Research Report

CHMP4B, ESCRT-III associating protein, associated with neuronal apoptosis following intracerebral hemorrhage

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\textbf{A R T I C L E   I N F O}

Article history:
Accepted 20 November 2014
Available online 3 December 2014

Keywords:
Intracerebral hemorrhage
CHMP4B
Apoptosis
Rat
Neuron

\textbf{A B S T R A C T}

Charged multivesicular body protein (CHMP) represents a family of small helical proteins that contain an N-terminal basically charged region and a smaller C-terminal acidic region, which are highly conserved in all eukaryotes. CHMP4B, a core component of the endosomal sorting complex required for transport (ESCRT)-III, is requisite for endosomal sorting and other biological processes. Here, we demonstrate that CHMP4B may be involved in neuronal apoptosis in the processes of intracerebral hemorrhage (ICH). From the results of Western blot, immunohistochemistry and immunofluorescence, we obtained a significant up-regulation of CHMP4B in neurons adjacent to the hematoma following ICH. Increasing CHMP4B level was found to be accompanied by the up-regulation of Fas receptor (Fas), Fas ligand (FasL), active caspase-8, and active caspase-3. Besides, CHMP4B co-localized well with Fas and active caspase-3 in neurons, indicating its potential role in neuronal apoptosis. What’s more, our in vitro study, using CHMP4B RNA interference in PC12 cells, further confirmed that CHMP4B might exert its pro-apoptotic function on neuronal apoptosis through extrinsic pathway. Thus, CHMP4B may play a role in promoting the brain damage following ICH.

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1. Introduction

Intracerebral hemorrhage (ICH) is a devastating stroke subtype resulting from bleeding within the brain parenchyma (Andrews et al., 2012). It has an incidence of 10–30 cases per 100,000 people/year, which is increasing and expected to double by the year 2050 (Qureshi et al., 2001). Evidences during the past decades demonstrate that the fatality rate

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of ICH at 1 month is approximately 40%, which likely results from a lack of effective therapeutic schedule after ICH (Brouwers and Greenberg, 2013; Keep et al., 2012). Though multiple resources have been invested into clinical and basic researches, this high rate of mortality seems still not changed (Kuramatsu et al., 2013). Rupture of blood vessels within the brain parenchyma lead to primary and secondary injuries. The primary damage is induced by the dynamic of hematoma expansion associated with mass effect; the secondary damage comprised of many parallel pathways including cytotoxicity of blood, inflammation, oxidative stress and so on together lead to irreversible injury, eventually cause the disability or even the death (Aronowski and Zhao, 2011; Zhou et al., 2014). Pathologic changes of ICH include neuronal apoptosis, astrocyte proliferation and oligodendrocyte death, which all implicated in the above processes (Bradl and Lassmann, 2010; Gong, 2001). Among them, neuronal death is regarded as one of the most crucial events which depend on complex pro-apoptotic and subtle anti-apoptotic modulation (L. Li et al., 2013). We here have only a sketchy understanding of the underlying molecular and cellular mechanisms of ICH, lots of studies remains to be done.

Endosomal sorting complex required for transport (ESCRTs) and their associated proteins (called CHMPs) are conserved from yeast to humans, and function in the sorting of ubiquitinated cargoes into intraluminal vesicles, which are generated by inward budding of the endosomal membrane (also called multivesicular bodies (MVBs), or endosomes) (Horii et al., 2006; Katoh et al., 2003). On the way toward degradation, ubiquitinated cargoes trigger the sequential formation of protein hetero-oligomeric complexes called ESCRT I, II, and III, which then help sorting their incorporation into vesicles budding inside MVBs (Katoh et al., 2003). During the processes, charged multivesicular body protein CHMP4B is a core subunit of ESCRT-III, and facilitates the biogenesis of MVBs and implicates in playing roles in multivesicular body sorting (Hurley and Emr, 2006). Thereafter, the vesicles and their cargoes will be hydrolyzed after the MVB fusion with the lysosome (Horii et al., 2006; Hurley and Emr, 2006; Katoh et al., 2003).

In majority conditions, CHMP4B is part of the ESCRT-III, and functions in the sorting of endocytosed cell-surface receptors into MVBs, in the final abscission stage of cytokinesis, or in the budding of enveloped viruses such as HIV-1 (Hurley and Hanson, 2010; Raiborg and Stenmark, 2009). However, in pathological conditions, CHMP4B, ESCRT-III associating protein, interact with programmed cell death 6 interacting protein (PDCD6IP), also known as ALG-2 (apoptosis-linked gene 2)-interacting protein X (ALIX), an adaptor protein involved in the regulation of the endolysosomal system, might participate in involvement with autophagy-associated degenerative diseases or cell apoptosis (Mahlul-Mellier et al., 2006, 2009, 2008; Sagona et al., 2014). For example, Sagona et al. (2014) indicated that CHMP4B was a novel common component of chromosome bridges and micronuclei, participating in the autophagolysosomal degradation of micronuclei during lens cell differentiation and cataract formation (Sagona et al., 2014). Mahul-Mellier et al. (2009) demonstrated that overexpression of ALIX is sufficient to induce cell death of motoneurons, whereas it prevented early programmed cell death when mutated the binding sites of CHMP4B (Mahul-Mellier et al., 2009). And the study by Mahul-Mellier et al. (2008) uncovered that ALIX and ALG-2 regulate neuronal death in ways involving interactions with proteins of ESCRT via forming a complex with apical caspases and with the endocytosed death receptor TNFR1 (tumor necrosis factor α receptor 1) in the presence of calcium (Mahul-Mellier et al., 2008). Additionally, there is also evidence indicated that dysfunction of ESCRT-III was associating with frontotemporal dementia linked to chromosome 3 (FTD3), which caused autophagosome accumulation and dendritic retraction before neurodegeneration in cultured mature cortical neurons, whereas its potential relationship with CHMP4B is not clear (Lee and Gao, 2009). As CHMP4B involves in multiple cellular activities, whether it takes part in the pathophysiologic processes following ICH remains to be investigated.

To date, we know little about CHMP4B, especially involving in neuronal death in the central nervous system (CNS). Since CHMP4B is indispensable for participating in cellular death, we hypothesize that CHMP4B may contribute to brain injury following ICH, and associate with neuronal apoptosis via caspase-dependent or -independent manner. In the present study, we for the first time investigated the expression and distribution of CHMP4B in rat basal ganglia adjacent to the hematoma after ICH. This study was performed to gain an insight into the function of CHMP4B in the adult CNS and might make a foundation for further research and be applied to clinical treatment for its role in injury and repair after ICH.

2. Results

2.1. Expression variations of CHMP4B following ICH by western blot analysis

Rats in the sham and ICH groups were subjected to forelimb placing and corner turn test at different survival times, respectively. Rats of ICH with significant neurological function deficits were chosen for subsequent experiments (Fig. 1A, B). Western blot was performed to investigate the temporal levels of CHMP4B in rat basal ganglia adjacent to the hematoma at various time points after ICH. CHMP4B protein level was low in the sham group, then progressively increased from 6 h after ICH and peaked at day 2 (Fig. 1C, D) (*p<0.05). The data indicate that CHMP4B protein profiles had a temporal change after ICH.

2.2. Expression and distribution of CHMP4B immunoreactivity

To identify the variation and distribution of CHMP4B after ICH, immunohistochemistry was applied to detect CHMP4B at 2 days following ICH. The sham-operated group showed low CHMP4B-positive staining (Fig. 2A, B), which consisted the result from Western blot. By contrast, the number of CHMP4B in the ipsilateral group was upregulated adjacent to the hematoma (Fig. 2C, D). However, there is no significant change of CHMP4B staining in the contralateral group compared with the sham group (Fig. 2E, F). Additionally, no positive signal was observed in the negative control (Fig. 2G). The expression of CHMP4B was
assessed by cell counting among the above three groups, and the statistical data indicated that the upregulation of CHMP4B appeared adjacent to the hematoma (Fig. 2H).

2.3. Phenotype of CHMP4B-positive cells

To further identify which kind of cell types may be relevant with the increased expression of CHMP4B after ICH, we used double immunofluorescent microscopy studies on transverse cryosections of brain tissues by labeling CHMP4B (Fig. 3B, F, J, N) with different markers, such as NeuN (Fig. 3A), CNPase (Fig. 3E), GFAP (Fig. 3I), and Iba-1 (Fig. 3M). In the sham-operated group, the staining of CHMP4B was merely restricted to NeuN-positive cells, indicative of neurons (Fig. 3D). Following ICH, intensive CHMP4B staining was observed in neurons (Fig. 3C), while not in CNPase (Fig. 3G, H), GFAP (Fig. 3K, L), or Iba-1-positive cells (Fig. 3O, P), indicative of oligodendrocytes, astrocytes or microglias. No positive signals were detected in the negative control (Fig. 3Q, R).

To calculate the proportion of NeuN-positive cells expressing CHMP4B, a minimum of 200 phenotype-specific marker positive cells were counted between the sham-operated group and the group 2 days following ICH. CHMP4B expression was significantly increased in neurons compared with the sham group (Fig. 3S).

2.4. CHMP4B might be relevant to neuronal apoptosis after ICH

Neuronal apoptosis is regarded as one of the most crucial events after ICH (Wu et al., 2008). Our observation above revealed that the up-regulation and distribution of CHMP4B after ICH appeared in neurons, hence it is reasonable for us to investigate whether CHMP4B is relevant to neuronal apoptosis after rat ICH. Based on this hypothesis, we detected the protein levels of active caspase-3. The expression of active caspase-3 increased after ICH, and peaked at days 2 and 3, which was relevant with the expression profiles of CHMP4B in a time-dependent manner (Fig. 4A). In accordance with the result of Western blot, the immune-activity of active caspase-3 was remarkably increased in the ICH group compared with the sham group (Fig. 4C, G).

The death receptor Fas and its specific ligand (FasL) have gained widespread recognition as an apoptotic mediator. Moreover, CHMP4B participates in neuronal apoptosis might be via caspase-dependence manner in the previous reports (Mahul-Mellier et al., 2009, 2008). We then examined the protein levels of Fas receptor (Fas), Fas ligand (FasL) and active caspase-8. Expectedly, the levels of Fas, FasL and active caspase-8 remarkably increased following ICH, and peaking at days 2 and 3, respectively (Fig. 4B), which were consistent of the temporal profile of CHMP4B expression.
To test the upregulation of CHMP4B, active caspase-3, Fas and FasL whether occurred in neurons, double immunofluorescent staining was employed after ICH adjacent to the hematoma. As shown in Fig. 4D–F, H–J, the majority of neurons was active caspase-3-positive, and the colocalization of active caspase-3 and CHMP4B was also observed at 2 days after ICH. Moreover, Fig. 4K–P shows CHMP4B colocalized well with Fas, but just partly localized to FasL in neurons, indicating that FasL was partly occurred in neurons. A possible explanation would be that FasL was also/or mainly secreted from other cell types, such as astrocytes, macrophages, neutrophils and microglias, which were reported in previous studies (Krzyzowska et al.,

Fig. 2 – Representative microphotographs for CHMP4B immunohistochemistry adjacent to the hematoma. Low level of CHMP4B signal was found in the sham-operated group (A, B). At 2 days after ICH, the ipsilateral group showed increasing CHMP4B signals (C, D), while the contralateral group showed no significant difference in CHMP4B compared to sham-operated group (E, F). (G) No positive signal was detected in the negative control. (H) The number of CHMP4B cells was largely increased comparing the ipsilateral group with the sham-operated and contralateral groups. *p < 0.05 indicated the ipsilateral group was different from the sham-operated and contralateral group with statistical significance. Scale bar left column, 100 μm; right column, 50 μm.
2014; Yu and Fehlings, 2011). Based on above, the results together indicated that CHMP4B might implicate in neuronal apoptosis following ICH.

2.5. Detection change of CHMP4B and cellular apoptosis in vitro

To further investigate the role of CHMP4B on neuronal apoptosis after ICH, we established hemin (heme with a chloride ligand)-induced apoptosis model in PC12 cells in vitro. As hemin is released from hemoglobin accumulates in blood outside vessel, which is thought to play an important role in intracerebral hemorrhage (Levy et al., 2002; Lin et al., 2012; Wang and Dore, 2008). We initially incubated PC12 cells with different concentration of hemin at indicative time points to determine the most appropriate stimulant concentration (25, 50, 75, 100 and 150 μM) to induce apoptosis. From CCK-8 assay, a significant reduction in cell viability was noted 18 h after treatment with 75 μM hemin compared to untreated neurons ($p<0.05$) (Fig. 5A), and at this concentration, cell viability decreased in a time-dependent manner (Fig. 5B). Also, we detected the level of apoptotic marker, active caspase-3, with Western blot and found that active caspase-3 levels increased concomitantly with cell viability loss (Fig. 5C). Based on the in vitro ICH model, we examined the CHMP4B, Fas, Fasl and caspase protein levels. Western blot showed the concomitant upregulation of these proteins expression in a time-dependent way (Fig. 5D). Since Fasl is generally the membrane-associated ligand, soluble Fasl (sFasl) in the conditioned medium was also measured by ELISA. From Fig. 5E, sFasl in conditioned medium of PC12 cells treated with hemin exhibited progressively higher concentrations compared to untreated group. And the concentration values of

Fig. 3 – Double immunofluorescence staining for CHMP4B with different phenotype-specific markers adjacent to the hematoma. Sections were labeled with CHMP4B (red, B, F, J, N), neuronal marker NeuN (green, A), oligodendrocyte marker CNPase (green, E), astrocyte marker GFAP (green, I) and microglia marker Iba-1 (green, M). The yellow color visualized in the merged images represented the colocalization of CHMP4B with different phenotype-specific markers (C, D, G, H, K, L, O, P). No positive signal was found in the negative control (Q, R). The number of NeuN-positive cells expressing CHMP4B (%) was significantly increased in the ICH group compared with the sham-operated group (S). *$p<0.05$. Scale bars 50 μm (A–R).
Fig. 4 – Correlations of CHMP4B with cellular apoptosis following ICH. (A) Western blot analysis showed the expression of active caspase-3 increased, peaking at day 2, following ICH. (B) Western blot analysis showed the protein levels of Fas, FasL, active caspase-8 increased from 6 h after ICH, and peaked at days 2, 3. The bar graph indicated the density of active caspase-3/Fas/ FasL/active caspase-8 versus GAPDH at each time point. Data are presented as mean ± SEM (*, #, \( \hat{p} \) < 0.05). Compared with the sham group, active caspase-3 was intense (green, C, G) via single immunofluorescent staining. Double immunofluorescent staining showed the colocalization of active caspase-3 (E)/NeuN (D) and active caspase-3 (I)/CHMP4B (H) in brain basal ganglia adjacent to the hematoma at 2 days after ICH (F, J, arrows). Sections labeled with CHMP4B (K, N), Fas (L), and FasL (O), and the colocalization of CHMP4B/Fas (M, arrows) and CHMP4B/FasL (P, arrows) adjacent to the hematoma at day 2 after ICH. Scale bars 50 μm (C–P).
sFasL achieve the highest level on 18 h after hemin treatment. Furthermore, cell immunofluorescent staining was also performed to investigate the sub-localization of CHMP4B with neuron and active caspase-3. Fig. 5F showed that CHMP4B co-localized well with tublin-IIIβ, indicative of neurons, in the presence or absence of hemin treatment, whereas active
caspase-3 elevated only after hemin treatment, and colocalized with CHMP4B. Taken together, the results above indicated that CHMP4B might implicate in the processes of cellular apoptosis induced by hemin stimulation, and might be via extrinsic apoptotic pathway.

2.6. CHMP4B regulates cellular apoptosis in vitro

To further explore the exact role of CHMP4B playing in neuronal apoptosis following ICH, RNAi-specific to CHMP4B was employed to knock down CHMP4B in PC12 cells. Western blot analysis showed that RNAi-specific to CHMP4B had a significant effect in reducing hemin-induced CHMP4B expression in cells compared with nonspecific-RNAi (Fig. 6A, B). Concomitantly, levels of Fas, FasL and active caspase-3 were significantly downregulated after CHMP4B silencing in the presence of hemin stimulation (Fig. 6C, D). Taken together, these results demonstrate that CHMP4B played an important part in cellular apoptosis induced by hemin via extrinsic apoptotic pathway.

3. Discussion

ICH is a devastating stroke subtype with about 50% of patients dying within the first month and only 20% of patients living independently at 6 months (Sansing et al., 2009). At present, the treatment options are limited with only supportive care and rehabilitation shown to improve the outcome after ICH (Hwang et al., 2011). Therefore, getting a better understanding about the
underlying molecular and cellular mechanisms of damage following ICH is urgent for individuals and societies. Based on the amount of bleeding and the predilection site in clinical scenarios, the present study established an ICH rat model to simulate clinical ICH. In this study, CHMP4B was upregulated adjacent to the hematoma of ICH and the temporal changes were striking in neurons, but not in astrocytes, oligodendrocytes or microglia. It reminded us that the elevated expression of CHMP4B might be associated with neuronal apoptosis after ICH. As expected, we found the expression patterns of Fas, FasL, active caspase-8 and active caspase-3 parallel with that of CHMP4B in vivo and in vitro studies. In addition, silencing of CHMP4B by siRNA in PC12 cells can suppress hemin-induced expression of Fas, FasL and active caspase-3. Based on the above, we speculated that CHMP4B might be involved in neuronal death via extrinsic apoptotic pathway.

The ESCRT machinery including ESCRT-0, -I, -II, -III, and work in the sorting of ubiquitinated proteins in the process of the invaginating endosomal membrane toward the lumen, and this compartment containing inner vesicles is called the MVB (endosome) (Ichioka et al., 2008; Katoh et al., 2003; Mahul-Mellier et al., 2006). At the endosome, ubiquitinated proteins are first recognized by vacuolar protein sorting (Vps)-27, a factor required for MVB formation, and are then transferred to recruited ESCRT-I during MVB sorting. ESCRT-I is then activating functions of ESCRT-II in the recruitment and oligomerization of CHMPs to form ESCRT-III. During the processes, CHMPs are components of ESCRT-III complex, and CHMP4B is a core subunit of ESCRT-III for both the formation of MVB luminal vesicles and the sorting of ubiquitinated cargo protein into these vesicles (Mahul-Mellier et al., 2006; Trioulier et al., 2004). Previous studies indicated that CHMP4s can delineate and generate vesicles within the lumen of MVB and participate in the membrane scission proves (Hanson et al., 2008). Apart from the sorting for proteins, the ESCRT machinery is also involved in other disease processes, such as virus budding, neuronal development and some degenerative diseases (Moberg et al., 2005; Vaccari and Bilder, 2005). For example, X. Li et al. (2013) discovered that CHMP4B/C can interact with Newcastle disease virus (NDV) matrix protein to facilitate viral replication (X. Li et al., 2013). Sagona et al. (2014) demonstrated that CHMP4B, through its association with chromatin, participated in the autophagolysosomal degradation of micronuclei and other extranuclear chromatin that might participate in cataract formation (Sagona et al., 2014). In addition, numerous observations suggest that, in several neurodegenerative diseases, including
ALS (amyotrophic lateral sclerosis), AD (Alzheimer’s disease) and Niemann–Pick disease, affected neurons display an early impairment in the endosomal system. For example, in AD brains, endosome abnormalities appear in neurons long before amyloid plaque and neurofibrillary tangle formation (Nixon, 2005). However, the exact role of ESCRT-III in these processes remain poorly understand. There is also evidence demonstrating that CHMP4B is indispensable for neuronal apoptosis, as Mahul-Mellier et al. (2006) showed that ALIX overexpression is sufficient to induce cell death in neuroepithelial cells, while expression of ALIX mutants lacking the CHMP4B binding sites prevents early programmed cell death in cervical motoneurons at day 4.5 of chick embryo development (Mahul-Mellier et al., 2006). Consequently, on the basis of previous studies on CHMP4B, we wonder whether CHMP4B can induce neuronal/cellular activities through some other ways, such as promoting neuronal apoptosis after ICH.

Primary and secondary damage both make up the injury after the onset of ICH. Excluding the primary damage caused by mass effect, the resulting hematoma can trigger a series of unfavorable events which lead to secondary insults and finally severe neurological deficits or death (Aronowski and Zhao, 2011; Zhou et al., 2014). Neuronal apoptosis, astrocyte proliferation, oligodendrocyte death and microglia activation all participate in these processes (L. Li et al., 2013). Among them, neuronal apoptosis is one of the most severe consequences after ICH. Apoptosis, also known as programmed cell death, comprises both extrinsic and intrinsic pathways (Elmore, 2007). By comparison, the extrinsic pathway activates the subsequent caspase cascade more directly. Pro-apoptotic agents include Fas, Fasl, active caspase-8, and active caspase-3 that are upregulated in our ICH model. Moreover, immunofluorescence showed that active caspase-3, Fas localized well with CHMP4B in neurons, while just part of Fasl was co-localized with CHMP4B in neurons. A possible explanation would be that FasL might be secreted from astrocytes, macrophages, neutrophils and microglia, which were reported in previous studies (Krzyzowska et al., 2014; Yu and Fehlings, 2011). Meanwhile, the experimental data suggest that CHMP4B may also represent one of the apoptotic aspects in ICH. Therefore, it is reasonable for us to understand that upregulation of CHMP4B might be associated with neuronal apoptosis following ICH.

Taken together, all our in vivo and in vitro data indicated the association of CHMP4B in neuronal apoptosis following ICH. However, the exact role of CHMP4B on neuronal apoptosis remains to be further explored in vivo, so as to seek the underlying cellular and molecular mechanisms and therapeutic potentials of CHMP4B for ICH.

4. Experimental procedures

4.1. Animals and the ICH model

Male Sprague–Dawley rats (240–260 g) provided by the Department of Animal Center, Medical College of Nantong University were used in this study. All animals were maintained in a temperature controlled room (22 ± 1 °C) on a 12 h light–dark cycle and the food and water were available ad libitum. The number of animals studied was the minimum to obtain significant results, and all efforts were made to minimize their discomfort caused by the experimental procedures. All surgical procedures and postoperative care were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory. Animals were approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch.

For ICH model, the rats were deeply anesthetized with 10% chloral hydrate, the right basal ganglia was exposed stereotaxically (David Kopf Instruments, Tujunga, CA, USA) using the Paxinos and Watson’s atlas. With the incisor bar placed at 3.3 mm below the interaural line (horizontal zero), the coordinates from bregma to the guide the cannula were 0.2 mm anterior, 3.5 mm lateral, and 5.5 mm dorsoventral. Autologous whole blood (50 μl) was collected by cutting the tail tip, and drawing into a sterile syringe. The syringe was secured in the frame, and the needle was quickly introduced into right basal ganglia. The sham group only had a needle insertion. After injection, the sterile syringe was left in situ for over 10 min, and then closed the skin incision. The animals were allowed to recover from surgery (Xue and Del Bigio, 2000). Experimental animals (n=6 per time point) were anesthetized to extract the protein for Western blot analysis at indicated time points (6 and 12 h, 1, 2, 3, 5, 7 and 14 days) following ICH.

4.2. Behavioral testing procedures

4.2.1. Forelimb placing test

The rats were held by torsos, thus the forelimb could hang free. Independent testing of each forelimb was elicited by brushing the respective vibrissae on the corner edge of a countertop. Intact animals place the forelimb quickly onto the countertop. Intact animals place the forelimb quickly onto the countertop. According to the extent of injury, the ICH rats placing of the forelimb contralateral to the infusion may be impaired. During the experiments, each rat was tested 10 times for each forelimb, and the percentage of trials in which the rat placed the left forelimb was calculated as the forelimb placing score (Hua et al., 2002).

4.2.2. Corner turn test

The rats were allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the rat should turn either to the left or to the right. Only the turns involving full rearing along either wall was included. Depending on the extent of injury, rats may show a tendency to turn to the side of the injury and the percentage of right turns was scored. The trial was repeated 10 times, with at least 30 s interval for each trial (Karabiyikoglu et al., 2004).

4.3. Western blot analysis

Rats were sacrificed at different time points by injecting overdose of chloral hydrate. Tissues surrounding the hematoma (2 mm) from the ICH or corresponding areas from the sham rats were dissected and flash-frozen at −80 °C. For brain tissue proteins, the samples were weighted and cut into pieces, then homogenized in modified RIPA lysis buffer.
(150 mM NaCl, 50 mM Tris, pH 7.5, 1% Nonidet P-40, 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 5 mM EDTA), phosphatases inhibitor cocktails and protease inhibitor cocktail tablet. The supernatant was collected by centrifuging tissue homogenate at 12,000 rpm for 15 min in a microcentrifuge at 4 °C. After the determination of concentration with the Bradford assay (Bio-Rad), the samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) filter membrane by a transfer apparatus at 300 mA for 1.5 h. The membranes were blocked with 5% non-fat milk and incubated with primary antibody against CHMP4B (rabbit; 1:1000; Santa Cruz), FasL (mouse; 1:500; Santa Cruz), Fas (mouse; 1:500; Santa Cruz), active caspase-3 (mouse or rabbit; 1:1000; Santa Cruz), active caspase-8 (rabbit; 1:1000; Santa Cruz), active caspase-9 (mouse; 1:500; Santa Cruz), GAPDH (rabbit; 1:1000; Santa Cruz) and β-actin (mouse; 1:1000; Santa Cruz) overnight. After washing with TBST, the blots were incubated with secondary antibodies for 1.5 h at room temperature (RT) and detected using either an enhanced chemiluminescence (ECL) kit (Pierce) or scanned with the Odyssey infrared imaging system (LI-COR Bioscience). All results shown are representative of at least three independent experiments.

4.4. Sections and immunohistochemistry

Rats two days after autologous whole blood injection or from the sham group were anesthetized and perfused with 500 mL of 0.9% saline, followed by 4% paraformaldehyde. After perfusion, the brains were removed and post-fixed in the same fixative for 24 h and transferred to 20% sucrose for 2–3 days, followed by immersion in 30% sucrose for another 2–3 days, respectively. The Tissues were then cut at 5 μm with a cryostat and the sections were stored at −20 °C until use.

For immunohistochemistry, brain sections were washed in phosphate-buffered saline (PBS), and treated with 3% H2O2 for 10 min at RT to reduce endogenous peroxidase activity. The sections were blocked with confining liquid consisting of 10% donkey serum, 1% bovine serum albumin (BSA). 0.3% Triton X-100, and 0.15% Tween-20 for 2 h at RT, then incubated with anti-CHMP4B antibody (rabbit; 1:100; Santa Cruz) for 2 h. After washing in PBS, the sections were incubated in secondary antibodies at 37 °C for 30 min. In the final, the sections were color-reacted with the liquid mixture (0.02% diaminobenzidine tetrahydrochloride (DAB), 0.1% PBS and 3% H2O2). Then, the sections were air-dried, dehydrated, covered with coverslips and examined using Leica fluorescence microscope (Wetzlar, Germany).

4.5. Immunofluorescent staining

For double immunofluorescent labeling, sections were prepared as previously. After air-dried for 2 h, sections were first blocked with normal serum blocking solution (10% donkey serum, 1% bovine serum albumin (BSA), 0.3% Triton X-100, and 0.15% Tween-20) for 2 h at RT. Then, the sections were incubated with primary antibodies against CHMP4B (rabbit; 1:100; Santa Cruz), NeuN (mouse; 1:300; Chemicon), CNPase (mouse; 1:200; Sigma), GFAP (mouse; 1:100; Sigma), Iba-1 (mouse; 1:100; Santa Cruz), FasL (mouse; 1:50; Santa Cruz), Fas (mouse; 1:50; Santa Cruz) and active caspase-3 (mouse or rabbit; 1:200; Santa Cruz). Briefly, sections were incubated with both primary antibodies overnight at 4 °C, followed by a mixture of FITC- and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) for 2 h at 4 °C. Images were captured using a Leica fluorescence microscope (Wetzlar, Germany).

4.6. Quantitative analysis

Cells double labeled for CHMP4B and cell phenotypic markers used in the experiment were quantified. To identify the ratio of each phenotype-specific marker-positive cells expressing CHMP4B, at least 200 phenotype-specific marker-positive cells were counted surrounding the hematoma in each section. Then, doublelabeled cells for CHMP4B and phenotype-specific markers were recorded. A total of three adjacent sections per animal were sampled.

4.7. Cell culture procedures and experimental treatments

Rat pheochromocytoma cells (PC12) were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Science (Shanghai, China) and cultured in Ham’s F12 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (Invitrogen) at 37 °C in a 5% CO2 humidified incubator. To study neuronal apoptosis in vitro, PC12 cells were induced for differentiation using 100 μg/ml nerve growth factor (NGF, R&D system) supplemented with 1% FBS in F12 medium for 3 days, and then exposed to hemin for indicative times.

4.8. CCK-8 assay

The viability of PC12 cells under various treatment conditions was assayed using the commercial cell counting kit-8 (CCK-8) in accordance with the manufacturer’s protocol (Dojindo, Kumamoto, Japan). For the dose experiment, PC12 cells were exposed to hemin (25, 50, 75, and 150 μM) for indicative time points (3, 9, and 18 h) in treatment medium. CCK-8 solution was added to each well at a final concentration of 10% (v/v) and incubated for 2.5 h at 37 °C. The absorbance was measured at 490/630 nm by the microplate reader (Bio-Rad). For measurement of neurotoxicity, PC12 cells were exposed to hemin for different time point, and then CCK-8 assay was performed. The experiments were repeated at least 5 replicates of each treatment.

4.9. Quantification of sFasL in the supernatant of PC12 cells

According to the manufacturer’s instructions, enzyme-linked immunosorbent assay (ELISA) kits with an antibody that recognizes rat FasL (R&D Systems, Minneapolis, USA) was used to determine the concentration of FasL in the supernatant of cultured PC12 cells (1 × 106 cells/well). To quantify the concentration of FasL in cultured medium of PC12 cells, the supernatant was collected after treatment with hemin for indicative time points. The sample without treatment was used to construct a standard curve and to obtain absolute values for calibration. The concentration was determined in
quintuple in each sample and the average measurement was considered to be the final concentration.

5.  10 siRNA and transfection

The rat CHMP4B-specific RNAi duplex with 3 concentration of Fassynthesized in invitrogen. The siRNA target for CHMP4B was GCUCGGAGAACUGGACACAATT. For transient transfection, the CHMP4B siRNA vector or the non-specific vector was transfected using lipofectamine 2000 and plus reagent in OptiMEM (Invitrogen). Transfected cells were cultured for 30 h before use.

5.1  1 Statistical analysis

All data were analyzed with Stata 8.0 statistics software. Values were expressed as means ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey’s post-hoc multiple comparison tests. p < 0.05 was considered significant. Each experiment consisted of at least three replicates per condition.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (Nos. 81371299, 31300902); the Colleges and Universities in Natural Science Research Project of Jiangsu Province (13KJB310009); A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); Technology Innovation Programme of Nantong University (Nos. YKC13075, YKC13086).

REFERENCES