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Bex1 attenuates neuronal apoptosis after intracerebral hemorrhage

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Abstract

Bex1 belongs to a family of small proteins of unknown function, but with several features suggesting roles as adaptors or modulators of intracellular signaling pathways. Previous studies have indicated that Bex1 was present at high levels in several populations of CNS neurons. But its distribution and function in the brain remains unclear. In this study, we explored the roles of Bex1 protein in the pathophysiology of intracerebral hemorrhage (ICH). From the results of western blot, immunohistochemistry, and immunofluorescence, we found an obvious up-regulation of Bex1 in neurons adjacent to the hematoma after ICH. Furthermore, we also found that the increase of Bex1 expression was accompanied by the enhanced expression of Bax and active caspase-3, and decreased expression of Bcl-2 in the pathological process of rat ICH. What's more, our *in vitro* study, using Bex1 RNA interference in PC12 cells, suggested that Bex1 might exert its anti-apoptotic function in neuronal apoptosis. Therefore, Bex1 may play a role in protecting the brain from secondary damage following ICH.

Keywords: ICH; Bex1; neuron; apoptosis, Bcl-2

Introduction

Intracerebral hemorrhage (ICH) is defined as bleeding into the brain parenchyma, which accounts for 10-15 % of total strokes and the percentage is even larger among Asians (1-3). Although ICH is a horrible disease with a high rate of morbidity and mortality, there still lacks effective medical and surgical strategies for ICH treatment (2, 3).

Rupture of blood vessels within brain parenchyma leads to primary and secondary injuries. Hematoma formation, brain edema, and the activation of cytotoxic, oxidative, and inflammatory pathways all induce the brain injury after ICH (4). These parallel pathological pathways lead to neuronal cell death and subsequent neurological impairment; besides, neuronal apoptosis is considered as one of the most crucial events which is divided into extrinsic pathway and intrinsic pathway (5). The extrinsic cell death pathway requires cell surface receptors which bind to their specific ligands and then activate caspase-8 (6). However the intrinsic cell death pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) events, in which B-cell lymphoma 2 (Bcl-2) family members play vital roles. Bax, belonging to the Bcl-2 family, promotes apoptosis by transferring from the cytosol to the mitochondrial outer membrane (MOM) (7). On the contrary, Bcl-2 is an inhibitor of the mitochondrial apoptotic pathway, which prevents the release of cytochrome *c* and caspase activation by blocking the effects of pro-apoptotic proteins (8). Undoubtedly, the increase in the proportion of Bax/Bcl-2 facilitates additional release of cytochrome *c* and cell apoptosis. Besides, caspase-3 is a member of the caspase family which is a key mediator of neuronal programmed cell death and can be usually activated by both pathways (9). Although a large amount of resources have been invested into clinical and basic researches, the prognosis of patients suffering from ICH remains poor (10). Therefore, there is an urgent need to explore the possible mechanism during ICH and then develop the new treatments for ICH.

Bex1 belongs to a small growing family including six members with high homology in gene sequences and structures but distinct in their expression patterns and subcellular localization (11). Until now, the functions of Bex1 have been largely unknown. Bex1 has been identified as a candidate tumor suppressor gene because its inactivation is associated with the development of various types of tumors (12-14). Additionally, Bex1 has been recently proposed to play key roles in the formation of multiple signaling network hubs (15). In particular, Bex1 has been identified as a regulator of neuron

regeneration, as Bex1 knockout mice are deficient in axon regeneration after sciatic-nerve injury (16). In addition, Bex1 levels are cell-cycle dependent in PC12 neuronal cells, with the lowest expression level in G1 phase and the highest level in S phase. Moreover, down-regulation of Bex1 is necessary for the cell cycle exit of neural progenitor cells, as over-expression of Bex1 results in sustained proliferation even under growth-arresting conditions (17).

In this study, we indicated that Bex1 might play a role in the pathological process after ICH. We identified the expression and function of Bex1 in the ICH models *in vivo* and *in vitro*. Our data suggest that Bex1 attenuates neuronal apoptosis after ICH.

Methods and Materials

Animals and the ICH model

Male Sprague-Dawley rats (230-275g) provided by the Department of Animal Center, Medical College of Nantong University were used in this study. They were kept in a temperature-controlled environment (21 °C) on a 12 h light-dark cycle. Rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) positioned in a stereotaxic frame, and a cranial burr hole (1 mm in diameter) was drilled near the right coronal suture 3.5 mm lateral to the midline. Autologous whole blood (50 µl) was collected from its tail tip to a sterile syringe. The sterile syringe was inserted stereotactically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma) (18, 19). The autologous blood was injected at the rate of 10 µl/min. 10 min later, the needle was removed, the skin incision closed, and the animals were allowed to recover. Sham-operated rats only had a needle insertion. Experimental animals (n=3 per time point) were sacrificed to extract the protein for western blot analysis at 6 and 12 h, 1, 2, 3, 5, 7 and 14 day(s) after ICH, respectively. The sham-operated animals were sacrificed on the third day. Additional experimental animals at each time point were killed for pathologic studies. Experiments were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory and approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch. All efforts were made to minimize the number of animals used and suffering.

Behavioral testing procedures

Forelimb placing and corner turn tests were used to assess the neurological deficits induced by ICH.

Forelimb placing test

Forelimb placing test was performed as described (20). The rats were held by torsos, allowing the forelimb to hang free. Testing of each forelimb was performed by brushing the vibrissae on the corner edge of a countertop. Intact rats placed the forelimb quickly onto the countertop. According to the severity of injury, placing of the forelimb contralateral to the injury was impaired. During the experiments, every rat was tested 10 times for each forelimb. The percentage of trials in which the rat placed its left forelimb was calculated.

Corner turn test

Corner turn test was performed as described (19). Rats proceed into a corner, whose angle was 30°. In order to exit the corner, the rats should turn to the left or the right, only the turns involving full rearing along either wall were involved (a total of eight per animal). Injured rats would show a tendency to turn to the side of the injury. The percentage of right turns was used as the corner turn score. After each turn, the rats were not picked up immediately, so that they would not develop an aversion for their prepotent turning response.

Protein extraction and western blot analysis

After injected an overdose of sodium pentobarbital (50 mg/kg), rats were executed at different time points postoperatively, and the brain tissue around the hematoma (extending 2 mm to the incision) as well as an equal part of the normal, sham-operated, and contralateral cortex were dissected out and stored at -80 °C until use. To prepare the lysates, frozen samples were weighed and minced on ice. The samples were then homogenized in lysis buffer (1 % NP-40, 50 mmol/l Tris, pH =7.5, 5 mmol/L EDTA, 1 % SDS, 1 % sodium deoxycholate, 1 % Triton X-100, 1 mmol/l PMSF, 10 µg/ml aprotinin, and 1 µg/ml leupeptin) and centrifuged at 12,000 rpm and 4 °C for 20 min to collect the supernatant (21). After ascertain of its protein concentration with the Brad-ford assay (Bio-Rad), protein samples were sustained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride filter (PVDF) membrane by a transfer apparatus at 250 mA for 2 h. The membranes were blocked with 5 % non-fat milk for 2 h and incubated with primary antibody against Bcl-2 (1:1000; Abcam), active caspase-3 (rabbit, 1:1000, Cell Signaling Technology), GAPDH (rabbit, 1:2000, Abcam), Bcl-2 (mouse, 1:1000, Abcam), and Bax (rabbit, 1:1000, Cell Signaling Technology) at 4 °C overnight. At last, the membrane was incubated with a second antibody for 2 h and visualized using an enhanced chemiluminescence system (Pierce Company, USA) (22).

Immunohistochemistry

Rats were deeply anesthetized and perfused with saline and then 4 % paraformaldehyde through the ascending aorta as the survival times determined. After perfusion, the brains were took away and post-fixed in the same fixative for 3 h and then replaced with 20 % sucrose for 2-3 days, followed by 30 % sucrose for another 2-3 days. Tissues were then cut at 7 μ m with a cryostat. All sections were stored at -20 °C before used. Slide-mounted sections were picked up from the freezer, kept in an oven at 37 °C for 30 min, and rinsed twice in 0.01M PBS for 5 min. The sections were handled with 10 mmol/l citrate buffer (pH=6.0) and heated to 121°C in an autoclave for 3 min to retrieve the antigen. The sections were taken from the pressure cooker and cooled to room temperature (RT) spontaneously. Then, we blocked the sections with confining liquid which including 10 % donkey serum, 1 % BSA, 0.3 % Triton X-100 and 0.15 % Tween-20 for 2 h at RT, then incubated with anti-Bex1 antibody (mouse, 1:300, Abcam) overnight at 4 °C. Following incubation in the secondary antibody at 37 °C, the sections were color-reacted with 0.02 % diaminobenzidine tetrahydrochloride (DAB), 0.1 % phosphate buffer solution (PBS), and 3 % H₂O₂. At last, slides were counterstained with hematoxylin, dehydrated, and coverslipped. Bex1 staining was assessed under a Leica light microscope (Germany). Cells with strong or moderate brown staining were believed as positive; cells with no staining were rated as negative, while cells with weak staining were scored separately.

Double Immunofluorescent Labeling

Additional sets of sections were used for multiple fluorescence staining. After air-dried for 1 h, sections were first blocked with 10 % normal donkey serum blocking solution species the same as secondary antibody, containing 3 % (w/v) bovine serum albumin (BSA), 0.1 % Triton X-100 and 0.05 % Tween 20 for 2 h at RT in order to avoid unspecific staining. The sections were then incubated with primary antibodies against Bex1 (mouse, 1:100; Santa Cruz), NeuN (rabbit, 1:300; Chemicon), glial fibrillary acidic protein (GFAP; rabbit, 1:300; Sigma), CD11b (rabbit, 1:500; Abcam), Active caspase-3 (rabbit, 1:500; Cell Signaling Technology) overnight at 4 °C, followed by a mixture of FITC- and TRITC-conjugated seconary antibodies (1:1000, Life Technology) for 2 h at 4 °C. The stained sections were examined with Leica fluorescence microscope (Germany).

Quantitative Analysis

Cell quantification was performed according to the principles described by Koep *et al* (23). Cells double labeled for Bex1 and phenotypic markers used in the experiment were counted. To identify the proportion of each phenotype-specific marker-positive cell expressing Bex1, a minimum of 200

phenotype-specific marker-positive cells were counted in adjacent to the hematoma in each section.

Three adjacent sections per animal were sampled.

Cell Cultures and Stimulation

Rat pheochromocytoma (PC12) cells were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum and 1 % penicillin/streptomycin at 37 °C under 5 % CO₂ and 95 % oxygen in humidified air. The medium was changed every 2 days. In order to study apoptosis, PC12 cells were seeded onto 60-mm dishes and incubated in a low concentration of serum (1 % horse serum) for 24 h prior to treatment with hemin (100 µmol/L) for different time points.

siRNAs and Transfection

The DNA target sequence for the Bex1 siRNA construct was as follows: 5'-GTTAGGCAGCCCATTTCCTACTATA-3'. For transient transfection, the Bex1 siRNA vector and the nonspecific vector were carried out using lipofectamine 2000 (Invitrogen), and transfected cells were cultured for at least 48 h before use.

Apoptosis analysis

PC12 cells were treated with control or hemin (100 µmol/L) for 12 h and then stained with Annexin V-fluorescein isothiocyanate/PI (BD Biosciences, San Jose; Clontech, Mountain View, CA), followed by quantification of apoptotic cells using FACS Calibur (BD Biosciences).

Statistical Analysis

All data in this paper were analyzed with Stata 8.0 statistical software. All values are expressed as mean ± SEM. The statistical analysis was determined by One-way ANOVA followed by the Tukey's post hoc multiple comparison tests. P < 0.05 was considered statistically significant. Each experiment consisted of at least three replicates per condition.

Results

Bex1 protein expression after ICH

According to previous reports, a series of behavior tests were applied to assess acute and chronic changes in sensorimotor function and plasticity in rat models of unilateral brain injury such as ICH (22). In this study, forelimb placing and corner turn tests were employed to evaluate the neurological deficits

1 at different time points following ICH. As shown in Fig. 1, the ICH group was obviously worse
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3 impaired when compared with the sham-operated group over the first 5 days (*p < 0.05). By 7 days and
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5 thereafter, neurological test scores of the rats went back to baseline.
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7 Next, in order to detect whether Bex1 is involved in ICH, western blot was performed to measure
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9 Bex1 protein expression at various time points. As shown in Fig. 2 , Bex1 level was low in
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11 sham-operated group, progressively increased from 6 h, reached the peak at day 3, and then gradually
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13 returned to baseline after ICH (Fig. 2A, B). These findings revealed that the expression of Bex1
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15 undergoes a substantial alteration after rat ICH.
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18 **Expression and distribution of Bex1 immunoreactivity**

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20 To determine the expression and distribution of Bex1 after ICH, we performed immunohistochemistry
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22 staining 3 days after ICH. The sham group showed a low level of Bex1 staining (Fig. 3A, B), similar to
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24 the profiles in the contralateral side of the experimental brains (Fig. 3C, D). The number of
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26 Bex1-positive cells was observably increased in the brain tissue surrounding the hematoma at day 3
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28 after ICH (Fig. 3E, F, G) and these results were consistent with western blot results.
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31 **Phenotype of Bex1-positive cells**

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33 To further explore the cell types expressing Bex1 following ICH, double Immunofluorescent staining
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35 was performed with different markers: NeuN, GFAP, CD11b, which represent neurons, astrocytes, and
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37 microglia, respectively. Microscopy detected a strongly enhanced co-localization of Bex1 and NeuN
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39 (Fig. 4C, D). However, colocalization was not observed in microglia or astrocytes (Fig. 4E-L). To
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41 identify the proportion of neurons expressing Bex1, at least 200 NeuN-positive cells were counted.
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43 Bex1 expression was markedly increased in neurons (the NeuN-positive cells) after ICH compared
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45 with the sham group, which were consistent with the results of immunohistochemistry staining (Fig.
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47 4O). The expression and distribution of Bex1 in NeuN positive cells indicated that Bex1 might be
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49 associated with the changes of biological function of neurons after ICH.
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51 **Association of Bex1 with Neuronal Apoptosis After ICH**

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53 Neuronal apoptosis is regarded as one of the most crucial events after ICH (24, 25). Our research above
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55 found that the distribution of Bex1 after ICH is appeared in neurons, hence it is reasonable for us to
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57 investigate whether Bex1 is correlated with neuronal apoptosis following ICH. Then, we examined the
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59 expression profiles of active caspase-3, Bax and Bcl-2 by western blot. As shown in the results, the
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1 expression of active caspase-3 and Bax increased after ICH, peaked at day 3 and 2 respectively, which
2 was relevant with the expression of Bex1 in a time-dependent manner (Fig. 5A, C). Meanwhile, the
3 level of Bcl-2 was decreased after ICH, with minimum value at day 2 (Fig. 5C). In addition,
4 immunofluorescent labeling showed that Bex1 co-localized well with active caspase-3 in neurons (Fig.
5 5E).
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10 **Detection change of Bex1 and cellular apoptosis *in vitro***

11 To further explore the role of Bex1 on neuronal apoptosis after ICH, hemin-induced apoptosis model in
12 PC12 cells was built as described. We stimulated PC12 cells with 100 $\mu\text{mol/L}$ hemin at different time
13 points and then measured the protein level of Bex1, active caspase-3, Bax, and Bcl-2. It found that
14 active caspase-3 and Bax were up-regulated, the tendency was consistent with Bex1 after ICH. Bcl-2
15 was reduced, which was opposite with those of Bex1, active caspase-3, and Bax (Fig. 6A, B).
16 Additionally, RNAi specific to Bex1 was employed to knock down Bex1 in PC12 cells (Fig. 7A, B).
17 Down-regulation of Bex1 expression obviously increased the expression of active caspase-3 and Bax,
18 while increased the level of Bcl-2 in the presence of hemin stimulation (Fig. 7C, D). Eventually, to
19 confirm the above views, PC12 cells were treated with hemin (100 μM) for 12 h, and then stained with
20 Annexin V/PI. Bex1 induced a marked reduction in both early (Annexin V⁺/PI⁻) and late (Annexin
21 V⁺/PI⁺) apoptotic cell populations. On the contrary, Bex1 siRNA increases hemin-induced neuronal
22 apoptosis (Fig. 7E). In total, these data indicated that Bex1 might play an anti-apoptotic role on neurons
23 following ICH.
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42 **Discussion**

43 ICH is a fierce and devastating CNS disease with high morbidity and mortality (26). Basal ganglia is
44 one of the most common sites where human ICH often takes place. Despite a tremendous amount of
45 research on the events after ICH, its molecular and cellular mechanisms of damage have not been well
46 understood. Therefore, to better understand about ICH, we established an *in vivo* and *in vitro* ICH to
47 simulate clinical ICH. In the present study, Bex1 was up-regulated surrounding the hematoma after
48 ICH, and the temporal changes were striking in neurons rather than in microglia or astrocytes;
49 meanwhile, there was a concomitant increase of active caspase-3 and Bax, and down-regulation of
50 Bcl-2 *in vivo* and *in vitro* studies. Furthermore, silencing of Bex1 by siRNA in PC12 cells could
51 increase hemin-induced expression of active caspase-3 and Bax, and could attenuate the expression of
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1 Bcl-2. Based on our results, we hypothesized that Bex1 might play an anti-apoptotic role in neurons
2 following ICH.
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4 Bex1 belongs to a family of small proteins of unknown function, but with several features
5 suggesting roles as adaptors or modulators of intracellular signaling pathways. Bex1 is primarily
6 expressed in nerve cells (11). Bex1 is involved in the p75^{NTR}/TrkA/B pathway (17). Currently, five
7 highly homologous members have been found, namely Bex1 (Rex3/NADE4), Bex2 (NADE5), Bex3,
8 Bex4 and Bex5. Bex1 and Bex2 show 87 % homology in their amino acid sequences (27). Bex1 and
9 Bex2 were shown to be present at high levels in rat and mouse retinal-ganglion cells (RGC) in both the
10 soma and the axons (28) and in olfactory-sensory neurons as well as in several populations of CNS
11 neurons (29). Interestingly, it has been shown that expression of Bex1 and Bex2 increases in RGCs
12 after axonal damage resulting from stroke (28). This is in agreement with our results showing that Bex1
13 is up-regulated in neurons of brain after ICH. Except the expression patterns within the CNS, Bex1
14 reveals a high expression level also in peripheral tissues (11). For example, Bex1 is also expressed in
15 the hematopoietic system and in various tumor cells (30-32). A recent study suggested that Bex1
16 functions as a tumor suppressor gene (33). However the definite function of Bex1 in brain neurons
17 remains poorly understood. Based on the above results, we found Bex1 is elevated in ICH, and it
18 attenuates neuronal apoptosis following ICH.
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35 In addition to the primary damage, ICH gives rise to a series of molecular and cellular events. A
36 lot of mechanisms have been implicated in the above processes such as astrocyte proliferation,
37 microglia activation, oligodendrocyte death, and neuronal apoptosis. Among them, neuronal apoptosis
38 is one of the most severe consequences, and it can be divided into extrinsic pathway and intrinsic
39 pathway (34). The former is triggered by cell surface receptors which bind to their specific ligands and
40 then activate caspase-8 (6). The intrinsic one is dependent on mitochondria mediated apoptosis pathway.
41 There is no doubt that the increase in the proportion of Bax/Bcl-2 facilitates release of cytochrome *c*
42 and activation of caspases, and eventually gives rise to cell apoptosis (35, 36). Importantly, caspase-3
43 has been identified as a key mediator of neuronal programmed cell death (37). In this study, the
44 expression profile of active caspase-3 was increased in the ICH model which indicates that neuronal
45 apoptosis occurred. Moreover, double immunofluorescence staining in the rat cerebral cortex showed
46 that active caspase-3 co-localized well with Bex1 in neurons. Simultaneously, our data suggest that
47 Bex1 may represent one of the anti-apoptotic aspects in ICH. Therefore, our results indicated that
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up-regulation of Bex1 might play a negative role in neuronal apoptosis following ICH.

Quantities of former studies provide the evidence of neuronal apoptosis in ICH and suggest a vital role for anti-apoptotic agents in ICH. And the present study for the first time detected the expression and variation of Bex1 surrounding the hematoma; and all the data certificated the protective role of Bex1 in neuronal apoptosis following ICH. However, the protective role of Bex1 through which molecular mechanism for ICH is still unclear. Is it possible that Bex1 can work as an anti-apoptotic effector through regulating the degradation of Bax, active caspase-3, and Bcl-2? The underlying cellular and molecular mechanisms and therapeutic potentials of Bex1 for ICH remain to be elucidated.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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Figure Legends

Fig. 1 Estimations and scores of behavioral tests on rats suffering from ICH. Behavioral tests were executed in rats after ICH or sham operation. Forelimb placing (A) and corner turn testing scores (B) showed that the ICH group exhibited remarkable deficits compared with the sham-operated group over the first 5 days (*p < 0.05, significantly different from the sham-operated group), with no significant

1 difference at baseline or 5 days later.

2 **Fig. 2** Western blot analysis protein level change of Bex1 after ICH. Western blot was performed to
3 study the protein level of Bex1 surrounding the hematoma at various survival times. (A) Time courses
4 of Bex1 expression after ICH, peaked at day 3, and declined thereafter. (B) Quantification graphs
5 (relative optical density) of the intensity of staining of Bex1 to GAPDH at each time point. GAPDH
6 was used to confirm equal amount of protein was run on gel.

7 **Fig. 3** Representative microphotographs for Bex1 immunohistochemistry surrounding the hematoma.
8 Low level of Bex1 was detected in the sham group (A, B). At day 3 after ICH, the contralateral group
9 showed no significant difference in Bex1 (C, D) compared with the sham ones, while the ipsilateral
10 group (E, F) showed increased Bex1 expression. The number of Bex1 positive cells was largely
11 increased comparing the ipsilateral group with the sham and contralateral groups (G). Asterisk denotes
12 $p < 0.05$. Scale bar: left column, 50 μm ; right columns, 20 μm .

13 **Fig. 4** The colocalization of Bex1 with different cellular markers by double immunofluorescent
14 staining. In the adult rat caudate within 2 mm distance from the hematoma at the third day after ICH,
15 horizontal sections were labeled with Bex1 (green, A, E, I), different cell markers (red, B, F, J), such as
16 neuronal marker (NeuN), astrocyte marker (GFAP) and microglia marker (CD11b). The yellow color
17 visualized in the merged images represents the colocalization of Bex1 with different specific phenotype
18 markers (C, G, K). The colocalizations of Bex1 with different specific phenotype markers are also
19 shown in the sham-operated group (D, H, L). No positive signals were found in the negative control (M,
20 N). The number of NeuN-positive cells expressing Bex1 (%) was remarkably increased in the ICH
21 group compared with the sham group (O). Asterisk means $p < 0.05$. Scale bars 20 μm (A-N) .

22 **Fig. 5** Association of Bex1 with cell apoptosis after ICH. The expression of active caspase-3 and Bax
23 increased, peaked respectively at day 3 and 2 following ICH (A). The expression of Bcl-2 decreased
24 after ICH and reached valley at 2 days (C). The bar graphs indicated the relative density of active
25 caspase-3, Bax, and Bcl-2 versus GAPDH at each time point (B, D). Immunofluorescent staining
26 showed co-staining of NeuN (green) and Bex1 (green) with active caspase-3 (red) in rat brain around
27 hematoma (E). Scale bar 20 μm .

28 **Fig. 6** Modulation of Bex1 on cell apoptosis *in vitro*. PC12 cells were incubated with hemin at 100
29 $\mu\text{mol/L}$ for different times. Bex1, active caspase-3, and Bax were up-regulated, peaked at 12 h, while
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1 Bcl-2 had the opposite regulation (A). The bar graph indicated the relative density of Bex1, active
2 caspase-3, Bax, and Bcl-2 versus GAPDH at each time point (B), ^{#, \$, &, ^}p < 0.05, significantly different
3 from the control group.
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7 **Fig. 7** Bex1 protected cellular damage *in vitro* studies. Western blot analysis indicated siRNA silenced
8 Bex1 in PC12 cells (A); the bar chart indicates the density of Bex1 versus GAPDH (B). Knockdown of
9 Bex1 induced increasing levels of active caspase-3 and Bax and decreasing level of Bcl-2 (C); the bar
10 chart indicates the density of active caspase-3, Bax, and Bcl-2 versus GAPDH, respectively (D), ^{*, #, \$, &}p
11 < 0.05, indicated significantly different from the control. PC12 cells were treated with hemin (100 μM)
12 or control for 12 h, followed by flow cytometry assay for apoptosis with Annexin V/PI double staining
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