP enzymes are up-regulated in XBP1s-TG mice brains (Jiang et al., 2017). However, XBP1s, as a transcriptional factor, modulates many genes and associated pathways (Lee et al., 2003). To fully appreciate the protective effects of the XBP1 pathway in brain disorders, future research may be directed to identify XBP1s-regulated genes in the brain using current transcriptome analysis approaches. Stroke primarily impacts the elderly. As we proposed previously, a promising therapeutic approach in stroke is to target pro-survival pathways that are critical to promoting cell survival after stroke, but that, during aging, become functionally compromised (Yang and Paschen, 2017). Our data presented here further support this notion.

Authors’ contributions
Conceived and supervised the study and wrote the manuscript: UH, DSW, WP, WY. Designed and performed the experiments: ZW, XL, IS, LL, YS, XQ, HS, WP, WY. Analyzed the data: ZW, XL, IS, UH, DSW, HS, WP, WY.

Declaration of Competing Interest
None declared.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2021.113646.

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