Overexpression of Slit 2 decreases neuronal exitotoxicity, accelerates glymphatic clearance and improves the cognition in multiple microinfarcts

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Research

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Abstract

**Background:** Cerebral microinfarcts (MIs) lead to progressive cognitive impairment in elderly, but the mechanism has kept unknown. Dysfunction of GABAergic transmission induces excitotoxicity, which contributes to stroke pathology, but the mechanism has kept unknown. The secreted leucine-rich repeat (LRR) family protein slit homologue 2 (Slit 2) upregulates GABAergic activity and protects against global cerebral ischemia, but the neuroprotective efficacy of Slit 2 against MI has not been examined.

**Methods:** Middle-aged Wild type and *Slit 2-Tg* mice were divided into sham and MIs groups. MIs in parietal cortex of was induced by laser-evoked arteriole occlusion. Spatial memory was examined by Morris water maze, neuronal activity and glymphatic clearance in peri-infarct areas were monitored by two-photon imaging. GABAergic transmission and neuroinflammation were detected by immunofluorescent staining or western blotting.

**Results:** MIs increased the intracellular Ca$^{2+}$ amplitude and frequency, decreased the neuron survival and neuronal connectivity of parietal cortex, decreased the GABAergic transmission, induced the neuroinflammation, impaired the glymphatic clearance and cognition in middle-aged mice. Slit 2 overexpression attenuated dysfunctional neuronal Ca$^{2+}$ signaling, protected against the neuronal death in the peri-infarct area as well as loss of parietal cortex connectivity, increased the GABAergic transmission and attenuated the neuroinflammation, improved glymphatic clearance and eventually improved spatial learning and memory.

**Conclusion:** Our results strongly supported overexpression of Slit 2 protected against the dysfunction in MIs, which is a potential therapeutic target for cognition impairment in the elderly.

Introduction

Cerebral microinfarct (MI) is wedge-shaped ischemic lesion that result from occlusion of penetrating arteriole (Luo, et al. 2018; Coban et al. 2017). Microinfarcts (MIs) are common in the brains of patients with Alzheimer disease (AD) (Neuropathology Group, 2001), mild cognitive impairment (MCI) (Launer LJ, et al. 2011), and vascular dementia (VaD) (De Reuck J, et al. 2014), and MI load is associated with the severity of cognitive impairment and dementia in the elderly (Hartmann DA, et al. 2018). Accumulating evidence suggests that MI produce persistent brain inflammation (Sofroniew and Vinters, 2010) and disorganized axonal structure in both subcortical (Hinman et al., 2015) and cortical tissues (Coban et al., 2017), thereby expanding regional injury and dysfunction. However, MI is difficult to detect in living human brain and the extent of these lesions is only revealed by postmortem histological examination (Arvanitakis Z, et al. 2011). Population aging is currently increasing the global dementia burden, so it is critical to understand the etiology and pathophysiology of MI to aid in the development of effective and safe preventative treatments.

Excessive release of glutamate and concomitant overstimulation of glutamate receptors during and following ischemic stroke (termed excitotoxicity) induces both acute and delayed neuronal death due to
excessive calcium influx, oxidative stress, degradation of macromolecules, and activation of apoptotic pathways (Lai TW, et al. 2014). Release of gamma-aminobutyric acid (GABA) can counteract glutamate excitotoxicity by inhibiting glutamatergic transmission at presynaptic sites and counteracting glutamate-mediated depolarization in postsynaptic neurons, thereby reducing intracellular calcium deregulation and downstream processes leading to neuronal death (Louzada PR, et al. 2004). Indeed, augmenting GABAergic transmission can protect against ischemic damage (Costa C, et al. 2004; Bi M, et al. 2017). However, it is uncertain whether glutamate/GABA imbalance and ensuing excitotoxicity contributes to MI pathology during aging. In addition, research on excitotoxic neuronal damage in cerebral ischemia has focused mainly on the dynamics of excitatory mediators, and much less is known regarding the changes in GABAergic activity (Schwartz-Bloom and Sah, 2001).

The secreted leucine-rich repeat (LRR) protein slit homologue 2 (Slit 2) regulates the migration, development, and axonal path-finding of GABAergic interneurons by stimulating roundabout (Robo) receptors (Andrews W, et al. 2006). In addition to regulating GABAergic neuron development and circuit formation, recent studies have also implicated Slit 2 signaling in cellular senescence (Gupta KP, et al. 2015) and improved glymphatic clearance (Ge Li, et al. 2018) as well as inhibition of neuroinflammation and protection against global cerebral ischemia (Altay T, et al. 2007). In the present study, we examined the effects of Slit 2 on MI induced by two-photon irradiation in aging mouse brain.

**Materials And Methods**

**Animals**

Transgenic mice overexpressing human Slit 2 (slit 2-Tg) (Li G, et al. 2018) were generated and supplied by the Institute of Biochemistry and Cell Biology (CAS, Shanghai, China) (Han HX, et al. 2011). These slit 2-Tg mice were bred at Guangdong Animal Centre (Guangzhou, China). Wild type (WT) C57BL/6J mice were supplied by Guangdong Animal Centre (Guangzhou, China). Mice at 14 months of age were randomly divided into sham and microinfarct (MI) groups. Animals in sham groups received the same surgery except for irradiation by a femtosecond laser to induce microinfarct formation.

**Induction of microinfarcts**

Anesthesia was induced with 5% isoflurane and maintained by 2.5% isoflurane in oxygen, a 2 × 2 mm² cranial window was created using a microdrill over the right parietal cortex (Nishimura N, et al. 2010). Fluorescein isothiocyanate-dextran (FITC-d2000, 1.5% in saline) was injected into the tail vein to image the neurovasculature through a 25 × water immersion objective lens. Five penetrating arterioles (PAOs) of 20–25 µm diameter were selected as targets for photobleaching-induced clotting as described (Luo CM, et al. 2018, Nishimura N, et al. 2006). Successful microinfarct induction was confirmed at 24 h post-occlusion by two-photon imaging of target PAOs.

**Morris water maze**
Morris water maze testing was performed 1 week after MI modeling as described (He XF, et al. 2016). Briefly, mice were first examined for spatial learning during five consecutive days of hidden platform training with four trials per day. On day six, the platform was removed and each mouse was tested for spatial memory on a single 60-s probe trial. Swim paths were recorded, the latency to reach the platform during water maze training as well as the number of crossings over the former platform location (target area) and time spent in the target quadrant during the probe trial were analyzed.

**Two-photon Ca\(^{2+}\) imaging**

Two weeks after MI induction, the incision was re-opened and the agarose and coverslip over the cranial window were removed, intracellular Ca\(^{2+}\) imaging was performed on the region surrounding the infarct area (Fig. 2A & B) using a two-photon microscope (Leica, Wetzlar, Germany) as described previously (He XF, et al. 2015). Briefly, peri-ischemic target cells were stained with Oregon Green 488 BAPTA-1 AM (OGB-1 AM) at a concentration of 10 mM by multicell bolus loading (MCBL) (Garaschuk, et al. 2006). The dye solution was diluted 1:10 in standard pipette solution. We used sulforhodamine (SR) 101 (1 mg dilution into 4 mL standard pipette solution) to distinguish astrocytes from neurons. The combined staining mixture was injected at a depth of 200–300 µm below the pial surface. Fluorometric Ca\(^{2+}\) imaging was performed at 809 nm emission using a two-photon laser scanning microscope. The detection of Ca\(^{2+}\) transients in individual neurons was performed automatically by defining regions of interest (ROIs) in the collected videos and deleting the background fluorescence. Sixty cells located 200 µm below the pial surface (layer 2/3) and surrounding the clotting site were tested (Shih AY, et al. 2013). Calcium transient amplitudes above the baseline are presented as the relative change in fluorescence (), and calcium transient frequencies (as the number of fluorescence changes in 1 min (60 seconds).

**Assessment of the glymphatic efficiency**

Glymphatic clearance were evaluated as described (Ge Li, et al. 2018). Briefly, the animals were anesthetized, the incision was re-opened, FITC-conjugated dextran (40 kDa) dissolved in ACSF was injected into the subarachnoid space via cisterna magna puncture with a microsyringe pump controller, and 200 µL of rhodamine B (70 kDa) was injected intravenously immediately prior to imaging. Images were acquired 5, 15, 30, 45, and 60 min following intra-cisternal FITC-conjugated dextran injection. Mean pixel intensity of the FITC-tracer in the paravascular space was quantified to evaluate clearance by the glymphatic system and BBB permeability, respectively.

**Biotinylated dextran amine (BDA) injection and measurements of axon density**

To investigate the effect of microinfarcts on axonal connectivity, 0.5 µL BDA solution (5% in 0.1 M PBS) the neuroanatomical tracer biotin dextran amine (BDA, MW 10000) was injected into the ipsilateral (exposed/injured) right parietal cortex and imaged in three cortical target regions. Animals were perfused through the heart 2 weeks later, and brains were fixed, frozen, and coronally sectioned at 10 µm. Six
sections spaced 100 µm apart were stained with Alexa Fluor® 488 Streptavidin, embedded in Fluoroshield™ containing DAPI for nuclear counterstaining and enclosed under a coverslip.

**Histology**

Sections were treated with 0.3% Triton and 10% goat serum for 1 h at room temperature, then incubation overnight at 4 °C with the indicated primary antibody. Sections were then incubated with the indicated secondary antibodies at room temperature in PBS containing 10% normal goat serum for 1 h. Slices were mounted onto slides, embedded in Fluoroshield™ with DAPI, and enclosed under a coverslip. Images were acquired using a Nikon fluorescence microscope or a confocal microscope equipped with a 63× (N.A. 1.25) glycerol immersion objective.

**Western blot analysis**

20 µg total protein per lane separated by SDS-PAGE using 12% precast polyacrylamide gels at 120 V for 90 min. Separated proteins were then transferred to polyvinylidene fluoride membranes at 100 V for 2 h. Membranes were blocked with 5% BSA at room temperature for 1 h and incubated with the indicated primary antibodies overnight at 4 °C, followed by incubation with anti-rabbit or anti-mouse immunoglobulin G secondary antibody for 1 h.

**Data and statistical analyses**

Data were analyzed by an experimenter blinded to treatment history. All data are expressed as mean ± standard deviation. Immunohistochemical staining and western blotting were analyzed using ImageJ. Mean Slit 2 expression levels on western blots were compared by independent-samples t test while other group means were compared by two-way repeated measures ANOVA with Tukey’s post hoc tests for multiple comparisons. All statistical analyses were conducted using SPSS 19.0. A $P < 0.05$ (two tailed) was considered statistically significant for all tests.

**Results**

**Slit 2 was overexpressed in neurons and astrocytes but not in microglia of transgenic mice**

Western blotting was performed to confirm expression of the human Slit 2 transgene protein in transgenic (Tg) mouse brain. Expression was significantly elevated in slit 2-Tg mice compared to WT mice ($P < 0.001$) (Supplementary Fig. 1A & B). Co-immunofluorescence staining using anti-Flag for detection of Slit 2 and cell type-specific antibodies revealed overexpression in neurons (Supplementary Fig. 1C) and astrocytes (Supplementary Fig. 1D) but not in microglia (Supplementary Fig. 1E).

**Overexpression of Slit 2 improved Morris water maze performance in mice with parietal microinfarcts**
The posterior parietal cortex (PPC) is a multimodal association area involved in spatial navigation as evidenced by performance deficits in the MWM following PPC lesions (Olsen GM, et al. 2017). To explore the protective efficacy of Slit 2 against cognitive dysfunction due to PPC microinfarcts in aged (14-month-old) mice, we compared MWM performance between WT and *slit 2-Tg* mice following sham treatment or MI induction (Fig. 1A). As shown in Fig. 2B, on days 4 and 5 during training, there were no significant differences in escape latencies between WT sham and *slit 2-Tg* sham groups (Both *P* > 0.05), but the escape latencies in *slit 2-Tg* MIs mice exhibited shorter than WT MI mice (*P* < 0.05 and *P* < 0.001, respectively). These findings suggest that Slit 2 overexpression protects against spatial learning impairment following MIs induction.

During the probe trial (Fig. 1C & D), the number of crossings was significantly reduced in MIs group compared with sham group, for WT mice (*P* < 0.05) but not for *slit 2-Tg* mice (*P* > 0.05), which was significantly reduced in the WT MIs group compared with *slit 2-Tg* MIs group (*P* < 0.05). Similarly, the target quadrant time was significantly decreased in MIs group compared with sham group, for WT mice (*P* < 0.05) but not for *slit 2-Tg* mice (*P* > 0.05), which was shorter in WT MIs mice than WT MIs mice (*P* < 0.05). These results suggest that MIs cause spatial memory deficits that can be improved by Slit 2 overexpression.

**Overexpression of Slit 2 inhibited neuronal hyperactivation in the peri-infarct area**

Two-photon Ca\(^{2+}\) imaging in the peri-infarct area was performed two weeks after MI induction to assess excitatory-inhibitory balance (Fig. 2A & B). Amplitude was significantly greater in MIs mice than sham group, for WT mice (*P* < 0.001) (Fig. 2C, D, G), but not for *slit 2-Tg* mice (*P* > 0.05) (Fig. 2E, F, G). Frequency was significantly greater in MIs group compared to sham group, for WT mice (*P* < 0.05) (Fig. 2C, D, G), but not for *slit 2-Tg* mice (*P* > 0.05) (Fig. 2E, F, G). Collectively, these findings indicate that Slit 2 overexpression suppress neuronal Ca\(^{2+}\) transients in the peri-infarct area.

**Overexpression of Slit 2 protected against axonal damage after multiple cortical microinfarct induction**

The anatomical tracer BDA was injected into right parietal cortex and assessing transport to ipsilateral hippocampus, ipsilateral entorhinal cortex, and contralateral parietal cortex (Fig. 3A). The BDA-positive cell number in ipsilateral hippocampus was significantly higher in *slit 2-Tg* MIs mice compared to WT MIs mice (*P* < 0.05) (Fig. 3b1 & C). BDA-positive cell number in ipsilateral entorhinal cortex was significantly lower in WT MIs mice compared to *slit 2-Tg* MIs mice (*P* < 0.05) (Fig. 3b2 & C). Finally, BDA-positive cell number in contralateral parietal cortex was significantly higher in *slit 2-Tg* MIs mice compared to WT MIs mice (*P* < 0.05) (Fig. 3b3 & C). These results indicating disruption of cortical projections. However, Slit 2 overexpression preserved these projections following MIs induction.
Overexpression of Slit 2 increased GABAergic transmission in the peri-infarct area

Effect of Slit 2 overexpression on excitatory–inhibitory balance in peri-infarct areas were measured the immuno-expression of the GABAergic interneuron markers GAD67 (Fig. 4A) and vesicular GABA transporter (VGAT) (Fig. 4B). The number of GAD67-positive neurons surrounding the infarcts area was significantly lower in MIs group compared to sham group, for WT mice ($P<0.05$), but not for $slit\ 2$-$Tg$ sham mice ($P>0.05$) (Fig. 4C). VGAT intensity was significantly lower in MIs group than sham group, for WT mice ($P<0.05$), but not for $slit\ 2$-$Tg$ mice ($P>0.05$) (Fig. 4C). Finally, we performed western blotting to verify the expressions of GAD67 and VGAT in peri-infarct areas (Fig. 4D & E). GAD67 expression was significantly lower in WT MIs mice compared to WT sham mice ($P<0.01$) but did not differ between $slit\ 2$-$Tg$ MIs and $slit\ 2$-$Tg$ sham mice ($P>0.05$). Furthermore, GAD67 expression was significantly lower in WT MIs mice than $slit\ 2$-$Tg$ MIs mice ($P<0.01$). VGAT expression was significantly lower in WT MIs mice than WT sham mice ($P<0.01$) but did not differ between $slit\ 2$-$Tg$ MIs and $slit\ 2$-$Tg$ sham mice ($P>0.05$). Moreover, VGAT expression was significantly lower in WT MIs mice than $slit\ 2$-$Tg$ MIs mice ($P<0.01$).

Overexpression of Slit 2 attenuated peri-infarct neuroinflammation and protected against local neuronal loss

Neuronal number was significantly reduced in MIs groups compared to corresponding sham groups, both for WT mice ($P<0.0001$) and $slit\ 2$-$Tg$ mice ($P<0.001$) (Fig. 5A & B), but significantly greater in $slit\ 2$-$Tg$ MIs mice compared to WT MIs mice ($P<0.001$). Microglial number was significantly greater in both MIs groups compared to the corresponding sham controls, both for WT mice ($P<0.0001$) and $slit\ 2$-$Tg$ mice ($P<0.01$), but significantly lower in $slit\ 2$-$Tg$ MIs mice compared to WT MIs mice ($P<0.001$). Therefore, Slit 2 overexpression appears to protect neurons for MI-induced degeneration, possibly by quelling the ensuing neuroinflammatory response.

Overexpression of Slit 2 improved glymphatic clearance and increased the astrocytic AQP4 polarity

As shown in Fig. 6A, following intra-cisternal injection, FITC-dextran tracer moved along the paravascular space and rapidly entered the interstitium of the parenchyma. The FITC intensity at 5 min showed no significant differences among the WT sham, WT MIs, $slit\ 2$-$Tg$ sham and $slit\ 2$-$Tg$ MIs groups (Fig. 6B & C). In all groups, parenchymal/perivascular FITC-dextran fluorescence intensity gradually increased over the first 45 min after injection. Thereafter, intensity continued to increase in the WT MIs group, indicating dysfunction of glymphatic clearance, but decreased in the other three groups. At 60 min after FITC-dextran injection (Fig. 6C), FITC intensity was significantly higher in MIs mice compared to sham mice, both for WT mice ($P<0.01$) and $slit\ 2$-$Tg$ mice ($P<0.05$), Further, FITC intensity was significantly lower in
slit 2-Tg MIs mice compared to WT MIs mice ($P < 0.05$) (Fig. 6C), suggesting that Slit 2 overexpression sustained glymphatic clearance following MIs induction.

Reactive astrocyte number was significantly greater in peri-infarct areas of MI groups compared to corresponding sham groups, both for WT ($P < 0.001$) and Slit 2-Tg mice ($P < 0.05$) (Fig. 6D & E). Astrocyte number was significantly lower in slit 2-Tg MIs mice compared to WT MIs mice ($P < 0.01$), suggesting that Slit 2 overexpression suppressed astrocyte reactivity in response to MIs induction. AQP4 polarity was significantly lower in MIs groups compared to corresponding sham groups, both for WT mice ($P < 0.001$) and slit 2-Tg mice ($P < 0.01$) and significantly greater in slit 2-Tg MIs mice compared to WT MIs mice ($P < 0.05$). These results indicate that MIs induction impaired glymphatic clearance and disrupted astrocytic AQP4 expression function, which was protected against by Slit 2 overexpression.

**Discussion**

The ensuing imbalance between excitatory and inhibitory transmission is the primary pathogenic mechanism for cell death in stroke (Costa C, et al. 2004), and inhibiting the excitotoxicity protects against the brain damage. Numerous inhibitors of glutamatergic transmission have been tested as neuroprotective agents following experimental stroke, but none has been shown to be of clinical value (Green AR, et al. 2000). GABA is the primary inhibitory neurotransmitter in the mammalian brain and has been demonstrated to counteract excessive glutamatergic excitation, thereby protecting neurons from excitotoxicity during stroke (Galeffi F, et al. 2000). However, many compounds shown to increase GABA function do not possess robust neuroprotective efficacy in focal stroke models (Green AR, et al. 2000).

Slit-Robo signaling regulates the development of interneuron populations in cerebral cortex (Andrews W, et al. 2008; Marín O and Rubenstein JL. 2003), we demonstrate that Slit 2 overexpression can increase peri-infarct GABAergic activity, which was protective in focal microstroke. It was reported that curcumin upregulate Slit 2 expression (Sirohi VK, et al. 2017), which protect against stroke-mediated brain damage (Bhat A, et al. 2019). Curcumin is widely consumed as turmeric (Bavarsad K, et al. 2019), which may be a potential treatment to upregulate GABAergic activity around microinfarcts, a possibility that we will study in further study.

In addition, Slit 2 was reported to reduce inflammatory responses following ischemic insults (Altay T, et al. 2007). Consistent with these findings, overexpression of Slit 2 inhibited local microglial and astrocytic activation and protected against peri-infarct neuronal loss. Slit 2 is expressed in multiple brain cell types, including neurons, astrocytes, endothelial cells (Wu JY, et al. 2001), and pericytes (Guijarro-Muñoz I, et al. 2012), and different ischemic models induce distinct cell type-specific changes in Slit 2 expression. For instance, Park et al. (Park JH, et al. 2016) reported that Slit 2 was constitutively expressed in neurons of control rats and that transient forebrain ischemia upregulated Slit 2 ligand in reactive astrocytes but not neurons or activated microglia of the hippocampus. Fang et al. (Fang M, et al. 2010) reported that Slit 2 was expressed mainly by neurons of the temporal lobe during the acute and latent phases of temporal lobe epilepsy (TLE) in rats, but mainly in astrocytes during the chronic phase. In our study, Slit 2 was
expressed in both cortical neurons and astrocytes but not in microglia of control mice, while MI increased astrocytic expression and reduced neuronal expression. Slit-Rob signaling is high in interneurons, so reduced in Slit 2 expression likely reflected loss of peri-infarct interneurons and ensuing local excitotoxicity. Indeed, Slit 2 overexpression protected against interneuronal loss and reduced excitotoxic sequela.

Slit 2 was reported to be overexpressed in these Tg mice throughout life (Han HX, et al. 2011, Li JC, et al. 2018; Li G, et al. 2018), which promoted paravascular clearance (Li G, et al. 2018). Consistent with these findings, we demonstrated that microstroke impaired AQP 4 function, paravascular clearance (Gaberel T, et al. 2014), and BBB permeability (Chen X, et al. 2018), all of which were protected by Slit 2 overexpression. Finally, we demonstrated that Slit 2 overexpression promoted neuronal plasticity after cortical microinfarct induction. Slit 2 has been reported to promote both axonal elongation and branching (Ma L and Tessier-Lavigne M, 2007) or dendritic growth and branching in developing cortical cells (Whitford KL, et al. 2002), as well as GABAergic function in mature brain. Collectively, these processes contribute to circuit recovery post-ischemia. Indeed, GABA-mediated inhibition is a critical modulator of cortical remapping, which is required for functional recovery after stroke (Hui T, et al. 2016). It is worthy of note that parietal cortex contributes to route learning using proximal salient cues in the water maze task (Solari N, et al. 2018). Thigmotaxis (swimming alone the tub edge) is indicative of spatial learning failure (Vorhees CV, et al. 2006) and we observed thigmotaxis in WT MIs mice, but not Slit 2-Tg MI mice, suggesting that Slit 2 overexpression protected against the spatial learning impairment induced by parietal microinfarcts.

In summary, we demonstrate that Slit 2 overexpression can preserve the excitatory–inhibitory balance in peri-infarct regions of parietal cortex by promoting GABAergic transmission, thereby protecting against excitotoxicity and local neuroinflammation. Furthermore, Slit 2 overexpression protected against glymphatic system and BBB dysfunction, attenuated local neuronal loss, and ultimately prevented cognitive decline induced by parietal microinfarcts.

Abbreviations

Slit 2: secreted LRR protein slit homologue 2; PBS: phosphate-buffered saline; GABA: γ-aminobutyric acid; Vesicular GABA Transporter: VGAT; MBP: myelin basic protein; MIs: Microinfarcts; AQP4: Aquaporin.

Declarations

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Declarations

Ethics approval:
The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Guangdong Laboratory Animal Monitoring Institute (Guangzhou, China. IACUC NO. 2015023).

Consent for publication:
Not applicable.

Author Contributions
Xiao-fei He, Ge Li, Li-li Li and Feng-yin Liang performed the experiments. Xiao-fei He, Ge Li, and Ming-yue Li drafted the manuscript. Xi-quan Hu conceived and designed the research. Xi Chen edited and revised the manuscript. Xi-quan Hu and Xi Chen approved the final version of the manuscript.

Data Availability
Data openly available in a public repository that issues datasets with DOIs.

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**Figures**
Figure 1

Overexpression of Slit 2 improves spatial learning and memory deficits caused by cerebral microinfarcts induction. A. Experimental timeline of BDA injection and Morris water maze (MWM) testing, Two-photon imaging and pathological analysis. B. Latencies to the hidden platform (escape latencies) during training trials by wild type (WT) sham, WT cerebral microinfarcts (MIs), slit 2-Tg sham, and slit 2-Tg MIs groups. C. Swim paths during the probe trial. D. Number of target area crossings and time spent in the target quadrant during the probe trial. Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.
Figure 2

Overexpression of Slit 2 reduces Ca2+ signal amplitude and frequency in the peri-infarct region. A-B. Diagram showing the site of microinfarcts (Mls) induction and two-photon Ca2+ imaging. C. Representative Ca2+ images in the peri-infarct area of a WT mouse. White circles show six representative neurons monitored for Ca2+ signals over 60 s. D. Representative Ca2+ signals in the six representative neurons (regions of interest, ROIs) indicated in C. E. Representative Ca2+ images of the peri-infarct area in a slit 2-Tg mouse. White circles show the six representative neurons monitored for 60 s. F. Representative Ca2+ signals in the OGA-1AM positive neurons (ROIs) indicated in E. G. Comparisons of Ca2+ signals between sham and Mls groups of WT and slit 2-Tg mice. Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.
Figure 3

Overexpression of Slit 2 protects against MI-induced loss of axonal connectivity. A. Representative image showing BDA injection. B. Images of BAD-positive neurons and axons in ipsilateral hippocampus (b1), entorhinal cortex (b2), and contralateral parietal cortex (b3). C. Comparison of BDA-positive cells in ipsilateral hippocampus, ipsilateral entorhinal cortex, and contralateral parietal cortex among sham and MIs groups of WT and slit 2-Tg mice (20×, 1 field/slice, 5 slices per mouse). Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.
Overexpression of Slit 2 increases peri-infarct GABAergic activity. A. The GABAergic interneuron marker GAD67 and the neuronal marker Neun in peri-infarct areas (40 ×). B. Immunofluorescence staining of VGAT in peri-infarct areas (63 ×, zoomed in 3). C. Comparison of GABAergic interneuron number and mean VGAT immunofluorescence intensity among sham and MI groups of WT and slit 2-Tg mice. D. Chemiluminescence imaging of GAD67 and GAPDH, VGAT and GAPDH in peri-infarct area. E. Comparison of GAD67/GAPDH and VGAT/GAPDH expressions in the peri-infarct area among sham and MIs groups of WT and slit 2-Tg mice. Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.
Figure 5

Overexpression of Slit 2 reduces peri-infarct neuronal loss and microglial activation. A. Immunofluorescence staining of neurons and microglia (20×). B. Comparison of the neuron and microglial numbers among WT sham, WT MI, slit 2-Tg sham, and slit 2-Tg MIs groups (20×, 1 field/slice, 5 slices per mouse). Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.
Figure 6

Overexpression of Slit 2 improves paravascular clearance in the per-infarct area. A. Representative xyz overlaid images of the cortical vascular (Rhodamine B: red color) and paravascular spaces (FITC-dextran: green color) in the peri-infarct area at 5, 15, 30, 45, and 60 min after FITC-dextran injection (25 × objective). B. Linear analysis of the FITC-dextran fluorescence emission intensity in the paravascular space. C. Histograms of FITC-dextran fluorescence intensity in the paravascular space at 5 and 60 min after cisterna magna injection among WT sham, WT MI, slit 2-Tg sham, and slit 2-Tg MIs groups mice. D. Immunofluorescence staining of AQP4 and the astrocyte marker GFAP (25 × objective, magnified by 3). E. Comparison of GFAP+ astrocyte numbers and AQP4 polarity among WT sham, WT MI, slit 2-Tg sham, and slit 2-Tg MIs group. Each dataset is expressed as mean ± SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. n=6 mice.

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