Neurobiology of Disease 127 (2019) 570–581

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Therapeutic potential of a TrkB agonistic antibody for ischemic brain injury

Fang Han[a,b], Xiaoming Guan[c], Wei Guo[a,b,**], Bai Lu[a,b,*]

[a] School of Pharmaceutical Sciences, IDG/McGovern Institute for Brain Research, Tsinghua University, Beijing 100084, China
[b] R&D Center for the Diagnosis and Treatment of Major Brain Diseases, Research Institute of Tsinghua University in Shenzhen, Shenzhen, Guangdong 518057, China
[c] 4B Technologies Limited, Suzhou BioBay, Suzhou, Jiangsu 215123,China

ARTICLE INFO

Keywords:
Receptor tyrosine kinase
TrkB agonist
Monoclonal antibody
Drug discovery
Stroke
Apoptosis
Necroptosis

ABSTRACT

The clinical trials employing neuroprotectants targeting single, early pathogenic mechanisms in stroke have so far been barely successful. We found in human *postmortem* stroke brains that in addition to apoptosis, necroptosis also contributed to neuronal damage. Thus, a new strategy targeting both mechanisms might be necessary. While brain-derived neurotrophic factor (BDNF) is a potent survival factor for neurons, its poor bioavailability including low diffusion rate and short half-life makes it unlikely a therapeutic agent. We recently developed a TrkB agonistic antibody (Ab4B19) that mimicked BDNF functionally but exhibited better physicochemical and pharmacological features. We showed that Ab4B19 halted neuronal death *in vitro* under multiple conditions that simulate ischemia/reperfusion injury, including oxygen-glucose deprivation (OGD), glutamate toxicity, oxidative stress and nutrient deprivation. In a rat model of ischemia/reperfusion, Ab4B19 suppressed both apoptosis and necroptosis, leading to a reduction in infarct volume and acceleration of functional recovery from sensorimotor impairments. In neurons derived from human embryonic stem cells (hESCs), Ab4B19 activated TrkB and its downstream signaling, and rescued neuronal death from OGD at a similar level as that in mouse neurons. Together, our study revealed necroptosis in human stroke brain, and demonstrated a BDNF-based strategy targeting both apoptosis and necroptosis for ischemic stroke treatment.

1. Introduction

As the second biggest killer of all human illnesses, stroke affects almost 25.7 million people while taking 6.5 million lives each year, with more than half being attributed to ischemic stroke (Feigin et al., 2015). The only FDA-approved drug for ischemic stroke is tissue plasminogen activator (tPA) which dissolves clots in brain vasculature through thrombolysis (Sandercock et al., 2012). However, the utility of tPA is very limited due to its requirement for treatment within 4.5 h of stroke attack. Neuronal damage continues after ischemic stroke (Onwuekwe and Ezeala-Adikaibe, 2012), and till now, no drug targeting neuronal damages after the attack has been developed.

Various cytotoxic stimuli, such as oxygen-glucose deprivation, glutamate excitotoxicity and oxidative stress, contribute to ischemia/reperfusion injury (Onwuekwe and Ezeala-Adikaibe, 2012). Thus, neuroprotectants, which block these deleterious downstream cascades evoked by ischemia/reperfusion, were tested with the hope to slow down neuronal death. Unfortunately, nearly all neuroprotective drug candidates with positive effects in animal stroke models have so far been unsuccessful in human clinical trials (Onwuekwe and Ezeala-Adikaibe, 2012), and the neuroprotection strategy has now been challenged (Feuerstein and Chavez, 2009). There could be two major limitations in this strategy. First, the cytotoxic signaling pathways are initiated immediately after stroke while it generally takes hours or even days for stroke patients to receive proper treatments, and brain damages have already taken place when patients arrive at hospitals (Ginsberg, 2009). Second, the pathogenesis of stroke may involve multiple mechanisms, and blocking one particular mechanism might not be sufficient to stop pathological progression. Thus, a reasonable approach for stroke therapy could be to focus on later events in the pathologic cascade and halt brain damages rather than preventing the damages from occurring in the first place. Here, we propose BDNF-based neurorepair as a novel strategy to supplement neuroprotection in ischemic stroke treatment, focusing primarily on cell death and aiming to confer persistent neurotrophic support after the initial events of ischemia.

BDNF is a well-characterized member of the neurotrophin family (Nagahara and Tuszynski, 2011). BDNF binds its high affinity receptor TrkB (Squinto et al., 1991; Kaplan and Stephens, 1994), and activates its downstream MAPK, PI3K, and phosphoinositide phospholipase C-γ

* Correspondence to: B. Lu, School of Pharmaceutical Sciences, Room B303, Tsinghua University, Beijing, 100084, China.
** Correspondence to: W. Guo, School of Pharmaceutical Sciences, Room B302, Tsinghua University, Beijing, 100084, China.
E-mail addresses: wguo@tsinghua.edu.cn (W. Guo), bai_lu@tsinghua.edu.cn (B. Lu).

https://doi.org/10.1016/j.nbd.2019.04.009
Received 24 January 2019; Received in revised form 4 April 2019; Accepted 10 April 2019
Available online 11 April 2019
0969-9961/

(PLCγ) pathways (Kaplan and Miller, 2000; Huang and Reichardt, 2001), leading to multiple cellular functions including neuronal survival, dendritic growth and synapse formation. Extensive studies have shown a strong neurotrophic effect of BDNF in a variety of models for neurological disorders (Zuccato and Cattaneo, 2009; Nagahara and Tuszynski, 2011; Lu et al., 2013). In patients after ischemic stroke, it has been reported that low circulating concentration of BDNF is associated with poor functional outcome (Stanne et al., 2016; Wang et al., 2016), suggesting a role of BDNF in stroke pathogenesis and prognosis. Moreover, a number of studies have reported that intravenous administration of BDNF prior to stroke onset reduced infarct volume and improved functional recovery in animal models of stroke, partially through apoptosis suppression (Schabitz et al., 2000; Zhang and Pardridge, 2001a,b; Schäbitz et al., 2004; Zhang and Pardridge, 2006; Schäbitz et al., 2007; Lu et al., 2016). Despite all the positive modulatory and repair activities of BDNF, its short half-life, minimal tissue penetration (Dittrich et al., 1996), and non-specific activation of p75 neurotrophin receptor ($p75^{NTR}$) (Kraemer et al., 2014) hinder the development of BDNF as a therapeutic agent against ischemic stroke.

Neuronal cell death could be mediated by multiple mechanisms. In addition to apoptosis, necroptosis has now been recognized as a new player in stroke pathogenesis. Degterev et al. defined necroptosis as a type of necrotic cell death in the absence of apoptotic signaling (Degterev et al., 2005a). After the pathway is initiated, receptor-interacting protein 1 (RIP1) and RIP3 are activated to build necrosome, the core machinery of necroptosis. The executor in this necrosome, mixed-lineage kinase domain-like protein (MLKL), is then phosphorylated and oligomerized to form membrane-rupturing pores, leading to organelle swelling and eventually cell death (Hanson, 2016). Recent evidence from animal studies suggests that necroptosis also contributes to the brain infarct in ischemic strokes and necroptosis inhibitors could reduce the ischemic damage (Degterev et al., 2005a; Rami et al., 2008; Fayaz et al., 2016; Yang et al., 2017a; Cruz et al., 2018; Li et al., 2018). Moreover, combined treatments suppressing both apoptosis and necroptosis exhibited even higher levels of neuronal protection than single treatments (Xu et al., 2010b; Tian et al., 2018a). While the anti-apoptotic effect of BDNF has been well documented, whether BDNF also suppresses necroptosis has not been investigated.

To bridge the gap between animal research and human clinical studies and to develop new therapies for stroke, the present study is designed to address a number of questions: 1) whether necroptosis also exists in human stroke brain; 2) whether a BDNF-based therapy could ameliorate necroptotic cell death *in vitro* and *in vivo*; 3) whether a TrkB agonistic antibody with properties superior to BDNF could be used as a therapeutic agent in ischemic stroke. We have recently developed a TrkB agonistic antibody (Ab4B19, see the submitted accompanying article by Guo et al.) and, in this study, tested both *in vitro* and in an animal model of stroke for its therapeutic efficacy under a number of mechanisms involved in neuronal death in ischemic brains. We have also validated the mechanisms in *postmortem* human brain tissues and in human neurons derived from embryonic stem cells. Our findings may provide new insights into the mechanisms of stroke as well as the BDNF-based therapy, and pave the way for a new strategy for ischemic stroke treatment.

2. Materials and methods

2.1. Cell culture and survival assays

Primary cortical cultures were prepared from E17.5-E18.5 mouse or E18-E19 rat embryonic cortices. After dissection, the cortical tissues were digested in 0.125% trypsin at 37 °C for 20 min. Cell suspension was filtered through a 40 μm cell strainer, diluted and added to poly-D-lysine-coated plates. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% GlutaMax and 1% penicillin/streptomycin. 4 to 6 h post plating, the medium was replaced by maintenance medium, namely Neurobasal Medium supplemented with 2% B27, 1% GlutaMax and 1% penicillin/streptomycin (all reagents were from Gibco). Half of the medium was changed every 3 to 4 days. The cultures were used at DIV7 to DIV10 and were confirmed to have > 90% Tuj1-positive cells (Fig. S2A).

The hESC-derived neurons were kindly provided by S. Ding lab (Tsinghua University) following the previously reported protocol (Li et al., 2011). After 14 days of spontaneous differentiation, the cells were used for experiments.

For OGD and re-oxygenation, cells were exposed to glucose-deprived buffer (prepared as described previously (Liu et al., 2010)) in a chamber infused with 95% nitrogen and 5% carbon hydroxide for 30 min. The plate was then sealed for another 3.5 h to maintain the OGD condition. At re-oxygenation, the OGD buffer was replaced with maintenance medium containing BDNF, Ab4B19 (both 3 nM) or 0.1% bovine serum albumin (BSA) and the cells were transferred to an incubator with normal oxygen supply for another 24 h before cell viability measurement. The normal group of cells were treated identically without oxygen or glucose deprivation. To block TrkB, K252a (200 nM) was maintained in the medium throughout the experiment. To test the effect of BDNF on necroptosis, 100 μM *Z*-VAD-FMK (Selleck) and 50 μM BV6 (MCE) were added 30 min before OGD and throughout the experiment in hESC-derived cultures.

In B27-deprivation test, B27 was removed from the maintenance medium for 3 days to simulate a nutrient-deficient environment for primary neurons. BDNF, Ab4B19 (both 3 nM) or 0.1% BSA was applied to the culture at the same time of B27-deprivation. Cell viability was measured 72 h after treatment.

In glutamate-induced excitotoxicity test, a final concentration of 30 μM glutamate was maintained in the culture for 3 h to induce excitotoxicity. To rescue cell death, the glutamate-containing medium was replaced with maintenance medium containing BDNF, Ab4B19 (both 3 nM) or 0.1% BSA. Cell survival was measured 24 h afterwards.

In hydrogen peroxide-induced toxicity test, a final concentration of 500 μM hydrogen peroxide was applied to the culture together with BDNF, Ab4B19 (both 3 nM) or 0.1% BSA for 24 h before cell survival was measured.

For OGD and B27-deprivation assays, relative cell survival was quantified using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions. Hoechst33342 (Thermo Scientific) and propidium iodide (PI, P4170, Sigma) were used to label nuclei and non-viable cells and images were capture by Opera Phenix™ High Content Screening System (Perkin Elmer). For glutamate and hydrogen peroxide-induced toxicity assays, relative cell survival was measured using CCK8 reagents (Beyotime) according to the manufacturer's instructions.

2.2. Surgical procedure and drug administration

Male Sprague-Dawley rats (200–300 g) were used for *in vivo* experiments. All animal protocols were approved by Tsinghua University Animal Care and Use Committee. The MCAO surgery was performed as previously described (Longa et al., 1989). Briefly, animals were anaesthetized by isoflurane. A scission was made on the front skin of the neck and muscles were bluntly dissected to expose the right carotid artery. A suture with silica gel covering the tip (Jialing Biotech, Guangzhou) was inserted through a scission on the external carotid artery toward the internal carotid artery. The suture stopped proceeding when the middle cerebral artery was reached, about 20 mm from the scission. 2 h after occlusion, the suture was retracted to achieve reperfusion. At the initiation of reperfusion, Ab4B19 (1 or 0.2 mg/kg) or normal rabbit IgG (1 mg/kg) was injected through the tail vein. Animals of sham operation underwent the same surgery only with the suture retracted immediately after reaching MCA. These animals were injected with 1 mg/kg normal rabbit IgG except for the quantification analysis of Ab4B19 in the brain tissue, where 1 mg/kg

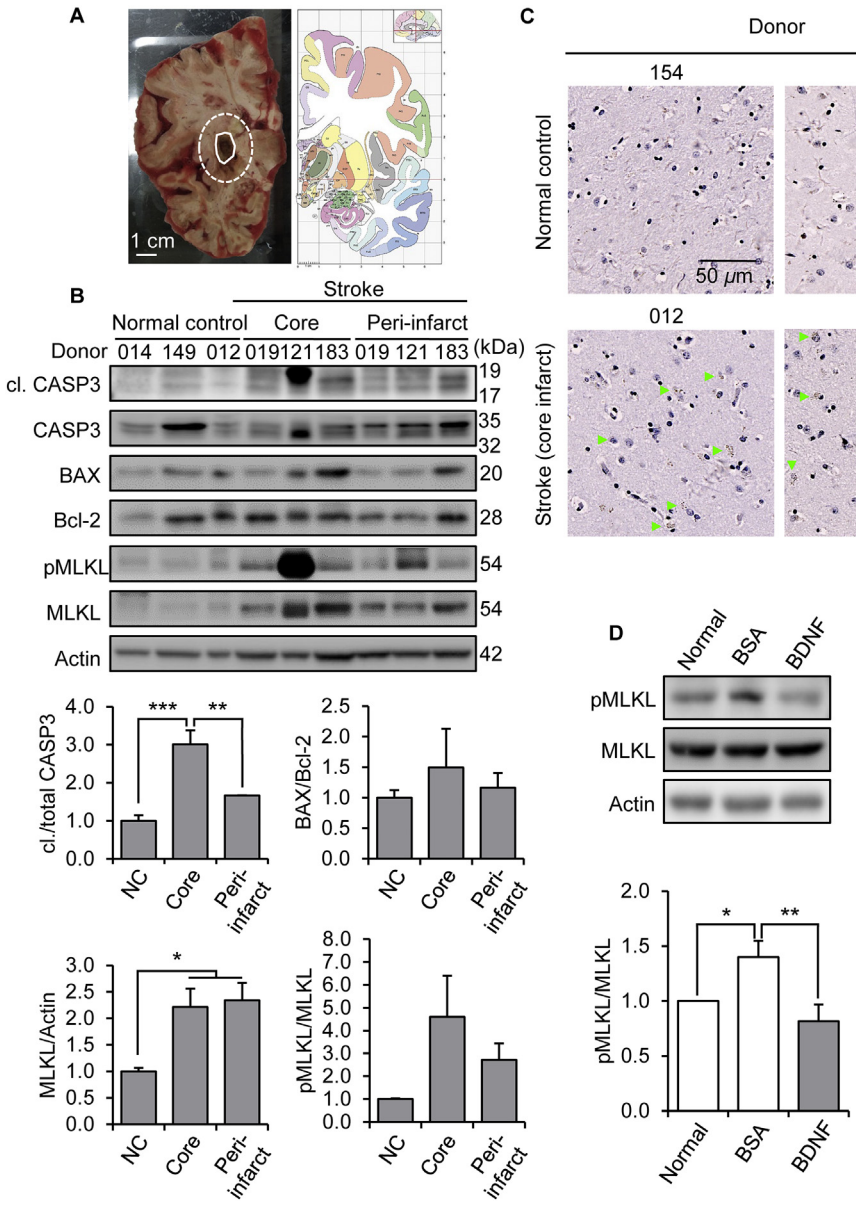

Fig. 1. Both apoptosis and necroptosis were prominent in human stroke tissues , and BDNF potentially suppressed necroptosis. (A) A representative coronal section of *postmortem* brain tissues (core, solid line; peri-infarct, dashed line) and a schematic diagram of the contralateral hemisphere (*right*). (B) Western blots (*upper*) and quantitative analyses (*lower*) of cleaved CASP3, total CASP3, BAX, Bcl-2, pMLKL and MLKL ($n = 3$). Note that normal control samples were either obtained from non-stroke donors (donor 014 and 149) or the non-stroke hemisphere of the stroke patient (donor 012). Core and peri-infarct tissues were from stroke donors 019, 121, 183. (C) Immunohistochemical staining of pMLKL in normal control and stroke brain tissues. Green arrowheads indicate cells with brown dotted pMLKL signal, which were observed in stroke brain samples but rare in control samples. Labels associate with each sample correspond to information in Table S1. (D) Effect of BDNF on MLKL phosphorylation. Neurons derived from human embryonic stem cell (hESC) were cultured under normal or oxygen-glucose deprivation (OGD) conditions for 4 h in the presence of *Z*-VAD-FMK (100 μM) and BV6 (50 μM). 0.1% BSA or BDNF (3 nM) was applied to the culture at re-oxygenation, and cell lysate was collected 16 h thereafter for MLKL phosphorylation measurement. Note that BDNF reduced MLKL phosphorylation induced by OGD and Z-VAD-FMK and BV6 treatment. Cell data were pooled from 4 independent experiments. One-way ANOVA followed by the Dunnett's multiple comparisons test was used for statistical analyses. For this and all other figures, data were presented as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ab4B19 was injected for both MCAO and sham-operated animals. For Fig. S4B and Fig. S5A, B and C, animals underwent MCAO but were not treated with any antibody so as to avoid detecting exogenous antibody in immunostaining.

2.3. Adhesive-removal test

This behavioral test was carried out as previously described with modifications (Komotar et al., 2007). Before testing, the animals were given 15 min to habituate in the testing cage. In each trial, adhesives of 7 mm × 7 mm were stick to the palms of the forelimbs, one on each side. The animal was then immediately transferred to the testing cage. Time-to-contact and time-to-remove were recorded by experimenters to whom the information of the animal was unknown. Four trials were given per test day and the average of time from the four trials were calculated as the outcome for each animal. Before MCAO, the animals were trained for 3 consecutive days.

2.4. Cylinder test

Following the previously described protocol (Hua et al., 2002), each animal was placed in a clear acrylic cylinder (diameter: 20 cm, height: 30 cm) on top of a clear plate. A mirror under the plate was used to reflect the movement of the animal when not facing the camera. A camera was used to record the rearing behaviors of the animals. The counts of contacts onto the cylinder wall with the ipsilateral forelimb (I), contralateral forelimb (C) or both forelimbs (B) were recorded by an experimenter blind to the assignment of the animal. Score (%) = (I - C)/(I + C + B) *100%.

2.5. TTC (triphenyltetrazolium chloride) assay

At 14 days post MCAO, the animals were sacrificed for infarct volume measurements. Each brain was sectioned into 2 mm slices using a brain mold. The slices were immersed in 0.2% TTC (Sigma) and incubated for 15 min in 37 °C water bath. All brain sections were scanned and analyzed using Image-Pro Plus. Infarct volume (%) = total infarct area/(2 * sum of contralateral hemisphere area) * 100%.

2.6. Ab4B19 measurement in the brain

After a thorough PBS perfusion, brain slices and striatal tissues of

both hemispheres were collected separately for immunostaining and protein extraction, respectively. For immunostaining, Alexa Fluor 488 goat anti-rabbit IgG was used to probe Ab4B19 and images were captured by Axio Scan.Z1 (Zeiss). For ELISA, human TrkB ectodomain was used to coat 96-well plates. Goat anti-rabbit antibody conjugated with HRP was used as the secondary antibody. After development using TMB, signals were measured by Cytation BioTeK plate reader. The amount of Ab4B19 was extrapolated from a standard curve prepared in the same plate. All samples were tested in duplicates. Values were calculated by the amount of Ab4B19 divided by the protein concentration measured using the Pierce BCA Protein Assay Kit (Thermo Fisher).

2.7. Human postmortem samples

Human *postmortem* brain samples were obtained with permission from the Chinese Academy of Medical Sciences and Peking Union Medical College Human Brain Bank (Zhang et al., 2018). The present study was approved by the Institutional Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Approval Number: 009–2014). For Western blotting analysis, normal controls were from tissues of 2 non-stroke brain donors (PTB014 and PTB149) and the non-stroke hemisphere of donor PTB012, and stroke tissues were obtained from 3 stroke donors, PTB019, PTB121 and PTB183. For the stroke tissues, the infarct core and peri-infarct tissues were collected separately under guidance of experienced physicians. For immunohistochemistry analysis, formalin-fixed tissues were obtained from 2 non-stroke donors PTB154 and PTB190, and the stroke tissues were obtained from the infarct region of donors PTB012 and PTB106. Further information of donors can be found in Table S1. The schematic diagram of human brain coronal section (Fig. 1A, *right*) was modified from http://www.thehumanbrain.info.

2.8. Immunofluorescence and immunohistochemistry

Tissue sections were blocked with blocking buffer (5% goat serum, 0.3% Triton ™ X-100 in 0.1 M PBS) for 1 h at room temperature followed by sequential incubation of primary and secondary antibodies overnight at 4 °C. Antibodies used for immunostaining were Tuj-1 (AT809, Beyotime) and pMLKL (ab187091). Hoechst33342 was used to indicate nuclei. The slices were mounted in mounting medium (H-1400, Vector Laboratories, InC.) for imaging. Axio Scan.Z1 (Zeiss) and Zeiss LSM780 were used for widefield and confocal imaging respectively.

2.9. Western blotting

Proteins were separated by electrophoresis in 10% or 15% SDS-PAGE gel and transferred onto PVDF membranes (BIO-RAD). Membranes were blocked for 1 h at room temperature, incubated at 4° C overnight in the primary antibody solution and incubated at room temperature for another hour in the secondary antibody solution. SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific) was used for blot development and signals were detected by Tanon 5200. The bands were analyzed using ImageJ. The primary antibodies used for immunoblotting were TrkB (Rabbit, CST), pTrkB (Rabbit, CST), Akt (Rabbit, Easybio), pAkt (Rabbit, CST), PLCγ (Rabbit, CST), pPLCγ (Rabbit, CST), ERK (Rabbit, CST), pERK (Rabbit, CST), GAPDH (Mouse, Easybio), BAX (ab32503, Abcam), Bcl-2 (sc-7382, Santa Cruz), MLKL (E2A7412-1-2-3, EnoGene), pMLKL (ab196436 for mouse and rat antigen and ab187091 for human antigen, Abcam), cleaved CASP3 (9664, CST), RIP1 (610,459, BD Transduction Laboratories), GFAP (ab134436, Abcam), TrkB (sc-377,218, Santa Cruz) and Actin (AA128, Beyotime).

2.10. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis were performed with GraphPad Prism using one-way ANOVA followed by the Dunnett's *post hoc* multiple comparisons test, or two-way ANOVA followed by the Holm-Sidak's *post hoc* multiple comparisons test. Asterisks were used to indicate significance of difference (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$).

3. Results

3.1. Human stroke tissues exhibited both apoptosis and necroptosis

Although recent studies have suggested apoptosis as well as necroptosis are involved in neurodegenerative diseases such as Alzheimer's disease (Caccamo et al., 2017), few studies have validated in human disease samples that necroptosis also occurred in stroke. To identify cell death mechanisms involved in human stroke, we have obtained *postmortem* specimens of stroke and non-stroke brain tissues and examined cell death markers using immunohistochemistry and Western blotting analyses. We found that apoptosis and necroptosis were highly prevalent in the core regions (darkened focal area on the *postmortem* sample) of the stroke brain tissues (Fig. 1A to C). A marker for apoptosis, namely the ratio of cleaved CASP3 to total CASP3 was significantly increased in the core area, but not in the peri-infarct regions, comparing to the normal control (3.0-fold, Fig. 1B). There was also a change in another apoptosis marker - the ratio between Bcl-2 (B-cell lymphoma 2) and BAX (Bcl-2-associated X protein), although it did not reach the statistically significant level due possibly to the small sample size and the variability in the disease conditions of the samples (Fig. 1B**).**

The protein level of MLKL, the executor of necroptosis, was also increased in the core regions of stroke (2.2-fold, Fig. 1B). Unlike the apoptosis marker, the level of MLKL was higher in the peri-infarct regions as well, suggesting the existence of necroptosis in both regions (2.3-fold, Fig. 1B). The pMLKL/MLKL ratio, another way of revealing necroptosis, was also higher in the core and penumbra regions (Fig. 1B), although not statistically significant possibly due to the aforementioned reasons. In addition, we found that many cells in the core region of stroke brain tissues were positively stained with pMLKL (Fig. 1C). Additionally, we discovered that RIP1, an upstream regulator of both apoptosis and necroptosis (Degterev et al., 2008), was significantly upregulated in the core region of tissues derived from stroke patients (Fig. S1). Thus, our data support the notion that necroptosis may contribute to neuronal damages in the infarct region. These results have provided, to our knowledge, the first direct evidence for a role of necroptosis in human stroke.

3.2. BDNF elicited necroptosis-suppressing effects in human ESC-derived neurons

BDNF has previously been shown to be anti-apoptotic under ischemic conditions (Van Kanegan et al., 2014). As a first step toward identifying means that could ameliorate necroptosis during ischemic stroke, we determined whether BDNF could also be anti-necroptotic. In human embryonic stem cell (hESC)-derived neurons with mesencephalic regional identity (Li et al., 2011), necroptosis was significantly induced by oxygen-glucose deprivation (OGD) together with the treatment of BV6, a mitochondria-derived activator of caspase (smac) mimetic (Laukens et al., 2011), when apoptosis was completely inhibited by the caspase inhibitor *Z*-VAD-FMK. BDNF (3 nM) or bovine serum albumin (BSA, 0.1%) was applied to the culture at the time of re-oxygenation, and the level of necroptosis, indicated by MLKL phosphorylation was measured 16 h thereafter. Under these conditions, the ratio of pMLKL/MLKL was significantly elevated in the BSA control group (1.4-fold *vs* normal, Fig. 1D). In contrast, in cells treated with

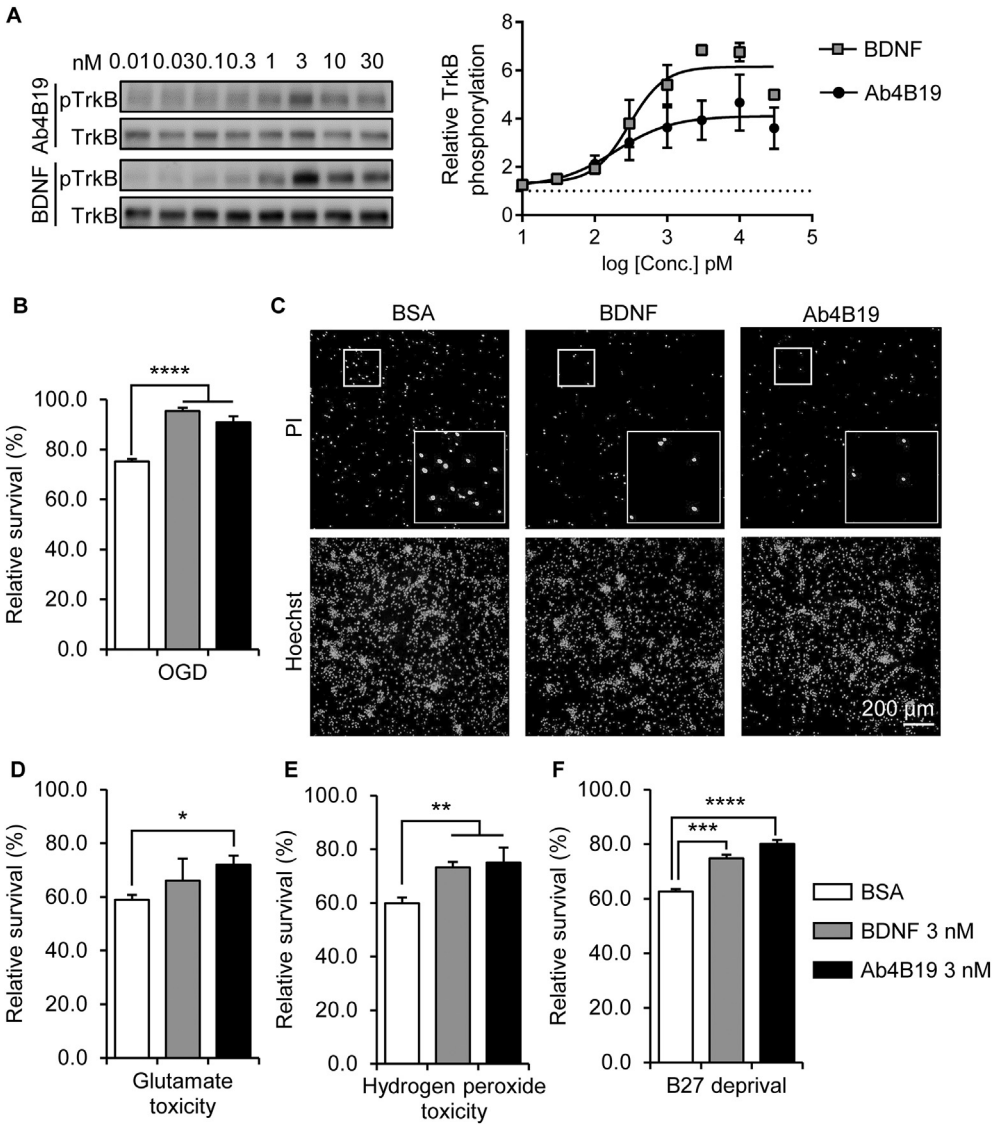

Fig. 2. Ab4B19 activated TrkB and rescued neuronal death under various cytotoxic conditions. (A) Dose-response curve for TrkB activation by BDNF or Ab4B19. In cultured cortical neurons, Ab4B19 (filled circles) induced dose-dependent activation of TrkB in a similar manner as BDNF (grey squares). BDNF or Ab4B19 (both 3 nM in this and all other panels) was applied to the culture (DIV10) and cells were harvested and processed 30 min later for Western blotting (WB) using anti-phospho-TrkB and anti-TrkB antibodies. (B-C) Ab4B19 suppressed neuronal death induced by OGD. After 4 h of OGD, Ab4B19 or BDNF was applied to the cultured cortical neurons (DIV 7–10) and cell viability was measure by both ATP-based (B) and plasma membrane integrity-based assays (C). (D-F) Ab4B19 improved neuronal survival under various insults, including (D) glutamate toxicity (30 μM), (E) hydrogen peroxide (500 nM) and (F) nutrient (B27) removal. BDNF or Ab4B19 was applied to the cultures 3 h after (D) or at the onset (E and F) of the insult. Data were from at least triplicates and were representative of at least three independent experiments. One-way ANOVA followed by the Dunnett's multiple comparisons test was used.

BDNF, the same OGD treatment failed to induce the elevation of pMLKL/MLKL (0.8-fold *vs* normal, Fig. 1D), suggesting that BDNF potentially suppressed necroptotic signaling under this ischemia/re-oxygenation condition.

3.3. Ab4B19 protected primary neurons from ischemia/reperfusion-relevant cytotoxic challenges

BDNF is not an ideal molecule for therapeutic use given its highly basic nature, poor pharmacokinetic properties, and its activation of the death receptor $p75^{NTR}$ (Hempstead, 2002). Using the ectodomain of human TrkB protein, we have developed a number of highly specific and potent agonistic monoclonal antibodies that could mimic BDNF and activate TrkB without affecting $p75^{NTR}$ (Guo et al., submitted). A series of experiments were performed to determine whether these TrkB agonistic antibodies have the potential to be therapeutic agents for stroke. Among the antibodies, TrkB agonist Ab4B19 was a highly specific and potent agonistic antibody with good drug-like properties (Guo et al., submitted). Ab4B19 binds exclusively to TrkB and not to any other neurotrophin receptors (Guo et al. submitted). Application of Ab4B19 to primary cortical neurons elicited a marked activation of TrkB in a manner very similar to BDNF, as revealed by the Western blots showing phosphorylation of TrkB on tyrosine 516 (Fig. 2A). Dose-response experiment revealed that Ab4B19 and BDNF stimulated the peak of TrkB phosphorylation at around 10 nM and 3 nM, respectively (4.7- and 6.8-fold *vs* BSA control, Fig. 2A). Ab4B19 also achieved comparable activation of TrkB downstream molecules, including PLC-γ, protein kinase B (Akt) and extracellular signal–regulated kinase (ERK) (Fig. S2B, C and D, respectively), as BDNF.

To investigate the effects of Ab4B19 on neuronal survival under stroke conditions, we challenged mouse cortical neurons in culture with multiple cytotoxic conditions including OGD (Fig. 2B and C), excessive glutamate- (Fig. 2D) and hydrogen peroxide-induced toxicity (Fig. 2E) and nutrient-deprivation (Fig. 2F) that mimic the multiple mechanisms contributing to ischemia/reperfusion brain injury. Ab4B19 effectively attenuated neuronal damages under all these challenges (Fig. 1B to F) when added 3 or 4 h after (glutamate toxicity and OGD) or at initiation of the insult (hydrogen peroxide toxicity and B27-deprivation). In addition, the pro-survival effects elicited by BDNF and Ab4B19 were completely abolished by the pan Trk inhibitor, K252a (200 nM), which suggested that the effects were mediated by the activation of TrkB (Fig. S2E). These results demonstrated the capacity of Ab4B19 to efficiently activate TrkB signaling, and revealed its potential in rescuing neuronal survival under multiple cytotoxic mechanisms contributing to ischemia/reperfusion injury.

3.4. Ab4B19 penetrated into ischemic brain tissues and activated TrkB

While it is generally difficult for large molecules such as antibodies to pass the blood-brain barrier (BBB), it is reported that BBB is compromised after ischemia/reperfusion (Shi et al., 2016). Therefore, it was crucial to determine whether Ab4B19 can reach the brain parenchyma under ischemia/reperfusion. We used rat middle cerebral artery occlusion (MCAO) as a stroke model to examine the CNS (central nervous system) delivery of Ab4B19. After a 2-h ischemia, the suture was retracted from the MCA to achieve blood reperfusion. Ab4B19 (1 mg/kg body weight) was administered intravenously at the same time through tail-vein (Fig. 3A). Indeed, BBB was significantly compromised on the side of MCAO. After a thorough cardiac perfusion to remove circulating Ab4B19, immunostaining with a goat anti-rabbit IgG antibody was applied to visualized the diffusion of the therapeutic IgG (Ab4B19) into the parenchyma from the occluded arteries, which showed drug delivery in the focal area (dashed lines in Fig. 3B). In contrast, neither the sham operated brain nor the self-controlled contralateral side of the ischemic brain showed any notable fluorescent signal, indicating that the amount of residual Ab4B19 attached to the capillary was negligible compared to that diffused into the parenchyma. We also discovered that Ab4B19 diffused into the ischemic area overlapped with the elevated TrkB signal (Fig. S4A), suggesting successful target binding. These TrkB-upregulated cells were not labeled by either the microglial cell marker Iba1 or the astrocyte marker GFAP (Fig. S4B). Rather, these cell exhibited morphologic features of neurons, which implies that Ab4B19 acted primarily on neurons in the ischemic region, and was 'engaged' with its target, TrkB. To quantify the Ab4B19 penetration, we collected ipsilateral and contralateral striatal tissues of sham-operated and MCAO animals at 6 h post reperfusion. MCAO significantly damaged the BBB and increased Ab4B19 retention (0.2 and 1.4 ng Ab4B19/g protein for sham and MCAO operation, respectively) in the ipsilateral striatum after 6 h of reperfusion (Fig. 3C). In addition, MCAO specifically opened up BBB of the focal area on the ipsilateral side but not the contralateral side (1.4 and 0.1 ng Ab4B19/g protein for the ipsilateral and contralateral side, respectively), as measured by ELISA using a standard curve of Ab4B19 (Fig. 3C).

To examine whether peripheral administration of Ab4B19 indeed induced TrkB signaling in the brain *in vivo*, we measured the expression level of EGR1, a downstream gene often used as a biomarker for TrkB activation and TrkB target engagement (Merkouris et al., 2018), in the ipsilateral cortices of the animals 24 h after surgery. As previously reported, EGR1 expression was slightly elevated in MCAO groups due to ischemia (Honkaniemi and Sharp, 1996) (Fig. 3D, first two columns in the bar graph). In contrast, the EGR1 levels were dramatically increased after Ab4B19 treatment (Fig. 3D), suggesting that TrkB activation was markedly induced by Ab4B19.

3.5. Ab4B19 accelerated sensorimotor recovery in the rat stroke model

To evaluate the therapeutic potential of Ab4B19 for functional recovery after stroke, the effects of Ab4B19 at two intravenous injection doses (0.2 and 1 mg/kg) were evaluated in the MCAO model. Sham-operated animals injected with normal IgG (1 mg/kg) served as the healthy controls. 24 h after reperfusion, all animals were scored using the Bederson scale to validate the successful model establishment (Bederson et al., 1986). A higher score indicates a more severe neurologic deficit. At 24 h post operation, the Bederson score of animals underwent MCAO jumped to 2 while remained 0 among animals of sham operation. No statistically significant difference was observed between MCAO animals treated with IgG and Ab4B19 at this time point (Fig. S3A).

We assessed sensorimotor functions and spontaneous forelimb use by the adhesive-removal test and the cylinder test (Komotar et al., 2007; Wang et al., 2010). In the adhesive-removal test, sensory and motor functions were measured respectively by the time an animal used to sense and remove adhesives placed on each of its forelimb palms. For the ipsilateral forelimbs (relative to the injured brain), the sensory and motor functions were slightly affected at day 3 post surgery, but recovered quickly at day 5 (Fig. S3B and S3C). In contrast, the sensorimotor function of the contralateral forelimbs (controlled by the damaged brain) was severely impaired, as was shown by the increased time used to sense and remove the adhesives (Fig. 4A and B). Treatment with Ab4B19 at both 0.2 and 1 mg/kg doses significantly reduced "time-to-contact", suggesting the recovery of sensory function (Fig. 4A). Ab4B19 treatment at 1 mg/kg but not 0.2 mg/kg also elicited better motor function on day 3 and 5 post surgery (Fig. 4B). Similarly, in the cylinder test, animals after MCAO preferably used the forelimbs controlled by the unlesioned brain. We observed on day 5 that 1 mg/kg but not 0.2 mg/kg Ab4B19 treatment ameliorated the preferred use of the unimpaired forelimb (reduced cylinder test score), suggesting a better recovery of the impaired motor cortex (Fig. 4C). Thus, adequate Ab4B19 treatment was able to accelerate functional recovery in animals after ischemia/reperfusion.

3.6. Ab4B19 suppressed both apoptotic and necroptotic cell death induced by ischemia/reperfusion

The loss of sensory motor function is directly associated with infarct volume in the ischemic brain. We thus measured the infarct volumes after surgery by TTC staining. TTC is a redox indicator that turns red when reduced by dehydrogenase in living tissues. While the brain sections turned red in the sham group of animals, the infarcts in MCAO animals remained as white areas or liquefactive tissue loss (Fig. 5A). Quantification of the infarct volume over total volume of brain sections showed that 1 mg/kg Ab4B19 conferred a significant infarct reduction of 11.7%, comparing to the normal IgG-treated animals (21.4% *vs.* 33.1%, $P = .035$). Treatment with 0.2 mg/kg Ab4B19 also reduced the infarct ratio by 8% (25.1% *vs.* 33.1%), although the reduction did not reach a statistically significant level (Fig. 5A).

To investigate the molecular mechanisms of Ab4B19 rescuing cell death, we firstly examined apoptosis pathways by measuring protein expression and activation of apoptosis markers. Bcl-2 and BAX are crucial mediators of apoptosis and the ratio of BAX/Bcl-2 is often used as an indicator of the level of apoptosis (Liu et al., 2013). In this case, only the ipsilateral cortical tissues from MCAO animals treated with IgG control exhibited a significant 4.2-fold elevation in the ratio of BAX/Bcl-2 comparing to the sham group ($P = .002$). Ab4B19 treatment significantly suppressed this increase to a level of 1.2-fold (Fig. 5B, $P = .001$). Similarly, activation level of the apoptosis executor, CASP3, was significantly elevated (1.6-fold, $P = .005$ comparing to the sham group) in the ipsilateral tissues of IgG-treated animals as was delineated by the ratio of cleaved CASP3 over total CASP3 (Fig. 5B). This ratio was dramatically reduced in the tissues of Ab4B19-treated stroke animals, compared with the IgG-treated animals, reaching a level similar to the sham group (Fig. 5B). These results indicate that Ab4B19 suppressed apoptosis after brain ischemia/reperfusion injury.

To determine whether Ab4B19 also suppressed necroptotic cell death, we measured the level of necroptosis signaling in the ipsilateral cortices of animals with or without Ab4B19 treatment. In line with our results in human *postmortem* samples, RIP1 was upregulated in cortical tissues subjected to ischemia/reperfusion injury (Fig. S5D). Interestingly, Ab4B19 treatment had no effect on this elevation of expression (Fig. S5D). Similar to previous reports (Yang et al., 2017a; Chen et al., 2018), MLKL phosphorylation was significantly increased in the IgG-treated MCAO animals (1.8-fold comparing to the sham group, $P = .002$). Consistent with our data acquired in cultured neurons treated with BDNF, MLKL phosphorylation was significantly suppressed by Ab4B19 treatment ($P = .0004$, Fig. 5C) in stroke animals. Thus, it is possible that TrkB activation by Ab4B19 suppressed necroptosis in the downstream execution stage rather than the early stages of necroptosis.

To determine the cell types that underwent apoptosis and

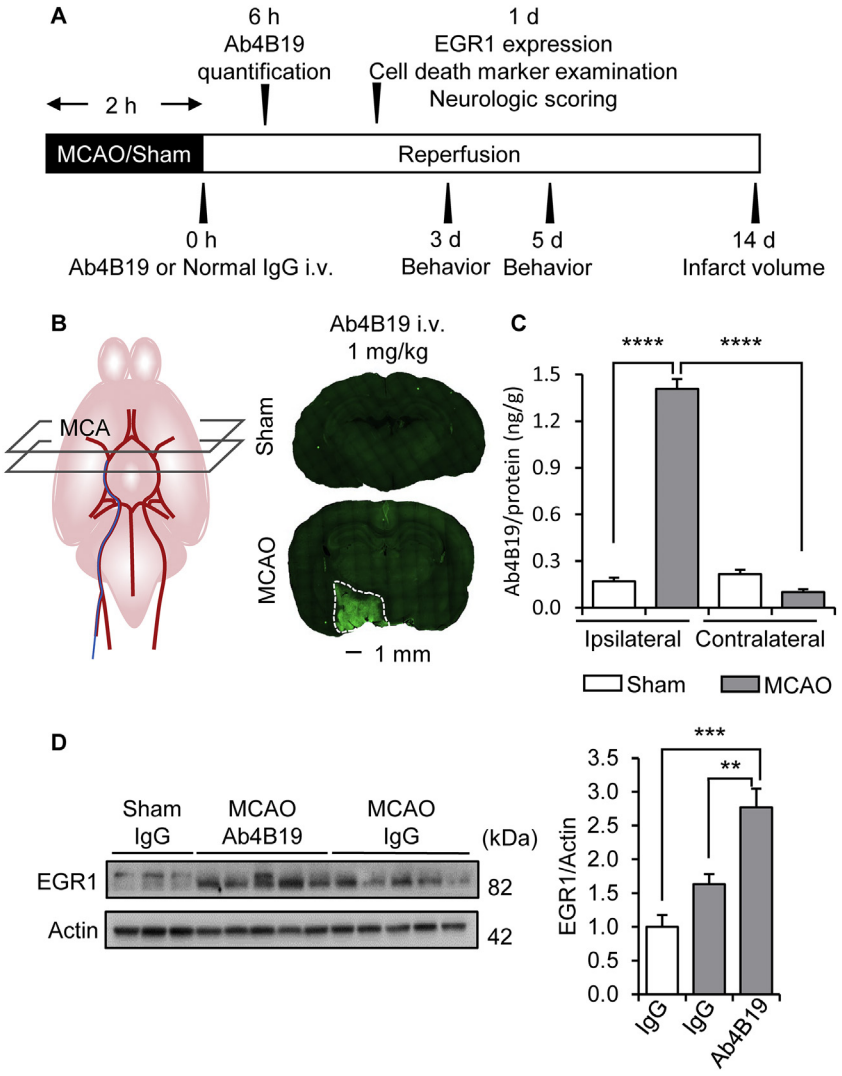

Fig. 3. Experimental design and target engagement in animals underwent ischemia/reperfusion. (A) Experimental design. Animals underwent MCAO (or sham operation) for 2 h before reperfusion and drug administration. Behavioral and pathological experiments were carried out at indicated time points. (B) Penetration of Ab4B19 (white dashed area) into the brain parenchyma. *Left*, schematic diagram of MCAO (modified from Zhang et al.. (Zhang et al., 2015)). *Right*, immunostaining of Ab4B19 detected by a fluorophore-conjugated secondary antibody. (C) Quantification of Ab4B19 penetration in both sides of striatal tissues from sham (the open bar) and ischemia/reperfusion animals (filled grey bars) by ELISA. (n = 3) (D) Western blots (*left*) and quantitative analysis (*right*) of Ab4B19-induced EGR1 expression in injured cortical tissues (n = 3 to 5 rats). Statistical analyses were carried out using two-way ANOVA followed by the Holm-Sidak's multiple comparisons test and one-way ANOVA followed by the Dunnett's multiple comparisons test for (C) and (D), respectively.

necroptosis, we planned to perform double-staining experiments using both the cell death and cell type markers in MCAO animals after treatment with Ab4B19 which is a rabbit monoclonal antibody. Unfortunately, the only commercially-available primary antibody for cleaved CASP3 and pMLKL were also of the rabbit origin. Signal cross-interference made it impossible to directly perform such experiments in Ab4B19-treated animals. We therefore took an indirect approach: to detect cell death markers in stroke animals without Ab4B19 treatment. In terms of necroptosis, notable pMLKL signal was detected 6 h post reperfusion in the cortex and hippocampus of stroke brains (Fig. S5A and S5B). The signal was more intense in the ipsilateral side of injury, where the signal intensity of NeuN-labeled neurons decreased due to cell death (Fig. S5A, third column, compare left and right). Through co-labeling analysis, we found that only a small proportion of pMLKL-positive cells (11.6%) were GFAP positive while 72% of pMLKL-positive cells were co-labeled by NeuN, indicating that neurons are more prone to necroptosis than astrocytes under this condition (Fig. S5C). We have also noticed that the pMLKL signal was mainly restricted to the nucleus, which is consistent with previous reports that MLKL was activated in the nucleus before exported for oligomerization (Yoon et al., 2016; Weber et al., 2018).

Given the extensive literatures reporting that neurons are susceptible to apoptosis after ischemia/reperfusion injury, it could be similarly concluded that majority of apoptotic cells in the stroke brain were neurons as well. Taken together, these results suggest that it is primarily neurons, rather than glia, that undergo apoptosis and necroptosis after stroke attack.

3.7. Ab4B19 activated TrkB signaling and rescued OGD-induced cell death in cultured human neurons

To determine whether the pro-survival effects of Ab4B19 found in our animal studies have any relevance in human ischemia, we further employed hESC-derived neuronal cultures to validate the functionality of Ab4B19 in human cells. These hESC-derived neurons expressed the neuronal marker Tuj-1 (Fig. 6A), and exhibited neuronal morphologies after 2 weeks of differentiation under the previously reported protocol (Li et al., 2011). We found that application of Ab4B19 to the human neurons activated TrkB at 3 nM (Fig. 6B). Moreover, the PLC-γ, Akt and ERK pathways were also activated by Ab4B19 in levels close to BDNF (Fig. 6B). We then assessed the potency of Ab4B19 in rescuing hESC-derived neurons under a condition relevant to human ischemia. As described in Fig. 2B, these human neurons were incubated in OGD buffer for 4 h before returning to the normal culture condition. Treatment of this culture with Ab4B19 or BDNF at the onset of re-oxygenation significantly increased neuronal survival, achieving a similar level of rescue in these human neurons as in mouse cultures (Fig. 6C comparing to Fig. 2B). With the aforementioned functional and pathological results in animal stroke models, the results obtained from cells of human origin indicate that Ab4B19 is capable of rescuing

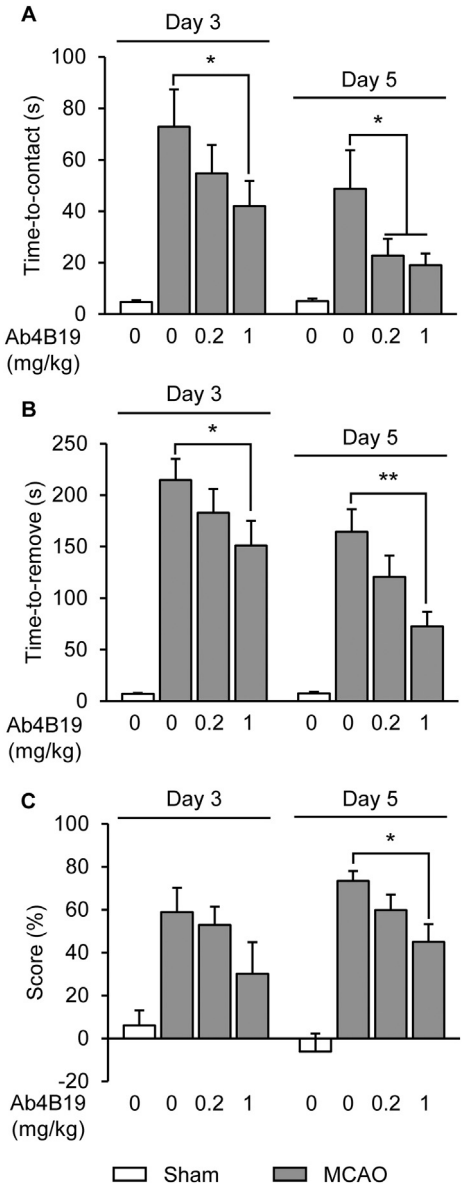

Fig. 4. Ab4B19 treatment improved sensorimotor functions of ischemia/reperfusion animals. Animals underwent sham operation or ischemic injury received intravenous injection of normal IgG (1 mg/kg) or Ab4B19 (0.2 or 1 mg/kg) right at reperfusion, which is 2 h post MCAO. Behavioral tests were done on day 3 and day 5 post surgery. Tactile response (A) and motor functions of the forelimb (B) were assessed by time-to-contact and time-to-remove in the adhesive-removal test. Spontaneous forelimb use was assessed by the score of ipsilateral and contralateral limb using in the cylinder test (C). Animals not treated with Ab4B19 were administered 1 mg/kg normal IgG instead. $n = 9$ to 16 for each group of animals. Two-way ANOVA followed by the Holm-Sidak's multiple comparisons test was used for statistical analyses.

neuronal death and activating TrkB of both species, suggesting a potential of Ab4B19 as a therapeutic agent for ischemic stroke in human.

4. Discussion

The treatments of stroke based on neuroprotection strategy have nearly all failed to be translated in the clinic (Neuhaus et al., 2017). This is due at least in part to the fact that virtually all of these approaches focused on a single mechanism of neuroprotection, while ischemic stroke is a complex and evolving process that involves multiple pathological mechanisms. In this study, we demonstrated that necroptosis, besides apoptosis, may be one of the key mechanisms underlying neuronal damages in stroke patients. An alternative strategy, therefore, would be to focus on enhancing neuronal survival so that the disease progression could be halted or slowed down regardless of its causes. Mounting evidence suggests that the BDNF-TrkB signaling pathway is an ideal candidate for this approach. However, attempts to use BDNF as a pharmacological agent have been unsuccessful because of the intrinsic problems of BDNF protein itself (Ochs et al., 2000; Kalra et al., 2003; Beck et al., 2005). Here, we tested whether the BDNF-based strategy as well as a newly discovered BDNF mimetic could be used to treat ischemic stroke. Ab4B19 is a TrkB-activating therapeutic antibody with pharmacological properties superior to BDNF. In the rat ischemia/reperfusion model, Ab4B19 decreased infarct volumes, suppressed both apoptotic and necroptotic signaling, and rescued behavioral deficits. The effects of Ab4B19 on halting ischemic neuronal death were validated in cultured neurons derived from hESCs. Taken together, these results pave the way for a new therapy for stroke using Ab4B19.

There were over 1000 neuroprotectants studied in clinical trials (Xiong et al., 2018); besides tPA, none has been approved by FDA for stroke treatment. A large portion of neuroprotectants target specific toxic pathways effecting in the early stages of stroke (Wahlgren and Ahmed, 2004). Among these, the most prominent ones in pre-clinical studies include ion channel blockers (*e.g.* nimodipine), NMDA antagonists (*e.g.* dextrorphan), antioxidants (*e.g.* NXY-059) and immune response-suppressing agents (*e.g.* enlimomab) (Wahlgren and Ahmed, 2004). Lack of clinical success calls for alternative strategies of combination therapies targeting multiple pathways and different stages of stroke progression. (Majid, 2014).

Apoptosis is one of the major cell death pathways and was believed to be both a critical player in stroke pathogenesis and a potential stroke therapy target (Sun et al., 2015). However, studies with apoptosis inhibitors have failed to reduce neuronal injury in global ischemia (Li et al., 2000), indicating that mechanisms other than apoptosis may also contribute significantly to neuronal death during stroke. Indeed, recent studies have identified a new form of cell death known as necroptosis that is mechanistically distinct from apoptosis (Degterev et al., 2005b). During necroptosis, the intracellular signaling molecules RIP1, RIP3 and MLKL were activated through sequential phosphorylation (Sun et al., 2012). As the mechanism of necroptosis and the executive function of MLKL were revealed (Dondelinger et al., 2014; Wang et al., 2014), the role of this form of cell death has been examined in several neurological disorders, such as amyotrophic lateral sclerosis (ALS) (Ito et al., 2016) and Alzheimer's disease (AD) (Caccamo et al., 2017). For example, it was reported that the ratio of pMLKL over MLKL is elevated in the 5xFAD AD mouse model and the expression level of necroptosis markers was increased in AD patients (Caccamo et al., 2017). A number of recent studies suggest that necroptosis may be involved in stroke. The expression of RIP1, RIP3 and MLKL were elevated in primary neurons under OGD (Vieira et al., 2014; Qu et al., 2016; Kong et al., 2017; Yang et al., 2017b; Yang et al., 2017c). Ischemic stroke in rodents also results in up-regulations of RIP1, RIP3(Vieira et al., 2014) and MLKL as well as the activation of MLKL (Yang et al., 2017c; Chen et al., 2018; Ryan et al., 2018). Most importantly, inhibition of necroptotic signaling attenuated ischemia/reperfusion brain injury in animal models of stroke (Degterev et al., 2005a; Qu et al., 2016; Kong et al., 2017; Tian et al., 2018a). In addition, it was reported that the combination of anti-apoptosis and anti-necroptosis agents could elicit a better treatment effect on ischemic injury (Xu et al., 2010b; Tian et al., 2018b). However, there lacks evidence for sensorimotor function improvement in these studies. Further, evidence for necroptosis in human stroke remains lacking. The present study demonstrated for the first time using *postmortem* human brain samples that both apoptosis and necroptosis signaling pathways were prominently activated in stroke tissues from human patients. This result has laid foundation for a strategy of stroke treatment targeting both pathways.

In stroke brains, neurons die at various stages due to multiple

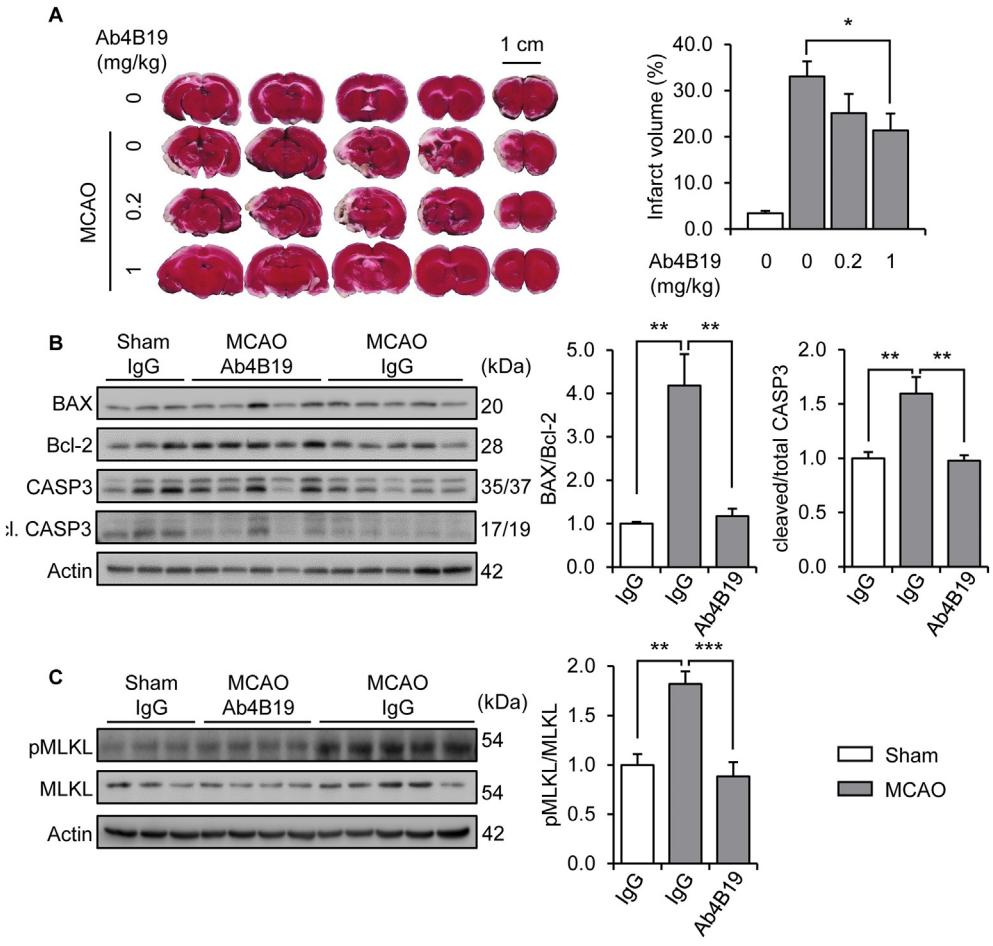

Fig. 5. Ab4B19 rescued cell death induced by ischemia/reperfusion *in vivo* through suppression of both apoptosis and necroptosis. (A) Representative TTC staining of brain coronal sections (*left*) and the quantified relative infarct volumes (%) (*left*). Animals were sacrificed 2 weeks after surgery for TTC (0.2%) staining of the brain slices (2 mm). Note that treatment with Ab4B19 at 1 mg/kg significantly reduced infarct volume. (B) Western blots (*left*) and the quantified relative intensities (*right*) of apoptotic markers, including cleaved CASP3, total CASP3, BAX and Bcl-2. (n = 3 to 5) (C) Western blots (*left*) and the quantified relative intensities (*right*) of the necroptotic markers, pMLKL and MLKL (n = 3 to 5). One-way ANOVA followed by the Dunnett's multiple comparisons test was used for statistical analyses.

mechanisms including lack of oxygen, glutamate toxicity, oxidative stress, short of nutrients, *etc.* (Zhou et al., 2018). Instead of specifically targeting these early events, our strategy is to halt neuronal deaths (both apoptosis and necroptosis) regardless of the cytotoxic stimuli, by activating the BDNF receptor TrkB, which is well known to be critical for neuronal survival (Nagahara and Tuszynski, 2011). Activation of TrkB has additional benefits of stimulating dendritic growth and promoting synaptic connections, and thereby repairing damages of neural circuits and restoring neuronal functions (Horch and Katz, 2002; Nagahara et al., 2009). This rationale is supported by numerous previous studies showing that TrkB activation may have therapeutic potential for many neurologic disorders including AD, ALS and Huntington's disease (HD) (Zuccato and Cattaneo, 2009; Nagahara and Tuszynski, 2011). In this study, we took advantage of the anti-apoptotic property of BDNF, and found for the first time that BDNF-based therapy suppressed necroptosis in both *in vitro* and *in vivo* models of stroke. Our results, together with previous findings of the beneficial effects of elevating BDNF level in stroke brains (Schabitz et al., 2000; Zhang and Pardridge, 2001a; Schäbitz et al., 2004; Zhang and Pardridge, 2006; Schäbitz et al., 2007), support the treatment of stroke using BDNF-based therapies.

Several means of enhancing BDNF/TrkB activities have been evaluated for their therapeutic efficacy in a number of disease models including stroke. For example, expression of the BDNF gene in stroke tissues using adeno-associated virus (AAV) has been shown to enhance cell survival, facilitate migration of cells from the subventricular zone (SVZ) and facilitate functional recovery (Andsberg et al., 2002; Yu et al., 2013). It was also reported that multipotent cells such as mesenchymal stem cells and human neural stem cells overexpressing BDNF reduced infarct volume and improved sensorimotor functions (Kurozumi et al., 2004; Kurozumi et al., 2005; Lee et al., 2010; Jeong et al., 2014). Unfortunately, the field is still in its infancy and the hurdles for safety and patient compliance for gene therapy using viral vectors or stem cells remain high. BDNF protein itself has been proven to be a non-viable drug candidate due to its short half-life and poor distribution property in target tissues.

An alternative approach to elevating brain BDNF is to activate TrkB directly by pharmacological means. While small molecule TrkB agonists have been reported to bind and activate TrkB (Jang et al., 2010a; Jang et al., 2010b; Schmid et al., 2012; Obianyo and Ye, 2013), more rigorous studies have failed to replicate these findings (Todd et al., 2014; Boltaev et al., 2017). A promising strategy is to activate TrkB by agonistic antibodies. A handful of studies have so far identified a number of TrkB agonistic antibodies (Qian et al., 2006; Tsao et al., 2008; Sahenk et al., 2010; Xu et al., 2010a; Traub et al., 2017), and some of them have been employed in animal disease models (Lin et al., 2008; Tsao et al., 2008; Bai et al., 2010; Kim et al., 2014; Todd et al., 2014). We have now developed a TrkB agonistic antibody with several pharmacological features suitable for the treatment of ischemic stroke. In another manuscript being submitted elsewhere, we extensively characterized this antibody and confirmed its high binding affinity and specificity to TrkB (Guo et al., submitted). In the OGD-induced cell death experiment *in vitro*, we demonstrated that the pro-survival effects of Ab4B19 could be abolished by a pan Trk inhibitor, which verified that Ab4B19 elicited its therapeutic effect through TrkB. For a promising CNS drug, it is imperative to demonstrate adequate target engagement and elicitation of appropriate functional modulation of the target following drug administration (Blin et al., 2012). Indeed, we showed that intravenously administered Ab4B19 effectively penetrated into the brain, bound and activated the target TrkB in the stroke tissues

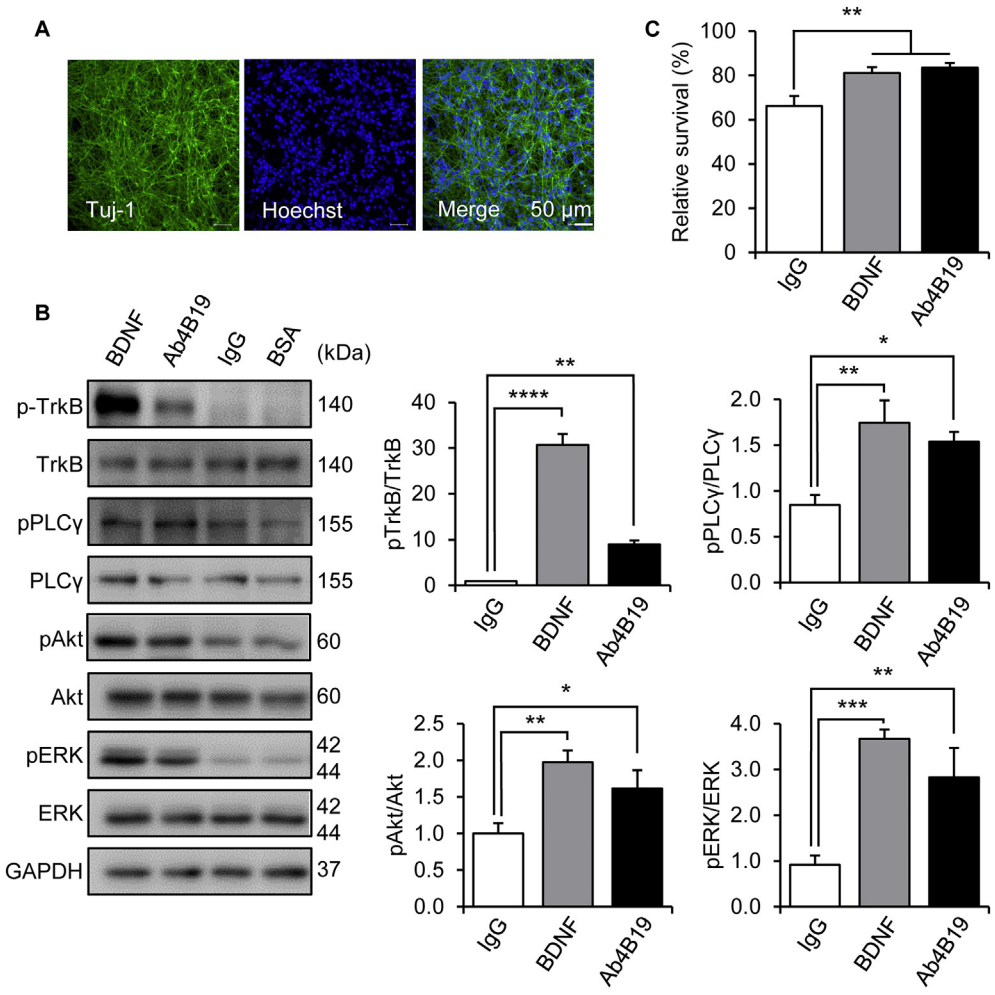

Fig. 6. Ab4B19 activated TrkB signaling and elicited pro-survival function in hESC-derived neurons in culture. (A) Images of hESC-derived neurons in culture. After 14 days of spontaneous differentiation, hESC-derived neurons expressed Tuj-1, a marker of mature neurons. (B) Western blots (*left*) and quantitative analyses (*right*) of Ab4B19 activating TrkB and its three major downstream signaling pathways: pPLC-γ, pAkt and pERK. (C) Ab4B19 elicited a significant pro-survival effect in human neurons under OGD/re-oxygenation. hESC-derived neurons were subjected to 4 h of OGD, and Ab4B19, BDNF (both 3 nM) or 0.1% BSA was applied to the culture at re-oxygenation.

by measuring the level of EGR-1. As a consequence, Ab4B19 was capable of suppressing multiple neuronal damages relevant to stroke, improving functional recovery and reducing the infarct volume in animals underwent ischemic stroke. Taken together, our results support the use of Ab4B19 as a BDNF mimetic with "drug-like" properties for stroke therapy.

5. Conclusions

In conclusion, here we provided evidence from *postmortem* samples that both apoptosis and necroptosis exist in human stroke, and explored to suppress these two forms of cell death employing the BDNF-TrkB signaling pathway using Ab4B19. We discovered that in both rodent and human cultures, this antibody was able to rescue neuronal death under ischemia/reperfusion-mimicking conditions. In a rat model of stroke, Ab4B19 treatment significantly accelerated functional recovery and reduced infarct volume, which could be attributed to the inhibition of both apoptosis and necroptosis. These results suggested that therapies targeting both forms of cell death hold great potential in stroke treatment and that TrkB agonist antibodies could serve as a promising candidate.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.04.009.

Acknowledgements

We are grateful to Dr. Sheng Ding, Yun Zhi, Dr. Jun Yao (all from Tsinghua University), Dr. Yi Sun, Huina Zhang and Chunxue Zhang (Tongji University) for the hESC-derived neuronal cultures; Dr. Liwei Zhang, Dr. Deling Li (Beijing Tiantan Hospital) and Dr. Wenying Qiu (Peking Union Medical College, PUMC) for access to the human *postmortem* samples; Dr. Jianmin Zhang and Dr. Yang Yang (PUMC) for instructions on MCAO; Dr. Hyunsoo Shawn Je (Duke-NUS) and Dr. Guan Wang (Tsinghua University) for advice on the manuscript; and Yihua Xu (Tsinghua University) for technical support.

Funding

This work was supported by National Natural Science Foundation of China, China (31730034, 81501105) and Shenzhen Science, Technology and Innovation Commission, China (JCYJ20170411152419928, JCYJ20180508152240368 and GJHZ20170314151528005) to B.L. and W.G., and Ministry of Education (Thousand Talent Program), China to B.L.

Author contributions

F.H., W.G., and B.L. initiated the project and designed the study. F.H. conducted the experiments and analyzed the data. F.H. and B.L. wrote and W.G. and X.G. edited the manuscript.

Declarations of interest

B.L is a scientific advisor and X.G. is an employee of 4B Technologies Limited. B.L and W.G are co-inventors of the filed patents related to Ab4B19.

References

Andsberg, G., Kokaia, Z., Klein, R.L., Muzyczka, N., Lindvall, O., Mandel, R.J., 2002. Neuropathological and behavioral consequences of adeno-associated viral vector-mediated continuous intrastriatal neurotrophin delivery in a focal ischemia model in rats. Neurobiol. Dis. 9, 187–204.

Bai, Y., Xu, J., Brahimi, F., Zhuo, Y., Sarunic, M.V., Saragovi, H.U., 2010. An agonistic TrkB mAb causes sustained TrkB activation, delays RGC death, and protects the retinal structure in optic nerve axotomy and in glaucoma. Invest. Ophthalmol. Vis. Sci. 51, 4722–4731.

Beck, M., Flachenecker, P., Magnus, T., Giess, R., Reiners, K., Toyka, K.V., Naumann, M., 2005. Autonomic dysfunction in ALS: a preliminary study on the effects of intrathecal BDNF. Amyotroph Lateral Scler Motor Neuron Disord. 6, 100–103.

Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R.L., Bartkowski, H., 1986. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 17, 472–476.

Blin, O., Davies, C.H., Lu, B., 2012. Clinical innovation for neurodegenerative diseases. Clin. Inv. 2, 663–665.

Boltaev, U., Meyer, Y., Tolibzoda, F., Jacques, T., Gassaway, M., Xu, Q., Wagner, F., Zhang, Y.L., Palmer, M., Holson, E., et al., 2017. Multiplex quantitative assays indicate a need for reevaluating reported small-molecule TrkB agonists. Sci. Signal. 10.

Caccamo, A., Branca, C., Piras, I.S., Ferreira, E., Huentelman, M.J., Liang, W.S., Readhead, B., Dudley, J.T., Spangenberg, E.E., Green, K.N., et al., 2017. Necroptosis activation in Alzheimer's disease. Nat. Neurosci. 20, 1236–1246.

Chen, Y., Zhang, L., Yu, H., Song, K., Shi, J., Chen, L., Cheng, J., 2018. Necrostatin-1 improves Long-term functional recovery through protecting oligodendrocyte precursor cells after transient focal cerebral ischemia in mice. Neuroscience 371, 229–241.

Cruz, S.A., Qin, Z., Stewart, A.F.R., Chen, H.H., 2018. Dabrafenib, an inhibitor of RIP3 kinase-dependent necroptosis, reduces ischemic brain injury. Neural Regen. Res. 13, 252–256.

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., Yuan, J., 2005a. Chemical inhibitor of non-apoptotic cell death with therapeutic potential for ischemic brain injury. Nat. Chem. Biol. 1, 112–119.

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., Yuan, J., 2005b. Chemical inhibitor of non-apoptotic cell death with therapeutic potential for ischemic brain injury. Nat. Chem. Biol. 1, 112–119.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I.L., Korkina, O., Teng, X., Abbott, D., Cuny, G.D., Yuan, C., Wagner, G., et al., 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.

Dittrich, F., Ochs, G., Grosse-Wilde, A., Berweiler, U., Yan, Q., Miller, J.A., Toyka, K.V., Sendtner, M., 1996. Pharmacokinetics of intrathecally applied BDNF and effects on spinal motoneurons. Exp. Neurol. 141, 225–239.

Dondelinger, Y., Declercq, W., Montessuit, S., Roelandt, R., Goncalves, A., Bruggeman, I., Hulpiau, P., Weber, K., Sehon, C.A., Marquis, R.W., et al., 2014. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep. 7, 971–981.

Fayaz, S.M., Suvanish Kumar, V.S., Davis, C.K., Rajanikant, G.K., 2016. Novel RIPK3 inhibitors discovered through a structure-based approach exert post-ischemic neuroprotection. Mol. Divers. 20, 719–728.

Feigin, V.L., Krishnamurthi, R.V., Parmar, P., Norrving, B., Mensah, G.A., Bennett, D.A., Barker-Collo, S., Moran, A.E., Sacco, R.L., Truelsen, T., et al., 2015. Update on the global burden of ischemic and hemorrhagic stroke in 1990-2013: the GBD 2013 study. Neuroepidemiology 45, 161–176.

Feuerstein, G.Z., Chavez, J., 2009. Translational medicine for stroke drug discovery: the pharmaceutical industry perspective. Stroke 40, S121–S125.

Ginsberg, M.D., 2009. Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke 40, S111–S114.

Hanson, B., 2016. Necroptosis: a new way of dying? Cancer Biol. Ther. 17, 899–910.

Hempstead, B.L., 2002. The many faces of p75NTR. Curr. Opin. Neurobiol. 12, 260–267.

Honkaniemi, J., Sharp, F.R., 1996. Global ischemia induces immediate-early genes encoding zinc finger transcription factors. J. Cereb. Blood Flow Metab. 16, 557–565.

Horch, H.W., Katz, L.C., 2002. BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat. Neurosci. 5, 1177–1184.

Hua, Y., Schallert, T., Keep, R.F., Wu, J., Hoff, J.T., Xi, G., 2002. Behavioral tests after intracerebral hemorrhage in the rat. Stroke 33, 2478–2484.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.

Ito, Y., Ofengeim, D., Najafov, A., Das, S., Saberi, S., Li, Y., Hitomi, J., Zhu, H., Chen, H., Mayo, L., et al., 2016. RIPK1 mediates axonal degeneration by promoting inflammation and necroptosis in ALS. Science 353, 603–608.

Jang, S.W., Liu, X., Chan, C.B., France, S.A., Sayeed, I., Tang, W., Lin, X., Xiao, G., Andero, R., Chang, Q., et al., 2010a. Deoxygedunin, a natural product with potent neurotrophic activity in mice. PLoS One 5, e11528.

Jang, S.W., Liu, X., Yepes, M., Shepherd, K.R., Miller, G.W., Liu, Y., Wilson, W.D., Xiao, G., Blanchi, B., Sun, Y.E., et al., 2010b. A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. Proc. Natl. Acad. Sci. U. S. A. 107, 2687–2692.

Jeong, C.H., Kim, S.M., Lim, J.Y., Ryu, C.H., Jun, J.A., Jeun, S.S., 2014. Mesenchymal stem cells expressing brain-derived neurotrophic factor enhance endogenous neurogenesis in an ischemic stroke model. Biomed. Res. Int. 2014.

Kalra, S., Genge, A., Arnold, D.L., 2003. A prospective, randomized, placebo-controlled evaluation of corticoneuronal response to intrathecal BDNF therapy in ALS using magnetic resonance spectroscopy: feasibility and results. Amyotroph Lateral Scler Motor Neuron Disord. 4, 22–26.

Kaplan, D.R., Miller, F.D., 2000. Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. 10, 381–391.

Kaplan, D.R., Stephens, R.M., 1994. Neurotrophin signal transduction by the Trk receptor. J. Neurobiol. 25, 1404–1417.

Kim, G.S., Cho, S., Nelson, J.W., Zipfel, G.J., Han, B.H., 2014. TrkB agonist antibody pretreatment enhances neuronal survival and long-term sensory motor function following hypoxic ischemic injury in neonatal rats. PLoS One 9, e88962.

Komotar, R.J., Kim, G.H., Sughrue, M.E., Otten, M.L., Rynkowski, M.A., Kellner, C.P., Hahn, D.K., Merkow, M.B., Garrett, M.C., Starke, R.M., et al., 2007. Neurologic assessment of somatosensory dysfunction following an experimental rodent model of cerebral ischemia. Nat. Protoc. 2, 2345–2347.

Kong, D., Zhu, J., Liu, Q., Jiang, Y., Xu, L., Luo, N., Zhao, Z., Zhai, Q., Zhang, H., Zhu, M., et al., 2017. Mesenchymal stem cells protect neurons against hypoxic-ischemic injury via inhibiting parthanatos, necroptosis, and apoptosis, but not autophagy. Cell. Mol. Neurobiol. 37, 303–313.

Kraemer, B.R., Yoon, S.O., Carter, B.D., 2014. The biological functions and signaling mechanisms of the p75 neurotrophin receptor. Handb. Exp. Pharmacol. 220, 121–164.

Kurozumi, K., Nakamura, K., Tamiya, T., Kawano, Y., Kobune, M., Hirai, S., Uchida, H., Sasaki, K., Ito, Y., Kato, K., et al., 2004. BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. Mol. Ther. 9, 189–197.

Kurozumi, K., Nakamura, K., Tamiya, T., Kawano, Y., Ishii, K., Kobune, M., Hirai, S., Uchida, H., Sasaki, K., Ito, Y., et al., 2005. Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. Mol. Ther. 11, 96–104.

Laukens, B., Jennewein, C., Schenk, B., Vanlangenakker, N., Schier, A., Cristofanon, S., Zobel, K., Deshayes, K., Vucic, D., Jeremias, I., et al., 2011. Smac mimetic bypasses apoptosis resistance in FADD- or caspase-8-deficient cells by priming for tumor necrosis factor alpha-induced necroptosis. Neoplasia 13, 971–979.

Lee, H.J., Lim, I.J., Lee, M.C., Kim, S.U., 2010. Human neural stem cells genetically modified to overexpress brain-derived neurotrophic factor promote functional recovery and neuroprotection in a mouse stroke model. J. Neurosci. Res. 88, 3282–3294.

Li, H., Colbourne, F., Sun, P., Zhao, Z., Buchan, A.M., Iadecola, C., 2000. Caspase inhibitors reduce neuronal injury after focal but not global cerebral ischemia in rats. Stroke 31, 176–182.

Li, W., Sun, W., Zhang, Y., Wei, W., Ambasudhan, R., Xia, P., Talantova, M., Lin, T., Kim, J., Wang, X., et al., 2011. Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. Proc. Natl. Acad. Sci. U. S. A. 108, 8299–8304.

Li, W., Liu, J., Chen, J.R., Zhu, Y.M., Gao, X., Ni, Y., Lin, B., Li, H., Qiao, S.G., Wang, C., et al., 2018. Neuroprotective effects of DTIO, a novel analog of Nec-1, in acute and chronic stages after ischemic stroke. Neuroscience 390, 12–29.

Lin, J.C., Tsao, D., Barras, P., Bastarrachea, R.A., Boyd, B., Chou, J., Rosete, R., Long, H., Forgie, A., Abdiche, Y., et al., 2008. Appetite enhancement and weight gain by peripheral administration of TrkB agonists in non-human primates. PLoS One 3.

Liu, C., Wu, J., Xu, K., Cai, F., Gu, J., Ma, L., Chen, J., 2010. Neuroprotection by baicalein in ischemic brain injury involves PTEN/AKT pathway. J. Neurochem. 112, 1500–1512.

Liu, G., Wang, T., Wang, T., Song, J., Zhou, Z., 2013. Effects of apoptosis-related proteins caspase-3, Bax and Bcl-2 on cerebral ischemia rats. Biomed. Rep. 1, 861–867.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.

Lu, B., Nagappan, G., Guan, X., Nathan, P.J., Wren, P., 2013. BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. Nat. Rev. Neurosci. 14, 401–416.

Lu, H., Liu, X., Zhang, N., Zhu, X., Liang, H., Sun, L., Cheng, Y., 2016. Neuroprotective effects of brain-derived neurotrophic factor and noggin-modified bone mesenchymal stem cells in focal cerebral ischemia in rats. J. Stroke Cerebrovasc. Dis. 25, 410–418.

Majid, A., 2014. Neuroprotection in stroke: past, present, and future. ISRN Neurol. 2014, 515716.

Merkouris, S., Barde, Y.A., Binley, K.E., Allen, N.D., Stepanov, A.V., Wu, N.C., Grande, G., Lin, C.W., Li, M., Nan, X., et al., 2018. Fully human agonist antibodies to TrkB using autocrine cell-based selection from a combinatorial antibody library. Proc. Natl. Acad. Sci. U. S. A. 115, E7023–E7032.

Nagahara, A.H., Tuszynski, M.H., 2011. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat. Rev. Drug Discov. 10, 209–219.

Nagahara, A.H., Merrill, D.A., Coppola, G., Tsukada, S., Schroeder, B.E., Shaked, G.M., Wang, L., Blesch, A., Kim, A., Conner, J.M., et al., 2009. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat. Med. 15, 331–337.

Neuhaus, A.A., Couch, Y., Hadley, G., Buchan, A.M., 2017. Neuroprotection in stroke: the importance of collaboration and reproducibility. Brain 140, 2079–2092.

Obianyo, O., Ye, K., 2013. Novel small molecule activators of the Trk family of receptor tyrosine kinases. Biochim. Biophys. Acta 1834, 2213–2218.

Ochs, G., Penn, R.D., York, M., Giess, R., Beck, M., Tonn, J., Haigh, J., Malta, E., Traub, M., Sendtner, M., et al., 2000. A phase I/II trial of recombinant methionyl human brain derived neurotrophic factor administered by intrathecal infusion to patients with amyotrophic lateral sclerosis. Amyotroph Lateral Scler Motor Neuron Disord. 1, 201–206.

Onwuekwe, I., Ezeala-Adikaibe, B., 2012. Ischemic stroke and neuroprotection. Ann. Med. Health Sci. Res. 2, 186–190.

Qian, M.D., Zhang, J., Tan, X.Y., Wood, A., Gill, D., Cho, S., 2006. Novel agonist

monoclonal antibodies activate TrkB receptors and demonstrate potent neurotrophic activities. J. Neurosci. 26, 9394–9403.
Qu, Y., Shi, J., Tang, Y., Zhao, F., Li, S., Meng, J., Tang, J., Lin, X., Peng, X., Mu, D., 2016. MLKL inhibition attenuates hypoxia-ischemia induced neuronal damage in developing brain. Exp. Neurol. 279, 223–231.
Rami, A., Bechmann, I., Stehle, J.H., 2008. Exploiting endogenous anti-apoptotic proteins for novel therapeutic strategies in cerebral ischemia. Prog. Neurobiol. 85, 273–296.
Ryan, F., Khodagholi, F., Dargahi, L., Minai-Tehrani, D., Ahmadiani, A., 2018. Temporal pattern and crosstalk of necroptosis markers with autophagy and apoptosis associated proteins in ischemic Hippocampus. Neurotox. Res. 34, 79–92.
Sahenk, Z., Galloway, G., Edwards, C., Malik, V., Kaspar, B.K., Eagle, A., Yetter, B., Forgie, A., Tsao, D., Lin, J.C., 2010. TrkB and TrkC agonist antibodies improve function, electrophysiologic and pathologic features in trembler J mice. Exp. Neurol. 224, 495–506.
Sandercock, P., Wardlaw, J.M., Lindley, R.I., Dennis, M., Cohen, G., Murray, G., Innes, K., Venables, G., Czlonkowska, A., Kobayashi, A., et al., 2012. The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the third international stroke trial [IST-3]): a randomised controlled trial. Lancet 379, 2352–2363.
Schabitz, W.R., Sommer, C., Zoder, W., Kiessling, M., Schwaninger, M., Schwab, S., 2000. Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke 31, 2212–2217.
Schäbitz, W.R., Berger, C., Kollmar, R., Seitz, M., Tanay, E., Kiessling, M., Schwab, S., Sommer, C., 2004. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke 35, 992–997.
Schäbitz, W.R., Steigleder, T., Cooper-Kuhn, C.M., Schwab, S., Sommer, C., Schneider, A., Kuhn, H.G., 2007. Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke 38, 2165–2172.
Schmid, D.A., Yang, T., Ogier, M., Adams, I., Mirakhur, Y., Wang, Q., Massa, S.M., Longo, F.M., Katz, D.M., 2012. A TrkB small molecule partial agonist rescues TrkB phosphorylation deficits and improves respiratory function in a mouse model of rett syndrome. J. Neurosci. 32, 1803–1810.
Shi, Y., Zhang, L., Pu, H., Mao, L., Hu, X., Jiang, X., Xu, N., Stetler, R.A., Zhang, F., Liu, X., et al., 2016. Rapid endothelial cytoskeletal reorganization enables early blood-brain barrier disruption and long-term ischaemic reperfusion brain injury. Nat. Commun. 7.
Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, S., Bianco, S.M., Radziejewski, C., Glass, D.J., Masiakowski, P., Furth, M.E., Valenzuela, D.M., et al., 1991. trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65, 885–893.
Stanne, T.M., Aberg, N.D., Nilsson, S., Jood, K., Blomstrand, C., Andreasson, U., Blennow, K., Zetterberg, H., Isgaard, J., Svensson, J., et al., 2016. Low circulating acute brain-derived neurotrophic factor levels are associated with poor long-term functional outcome after ischemic stroke. Stroke 47, 1943–1945.
Sun, L., Wang, H., Wang, Z., He, S., Chen, S., Liao, D., Wang, L., Yan, J., Liu, W., Lei, X., et al., 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148, 213–227.
Sun, Y., Xu, Y., Geng, L., 2015. Caspase-3 inhibitor prevents the apoptosis of brain tissue in rats with acute cerebral infarction. Exp. Ther. Med. 10, 133–138.
Tian, J., Guo, S., Chen, H., Peng, J.J., Jia, M.M., Li, N.S., Zhang, X.J., Yang, J., Luo, X.J., Peng, J., 2018a. Combination of Emricasan with Ponatinib synergistically reduces ischemia/reperfusion injury in rat brain through simultaneous prevention of apoptosis and necroptosis. Transl. Stroke Res. 9, 382–392.
Tian, J., Guo, S., Chen, H., Peng, J.J., Jia, M.M., Li, N.S., Zhang, X.J., Yang, J., Luo, X.J., Peng, J., 2018b. Combination of Emricasan with Ponatinib synergistically reduces ischemia/reperfusion injury in rat brain through simultaneous prevention of apoptosis and necroptosis. Transl. Stroke Res. 9, 382–392.
Todd, D., Gowers, I., Dowler, S.J., Wall, M.D., McAllister, G., Fischer, D.F., Dijkstra, S., Fratantoni, S.A., Van De Bospoort, R., Veenman-Koepke, J., et al., 2014. A monoclonal antibody TrkB receptor agonist as a potential therapeutic for huntington's disease. PLoS One 9.
Traub, S., Stahl, H., Rosenbrock, H., Simon, E., Florin, L., Hospach, L., Hörer, S., Heilker, R., 2017. Pharmaceutical characterization of tropomyosin receptor kinase B-agonistic antibodies on human induced pluripotent stem (hiPS) cell-derived neurons. J. Pharmacol. Exp. Ther. 361, 355–365.
Tsao, D., Thomsen, H.K., Chou, J., Stratton, J., Hagen, M., Loo, C., Garcia, C., Sloane, D.L., Rosenthal, A., Lin, J.C., 2008. TrkB agonists ameliorate obesity and associated metabolic conditions in mice. Endocrinology 149, 1038–1048.
Van Kanegan, M.J., He, D.N., Dunn, D.E., Yang, P., Newman, R.A., West, A.E., Lo, D.C., 2014. BDNF mediates neuroprotection against oxygen-glucose deprivation by the cardiac glycoside oleandrin. J. Neurosci. 34, 963–968.
Vieira, M., Fernandes, J., Carreto, L., Anuncibay-Soto, B., Santos, M., Han, J., Fernández-López, A., Duarte, C.B., Carvalho, A.L., Santos, A.E., 2014. Ischemic insults induce necroptotic cell death in hippocampal neurons through the up-regulation of endogenous RIP3. Neurobiol. Dis. 68, 26–36.
Wahlgren, N.G., Ahmed, N., 2004. Neuroprotection in cerebral ischaemia: facts and fancies - the need for new approaches. Cerebrovasc. Dis. 17, 153–166.
Wang, X., Han, W., Du, X., Zhu, C., Carlsson, Y., Mallard, C., Jacotot, E., Hagberg, H., 2010. Neuroprotective effect of Bax-inhibiting peptide on neonatal brain injury. Stroke 41, 2050–2055.
Wang, H., Sun, L., Su, L., Rizo, J., Liu, L., Wang, L.F., Wang, F.S., Wang, X., 2014. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol. Cell 54, 133–146.
Wang, J., Gao, L., Yang, Y.L., Li, Y.Q., Chang, T., Man, M.H., Zhang, X.Y., Guo, S.C., Li, L.H., 2016. Low serum levels of brain-derived neurotrophic factor were associated with poor short-term functional outcome and mortality in acute ischemic stroke. Mol. Neurobiol. 1–8.
Weber, K., Roelandt, R., Bruggeman, I., Estornes, Y., Vandenabeele, P., 2018. Nuclear RIPK3 and MLKL contribute to cytosolic necrosome formation and necroptosis. Commun. Biol. 1, 6.
Xiong, X.Y., Liu, L., Yang, Q.W., 2018. Refocusing neuroprotection in cerebral reperfusion era: new challenges and strategies. Front. Neurol. 9.
Xu, L., Zhang, Y., Cohen, S.B., Dipetrillo, K., 2010a. TrkB agonist antibody dose-dependently raises blood pressure in mice with diet-induced obesity. Am. J. Hypertens. 23, 732–736.
Xu, X., Chua, K.W., Chua, C.C., Liu, C.F., Hamdy, R.C., Chua, B.H.L., 2010b. Synergistic protective effects of humanin and necrostatin-1 on hypoxia and ischemia/reperfusion injury. Brain Res. 1355, 189–194.
Yang, M., Lv, Y., Tian, X., Lou, J., An, R., Zhang, Q., Li, M., Xu, L., Dong, Z., 2017a. Neuroprotective effect of beta-Caryophyllene on cerebral ischemia-reperfusion injury via regulation of Necroptotic neuronal death and inflammation: in vivo and in vitro. Front. Neurosci. 11, 583.
Yang, M., Lv, Y., Tian, X., Lou, J., An, R., Zhang, Q., Li, M., Xu, L., Dong, Z., 2017b. Neuroprotective effect of β-caryophyllene on cerebral ischemia-reperfusion injury via regulation of necroptotic neuronal death and inflammation: in vivo and in vitro. Front. Neurosci. 11.
Yang, X.S., Yi, T.L., Zhang, S., Xu, Z.W., Yu, Z.Q., Sun, H.T., Yang, C., Tu, Y., Cheng, S.X., 2017c. Hypoxia-inducible factor-1 alpha is involved in RIP-induced necroptosis caused by in vitro and in vivo ischemic brain injury. Sci. Rep. 7.
Yoon, S., Bogdanov, K., Kovalenko, A., Wallach, D., 2016. Necroptosis is preceded by nuclear translocation of the signaling proteins that induce it. Cell Death Differ. 23, 253–260.
Yu, S.J., Tseng, K.Y., Shen, H., Harvey, B.K., Airavaara, M., Wang, Y., 2013. Local administration of AAV-BDNF to subventricular zone induces functional recovery in stroke rats. PLoS One 8.
Zhang, Y., Pardridge, W.M., 2001a. Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. Stroke 32, 1378–1383.
Zhang, Y., Pardridge, W.M., 2001b. Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. Stroke 32, 1378–1384.
Zhang, Y., Pardridge, W.M., 2006. Blood-brain barrier targeting of BDNF improves motor function in rats with middle cerebral artery occlusion. Brain Res. 1111, 227–229.
Zhang, L., Zhang, R.L., Jiang, Q., Ding, G., Chopp, M., Zhang, Z.G., 2015. Focal embolic cerebral ischemia in the rat. Nat. Protoc. 10, 539–547.
Zhang, H., Chen, K., Wang, N., Zhang, D., Yang, Q., Zhang, Q., Liu, P., Wan, M., Gong, C., Hong, X., et al., 2018. Analysis of brain Donors' demographic and medical characteristics to facilitate the construction of a human brain Bank in China. J. Alzheimers Dis. 66, 1245–1254.
Zhou, Z., Lu, J., Liu, W.W., Manaenko, A., Hou, X., Mei, Q., Huang, J.L., Tang, J., Zhang, J.H., Yao, H., et al., 2018. Advances in stroke pharmacology. Pharmacol. Ther. 191, 23–42.
Zuccato, C., Cattaneo, E., 2009. Brain-derived neurotrophic factor in neurodegenerative diseases. Nat. Rev. Neurol. 5, 311–322.