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Rabies virus matrix protein induces apoptosis by targeting mitochondria

Jie Zan[1], Juan Liu[1], Jian-Wei Zhou[1], Hai-Long Wang[1], Kai-Kun Mo[1], Yan Yan[1], Yun-Bin Xu[1], Min Liao[1], Shuo Su[3], Rong-Liang Hu[4] and Ji-Yong Zhou[1,2,3*]

[1]Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University, Hangzhou, PR China

[2]Collaborative Innovation Center and State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University, Hangzhou, PR China

[3]Institute of Immunology, Nanjing Agricultural University, Nanjing, PR China.

[4]Laboratory of Epidemiology, Veterinary Institute, Academy of military Medical Sciences, Changchun, PR China

[*]Corresponding author: Ji-Yong Zhou, Key Laboratory of Animal Virology of Ministry of Agriculture, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, PR China. Tel: 86-571-8898-2698; Fax: 86-571-8898-2218. jyzhou@zju.edu.cn

Abstract

Apoptosis, as an innate antiviral defense, not only functions to limit viral replication by eliminating infected cells, but also contribute to viral dissemination, particularly at the late stages of infection. A highly neurotropic CVS strain of rabies virus induces apoptosis both *in vitro* and *in vivo*. However, the detailed mechanism of CVS-mediated neuronal apoptosis is not entirely clear. Here, we show that CVS induces apoptosis through mitochondrial pathway by dissipating mitochondrial membrane potential, release of cytochrome c and AIF. CVS blocks Bax activation at the early stages of infection; while M protein partially targets mitochondria and induces mitochondrial apoptosis at the late stages of infection. The α-helix structure spanning 67 to 79 amino acids of M protein is essential for mitochondrial targeting and induction of apoptosis. These results suggest that CVS functions on mitochondria to regulate apoptosis at different stages of infection, so as to for viral replication and dissemination.

Abbreviations

RABV, rabies virus; CVS, challenge virus standard-11 strain of fixed rabies virus; VSV, vesicular stomatitis virus; AIF, apoptosis-inducing factor; cyto *c*, cytochrome *c*; PARP, poly (ADP-ribose) polymerase; LDH, lactate dehydrogenase; TOMM20, translocase of the outer mitochondrial membrane 20; CLSM, confocal laser scanning microscopy; SRM, super-resolution microscopy.

Key words: Rabies Virus; Matrix protein; Mitochondria; Apoptosis

Introduction

Apoptosis is an active process of cellular self-destruction which is considered as a host innate defense mechanism by eliminating virus-infected cells against rampant viral replication. However, apoptosis may also contribute to release of viral progeny and avoid the immune response. Therefore, many viruses have evolved numerous strategies to either induce or suppress apoptosis at different stages of infection for their own benefits [1].

Virus infection can trigger several cellular signaling pathways that converge at activation of caspases and ultimately result in apoptosis. Two major apoptotic pathways leading to caspase activation have been well described: the extrinsic pathway (death receptor pathway) which is initiated by cell surface receptor and mediated by activation of caspase-8, and the intrinsic pathway (mitochondrial pathway) which is regulated by mitochondria and mediated by activation of caspase-9 [2, 3]. Both pathways eventually converge at the activation of caspase-3, which subsequently cleaves a variety of target substrates, such as poly (ADP-ribose) polymerase (PARP)

and results in nuclear fragmentation [4].

Mitochondria are multifunctional organelles which not only play essential roles in host immune response but also serve as an important control point in the regulation of apoptosis. Following apoptotic signals, mitochondria undergo loss of mitochondrial membrane potential and subsequently release pro-apoptotic factors [5]. This process is highly regulated by Bcl-2 family proteins, which consist of both anti-apoptotic and pro-apoptotic family members, depending on the presence of at least one of four conserved Bcl-2 homology (BH) domains. Pro-apoptotic multi-domain proteins Bax and/or Bak are critical to the induction of mitochondrial apoptosis [6]. Upon activation, Bax undergoes conformational changes and results in Bax translocating to mitochondria, where Bax form oligomers resulting in loss of mitochondrial membrane potential [7]. While in healthy cells, Bax is primarily in the cytoplasm as inactive monomer and is held in check by the anti-apoptotic protein Bcl-2 [8]. The BH3-only proteins, such as Bad, which possess only the BH3 domain, function as upstream sensors of signaling pathways and convey to other Bcl-2 family proteins to initiate apoptosis. Upon activation, Bad subsequently activates Bax and Bak or inhibits the anti-apoptotic function of Bcl-2 and then leads to release of pro-apoptotic factors such as cytochrome *c* (cyto *c*) and apoptosis-inducing factor (AIF) [9]. Cyto *c* in the cytosol activates caspase-9 and then leads to irreversible cell death, which represents the mitochondrial caspase-dependent apoptotic pathway; AIF is proteolytically cleaved and truncated AIF is released from mitochondria and then translocated into the nucleus to induce high-molecular weight DNA fragmentation and chromatin condensation, causing the so-called caspase-independent cell death [10]. Since the mitochondria represent a central crossroad, where pro- and anti-apoptotic signals are integrated, numerous viruses have been reported to regulate cell

apoptosis at the mitochondrial level [11].

Rabies virus (RABV), a member of the *lyssavirus* genus of *Rhabdoviridae* family, is the causative agent of rabies with severe neurological symptoms and almost 100% mortality [12]. The viral genome is a single non-segmented negative strand RNA and comprises five genes encoding viral proteins, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). The N, P and L proteins, together with viral genomic RNA, form a helical nucleocapsid, which is surrounded by a membrane composed of host lipids and two viral proteins, the M and G proteins [13]. Previous reports have shown that RABV induced apoptosis *in vitro* and *in vivo* [14-17]. However, the molecular mechanisms of RABV-induced apoptosis are still not fully understood. In this study, we show that RABV M protein partially targets mitochondria and induces mitochondrial apoptosis through caspase-dependent and caspase-independent pathways at the late stages of infection.

Materials and methods

Cells and viruses

Mouse neuroblastoma N2a cells, baby hamster kidney (BHK-21) cells and Human embryonic kidney epithelial (HEK) 293T cells were cultured in Dulbecco modified Eagle medium (DMEM, Life technologies Gibco, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100μg/ml) and 10% heat-inactivated fetal bovine serum (Gibco) at 37℃ with 5% CO_2. The challenge virus standard-11 strain of fixed rabies virus (CVS) was stored in our laboratory. Cells were infected with CVS at a multiplicity of infection (MOI) of 1when monolayers had reached 80% confluence. After 1 h of viral absorption, cells were gently washed with PBS. Fresh medium was

added and the cells were cultured for various periods at 37°C. Virus preparations were titrated on N2a cells, and then stored at -80°C.

Antibodies and reagents

Rabbit monoclonal antibodies (RabMAb) against AIF (ab32516), Prohibitin (ab75771), Cleaved PARP (ab32064), Bad (ab32445), mouse monoclonal antibody (MAb) against cyto *c* (ab13575), Bax6A7 (ab5714) and rabbit polyclonal antibody (PAb) against TOMM20 (ab78547) were all purchased from Abcam (Cambridge, MA, USA). RabMAb against Bak (D4E4) was purchased from Cell Signaling Technology. Rabbit PAb against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R1210-1), Bax (M1312-3) and MAb against Bcl-2 were purchased from HuaAn Biotechnology Co. Ltd. (Hangzhou, China). Mouse MAb and rabbit PAb to N protein of RABV, as well as mouse PAb to M protein of RABV, were prepared in our laboratory. Caspase-9 inhibitor (z-LEHD-FMK, 218761) and caspase-8 inhibitor Ⅱ (z-IETD-FMK, 218759) were purchased from Calbiochem (San Diego, CA). Caspase-3 inhibitor (Ac-DEVD-CHO, C1206) was purchased from Beyotime (Beijing, China). 4', 6'-diamidine-2-phenylindole (DAPI) was purchased from Roche (Mannheim, Germany).

Lactate dehydrogenase (LDH) release assay

The amount of LDH released from the cells at different times after infection was measured by a Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, Wis.) according to the manufacturer's instructions. All experiments were carried out in triplicate and conducted at least twice. Results were expressed as x-fold of control.

Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay (Beyotime, Beijing, China). Briefly, N2a cells were plated in 96-well plates at a density of 1×10^5 cells/cm^2 infected by CVS or not for different times. 10μl CCK-8 was added to each well, incubated for 2 h at 37°C, and then the absorbance was measured at 450 nm using automated ELISA reader (Bio-Tech Instrument, USA). All experiments were carried out in triplicate and conducted at least twice. Results were expressed as percentage of the infected group relative to the control group (set as 100%).

Measurement of ATP levels

Intracellular ATP levels were determined using an ATP Bioluminescence Assay kit (Beyotime, Beijing, China). Briefly, N2a cells cultured in 6-well plates were infected by CVS or not for different times. Cell culture media was aspirated and the cells were washed twice with PBS. Then lysis solution was added into each well to harvest the cellular ATP. The ATP levels were determined following the protocol of the assay kit. Results were expressed as percentage of control (set as 100%).

Caspase-3, -8, and -9 activities assay

Caspase-3, -8, and -9 activities were detected using the ApoAlert Caspase Fluorescent Assay Kits (Clontech, Palo Alto, CA) according to the manufacture's protocol. Briefly, cells were cultured in 6-well plates. At different time points, cells were collected by centrifugation at 400×*g* for 5 min,

and then resuspended in chilled cell lysis buffer on ice for 10 min. Cell lysates were centrifuged at maximum speed for 10 min at 4°C, and then the supernatant was collected. After the reaction buffer and corresponding caspase substrate were added, the mixture was incubated at 37°C for 60min in a water bath. The substrates of caspase-3, -8, and -9 were DEVD-AFC, IETD-AFC, and LEHD-AMC, respectively. Fluorometric detection for caspase-3 and caspase-8 was measured at 400-nm excitation and 505-nm emission wavelengths; fluorometric detection for caspase-9 was measured at 380-nm excitation and 460-nm emission wavelengths. Activities are expressed as fold change over the activity of the uninfected control cells.

TUNEL assay

Apoptotic cells were examined and quantified using an *In situ* Cell Death Detection Kit (Roche, Philadelphia, PA, U.S.). In brief, cells were seeded in 48-well plates. After infection or transfection for different times, cells were fixed in 4% paraformaldehyde for 1h at room temperature. After rinsing twice with PBS, cells were permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 5 min on ice. Cells were then incubated with the TUNEL reaction mixture for 1h at 37°C in a humidified atmosphere in the dark. Cells were then rinsed three times with PBS and stained DAPI for 10 min. After another three washes, stained cells were examined with a fluorescence microscope (Olympus). The amount of DAPI-positive cells were considered as the total cells number. Results were expressed as percentage of TMR red-positive cells relative to DAPI-positive cells.

JC-1 staining to measure mitochondrial membrane potential

Cells were stained with JC-1 dye (Beyotime, China) to detect a change in mitochondrial membrane potential. Due to that mitochondrial membrane potential is high in normal cells, JC-1 accumulates in the mitochondrial matrix to form red fluorescent J-aggregates. When cells undergo early apoptosis, the mitochondrial membrane potential is low which prevents JC-1 accumulation in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers) [18]. Briefly, cells were cultured in 12-well plates. After infected or transfected for different times, cells were stained with JC-1 (2μM final concentration) and incubated in the incubator (37°C, 5%CO_2) for 20 min. Following incubation, cells were washed once with PBS and then the fluorescence was detected with a fluorescence microscope (Olympus). J-aggregates and monomers were detected with an excitation wavelength of 525 nm or 490 nm and emission wavelength of 590 nm or 530 nm, respectively.

Construction of plasmids and cell transfection

Using specific primers (Table 1), full-length N, P and M genes were cloned from the cDNA of RABV-infected N2a cells. Bax and Bcl-2 were amplified from 293T cells. M gene was also cloned into pCMV-myc vector (Clontech, Palo Alto, CA). M truncated mutants were amplified from the full-length M with appropriate sequence specific primers and then cloned into pEGFP-C3 vector (Clontech). All constructs were verified by DNA sequencing and were designated pEGFP-C3-N, pEGFP-C3-P, pEGFP-C3-Bcl-2, pCMV-Myc-Bax, pCMV-Myc-M, pEGFP-C3-M, pEGFP-C3-M 67-79, pEGFP-C3-M 67-79/K77 and pEGFP-C3-M67-79/P74.

For cell transfection, cells were seeded into 6-well plates, 96-well plates (Corning, NY, USA) or 35-mm glass bottom dishes (Shengyou Biotechnology Co. Ltd., Hangzhou, China). On the

following day, recombinant plasmid was mixed with Exfect Transfection Reagent (Vazyme Biotech Co. Ltd., Nanjing, China), and the transfection procedure was carried out according to the manufacturer's instructions. Cells transfected with empty vector pEGFP-C3 or pCMV-Myc were used as control.

Subcellular fractionation and Western blot

The cytosolic and mitochondrial fractions were isolated using a ProteoExtract Cytosol/Mitochondria Fractionation Kit (Calbiochem) according to the manufacturer's instructions. Cells were collected by centrifugation at 600×*g* for 5min at 4°C. The collected cells were washed with ice-cold PBS and then resuspended with Cytosol Extraction Buffer Mix and incubated on ice for 10 min. After homogenization was completed, the homogenate was centrifuged at 700×*g* for 10min at 4°C. The supernatant was then collected and centrifuged at 10,000×*g* for 30min at 4°C. The cytosolic fraction in the supernatant was harvested and stored at -80°C. The mitochondria fraction was resuspended with Mitochondria Extraction Buffer Mix and then stored at -80°C before use.

Proteins were separated by 12% SDS-PAGE and then transferred onto 0.22 μm nitrocellulose membranes (GE Healthcare). After blocking with 5% skimmed milk in PBS containing 0.05% Tween 20 (PBST) for 1 h, the membranes were incubated with RabMAb against AIF, Cleaved PARP, Prohibitin, mouse MAb against to Cytochrome C, RABV-N or Rabbit PAb against β-actin overnight at 4°C. The membranes were then washed three times with PBST for 5 min each time, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) for 1 h at 37°C. Finally, the membranes were

washed again three times with PBST before visualization using SuperSignal West Pico chemiluminescent substrate (Thermo) under the conditions recommended by the manufacturer.

Confocal laser scanning microscopy (CLSM) and super-resolution microscopy (SRM)

Cells were grown overnight into 35-mm glass bottom dishes and then infected by CVS or not. After the indicated time points, cells were washed once with PBS, fixed with 4% paraformaldehyde for 60min, permeabilized with 0.5% Triton X-100 (in PBS) for 5 min on ice, and then washed three times with PBS. The fixed cells were incubated with a mixture of mouse MAbs against RABV-N or rabbit PAb to RABV-M and the above-mentioned antibodies (AIF, TOMM20, Bax and Cytochrome C) at 37°C for 60 min. Cells were then washed three times with PBS and incubated with the mixture of fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (KPL) and/or Alexa Fluor® 546-conjugated secondary antibodies (Invitrogen) at 37°C for 60 min. Cell nuclei were stained with DAPI. The stained cells were washed three times with PBS and subsequently examined by a LSM780 laser scanning confocal microscope (Zeiss, Oberkochen, Germany). For four-color CLSM, cells were labeled with MitoTracker® Red CMXRos (Cell Signaling Technology), incubated with a rabbit PAb to N protein of RABV and a mouse Ab to Bax6A7 as primary antibodies and then inoculated with FITC-conjugated donkey anti-mouse IgG (Invitrogen) and CF™ 647-conjugated donkey anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) as secondary antibodies. For SRM, cells were inoculated with rabbit anti-N pAb and mouse anti-Bax6A7 mAb as primary antibodies as well, followed by tandem dye pairs, Alexa Fluor® 405/Alexa Fluor® 647-conjugated donkey anti-rabbit and Cy™3/Alexa Fluor® 647-conjugated donkey anti-mouse IgG. Cells were viewed with the N-stochastic optical

reconstruction microscopy system (N-STORM) (Nikon, Tokyo, Japan).

Statistical analysis

Data were statistically analysed and graphed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All results were presented as means ± standard deviations. Statistically significant differences between groups were determined by the Student's t test. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Results

CVS-induced apoptosis is associated with caspase-9 activation

Neuronal process degeneration and apoptosis in adult mice, which is considered to play an essential role in the fatal neurological rabies, were induced by infection with CVS [15, 16, 19, 20]. N2a cells infected with CVS, which is widely used for the study of rabies pathogenesis for its low infection risk to human compared with wild type rabies virus, and strong neurotropism for experimental animals, were used as a model to explore the death mechanisms of neuronal cells. As shown in Fig. 1A, CVS did not significantly affect cell viability at the early stages of infection (24hour post-infection, h p.i.). With the infection prolonged, cell viability of infected cells dropped significantly to 55% at 72 h p.i. compared to mock-infected cells. Similarly, the intracellular ATP levels of infected cells progressively decreased in a time-dependent manner and were about 40% lower than that of mock-infected cells at 72 h p.i. (Fig. 1A). Subsequently, apoptosis was assessed by the TUNEL assay and LDH release. As shown in Fig. 1B, apoptotic cells were detected as early as 48h p.i. and then significantly increased at 72 h p.i. compared to mock. LDH release of CVS-infected cells was about 5.3-fold greater than that of mock-infected cells at 72 h p.i. These results confirmed that CVS induced apoptosis in N2a cells, consistent with previous reports [21].

To further elucidate the apoptotic mechanism during CVS infection, the activities of caspase-9, -8 and -3 processed during CVS infection were measured. Our results showed that caspase-9 and caspase-3 were activated in a time-dependent manner and the activities were markedly enhanced at 72 h p.i. in CVS-infected cells, compared to that of mock. By contrast, caspase-8 activities maintained relatively stable and only slightly increased at 72 h in CVS-infected cells (Fig. 1C). Furthermore, PARP cleavage, which is triggered by activated caspase-3 in the apoptotic events, was further detected with Western blot (Fig. 1D). Consistent with the activation of caspase-3, PARP was progressively cleaved during CVS infection. Furthermore, we determined the effects of caspase-8 specific inhibitor (z-IETD-FMK) or caspase-9 specific inhibitor (z-LEHD-FMK) on apoptotic signaling pathways in CVS-infected cells. As is shown in Fig. 1E, treatment of CVS-infected cells with z-LEHD-FMK, but not z-IETD-FMK, significantly suppressed the activation of caspase-3 and -8 at 72 h p.i. (Fig. 1E). Furthermore, z-LEHD-FMK or Ac-DEVD-CHO (a specific inhibitor of caspase-3) but not z-IETD-FMK, remarkably reduced CVS-induced apoptosis in N2a cells, as assessed by the TUNEL assay (Fig. 1F), suggesting that caspase-8 seems to play a minor role in initiating apoptosis and might be activated by active caspase-3, so as to amplify the apoptotic effects. Taken together, these results implicated that CVS-induced apoptosis in N2a cells at the late stages of infection is associated with activation of caspase-9.

CVS activates caspase-dependent and caspase-independent pathways at the late stages of infection

Activation of caspase-9, but not caspase-8 at 48 h p.i., in CVS-infected N2a cells implied that

CVS-induced apoptosis involved the mitochondrial intrinsic pathway (Figure. 1C and E). Changes of mitochondrial membrane potential which is an important parameter of mitochondrial function, are closely related to mitochondrial dysfunction and apoptosis [18]. Thus we used JC-1 dye to assess the changes of mitochondrial membrane potential during CVS-infection. As seen in Fig. 2A, green fluorescence (JC-1 monomers) was significantly enhanced and red fluorescence (JC-1 aggregates) was nearly undetectable at 48 and 72 h p.i. in CVS-infected cells, indicating that mitochondrial membrane potential was significantly dissipated at the late stages of infection. By contrast, red fluorescence remained relatively stable in mock-infected cells. Next, we assessed pro-apoptotic proteins cyto *c* distribution using Western blot and CLSM. As shown in Fig. 2B, cyto *c* in cytoplasm was notably increased at 72 h p.i. of CVS-infected cells; while, cyto *c* of mock-infected cells mainly existed in the mitochondrial pellets and did not notably reduce. Furthermore, cyto *c* in CVS-infected cells diffused into the cytoplasm from mitochondria at 72 hp.i.; whereas cyto *c* distributed exclusively in mitochondria in mock-infected cells (Fig. 2D). To further investigate the apoptotic events in CVS-infected cells, we then detected another pro-apoptotic protein AIF. AIF was progressively upregulated and proteolytically cleaved during CVS infection, whereas the expression level of AIF maintained relatively stable and no cleaved AIF was detected in mock-infected cells (Fig. 2C). As seen in Fig. 2E, AIF translocated into the nucleus in CVS-infected cells with a small amount of AIF still locating on the mitochondria. Whereas AIF of mock-infected cells exclusively localized on the mitochondria. Furthermore, release of AIF into the nucleus in CVS-infected cells was also confirmed by Western blot analysis (Supplementary Fig. 1). These results show that CVS dissipates mitochondrial membrane potential and induces cyto *c* and AIF release from mitochondria at the late stages of infection,

suggesting that CVS-induced apoptosis involves caspase-dependent and caspase-independent pathways.

CVS infection blocks Bax activation

The above results indicate that CVS-induced apoptosis is primarily via the mitochondrial pathway. Since the mitochondrial apoptotic pathway is highly regulated by the Bcl-2 family of proteins, we determined whether Bcl-2 family proteins play any roles in CVS-induced apoptosis. As observed in Fig. 3A, expression levels of pro-apoptotic Bcl-2 family members Bak, Bad and anti-apoptotic member Bcl-2 were not notably affected and maintained relatively stable during CVS infection. Bax levels were slightly downregulated at 24h p.i. and then recovered to the similar levels of that in mock control.

In viable cells, Bax is kept in an inactive state primarily in the cytoplasm. Upon activation, Bax undergoes conformational changes and translocates to mitochondria, resulting in loss of mitochondrial membrane potential and cell death [7, 22, 23]. Of these multiple steps, Bax conformational change can be detected by immunoreactivity with a conformation-specific antibody, Bax6A7 [24]. We observed that CVS infection did not activate Bax in the majority of infected cells. Although translocation of Bax to mitochondria, which were labeled with MitoTracker, was observed in only a few infected cells, the nucleus of these cells were integrity and no features of apoptosis (Fig. 3B). Interestingly, the viral N protein was also located on mitochondria and co-located with Bax in these cells (Fig. 3B). Furthermore, owing to the limited spatial resolution of CLSM, we analyzed the co-localization of activated Bax and N using SRM. The image showed that the viral N protein co-located with activated Bax in the manner of

filamentous, indicative of mitochondria (Fig. 3C), so as to seemly block activated Bax to function and provide a time window for viral replication. These results indicate that CVS blocks Bax activation, so as to inhibit the mitochondrial apoptotic pathway at the early stages of infection.

N protein reduces Bax-induced apoptosis

Next, we sought to determine which viral protein(s) was primarily responsible for blocking Bax activation and inhibiting apoptosis. Previous studies have indicated that overexpression of the viral G protein results in enhancement of apoptosis [25], therefore we focused on viral proteins N, P and M. N2a cells were cotransfected with plasmids encoding Myc-Bax and EGFP-N, EGFP-P, EGFP-M and EGFP-Bcl-2 (positive control). Apoptosis was then quantified in transfected (EGFP-positive) cells and detected with the TUNEL assay at 24 hours post-transfection (h p.t.). As shown in Fig. 4A, overexpression of Bax resulted in the artificial activation of Bax and cell apoptosis, which was well confirmed in previous study [26], and EGFP is not able to protect cells from apoptosis. However, when EGFP-Bcl-2 was overexpressed along with Bax, apoptosis was notably inhibited (Fig. 4A). Similarly, EGFP-N also attenuated Bax-induced apoptosis. Conversely, EGFP-P and EGFP-M did not protect against Bax-induced apoptosis, and surprisingly EGFP-M induced higher level of cell apoptosis than EGFP despite a lower expression level (Figure. 4A). Furthermore, we analyzed whether N protein influenced expression levels of Bcl-2 family members. As observed in Fig. 4B, expression of N protein did not affect the expression levels of Bak, Bax, Bad and Bcl-2.

M protein alone dissipates mitochondrial membrane potential and activates mitochondrial apoptotic pathway

CVS-induced apoptosis at the late stages of infection is associated with loss of mitochondrial membrane potential and activation of caspase-9 (Fig. 1B, 1C and 2A). Therefore, we sought to identify which viral protein(s) was primarily responsible for activating mitochondrial apoptotic pathway. We pay our attention to M protein, due to that expression of M protein induced more cell apoptosis compared to N or P protein (Fig. 4A) and that M proteins from Vesicular stomatitis virus (VSV) and Mokola virus (MOK), members of the *Rhabdoviridae* family, induces apoptosis in a number of cells types [27, 28]. As shown in Fig. 5A, expression of M protein induced N2a cells apoptosis in a time-dependent manner, compared to control. Similar results were also obtained with BHK-21 cells and 293T cells (data not shown). Next, the results showed that caspase-3 and -9 were activated within 24h of transfection with M and then the activities progressively enhanced at 48 and 72h; while caspase-8 activities were relatively stable at the similar level to that of control and only increased at 72h p.t. (Fig. 5B). Subsequently, expression of M protein alone resulted in loss of mitochondrial membrane potential in a time-dependent manner (Fig. 5C). These results reveal that matrix protein alone dissipates mitochondrial membrane potential and induces apoptosis associated with activation of caspae-9, similar to those described in the context of viral infection.

M protein targets mitochondria and induces apoptosis

Since matrix protein alone could disrupt mitochondrial membrane potential and trigger mitochondrial apoptotic pathway (Fig. 5B and 5C), it was hypothesized that M protein may locate to mitochondria and cause loss of mitochondrial membrane potential. To explore this possibility,

subcellular distribution of M protein in CVS-infected N2a cells was analyzed by CLSM. The results revealed that M protein partially co-localized with TOMM20, a mitochondrial protein maker, indicating mitochondrial localization of M protein during CVS infection in N2a cells; besides, the nucleus split into nuclear fragments, suggesting that cell apoptosis was undergoing (Fig. 6A). Furthermore, we also observed that M protein diffused around the nucleus and in the cytoplasm (Supplementary Fig. 2). Subsequently, to identify whether M protein alone targets mitochondria, independent of other viral proteins, N2a cells were co-transfected with EGFP-M and DsRed2-Mito, a specific mitochondrial reporter plasmid and then analyzed by CLSM (Fig. 6B). The result revealed a partial co-localization between the EGFP-M fluorescence signal and that of DsRed2-Mito, representing M protein alone could locate to mitochondria (Fig. 6B). Previous study reported that the sequences spanning 67 to 79 amino acids, which represents a hydrophobic α-helix flanked by positively charged residues, of MOK M protein is able to target this protein to mitochondria and induces apoptosis [29]. CVS M protein presents a 76.24% sequence similarity to MOK M protein. The sequences spanning 67 to 79 amino acids of CVS M protein and MOK M protein are quite similar, only the amino acid sequences at positon 70 and 77 are different. While the type of amino acid at positon 70 has been considered to have little impact on apoptosis [28, 29], therefore we generated plasmids that express amino acids 67 to 79 of CVS M (M67-79), the mutant M67-79/K77 (the arginine at position 77 replaced with lysine to obtain the MOK sequence) and M67-79/P74 (the histidine at position 74 replaced with a proline to disturb the structure of the α-helix) fused with EGFP. As shown in Fig. 6B and 6C, M67-79 and M67-69/K77 both exhibited a strict colocalization of the EGFP and DsRed2-Mito signals and induced levels of apoptosis similar to that for CVS-M. However, the M67-79/P74 variant did not

target mitochondria and induce apoptosis. These results revealed that CVS M protein targets mitochondria and induces apoptosis, and that the α-helix structure in the M protein is essential for mitochondrial targeting and induction of apoptosis.

Discussion

Apoptosis has been considered as a common defense mechanism upon viral infection, or serves as a viral strategy to induce cytopathogenicity or to maximize virus progeny. CVS has been reported to induce apoptosis in rat prostatic adenocarcinoma cells [19], mouse and human lymphocytes [14], mouse neuroblastoma cells [21], and in mouse embryonic hippocampal neurons [30]. Furthermore, intracerebrally inoculated with CVS showed prominent apoptotic death of neurons in the brains of mice of various ages [19, 31]. Pathogenicity of RABV has been considered to be inversely correlates with apoptosis and is affected by the viral proteins N, M and G [30, 32-34]. RABV G protein has an important role in the induction of apoptosis [25, 30]. A study reported that only RABV containing the G protein from the nonpathogenic RABV strain, but no the pathogenic strain CVS, was able to trigger the apoptosis of human cells [35]. The aim of the present study was to identify which viral protein(s) was involved in CVS-induced apoptosis and understand the mechanisms of CVS-induced apoptosis. We report here that CVS induces apoptosis in N2a cells at the late stages of infection, mainly through mitochondrial apoptotic pathway. Our results showed that caspase-9, -8 and -3 were activated evidently in CVS-infection N2a cells at 72h p.i.; Blocking caspase-9 activation, but not caspase-8 with its specific inhibitor, remarkably suppressed caspase-3 activation and apoptosis. Transfection of cells with the viral M protein induced apoptosis using pathways similar to those described in the context of viral infection. These results

suggest that caspase-9 is the main caspase involved in the cytopathic effects of CVS and caspase-8 might be activated by active caspase-3, so as to amplify apoptotic effects [36, 37]. However a laboratory attenuated CVS-B2C strain were reported to activate caspase-8 and -3, but not caspase-9 [38]. The discrepancy between our study and the afore-mentioned study could be due to differences in the virus strains as well as the cell types used. It is possible that different cells express different death receptors on their surfaces.

In response to various intracellular apoptotic signals, mitochondria undergo loss of inner mitochondrial membrane potential and subsequently release several pro-apoptotic proteins [5]. Among these, cytosolic cyto *c* participates with other pro-apoptotic factors in the formation of the apoptosome, which triggers the caspase-dependent proteolytic cascade [39], whereas AIF critically functions in caspase-independent apoptosis by relocating to the nucleus [10]. Evidence for the induction of caspase-dependent and -independent mitochondrial pathways by viral infection has been reported, such as human immunodeficiency virus and VSV [40, 41]. However, the translocation of AIF during viral infection is not general and for example, herpes simplex virus or bovine ephemeral fever virus-mediated apoptosis does not require AIF translocation [42, 43]. Recently, porcine epidemic diarrhea virus has been reported to induce apoptosis associated with translocation of AIF, but not cyto *c*. We demonstrate here that loss of mitochondrial membrane potential and release of cyto *c* and AIF from mitochondria are actively manifested during CVS infection, suggesting CVS induced apoptosis involves caspase-dependent and -independent mitochondrial apoptotic pathways.

The release of cyto *c* and AIF is ultimately controlled by the highly conserved Bcl-2 family of proteins, which regulate the integrity of mitochondria [8, 44]. Some viruses are found to induce

apoptosis through Bax and/or Bak activation, such as hepatitis C virus, West Nile virus and VSV [45-47]. Similarly, CVS has also been reported to induce expression of Bax [19]. However, our data show that Bax levels are slightly reduced at 24 h p.i., but not upregulated during the course of infection and CVS does not activate Bax in the majority of infected cells observed by CLSM at the early stages of infection. The discrepancy between our study and the afore-mentioned study could be due to differences in the cell types used. In addition, some RNA viruses-induced apoptosis is associated with interferon regulatory factor 3 (IRF3) activation, which interacts with Bax and translocates Bax to the mitochondria [48]. Numerous studies have reported RABV evades activation of RIG-I-mediated antiviral response and interferes with IRF-3 [32, 49-51]. It is possible that CVS initially suppresses apoptosis and avoids the immune response to maximize viral replication. Besides, many viruses have been reported to block mitochondrial apoptosis by inhibiting of Bax activation, such as M11L, encoded by myxoma virus, interacts with Bax and inhibits the conformational activation of Bax [52]; Rubella virus Capsid specifically bounds Bax to attenuate the pore-forming ability of Bax [24]; DPV022, encoded by Deerpox virus, binds and inactivates Bax to inhibit apoptosis [53]. Recently, a study reported small amount of CVS N protein was identified in the mitochondrial extracts of infected cells [54]. However, the roles of CVS N protein in the mitochondria are completely unknown so far. Although we observed CVS N protein co-located with activated Bax at the mitochondria, we could not confirm the interaction of the viral protein N and activated Bax by the coimmunoprecipitation assay (data not shown). Whether the viral protein N could bound activated Bax to attenuate the pore-forming ability of Bax, or play other functions needs further investigation.

Numerous viral proteins have been reported to directly target mitochondria for activating

mitochondrial apoptosis. These include Vpr, encoded by human immunodeficiency virus-1; X protein, encoded by hepatitis B virus; PB1-F2, encoded by influenza A virus; VP3, encoded by the avian encephalomyelitis virus; NSP4, encoded by rotavirus [55-58]. Our data reveal that CVS M protein targets mitochondria and is involved in the induction of apoptosis in neuronal cells. In addition, CVS M protein harbours a mitochondrial-targeting sequence within aa 67-79, which targets mitochondria and induces apoptosis. Sequence comparison revealed that the aa 67-79 within M proteins among different strains of lyssavirus are relatively conservative [29], suggesting that M protein induces apoptosis by targeting mitochondria may be a common feature of lyssavirus. However, most highly pathogenic strains of lyssavirus are R at position 77, such as CVS and Thailand virus [59, 60]; most low pathogenic strains are K at this position, such as MOK and Lagos bat virus[29, 61]. Therefore, residue K77 in CVS M protein not only may be related to induce apoptosis at the late stages of infection for viral dissemination, but also is probably associated with high pathogenicity. This also indicates that CVS-induced apoptosis is probably multigenic and not only related to the viral G protein [25]. M protein of the related vesiculovirus not only targets to mitochondria [62], but also interacts with nuclear pore components to block the nucleocytoplasmic transport of host RNA [63, 64]. The ability of M protein to induce apoptosis is genetically correlated with its ability to inhibit host gene expression [27]. Whether CVS M protein locating in the perinuclear region is related to inhibition of nucleocytoplasmic transport of host RNA, and then associated with induction of apoptosis needs further study. Thus, CVS M protein triggers cell death perhaps via multiple mechanisms and not only through the mitochondrial apoptotic pathway.

In summary, in this paper, we have given an insight into the molecular mechanisms of

CVS-induced apoptosis during the course of infection. We show that CVS blocks the activation of Bax, so as to delay apoptosis at the early stages of infection; while M protein targets mitochondria and triggers apoptosis at the late stages of infection. These findings deepen our understanding of the molecular mechanisms of CVS infection, suggesting that CVS perhaps have evolved a number of strategies both to inhibit and to activate apoptosis. Further investigations are required to identify the mitochondrial proteins that may interact with the viral M protein and explain the mechanisms of CVS induced apoptosis in more detail.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgements

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Figure 1. CVS-induced apoptosis is associated with caspase-9 activation. N2a cells were infected with CVS or not. After 24, 48 and 72 h p.i., cells were processed. (A) Cell viability and the intracellular ATP levels were measured. (B) Percentages of apoptotic cells and the amount of LDH released were detected. (C) Caspase-9 (Cas-9), caspase-8 (Cas-8), and caspase-3 (Cas-3) activities were detected. (D) Detection of cleaved PARP by Western blot, GAPDH as a loading control. (E) Caspase-3, -8 and -9 activities were detected in the presence of caspase-8 inhibitor (z-IETD-FMK, 50μM), caspase-9 inhibitor (z-LEHD-FMK, 50μM), or equivalent DMSO (as control) at 72 h p.i. (F) Apoptotic cells were detected in the presence of z-IETD-FMK (50μM), z-LEHD-FMK (50μM), caspase-3 inhibitor (Ac-DEVD-CHO, 50μM) or equivalent DMSO at 72 h p.i. Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

Figure 2. CVS disrupts mitochondrial membrane potential and promotes cyto *c* and AIF release. (A) JC-1 estimated mitochondrial membrane potential of CVS-infected cells at 24, 48 and 72 h p.i. (B) Detection of cyto *c* in mitochondrial (mito) and cytosolic (cyto) fraction by Western blot, prohibitin as a mitochondrial protein maker, and GAPDH as a cytosolic protein maker. (C) Western blotting analysis of AIF. (D and E) Distribution of cyto *c* and AIF observed by CLSM. CVS-infected N2a cells were fixed at 72 h p.i. and incubated with anti-cyto *c* antibody (green) (D)/anti-AIF antibody (green) (F) and anti-CVS-N antibody (red). Mock-infected cells served as controls. Nuclei (Nuc) were stained with DAPI (blue). Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

Figure 3. CVS infection blocks Bax activation. (A) N2a cells were infected with CVS or not. After 24, 48 and 72 h p.i., Bak, Bax, Bad, Bcl-2 and N were detected in immunoblotting experiments, GAPDH as a cytosolic protein maker. (B) N2a cells were infected with CVS for 24 h and then stained for CLSM using anti-Bax antibody (green), mito-tracker (red) and anti-CVS N (blue). Mock-infected cells served as controls. Nuclei (Nuc) were stained with DAPI (grey). A

higher magnification of an area is also presented (row 2 and 4). (C) Cells were fixed and immunostained for N-STORM. Images were taken and reconstructed to obtain 3D models of colocalization of CVS N (red) and activated Bax (green) proteins in the mitochondria. Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

Figure 4. N protein reduces Bax-induced apoptosis. (A) Percentages of transfected (EGFP-positive) cells undergoing apoptosis detected by the TUNEL assay. N2a cells were cotransfected with pCMV-Myc-Bax/pCMV-Myc vector (as control) and EGFP (V), EGFP-N, EGFP-P, EGFP-M or EGFP-Bcl-2. Apoptosis was detected at 24 h p.t. and the expression level of cotransfected genes was detected by Western Blot. (B) N2a cells were transfected with EGFP-N or EGFP vector for 24h. Cell lysates were probed with anti-N, anti-Bak, anti-Bax, anti-Bad, anti-Bcl-2 and anti-GAPDH, respectively in immunoblotting experiments. Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

Figure 5. M protein alone activates mitochondrial apoptotic pathway. (A) Percentages of transfected (EGFP-positive) cells undergoing apoptosis detected by the TUNEL assay. N2a cells were transfected with pEGFP-M or the pEGFP vector (as control), and processed for the TUNEL assay at 24, 48 and 72h h p.t. (B) Caspase-9, -8, and -3 activities were detected. N2a cells were transfected with pCMV-myc-M or the pCMV-myc empty vector (as control). After 24, 48 and 72 h p.t., cells were processed. (C) Effects of the viral M protein on mitochondrial membrane potential. N2a cells were transfected with pCMV-myc-M or the pCMV-myc empty vector (as control). After 24, 48 and 72 h p.t., cells were dyed with JC-1. Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

Figure 6. CVS M protein targets mitochondria. (A) Distribution of M protein observed by CLSM. CVS-infected N2a cells were fixed at 24 h p.i. and incubated with anti-TOMM20 antibody (green) and anti-CVS-M antibody (red). Mock-infected cells served as control. Nuclei (Nuc) were stained with DAPI (blue). (B) N2a cells were transfected with plasmids expressing EGFP-M or its truncated forms M 67-79, M 67-79/K77, and M67-79/P74, as well as the EGFP vector alone, and were cotransfected with plasmid vectors expressing DsRed2-Mito. The subcellular localization of

these constructs was observed at 24 h p.i. with CLSM. (C) Percentages of EGFP-M or its truncated forms, as well as the EGFP vector alone transfected cells undergoing apoptosis were measured by the TUNEL assay at 24 h p.t. Data are represented as means ± SD (n=3; *represents $P < 0.05$, **represents $P < 0.01$).

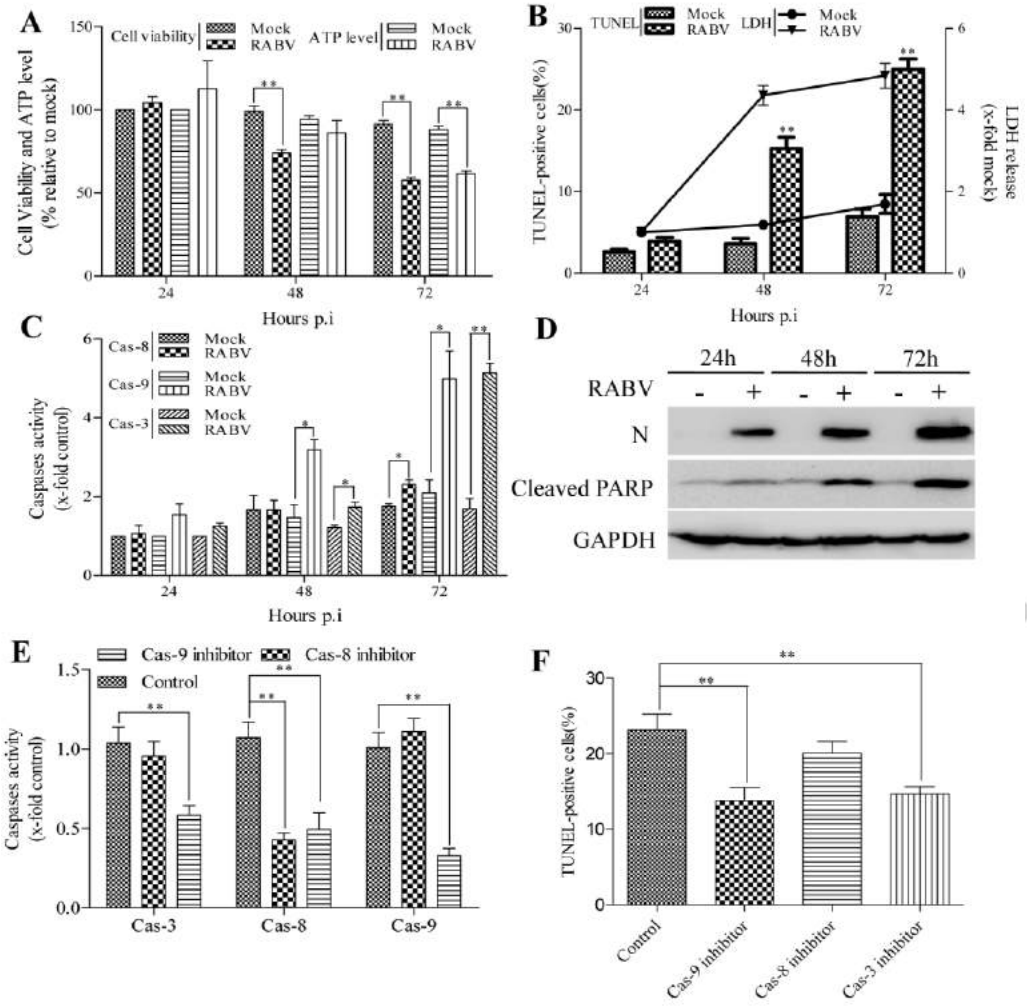
TABLE 1. Primers used for cloning

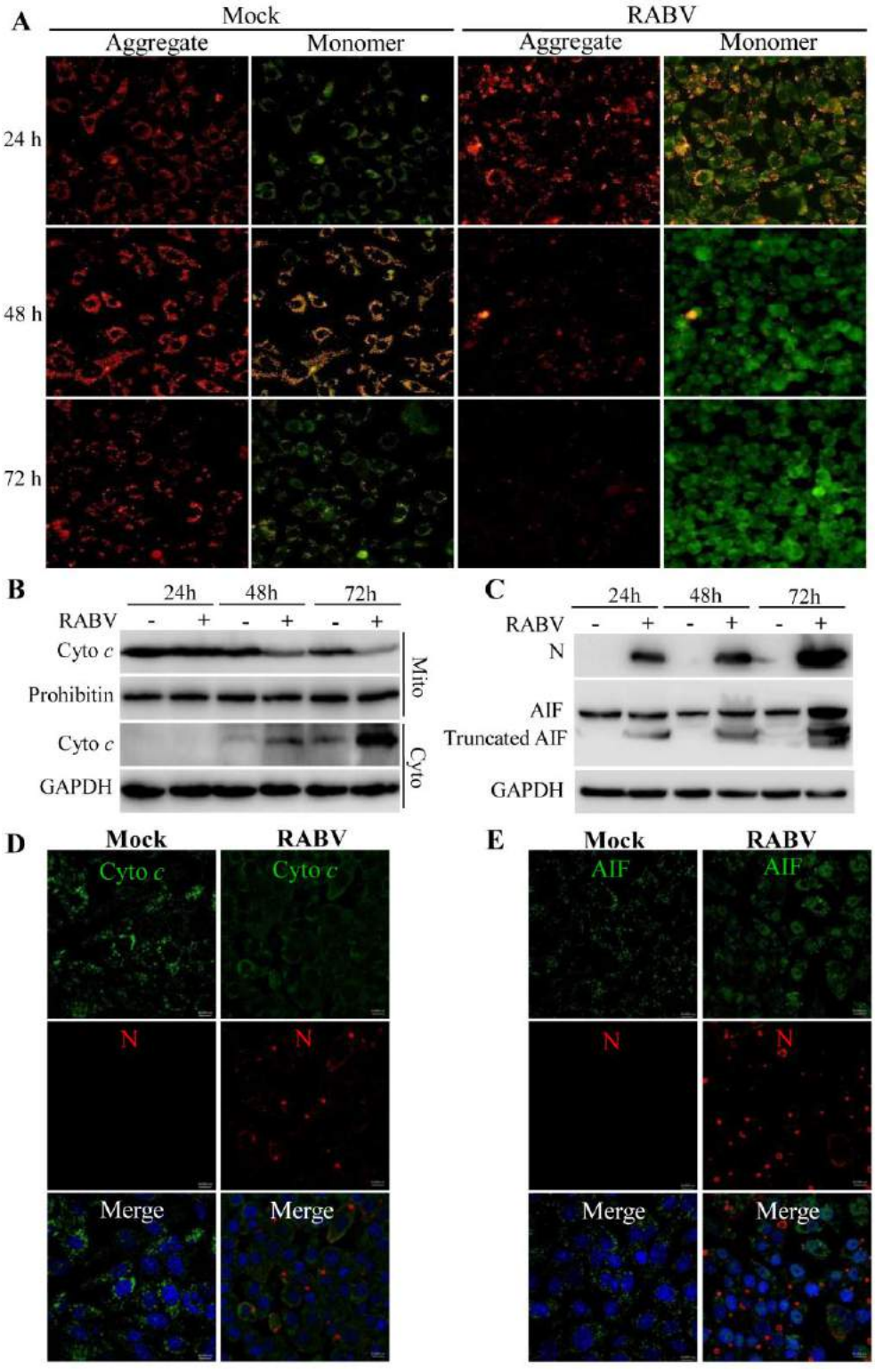
Recombinant Plasmid	Primers Sequnecse (5→3)	Restriction Site
pEGFP-C3-N	F:CCCAAGCTTATGGATGCCGACAAGA	*Hind* Ⅲ
	R:GGGGTACCTTATGAGTCATTCGAAT	*Kpn* Ⅰ
pEGFP-C3-P	F:CCCTCGAGATGAGCAAGATCTTTGTC	*Xho* Ⅰ
	R:GCGTCGACTTAGCAAGATGTATAGCG	*Sal* Ⅰ
pEGFP-C3-Bcl-2	F:CCCAAGCTTATGGCGCACGCTGGGAGAA	*Hind* Ⅲ
	R:GGGGTACCTCACTTGTGGCCCAGATAG	*Kpn* Ⅰ
pCMV-Myc-N-Bax	F:CGGAATTCGGATGGACGGGTCCGGGGAGCAG	*EcoR* Ⅰ
	R:GGGGTACCTCAGCCCATCTTCTTCC	*Kpn* Ⅰ
pCMV-Myc-N-M	F:ACGCGTCGACCATGAACGTTCTACGCAAGATA	*Sal* Ⅰ
	R:GGGGTACCTTATTCTAGAAGCAG	*Kpn* Ⅰ
pEGFP-C3-M	F:CCCAAGCTTATGAACTTTCTACGTAAGATAGT	*Hind* Ⅲ
	R:ACGCGTCGACTTATTCTAGAAGCAGAGAGGAA	*Sal* Ⅰ
pEGFP-C3-M 67-79	F:AGCTTATGTACTCGTTCAGGATCCTGCGGCACAT TCTGAGATCATTCTAAG	*Hind* Ⅲ *Sal* Ⅰ
	R:TCGACTTAGAATGATCTCAGAATGTGCCGCAGG ATCCTGAACGAGTACATA	
pEGFP-C3-M 67-79 K77	F:AGCTTATGTACTCGTTCAGGATCCTGCGGCACAT TCTGAAATCATTCTAAG	*Hind* Ⅲ *Sal* Ⅰ
	R:TCGACTTAGAATGATTTCAGAATGTGCCGCAGG ATCCTGAACGAGTACATA	
pEGFP-C3-M 67-79 P74	F:AGCTTATGTACTCGTTCAGGATCCTGCGG**CCG**AT TCTGAGATCATTCTAAG	*Hind* Ⅲ *Sal* Ⅰ
	R:TCGACTTAGAATGATCTCAGAAT**CGG**CCGCAGG ATCCTGAACGAGTACATA	

The restriction sites for cloning are underlined. F, forward; R, reverse.

Highlights

- A highly neurotropic CVS strain of rabies virus induces apoptosis in mouse neuroblastoma N2a cells via caspase-dependent and caspase-independent pathways.
- CVS blocks Bax activation at the early stages of infection to delay apoptosis, probably for viral replication.
- CVS M protein partially targets mitochondria and induces apoptosis at the late stages of infection.

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Mito
Cyto c
GAPDH
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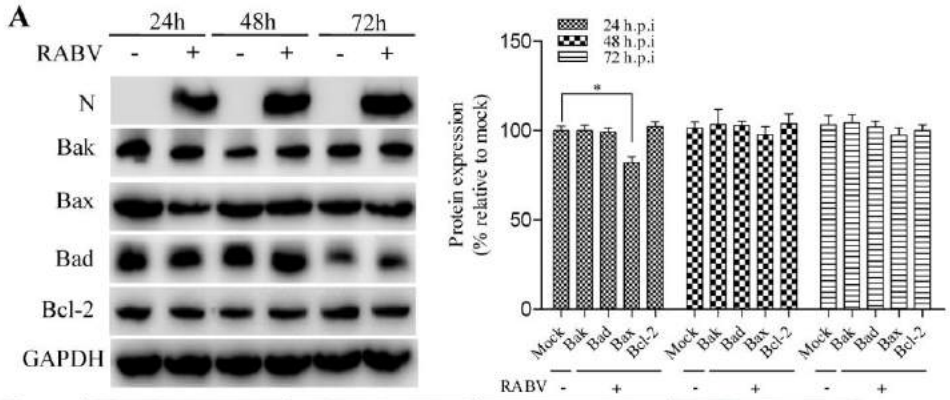

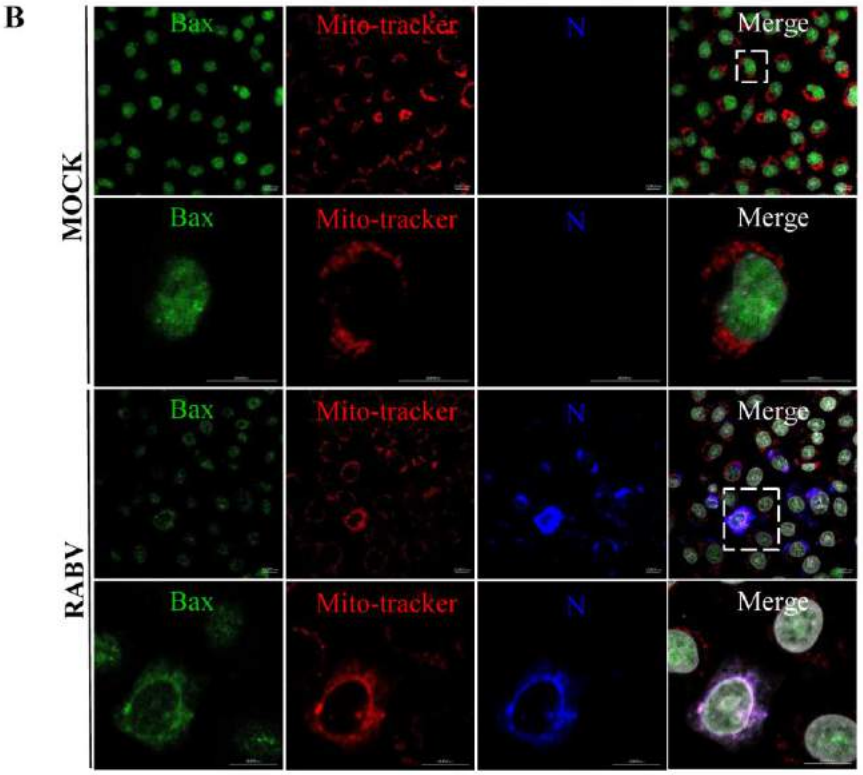

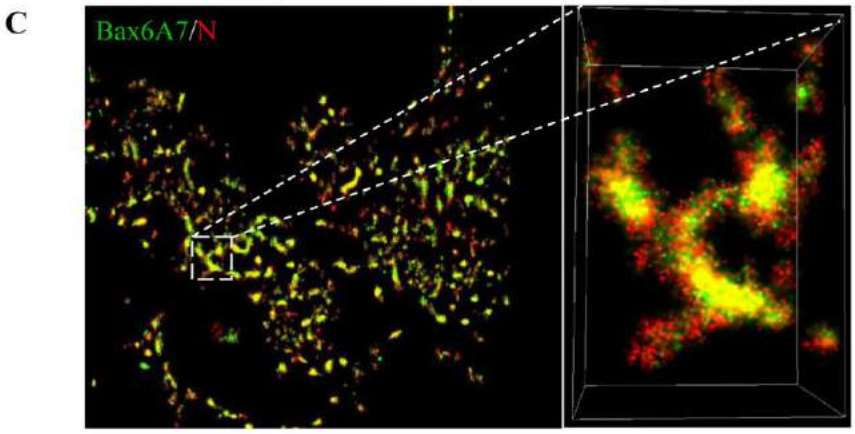

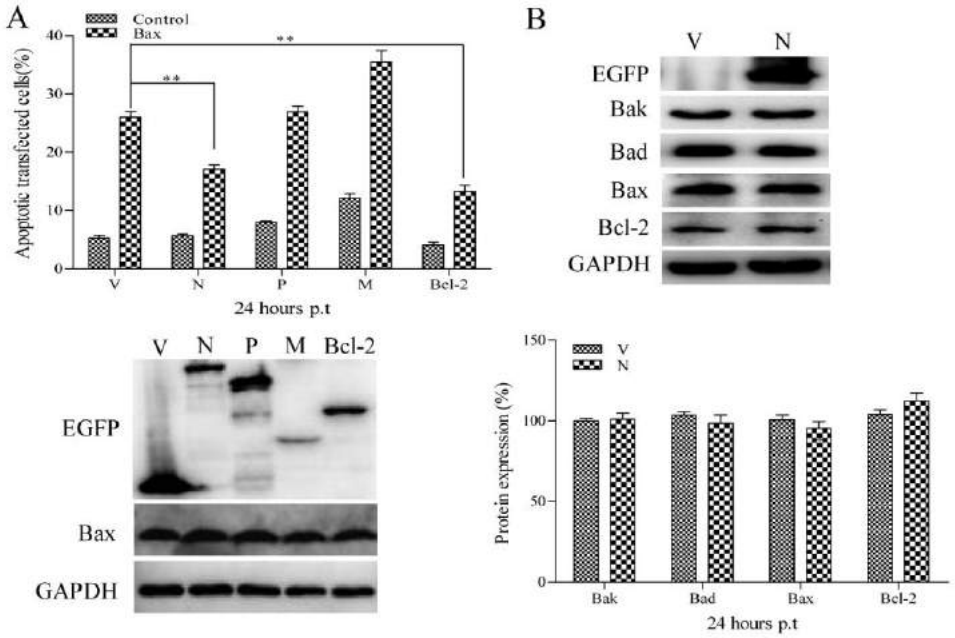

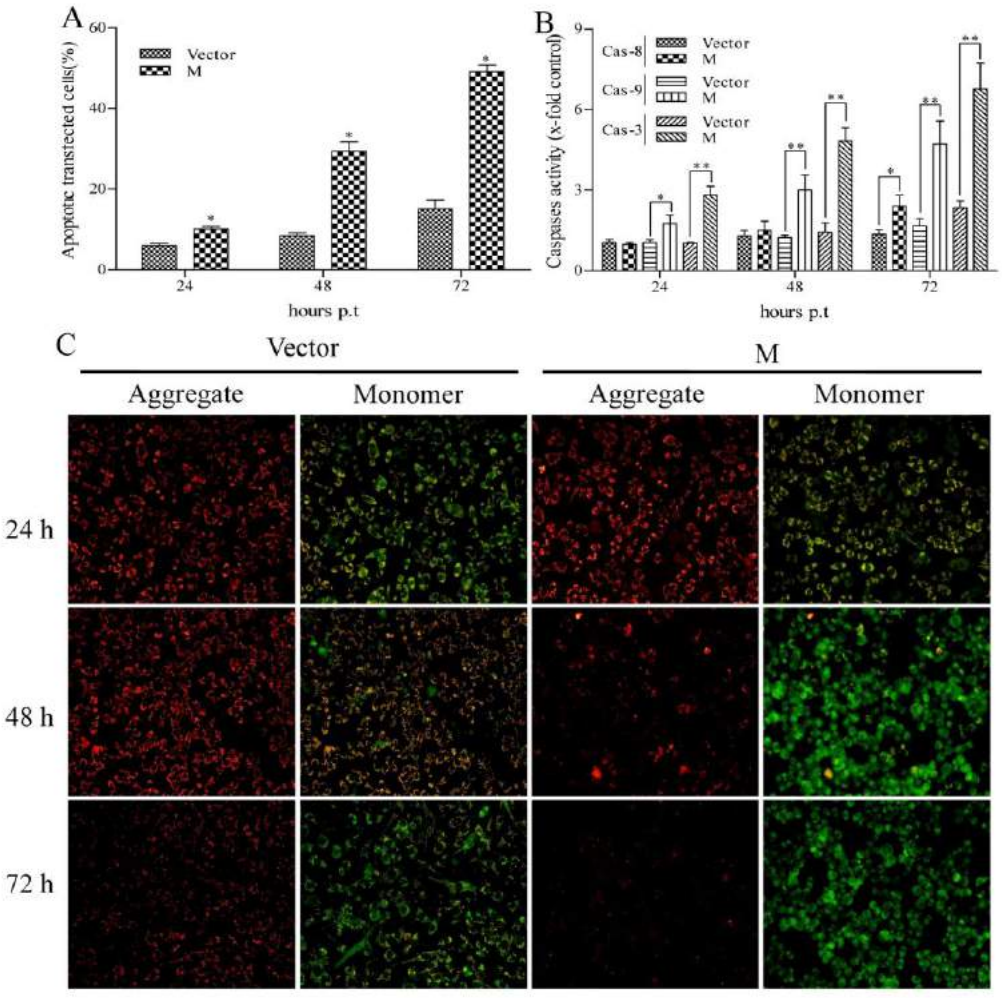
A
Apoptotic transfected cells(%)
Vector
M
hours p.t
B
Caspases activity (x-fold control)
Cas-8
Cas-9
Cas-3
Vector
M
hours p.t
C
Vector
M
Aggregate
Monomer
Aggregate
Monomer
24 h
48 h
72 h