

NEUROPROTECTIVE EFFECT OF ZNT3 KNOCKOUT ON SUBARACHNOID HEMORRHAGE

Abstract

Background: The pathophysiology of early brain injury (EBI) after subarachnoid hemorrhage (SAH) is poorly understood. The present study evaluates the influence of zinc transporter 3 (ZnT3) knockout and the depletion of vesicular zinc on EBI.

Methodology: SAH was induced in ZnT3 KO mice by internal carotid artery perforation. The changes in behavior were recorded at 24 hours after SAH. Hematoxylin-eosin, Nissl and TUNEL staining were performed to evaluate neuronal apoptosis. Data from mice with a score of 8-12 in intracerebral bleeding (i.e. moderate SAH), were analyzed.

Results: The degree of SAH-induced neuronal injury was directly correlated to the amount of blood lost, which in turn was negatively reflected in their behavior. The Wild Type (WT)-SAH group behaved poorly when compared to the knockout (KO)-SAH mice and their poor neurological score was accompanied by an increase in the number of apoptotic neurons. Conversely, the improvement of behavior in the KO-SAH group was associated with a marked reduction in apoptotic neurons.

Conclusions: These results suggest that ZnT3 knockout may have played a vital role in the attenuation of neuronal injury after SAH and that ZnT3 may prove to be a potential therapeutic target for neuroprotection in EBI.

Keywords

• Subarachnoid hemorrhage • Early brain injury • Zinc • ZnT3 knock out • Neuroprotection

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Introduction


Subarachnoid hemorrhage (SAH) is a severely debilitating condition where internal bleeding in the subarachnoid space exerts pressure on the brain causing tissue injury. Although SAH accounts for only 5% of brain stroke, the mortality is as high as 27.3%. [1-3] SAH is associated with vasoconstriction of cerebral arteries, and secondary complications leading to early brain injury (EBI), the primary cause of mortality in SAH. EBI encompasses the immediate injury to the brain as whole within the first 72 hours of the ictus, secondary to SAH. [4,5] One of the early brain injuries is the alteration of blood-brain barrier permeability after SAH. [4,6] Multiple factors have been found to be involved in the complex pathophysiology of EBI, such as delayed neuronal injury/death, impairment of neurovascular coupling, oxidative stress and inflammatory destruction of the parenchyma, and ischemic deficits

leading to cortical spreading depression. [1,7] Delayed cerebral vasospasm after SAH has been reported as a major factor in predicting a poor prognosis of SAH. [8] However, it should be noted that cerebral vasospasm (CVS) is different from EBI, and the remission of CVS may not predict the improvement of SAH. The current model of SAH mimics EBI, where the delayed neurological deficit (DIND) and neuronal death, the associated sequelae, are regarded as the markers for EBI.

Early brain injury was proposed to be a major factor influencing the prognosis of SAH, [4] where the severity of EBI shows a positive correlation with the clinical prognosis of SAH. Neurobehavioral changes in SAH patients are mainly attributed to the excitatory neurotoxicity. [5,9] There is ample evidence suggesting the role of excitatory amino acid neurotransmitters, such as N-methyl D-aspartate, in the apoptotic neuronal death that follows SAH. [10] Zinc, an abundant transition element in the mammalian

central nervous system, also plays a pivotal role in ischemic neurodegeneration. Ten percent to 15% of the total zinc is stored in the synaptic vesicles of glutamatergic neurons, [11,12] that are mainly distributed in the hippocampus and cortex. [13] Under physiological conditions, synaptic activation leads to the release of zinc into the synaptic cleft, where it acts as a neuromodulator for NMDA, AMPA, GABA and glycine receptor-mediated transmission. [14,15] Whereas in pathological conditions, such as stroke, cerebral ischemia, and Alzheimer's disease, excessive zinc is released from the synaptic vesicles, which in turn leads to a toxic influx of zinc through the glutamate receptors or calcium channels, resulting in the accumulation of zinc in the degenerating neurons, especially in the hippocampal hilus and CA1, cerebral cortex, thalamus, striatum, and amygdala. [16-18] Extracellular zinc has potent apoptotic activity and its concentration is tightly regulated to maintain homeostasis.

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Zinc transporters play a key role in the distribution of zinc.[19] ZnT3 is a major zinc transporter which is responsible for the transport of zinc into the synaptic vesicles. Furthermore, the vesicular zinc concentrations are sensitive to the amount of ZnT3 present on synaptic vesicle membranes.[18] ZnT3 gene knockout was found to block the aggregation of zinc in the synaptic vesicles of neurons in the hippocampus, but had no influence on the concentration of intracellular and extracellular zinc. With regard to the effect of ZnT3 gene knockout on behavior, the results have been conflicting. There have been reports that animals with ZnT3 gene knockout showed no behavioral abnormalities [18,20], however, it has also been reported that ZnT3 gene knockout mice that are older have cognitive loss, suggesting that the difference in the findings can be attributed to the age of the animals.[21]

The current study was undertaken to evaluate the role of ZnT3 transporter in attenuating EBI-induced neuronal death following SAH. ZnT3 knockout mice were used to establish the endovascular perforation model of SAH, and the neuronal injury and behavioral data were analyzed and reported herein. Our findings revealed the potential of ZnT3 as a novel target for the prevention of EBI-induced delayed neuronal death associated with SAH.

Materials and methods

Animal care, genotyping and grouping

ZnT3^{+/-} mice (Slc30a3^{tm1Rpa}; The Jackson Laboratory, ME, USA) used in this study were kindly provided by Prof. Chi ZH, Department of Pathophysiology, China Medical University. Details regarding the creation of the knockout ZnT3^{+/-} mice can be obtained at <http://jaxmice.jax.org/strain/005064.html>. Mice were given *ad libitum* access to water and food in an environment with 12:12 h light-dark cycle, and were allowed to mate freely. This study was carried out in strict accordance with the recommendations in the guidelines of the Department of Experimental Animals, China Medical University, Liaoning, China. The protocol was approved by the Committee on the Ethics of Animal Experiments of Sheng Jing

Hospital, affiliated to China Medical University.

Tail clippings of 2-month-old mice were collected and genotyped using PCR (Primers, BGI)-gel electrophoresis, as previously described. [18] Mice had a mixed genetic background of 129 and C57/BL6. Only Wild Type ZnT3 (ZnT-3^{+/+}) and ZnT3 knockout mice (ZnT-3^{-/-}) were included in the present study and were grouped into 3 groups, the WT-Con group (Wild Type mice without treatment, n=6); WT-SAH group (Wild Type mice with SAH, n=9); and ZnT3 KO-SAH group (ZnT3 KO mice with SAH, n=9).

Establishment of SAH animal model

In the WT-SAH group and ZnT3 KO-SAH group, the internal carotid artery puncture was used to establish SAH, as described elsewhere. [22-24] Endovascular perforation is described in both rats and mice, and is commonly used to model experimental SAH. Briefly, under general anesthesia with 5% chloral hydrate (0.6 mL/100 g; intraperitoneal) age-matched animals (2-month old) that weighted around 12-14 g were placed in supine position, and a midline incision (7 cm) was made in the neck, and glands were separated. We used chloral hydrate anesthesia instead of an inhalation anesthesia because of the tight regulations for conducting this experiment. Further, there are no reports on its differential effects on hemodynamic parameters in KO mice, as compared to the Wild Type. The external carotid artery was exposed, and its branches were electrocoagulated. The distal end of the external carotid artery was ligated completely, while the proximal end was loosely ligated with a 5-0 suture. A 6-0 nylon suture was inserted in the middle of the external carotid artery with ligation at both ends and was advanced until the carotid bifurcation. The suture was further inserted 2 mm into the internal carotid artery until a breakthrough sense was felt and the artery wall was perforated. The total length of suture in the artery was about 1 cm. The nylon suture was then retracted and the wound closed. The nylon wire is curved and thus its direction may be adjusted to avoid the pterygopalatine artery. The presence of SAH in our experimental mouse model also validated the accurate placement of the wire in the internal carotid artery. Besides, there

is no sense of breakthrough when the wire is inserted into the pterygopalatine artery. In the WT-Con group, the operating procedures were the same, except that the suture was removed before the perforation of the internal carotid artery. This experimental model (endovascular perforation) has been widely used to mimic SAH due to aneurysm rupture,[4,5] however the mortality rate is generally high (30-50%). The surviving animals were allowed to recover in a temperature-controlled facility with pain medications and free access to water and food.

Neurobehavioral evaluation and scoring of blood loss during SAH

At 24 h after surgery, neurobehavioral evaluation was performed and the scores were recorded using the method reported by Garcia et al.[25] After neurological scoring, the mice were sacrificed and brains were harvested quickly. Photographs of the ring of Willis and basilar artery were obtained and the regions for evaluation of blood volume were determined according to the protocol provided by Sugawara and colleagues.[22] Briefly, the basal cistern was divided into 6 segments and each segment was allotted a grade from 0-3 depending on the amount of subarachnoid blood clot in the segment as follows: Grade 0: no subarachnoid blood, 1: minimal subarachnoid blood, 2: moderate blood clot with recognizable arteries, 3: blood clot obliterating all arteries within the segment. The volume of blood collected in the subarachnoid spaces (intracerebral bleeding) was scored and recorded for each mouse. Only those mice with a score of 8-12 were used for further analysis, to avoid the influence of blood volume on the severity of SAH.

Histology

Samples that were harvested for observing blood volume scoring were fixed in 4% paraformaldehyde for 3 to 4 hours. Samples were then transferred to 30% sucrose at 4°C for 24 hours, following which they were placed in pre-labeled base molds filled with frozen tissue matrix (OCT®). Sections of the desired thickness (usually, 10-30 μm) were cut at -20°C using a cryostat (Leica CM 1850, Leica Biosystem, Germany) and mounted on slides. After drying the slides overnight at room

temperature, and quick fixation by immersion in cold acetone (-20°C) for 2 minutes or other suitable fixative (e.g. alcohol, formalin, etc.), the sections were air dried and stained for HE, Nissl staining (Beijing Leagene Biotech, Beijing, China) and TUNEL assay (Roche, USA). The Nissl staining and the TUNEL assay were performed according to the manufacturer's manual. Image analysis was done using Image Pro Plus 5.0. HE staining identifies neurons with pyknotic nuclei and nuclear condensation, suggesting cell damage. Quantification of Nissl bodies and apoptotic cells in the hippocampus CA1 region was performed by using the image analyzer by independent observers with the same objective and cells were identified according to their color and morphology.

Statistical analysis

Variables were represented as means and standard deviations (SDs). Kruskal-Wallis tests were performed to compare the differences among groups, with Mann-Whitney U tests adjusted by the Bonferroni method for pairwise group comparisons. Pearson correlation coefficient was estimated to investigate the linear correlation between SAH score and neurological score. A p value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 statistics software (SPSS Inc, Chicago, IL).

Results

Genotyping of ZnT3 mice

The primer sequence and PCR-gel electrophoresis for genotyping are shown in supplementary Figure 1. Mice were screened according to the instructions for the ZnT3 KO mice developed by the Jackson Laboratory (Bar Harbor, ME, USA). The knockout used in this study was a total knockout and no specific phenotypes were observed in these mice, as confirmed by the Jackson Laboratory and reports elsewhere.[18,20]

Scoring for SAH in terms of intracerebral blood loss

Intracerebral bleeding, as represented by the volume of blood lost, was scored using the grading method described previously.[22] SAH

mainly occurred at the base of the skull. Only those mice with a score of 8-12 were used for further analysis. Results indicate that the mean SAH scores were significantly higher in the WT-SAH and KO-SAH groups as compared to the WT-CON group (9.67 ± 1.34 and 9.73 ± 1.53 vs. 0.0 ± 0.0 , $p < 0.001$) (Figure 1A). Though the loss of blood volume was comparable between the WT-SAH and KO-SAH groups, a significant difference in their behavior was noted.

Neurobehavioral scoring after SAH

At 24 h after SAH, the behavior of mice in the WT-SAH and KO-SAH groups were markedly compromised. The mean neurological scores

were significantly lower in the WT-SAH and KO-SAH groups, in comparison to the WT-CON group (12.33 ± 1.54 and 14.07 ± 1.22 vs. 18.0 ± 0.0 , $p < 0.001$). However, the score in KO-SAH group was significantly higher than in the WT-SAH group, suggesting an improvement in the behavior in the knockout group (14.07 ± 1.22 vs. 12.33 ± 1.54 , $p = 0.003$) (Figure 1B).

Neurobehavioral scoring after 24 hours of surgery showed a significant correlation between intracerebral bleeding and the behavioral abnormalities. Significant changes in mouse behavior were noted with an increase in the volume of blood lost, especially in the WT-SAH group.

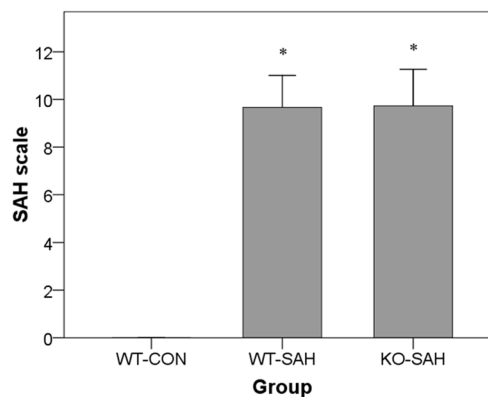


Figure 1A. The SAH scale is used to categorize the severity of SAH depending on the amount of blood lost. The severity of SAH is compared among the various experimental groups. WT-SAH and KO-SAH were significantly more severe in SAH as compared to the WT-CON group.

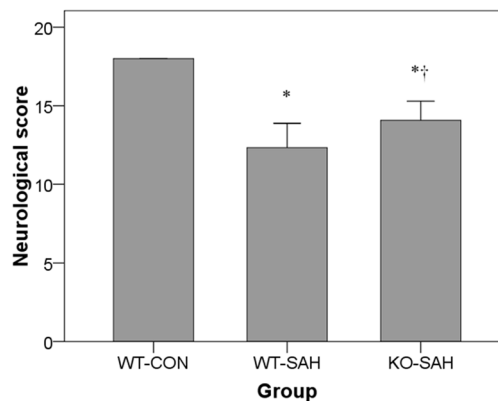


Figure 1B. Comparisons of neurological score among the groups. *, represent significant difference, as compared to the WT-CON group; †, represent significant difference, as compared to the WT-SAH group. (WT-CON group: N = 9; WT-SAH group: N = 15; KO-SAH group: N = 15).

Hematoxylin-Eosin staining

Hematoxylin and eosin (HE) staining was used to illustrate the changes in the morphology of cells in the CA1 region of the hippocampus as previously described.[26] As compared to the WT-SAH group (Figure 2B), apoptotic cells with apparent morphological changes were significantly reduced in the KO-SAH mice (Figure 2C). Apoptotic cells were round and had dark nucleus, concentrated cytoplasm, and mass-like chromatins (Figure 2).

Nissl staining

Differences in Nissl staining in the 3 experimental groups are represented in Figure 3. Under low magnification, Nissl bodies were stained as large blue dyed triangles. After apoptosis, the number of Nissl bodies decreased significantly. The number of Nissl bodies was significantly lower in the WT-SAH and KO-SAH groups (Figure 3B and 3C, respectively), as compared to the WT-CON group (42.25 ± 6.95 and 53 ± 4.32 vs. 71 ± 7.53 , $p \leq 0.029$), as shown in Figure 3A and 3D. Besides, a significant number of cells showed chromatin dissolution, dark nuclear staining and pyknosis in the SAH groups. Granular cells with vacuolar degeneration were also observed in the WT-SAH group (Figure 3B).

TUNEL assay

Images of the cortical brain region of mice in different groups, stained for TUNEL assay, were analyzed using the Image ProPlus, and were compared and presented in Figure 4. Mean number of cells with DNA breaks detected by TUNEL assay was significantly higher in the WT-SAH and KO-SAH groups (Figure 4B and 4C, respectively) than in the WT-CON group (24 ± 3.16 and 18 ± 2.94 vs. 1.25 ± 0.96 , $p \leq 0.029$) (Figure 4A and 4D).

Discussion

The present study is the first to use a ZnT3 knockout mouse SAH model to evaluate the effect of ZnT3 gene knockout on neurobehavior and delayed neuronal death associated with early brain injury. Our results reveal that neurobehavior was significantly compromised in WT-SAH mice, as compared

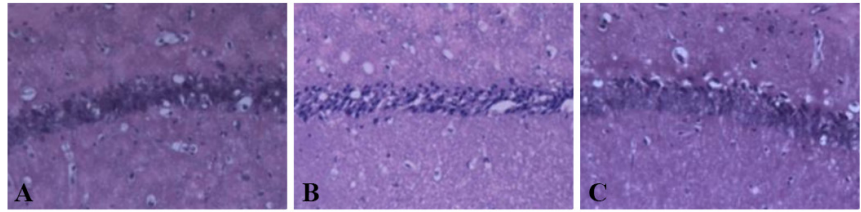


Figure 2. Hematoxylin-eosin staining of the CA1 region in hippocampus of (A) WT-CON; (B) WT-SAH; and (C) KO-SAH group. The HE staining displayed pyknotic nuclei and aggregate of nerve cells, suggesting cell damage. Figure B shows prominent nuclear condensation, the pyknotic nuclei were darker in color, and with a smaller size.

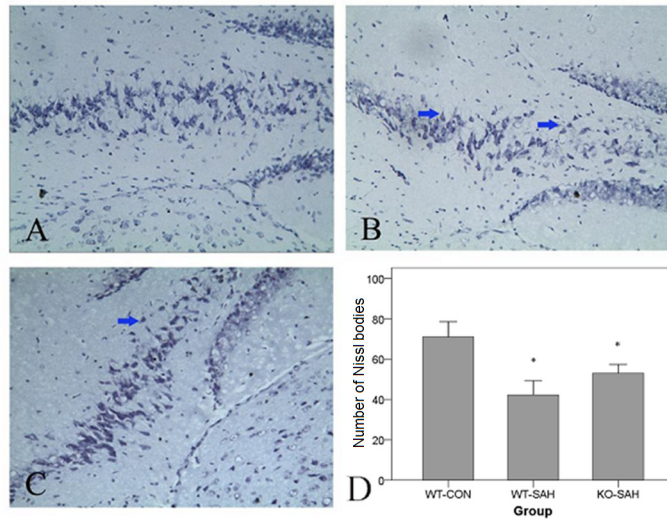


Figure 3. Nissl staining of the CA3 region (A) WT-CON; (B) WT-SAH; and (C) KO-SAH group; (D) comparison of Nissl staining among groups. The number of Nissl bodies decreased significantly after neuronal apoptosis. Blue arrows in Figure 3B and 3C, indicates Nissl bodies. *, represent significant difference, as compared to the WT-CON group. N = 4, in each group.

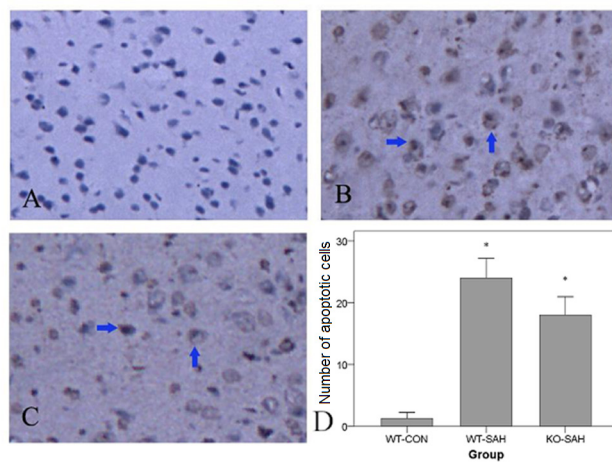


Figure 4. TUNEL assay showed neurons with DNA fragmentation (magnification, 20X). (A) WT-CON; (B) WT-SAH; (C) KO-SAH group; (D) Comparison among the groups. Condensed nuclei and shrunken cells represent the typical apoptotic cell morphology, and were indicated by the blue arrows. *, represent significant difference, as compared to the WT-CON group. †, represent significant difference, as compared to the WT-SAH group. N=4, in each group.

to the WT-control mice. However, the KO-SAH group showed significant improvement in their behavior as compared to their Wild Type counterparts. In addition, the cells in the hippocampus of WT-SAH and KO-SAH mice had fewer number of Nissl bodies, indicating SAH-induced neurodegeneration. These findings suggest that there is neuronal injury in the hippocampus after SAH, which is consistent with our previous findings on EBI after SAH. [27] Improvement in the neurobehavioral score and a significant reduction in the number of apoptotic cells in KO-SAH mice indicate that ZnT3 knockout attenuates the SAH induced injury to hippocampal neurons and may exert neuroprotection. In addition, our data suggest that uncontrolled zinc in the synapses after SAH might be one of the key factors causing EBI. The current model has a high translational value, as the majority of the patients presenting in the emergency care unit had acute SAH where the onset is sudden with severe symptoms. Ameliorating EBI can improve the prognosis of SAH in this group of patients.

Subarachnoid hemorrhage-induced EBI is generally modeled by internal carotid artery puncture, but the caveat of this method is the lack of control of intracerebral bleeding. In the present study, the scoring system described by Sugawara et al., was employed to evaluate the intracerebral bleeding (blood loss) after SAH, which ensures adequate control and normalization of the volume of blood lost.[22] Only those mice with a score of 8-12 were advanced further in studies. Neurobehavioral changes after SAH were evaluated using the method developed by Garcia and colleagues,[25] and the correlation of neurobehavioral score with the number of apoptotic cells was also analyzed. Our current results reveal that the degree of neuronal injury (score of 8-12 in blood volume loss; moderate SAH) was negatively associated with the neurobehavioral score. Indeed, neurobehavioral changes reflect injury to neurons, as previously reported, where the Garcia score was negatively related to the degree of bleeding in SAH rats.[25] The present data further corroborated this finding by Garcia et al.

Increased levels of glutamate and aspartate have been detected after SAH that correlate with the neurological status.[10] Glutamate-induced excitotoxicity leads to neuronal apoptosis, as confirmed by various studies. [28,29] Following synaptic activation and excessive release of glutamate, a large amount of free zinc is released into the synaptic cleft, which is then transported back to the neurons by ZnT3. Excessive Zn translocation and its accumulation in the postsynaptic neurons has been proposed as a molecular trigger of the cellular apoptotic cascade.[30] However, in a more recent study it was found that in rats with SAH there was reduced blood levels of Zn and increased loss of Zn in the urine.[31] It should also be noted that Zn in the injured brain may originate from other sources than synaptic vesicles and be independent of ZnT3. [32]. There is also conflicting evidence on the role of Zn based on a study of mice in which it was found that the ZnT3 knockout group had increased damage after TBI compared with WT control group. [15] There are very few studies on the role of free zinc in EBI secondary to SAH. Increased zinc concentrations have been found in patients with subarachnoid hemorrhage.[33] Zn accumulation is reported to induce selective and delayed degeneration of hippocampal CA1 neurons after focal and transient global ischemia.[11] Indirect evidence on the neuroprotective role of Zn in SAH comes from a recent study where endothelial nitric oxide synthase KO has been shown to decrease Zn release after SAH, thereby preventing neuronal degeneration.[34] The present study demonstrates the advantage of depleted synaptic zinc levels in attenuating the neuronal death during EBI. Though we did not measure the synaptic levels of Zn in the ZnT3 knockout mice, internal studies conducted by Jackson Laboratories confirm that histochemically reactive and immunoreactive synaptic vesicle zinc were undetectable in these mice (please refer to, <http://jaxmice.jax.org/strain/005064.html>). Reports elsewhere also demonstrate a reduction in synaptic Zn-concentration using ZnT3 knockout models.[18]

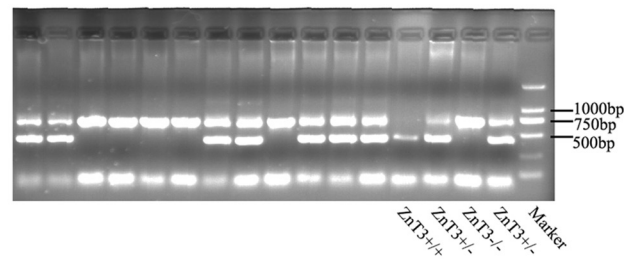
Evidence suggests that zinc chelators, like clioquinol (CQ), may attenuate neuronal

death by reduction of caspase-3 activation. [26] Dp-99 is another promising zinc chelator, which was found to be protective clinically in stroke patients, in addition to its efficacy in animal models.[17] However, a recent report on the role of Zn in chemical hypoxia-induced neuronal death suggests that zinc may play a neurotoxic or neuroprotective role depending on the hypoxic chemical used.[32] Though there is substantial evidence for zinc chelation-induced attenuation of excitotoxic injury, our model provides the first hand knowledge on the protective effect of ZnT3 gene knockout on EBI following SAH. Our current data reveals the correlation of synaptic zinc to neuroprotection and improvement in neurological score in EBI after SAH. Further studies are warranted to validate this preliminary report on the efficacy of zinc transporter gene knockout in early brain injury.

In conclusion, EBI following SAH is associated with the aggregation of free zinc. The synaptic release of Zn is dependent on the vesicular zinc concentration, and plays a key role in the release of excitatory neurotransmitters. Our results indicate that ZnT3 gene knockout prevents excitotoxic neuronal injury and preserves neurobehavior in a mouse model of SAH. This finding might be explained by the synaptic removal of Zn resulting in protective effects on SAH-induced brain injury. Zinc toxicity after SAH appears to be a putative mechanism in SAH-induced EBI, and the relevance of ZnT3 as a potential target for novel therapeutic interventions in SAH should be explored further.

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Supplementary Figure 1. PCR results for the genotyping of ZnT3 WT (+/+), ZnT3 heterozygous (+/-) and ZnT3 KO (-/-) mice.

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