Apoptosis-Induced Wnt2/β-Catenin Signaling Triggers Astrocytic Dedifferentiation and Facilitates Neurogenesis in Ischemic Cortex

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Abstract

Background: Reactive astrogliosis is a common pathologic change of various neurological disorders and usually takes some properties of neural progenitors. This dedifferentiation response of reactive astrocytes to injury is thought as an endogenous cellular attempt for neuronal regeneration, but the underlying mechanism remains largely unclear.

Methods: A focal cerebral ischemic model was adopted to assess the dedifferentiation of reactive astrocytes. Topgal mice (a Wnt signaling reporting mouse line) and Caspase-3-/− mice were used to evaluate the change and roles of Wnt signaling and apoptosis in this process. Virus mediated Wnt2, β-catenin, dnTCF4 and DAP5 manipulation was used to reveal the molecular mechanism of dedifferentiation. Ischemic cortical samples and Wnt2-5UTR sequences of macaca mulatta and human were analyzed to explore if the apoptosis-induced Wnt2 up-regulation was conserved.

Results: Focal ischemia induces rapid up-regulation of Wnt2 protein in apoptotic neurons in mice, primates and human, and activation of canonical Wnt signaling in reactive astrocytes. Local delivery of Wnt2 shRNA abolished the dedifferentiation response of astrocytes while over-expressing Wnt2 promoted progenitor marker expression and neurogenesis. Both the activation of Wnt signaling and dedifferentiation of astrocytes was compromised in ischemic caspase-3-/− cortex. Over-expressing stabilized β-catenin not only facilitated neurogenesis but also promoted functional recovery in ischemic caspase-3-/− mice. Apoptotic neurons up-regulated Wnt2 protein via internal ribosome entry site (IRES)-mediated translation. Knocking down death associated protein 5 (DAP5), a key protein in IRES-mediated protein translation, significantly diminished both Wnt activation and astrocyte dedifferentiation.

Conclusions: Our data demonstrated a novel apoptosis-initiated Wnt-activating mechanism which triggers the dedifferentiation of reactive astrocytes and facilitates neurogenesis in adult cortex, revealing a “SOS” mechanism for inducing astrocyte dedifferentiation and indicating Wnt2/β-catenin signaling as a potential therapeutic target for ischemic stroke.

Introduction

Reactive astrogliosis is a hallmark pathologic change of cerebral ischemia and various neurological diseases. Upon ischemia, astrocytes start to proliferate and become morphologically hypertropic. It has now been accepted that reactive astrocytes are heterogeneous and play diverse roles after injury [1]. Either be protective and reparative, or be neurotoxic and axon growth inhibiting, depending on the dynamics of different sub-populations of reactive astrocytes [2].

One outstanding feature of reactive astrocytes is that some reactive astrocytes up-regulate many genes associated with neural progenitors and can form neurospheres in vitro [3, 4], indicating certain degree of dedifferentiation. Considering that adult neural stem cells (NSCs) also share some properties of astrocytes and the lineage relationship between NSCs and astrocytes, it has been hypothesized that reactive astrocytes may undergo some degree of dedifferentiation [5, 6]. Elucidating how reactive
astrocytes acquire stem cell property, although partially acquired, is important for understanding how central nerve system (CNS) responds to injury and would shed light on facilitating repair/regeneration of CNS.

Several studies have attempted to disclose the mechanisms relating to the neurogenic potential of reactive astrocytes. In the striatum, Notch signaling which normally maintains the quiescence of adult NSCs is diminished after ischemia[7]. This liberates the neurogenic potential of local astrocytes [7]. In the cerebral cortex, Shh signaling has been demonstrated to be required for the in vitro neurosphere formation of reactive astrocytes [8]. Over-expressing Sox2 and NeuroD1 in ischemic cortex can directly reprogram astrocytes into neuroblasts and neurons, respectively [9, 10]. However, under ischemia, which local factor triggers the appearance of progenitor properties of reactive astrocytes, particularly in vivo, remains still unclear.

In the present study, we focused on Wnt/β-catenin signaling, a key niche signaling in maintaining the active status of adult NSCs and supports neurogenesis in the subventricular zone (SVZ) and hippocampus [11, 12], and explored the response of Wnt/β-catenin signaling to ischemia and its roles in the dedifferentiation of astrocytes and neurogenesis in cerebral cortex. Our data surprisingly revealed that apoptotic neurons up-regulates Wnt2 protein via internal ribosome entry site (IRES) mediated protein translation, which induces the dedifferentiation response of reactive astrocytes, and supports cortical neurogenesis.

**Materials And Methods**

**Mice and reagents**

The TOPgal mice (DasGupta and Fuchs, 1999) and caspase-3-/- mice (Kuida et al., 1996) mice were obtained from Jackson lab. All animal experiments were carried out under protocols approved by the Animal Care and Use Committees of Fourth Military Medical University.

Lentiviral vectors of stabilized β-catenin (EbC) and dominant-negative form of TCF-4 (dnTCF-4, also called EdTC) were kindly gifted by Dr. Fuerer Christophe and Dr. Roel Nusse (Stanford University). Retrovirus expressing GFP, lentivirus expressing Wnt2 shRNA, Wnt2, and DAP5 shRNA were purchased from Obio Biotech, Shanghai. Pan-caspase inhibitor v-ZAD was purchased from R & D System.

**Mice ischemia model**

Focal photochemic ischemia as described (Lee et al., 2007). Rose bengal (Sigma) was injected via tail vein at 25 mg/kg. A skull window ranging from 0.8 to 2 mm posterior to the Bregma, and 2 mm right to the midline was carefully made. For the behavior tests, the skull widow was made from 0.3-2.3 mm anterior to the Bregma, and 0.5-3.0 mm right to the midline. Brains were illuminated for 10-12 minutes using a cold light source (Zeiss FL1500 LCD). For neuroblasts detection, BrdU (100 mg/kg; Sigma) injection was started 12 h after ischemia, once a day for 6 days. Mice were sacrificed at 7 or 14 days post
ischemia (dpi). For BrdU/NeuN staining, BrdU injection was initiated at 12 h after the ischemia, once a day for 14 d, and mice were sacrificed at 21 dpi.

**Mice treatment**

Lentivirus expressing control shRNA, Wnt2 shRNA, DAP5 shRNA or dnTCF-4 (EdTC) was injected into the cortex 9 days before ischemia. Wnt2 shRNA sequences and DAP5 shRNA sequences are provided in the supplementary information.

For Wnt2 and β-catenin over-expression, lentivirus expressing Wnt2, EbC or control virus expressing luciferase was injected into the cortex 7 days before ischemia. For DCX/BrdU-staining, BrdU was injected once per day from 3 to 10 dpi, and mice were sacrificed at 14 dpi. For NeuN/BrdU-staining, BrdU was injected once per day from 3 to 17 dpi, and mice were sacrificed at 28 dpi.

**Monkey ischemia model**

Before ischemia, a healthy male macaca mulatta of 19 years old was anaesthesized by ketamine for anaesthesia induction and pentobarbital sodium for anaesthesia maintenance. Then, T1 and T2 MRI scanning were performed on both left and right motor cortex. According to the localization of MRI scanning, endothelin (E7764, Sigma, 5ug/ul) was injected into 3 points of one side motor cortex with intervals of 5 mm (5 μl per point, 4mm in depth) to induce ischemia. At 24h post ischemia, the monkey was perfused through heart by 4% PFA. The brain tissue was dissected immediately after perfusion and post-fixed by 4% PFA for about 2h. All the procedure and protocol of monkey experiments were performed under the approval of the Animal Care and Use Committees of Kunming Institute of Zoology, Chinese Academy of Sciences (animal ethic review number: IACUC19009).

**Human brain samples**

The study protocol involving human brain samples was reviewed and approved by the Ethics Committee of Xijing hospital (KY20202003). Ischemic cortical samples were obtained from the poststroke areas in two patients who met the following criteria: (1) requiring both decompressive craniectomy and partial lobectomy for diffuse cerebral infarction at 18-26 h post-ischemia; (2) > 20 years of age; and (3) willing to provide written informed consent. Control cortical biopsy samples from a patient with sever traumatic brain injury who underwent a decompressive craniectomy for his injury was resected from the pericontusional area of the temporal cortex. The diagnosis of cerebral infarction and trauma was made by neurosurgeons in the department of neurosurgery of Xijing hospital according to physical examination and neuroimaging (CT and MRI). Excluding criteria were set as: (1) presence of a malignant tumor, (2) presence and history of major infectious diseases, and (3) patients judged as unsuitable by the attending doctor.

**Western-blotting**
For the Western-blotting of cortical tissue, cortical samples including lesion center and 3-5 mm surrounding tissues were dissected out. Each sample was homogenized in RIPA buffer for about 20 min and incubated for another 40 min on ice, then centrifuged at 12 000 g at 4 °C. For the Western-blotting of primary cells, cells were rinsed by PBS, collected, and then lysed with RIPA buffer. Protein sample was boiled before sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were electrotransferred to polyvinylidene difluoride membrane and reacted with primary antibodies (as described in Supplementary materials) overnight at 4 °C, then with corresponding secondary anti-mouse, anti-rabbit, anti-goat, or anti-rat IgG peroxidase (1:5000) at room temperature for 50 min. The bands were visualized by an ECL kit (Millipore). For tissues and cells, β-actin was used as internal control. For the conditioned medium, BSA was used as internal control.

Real-time RT-PCR

For real-time RT-PCR, total RNA was extracted using Trizol agent. The RNA samples were treated with Dnase I to exclude the contamination of genomic DNA. After reverse transcription, semiquantitative PCR was carried out. The levels of target mRNA were normalized to the mRNA levels of the housekeeping gene Gapdh to allow comparisons using the $\Delta$Ct method. The primer information was listed in supplementary materials.

Behavioral tests

Glass sliding and footfault tests were used to assess the asymmetry in forelimb-use as described [13, 14]. The animals were videotaped in a transparent glass cylinder for 10 min. The numbers of contacts, and the numbers of sliding movements of each forelimb at the wall of the cylinder for every spontaneous stand-up were scored. Percentage of sliding was calculated by the following formula: the number of sliding/(number of contact + number of sliding) $\times$ 100. The asymmetry index was derived by subtracting the percentage of ipsilateral sliding from the % of contralateral sliding.

For footfault tests, animals were allowed 10 minutes to walk on a horizontally placed ladder (1.5 cm width between rungs). A step was considered a footfault if it was not providing support or a slip/miss occurred. Foot-faults for each limb were counted and compared to the overall step number taken by that limb. The observer was blind to experimental design and mouse genotypes. Thus, percentage of footfaults was calculated by: the number of foot-faults/(number of steps + number of foot-faults) $\times$ 100. The asymmetry difference was derived by subtracting the percentage of ipsilateral foot-faults from the percentage of contralateral foot-faults.

Electrophysiological recording

Retrovirus expressing GFP (Retro-GFP) was injected in caspase-3/- cortex with overexpression of EbC at 5-7 dpi and patch-clamp was performed at 21-24 dpi. Neurons in injured cortex expressing GFP were selected for recording. The electrode was filled with potassium based intrapipette solution with a resistance of about 7-9 MΩ. Cells with series resistance more than 30 MΩ at any time during the
recordings were discarded. Pipette offset current was zeroed immediately prior to contacting the cell membrane. The data were sampled at 20 kHz for current-clamp recordings and 10 KHz for voltage-clamp recording with pCLAMP 9 software and digidata 1322 (Axon Instruments). Action potentials were recorded in the presence or absence of 1 μM TTX.

**Immunohistochemistry**

For immunohistochemistry, serial coronal sections were prepared on a cryostat. Primary antibodies were incubated at room temperature overnight (detailed information is included in Supplementary information). Before adding primary antibodies, antigen retrieval was performed for Wnt2 immunostaining, and HCl treatment (2N HCl, 30-35 min at 37°C) was performed for BrdU staining. Corresponding secondary antibodies conjugated with Alexa Fluor 594 (donkey anti-mouse, anti-rabbit, anti-goat or anti-guinea pig IgG, 1:800, Jackson ImmunoResearch), Alexa Fluor 488 (donkey anti-mouse, anti-rabbit, or anti-goat IgG, 1:800, Jackson ImmunoResearch), Alexa Fluor 647 (donkey anti-rabbit IgG, 1:800, Jackson ImmunoResearch) were incubated with sections for 4 h at room temperature in dark.

TUNEL staining was performed according to the manual of DeadEND™ TUNEL system (Promega). For combination of TUNEL staining with immunohistochemistry, TUNEL staining was performed first, followed by individual immunostaining.

**Culture of cortical neurons and astrocytes, induction and inhibition of apoptosis, and collection of conditioned medium**

Cortical neurons were isolated from mouse embryos at embryonic days 15.5-16.5. For culturing caspase-3/- neurons and their WT controls, neurons were isolated from P0 pups. Astrocytes were primary isolated from P2-4 pups, and purified by shaking the flask at 260 rpm overnight at 37°C. Primary neurons were treated by oxygen-glucose deprivation (OGD) for 24 h to induce apoptosis. For inhibition of apoptosis, 25μM z-VAD was added into the culture 2 h before irradiation. Conditioned medium from normal cultured neurons (NNCM), apoptotic neurons (ANCM) and apoptosis-inhibited neurons (AICM) was collected at 18-20h after irradiation, centrifuged at 2000 rpm for 10min. Supernatants were stored in -70°C until use.

**In situ hybridization**

Antisense digoxigenin-labeled RNA probes for Wnt2, Wnt7a, Wn9a and Wnt10a were synthesized and in situ hybridization was performed as described previously (Wang et al., 2011b). All the probes were made by RT-PCR based in vitro transcription. The primer information is as the following: Wnt2: TGTACTCTGAGGACATGCTGGCT, CTTATGTGCAGCAGGTGGTTC; Wnt7a: TCAGCCTGGGCATAGTCTACCTCC, TTCTCCTCCAGGATCTTCCGACCC; Wn9a: CAAGTTTGTGTTTCTCTGG, TGTTGTTTGTAACCCTGTGCC; Wnt10a: CTGTTCTCTTCTTCTGCTGGCT, ATGTTCTCCATCACCACCGCTGCC. After hybridization and incubation with anti-digoxigenin antibody, color was development with HRP mediated reaction.

**Luciferase assay of Wnt-5UTR IRES activity.**
MCF-7 cells were routinely maintained. Wnt2-5UTR was PCR amplified from genome DNA of mice liver and cloned into plasmid pR-F. Mutations within the conserved stem-loop region of Wnt2-5UTR were introduced by overlap extension stitch PCR. The resulting mutation was confirmed by Sanger sequencing. Twenty-four hours after plasmids transfection, OGD was performed and lasted for 24h. Luciferase assay was conducted using the luciferase reporting kit (Promega) after 8h’s reoxygenation using GloMax. The ratio of LucF/LucR was used to indicate the activity of IRES.

**Statistical analysis**

*For in vivo* proliferation and neurogenesis, the numbers of immuno-double labeled cells were counted from every eighth section of ischemic region and at least three mice included for each comparison. Cell counting was performed by an investigator who was blind to experimental design and mouse genotypes. Data are presented as mean ± standard error. Statistical comparisons were made using the Student t test or analysis of variance (ANOVA) with Student Newman-Keuls post hoc analysis. P values less than 0.05 were considered as statistically significant.

**Results**

**Focal ischemia induces Wnt signaling activation and dedifferentiation of reactive astrocytes**

To probe the local signals that trigger the phenotype change of astrocytes, we adopted a photothrombosis focal ischemia model. Neural progenitor markers, Nestin and Sox2 were quickly up-regulated in the injury site from one day post ischemia (Fig. 1a). Both Nestin and Sox2 were mainly expressed by GFAP-positive astrocytes (Fig. 1b-d). BrdU-labeling showed that most of the Nestin- and Sox2-positive cells in the lesion area were proliferative (Suppl. Fig. 1a, 1b). These data showed that reactive astrocytes undergo certain degree of dedifferentiation upon ischemic injury.

To directly readout if canonical Wnt signaling were re-activated in reactive astrocytes, we adopted a widely-used Wnt signaling reporter mouse line, Topgal mouse [15]. There was no or very low expression of Wnt signaling reporter β-gal and Wnt signaling target gene Axin2 in intact cortex. After injury, β-gal and Axin2 was up-regulated from one day post injury (dpi) (Fig.1e), similar to the time course of Nestin and Sox2 up-regulation. To examine whether Wnt signaling were induced in reactive astrocytes, we performed triple-immunostaining of β-gal/GFAP/Nestin and β-gal/GFAP/Sox2. There were almost no triple-positive cells in control side of cortex, while a lot of triple-positive cells in ischemic cortex. Most of the β-gal-positive cells in ischemic cortex were Nestin/GFAP- or Sox2/GFAP-positive (Fig. 1f). These data suggested that canonical Wnt signaling is activated mainly in reactive astrocytes and in coincidence with the expression of progenitor markers.

**Apoptotic neurons up-regulate Wnt2 protein which activates Wnt signaling**

To probe the mechanism of Wnt activation, we first examined the expression of Wnt ligands in the cortex by real time RT-PCR. Among the 18 Wnts, Wnt2, Wnt4, Wnt7a, Wnt9a, and Wnt10a showed a relatively
high expression level in the intact cortex, as compared with house-keeping gene GAPDH in the same tissue (Suppl. Fig. 2a). Because Wnt4 acts as a non-canonical Wnt ligand in the CNS \([16, 17]\), we performed *in situ* hybridization of *Wnt2*, *Wnt7a*, *Wnt9a* and *Wnt10a*. Among these, *Wnt2* mRNA was expressed by more cells (Suppl. Fig. 2b). To identify the cell type which expresses Wnt2, we performed immunohistochemistry and Western-blotting using a Wnt2 antibody. Double staining of Wnt2 with NeuN or GFAP showed that Wnt2 is mainly expressed by neurons (Suppl. Fig. 2c). Western-blotting of cortical tissue showed a higher molecular weight of Wnt2 than predicted, possibly due to posttranslational modification. To test the specificity of Wnt2 antibody used, we injected cortex with lentivirus expressing Wnt2 shRNA, which significantly reduced the level of Wnt2 mRNA (Suppl. Fig. 2d) as well as Wnt2 protein (Suppl. Fig. 2e).

After ischemia, the levels of Wnt2 protein increased quickly in comparison with that in intact cortex (Fig. 2a, 2b). Interestingly, most of the cells with high level of Wnt2 immunoreactivity (Wnt2\(^{hi}\)) in the injury site were TUNEL positive (Fig. 2c). To confirm whether apoptotic neurons up-regulated Wnt2, we cultured primary neurons and treated neurons by oxygen-glucose deprivation (OGD) to mimic ischemia. Starting from 2 h post OGD treatment, the levels of Wnt2 and cleaved caspase-3 (CC3) increased simultaneously in cultured neurons (Suppl. Fig. 3a). Adding pan-caspase inhibitor z-VAD effectively blocked the up-regulation of Wnt2 in apoptotic neurons, as compared with control cells (Suppl. Fig. 3a). To investigate whether Wnt2 was released from apoptotic neurons, we analyzed the levels of Wnt2 protein in conditioned medium (CM) from normally-cultured neurons (NNCM), apoptotic neurons (ANCM) prepared with OGD treatment, and apoptotic neurons treated with Wnt2 shRNA (shRNA ANCM). Western-blotting showed that OGD induced notable large amount of Wnt2 (ANCM) in wild type (WT) neurons, but not in *Capase-3* deficient neurons (Fig. 2d). This induction of Wnt2 protein could be effectively blocked by pretreating neurons with Wnt2 shRNA (shRNA ANCM) (Fig. 2d). These data indicated that apoptotic neurons up-regulate and release Wnt2.

To investigate whether this Wnt2 was sufficient to activate Wnt/β-catenin signaling, we stimulated primary astrocytes with NNCM, ANCM, shRNA ANCM or CM from apoptosis-inhibited neurons (AICM). The results showed that ANCM significantly increased the amount of β-catenin in the cell nucleus in relative to NNCM, AICM, and shRNA ANCM (Fig. 2e), suggesting that Wnt2 released by apoptotic neurons was able to activate Wnt/β-catenin signaling in astrocytes *in vitro*. Further, we injected lentivirus-expressing Wnt2 shRNA into the cerebral cortex 9 days before ischemia and examined Wnt activation at 5 dpi. Lenti-virus expressing GFP was used to verify the cell types infected. Approximately 39.7% of lenti-GFP-labeled cells were GFAP-positive, 30.2% NeuN-positive, 7.8% NG2-positive, and 5.8% Iba-1-positive (Suppl. Fig. 3b). Both immunohistochemistry and Western-blotting revealed that the induction of Wnt reporter β-gal was remarkably attenuated in Wnt2 shRNA pre-treated cortex (Fig. 2f, 2g). These data indicated that ischemic injury-induced Wnt2, particularly from apoptotic neurons, activates Wnt signaling in the reactive astrocytes.

**Wnt2 is required for astrocyte dedifferentiation and facilitates neurogenesis**
To test whether Wnt2 was involved in the acquisition of stem cell properties of reactive astrocytes in vivo, we made focal ischemia in bilateral cortices, injected lentivirus expressing Wnt2 shRNA in the right cortex and lentivirus expressing control shRNA in the left cortex. Immunohistochemistry showed that Wnt2 shRNA dramatically down-regulated the expression of Nestin in the injured cortex without affecting the up-regulation of GFAP in the ischemic cortex (Fig. 3a). Western blotting confirmed the reduction of Nestin and Sox2 in injured cortex pretreated by Wnt2 shRNA (Fig. 3b), suggesting the requirement of Wnt2 for the acquirement of NSC property of reactive astrocytes. Previous studies have reported the migration of new-born neuroblasts, although very weak, from the SVZ to the cortex after ischemia [18, 19]. Considering that astrocytes in the SVZ play important roles for maintaining the neurogenic niche [20], we hypothesized that these Nestin-positive reactive astrocytes might provide supporting signals for neurogenesis in ischemic cortex. We then investigated the effects of Wnt2 knockdown on the post-ischemic cortical neurogenesis. Immunohistochemistry showed that, at 7 dpi, DCX/BrdU-double positive cells were significantly reduced in Wnt2 shRNA-treated cortex (Fig. 3c). To assess roles of the downstream signals of Wnt2 in this process, lentivirus expressing dominant-negative TCF-4 (dnTCF-4) was injected to cortex 9 days before ischemia. DnTCF-4 inhibits the activity of Wnt signaling by competing the interaction between β-catenin and TCF [21]. The efficacy of Wnt inhibition was confirmed by the down-regulation of β-gal (Fig. 3d). Similar to Wnt2 shRNA, dnTCF-4 significantly reduced the expression of Nestin and the number of BrdU/DCX-double positive cells in ischemic cortex at 7 dpi (Fig. 3d, 3e).

We next assessed the effects of Wnt2 over-expression by injecting lentivirus expressing Wnt2 (Lenti-Wnt2) into ischemic cortex. Increase of Wnt2-expression was confirmed by Western blotting (Fig. 4a). At 14 dpi when progenitor markers have declined in control virus-treated cortex, Lenti-Wnt2 sustained the high level expression of Nestin and Sox2 (Fig. 4b, 4c). Further analysis showed that there was a significant increase of BrdU/DCX-positive cells in Lenti-Wnt2-infected ischemic cortex (Fig. 4d). Notably, BrdU/NeuN-positive cells which could hardly be detected in the ischemic cortex under normal condition [22], were found in Wnt2-overexpressing cortex with the frequency of approximately one BrdU/NeuN-positive cell in one section on average at 4 weeks after ischemia (Fig. 4e). These data indicated that Wnt2/β-catenin signaling may serve as a local signal for triggering the dedifferentiation of reactive astrocytes and supporting neurogenesis in ischemic cortex.

**Caspase-3 mutation abolishes both Wnt activation and astrocytes dedifferentiation in vivo**

Since apoptotic neurons up-regulate Wnt2, we further asked whether caspase-3 ablation could affect the ischemia-induced Wnt signaling activation. Wnt2 expression in intact caspase-3−/− cortices was at a similar level to that in the intact cortex of WT mice (Fig. 5a), indicating that caspase-3 mutation had no significant effects on the basal expression of Wnt2. Interestingly, caspase-3 ablation significantly abolished the up-regulation of Wnt2 by ischemia (Fig. 5a). Consistently, the up-regulation of Axin2 (Wnt target gene) by the ischemia was also compromised in the injured caspase-3−/− cortex (Suppl. Fig. 4a). Furthermore, the expression of Wnt signaling reporter β-gal in the ischemic cortex of TOPgal:caspase-3−/− mice was significantly attenuated as compared to that in the lesion cortex of TOPgal:caspase-3+/+ mice.
(Fig. 5b). These results indicate that caspase-3 is required for the up-regulation of Wnt2 and activation of Wnt signaling in the ischemic cortex.

We next examined the expression of Nestin and cell proliferation in ischemic caspase-3\(^{-/-}\) cortex. Immunohistochemistry showed that both the expression of Nestin and the number of BrdU-positive cells was obviously reduced in the ischemic cortex of caspase-3\(^{-/-}\) mice, as compared to that of WT mice (Fig. 5c). Western-blotting confirmed the compromised induction of Nestin in injured caspase-3\(^{-/-}\) cortex (Fig. 5c). Further analysis showed that BrdU/DCX-positive cells in caspase-3-deficient cortices were also dramatically decreased (Fig. 5d). On the other hand, no differences of Ki67/Nestin-double positive cells were found in the SVZ between intact WT and caspase-3\(^{-/-}\) mice at 7 dpi (Suppl Fig. 4b), suggesting that the effects of Caspase-3 mutation on cell proliferation in cortex is lesion dependent. These data indicated that caspase-3 might be involved in the ischemia-induced astrocyte dedifferentiation through activating Wnt signaling.

**Over-expressing β-catenin restores stem cell properties of reactive astrocytes and promotes neurogenesis in Caspase-3\(^{-/-}\) cortex.**

We next examined whether enhancing Wnt signaling could restore astrocytic response and neurogenesis in caspase-3\(^{-/-}\) cortex by injecting lentivirus expressing stabilized β-catenin (EbC) [21]. EbC infection resulted in higher levels of Nestin and Sox2 by astrocytes in caspase-3\(^{-/-}\) cortex, in comparison with control virus (Fig. 6a, 6b). The number of BrdU/DCX-double positive cells was also increased in the EbC infected caspase-3\(^{-/-}\) cortex (Fig. 6c, 6d). Similar as Wnt2 overexpression, BrdU/NeuN-double positive cells, which could not be detected in control ischemic cortex, were found in the lesion area of EbC-infected caspase-3\(^{-/-}\) cortex (Fig. 6e, 6f).

We then examined whether the new-born neurons could have neuronal properties. Retro-virus expressing GFP was injected into EbC-treated cortex at 5 dpi. Whole cell patch-clamp recording of Retro-GFP positive cells was performed at 26-29 dpi (Fig. 7a). Biocytin was injected to make sure the cell recorded were Retro-GFP labeled neurons (Fig. 7a). Among the 23 successfully patched cells, action potentials were recorded from 9 cells which had an average input resistance of 415.4±87.3 MΩ and resting membrane potential of -56.8±2.5 mV (Fig. 7b). Three Retro-GFP-labeled cells showed spontaneous excitatory postsynaptic currents (sEPSCs) (Fig. 7b). To test whether EbC could enhance functional recovery in Caspase-3\(^{-/-}\) mice, we performed focal ischemia in the forelimb sensorimotor cortex and evaluated the locomotion recovery by foot-fault test and spontaneous forelimb activity. The results showed that stabilized β-catenin significantly improved the spontaneous activity of contralateral (to the injured cortex) forelimb and reduced the foot-miss of forelimbs on a horizontally placed ladder from 3 weeks after injury (Fig. 7c, 7d). These results indicated that activating Wnt/β-catenin signaling not only restores astrocyte dedifferentiation but also promotes neurogenesis and functional recovery in the ischemic caspase-3-deficient mice.

**Apoptotic neurons up-regulate Wnt2 protein via IRES-mediated alternative translation.**
Above data demonstrated that apoptosis-activated Wnt2 triggered astrocyte response. We then asked whether this apoptosis-induced Wnt2 expression could occur in other species. We analyzed the cortex sample of macaca mulatta and human patient at 24h after ischemia. Wnt2 was expressed by neurons in normal macaca mulatta and human cortex (Fig. 8a-d, left pannels). Obvious up-regulation of Wnt2 was detected in apoptotic neurons in both ischemic macaca mulatta and human cortex as showed by double-immunostaining of Wnt2 with cleaved caspase-3 (CC3) or NeuN (Fig. 8a-d, right pannels), suggesting that this apoptosis-induced Wnt2 up-regulation is a conserved phenomenon.

We next probed how apoptotic neurons up-regulated Wnt2. qPCR revealed no changes of Wnt2 mRNA under apoptotic conditions both in vitro and in vivo (Fig. 9a, Suppl. Fig.5a), suggesting that post-transcription mechanism may account for the up-regulation of Wnt2 protein. Intriguingly, 26S proteasome activator PD169316 did not reduce the level of Wnt2 protein (Fig. 9b), while protein translation inhibitor CHX significantly attenuated the OGD-induced Wnt2 up-regulation (Fig. 9c), indicating that apoptotic neurons may up-regulate Wnt2 at the level of protein translation.

In apoptotic cells, the translation of most proteins is suppressed except for few proteins which are crucial for cell survival or apoptosis, such as insulin-like growth factor (IGF) and X-linked inhibitor of apoptosis (XIAP) [23, 24]. The “escaped” protein translation is achieved mainly by cap-independent translation, and in most cases, through the internal ribosome entry site (IRES) sequence in the 5′-untranslated region (5UTR) of the mRNA [25]. Sequence analysis showed that the Wnt2-5UTR of mouse, macaca mulatta and human shared highly conserved sequence (Fig. 9d) which could form IRES-like secondary structure (Fig. 9e). To test the function of Wnt2-5UTR, we inserted the Wnt2-5UTR into a bicistronic reporter vector and examined the activity of Wnt2-5UTR in MCF-7 cells(which normally express Wnt2) (Fig. 9f). OGD treatment induced dramatic expression of luciferase downstream of Wnt2-5UTR as positive control XIAP-5UTR did (Fig. 9g). Mutation of conserved sequence in Wnt2-5UTR signicantly abolished this apoptosis induced reporter expression (Fig. 9d, 9e, and 9g). These data indicated that apoptotic neurons may up-regulate Wnt2 protein through IRES-mediated protein translation.

Death-associated protein 5 mediates Wnt2 up-regulation and astrocyte de-differentiation.

In apoptotic cells, death associate protein 5 (DAP5) is one of the key protein which recruits ribosome to the IRES sequence of target mRNA and facilitates translation [26, 27]. We then tested whether this mechanism were involved in the apoptosis-induced Wnt2 up-regulation. Western-blotting showed that DAP5 was up-regulated in ischemic cortex (Fig. 10a). Pretreating primary neurons with lentivirus expressing DAP5 shRNA significantly attenuated the translation initiating activity of Wnt2-5UTR under OGD (Fig. 10b, Suppl. Fig. 5b). Local injecting lentivirus expressing DAP5 shRNA to cortex or cultured neurons significantly attenuated the ischemia/apoptosis-induced Wnt2 up-regulation both in vivo and in vitro (Fig. 10c, Suppl. Fig. 5c). Accordingly, the ischemia-induced expression of Wnt reporter β-gal in astrocytes was significantly reduced in the cortex of Topgal mice infected by lentivirus expressing DAP5 shRNA (Fig. 10d).
We next asked whether DAP5 knockdown affected the dedifferentiation of astrocytes and neurogenesis post cerebral ischemia. Double-immunostaining showed that, similar as Wnt2 shRNA pre-treatment, the expression of Nestin and Sox2 was significantly reduced in DAP5 shRNA pre-treated cortex while the expression of GFAP remain not changed (Fig. 10e, Suppl. Fig. 5d). The number of BrdU/DCX-positive cells was significantly reduced in DAP5 shRNA pre-treated cortex (Fig. 10f). These data suggested that DAP5 shRNA can partially phenocopy the effects of Wnt2 knockdown in terms of astrocytes dedifferentiation and cortical neurogenesis.

Discussion

In the present study, by using a Wnt signaling reporting mouse line Topgal, we first demonstrated the activation of canonical Wnt signaling in reactive astrocytes. By lentivirus-mediated Wnt2 silencing and Wnt2 over-expression, we demonstrated a key role of Wnt2 in the dedifferentiation response of astrocytes and post-ischemic neurogenesis. The data from caspase-3 deficient mice revealed the requirement of caspase-3 for the ischemia-induced Wnt2 up-regulation and astrocyte dedifferentiation. In the end, we demonstrated that the up-regulation of Wnt2 was mediated by IRES-mediated protein translation.

The present study focused on the in vivo dedifferentiation response of reactive astrocytes and neurogenesis in cortex after ischemia. The up-regulation of neural progenitor markers by reactive astrocytes has been reported [3]. Because reactive astrocytes can form neurospheres which can differentiate into neurons in vitro, it has been proposed that reactive astrocytes may be neurogenic. Two recent studies revealed that the neural sphere-forming reactive astrocytes in cerebral cortex were mainly derived from SVZ progenitors [28, 29]. How the local reactive astrocytes up-regulate progenitor markers remained to be explored. The fact that local delivery of Wnt2 shRNA in ischemic cortex effectively reduces Nestin expression in reactive astrocytes suggested a role of Wnt2 in the local response of astrocytes, because the migrating SVZ reactive astrocytes have already expressed Nestin. The reduction of DCX-positive neuroblasts and increase of BrdU-labeled NeuN-positive mature neurons by Wnt2 manipulation indicated that the local dedifferentiation of Wnt-activated astrocytes may be neurogenic, or alternatively, provide niche signals for the supporting SVZ neurogenesis.

It is very interesting that neuronal Wnt2 up-regulation and astrocyte dedifferentiation is caspase-3 dependent. Injury induced-Wnt activation has been documented in multiple tissues [30], while how injury activates Wnt signaling remains unclear. In low animals, for example, in hydra, apoptosis-induced Wnt activation is crucial for initiating head regeneration [31]. In mammalian, two studies have reported that in mammalian skin and tumor tissues, apoptotic cells induce wound healing [32] and tumor cell repopulation [33] by releasing PGE₂, a potential Wnt signaling activator [34]. In adult cortex, it is still a controversial topic concerning whether apoptosis could induce cortical neurogenesis [35, 36]. Recently, a study reported that the proliferation of microglia is apoptosis-coupled [37]. Our data revealed that Wnt2 may act as an endogenous reparative signal released by dying neurons. As conventional Casapse-3 knockout mice and lenti-virus expressing Wnt2 shRNA/Wnt2 were used in this study, the involvement of caspase-3 and Wnt2 in other cell types is not excluded.
This IRES-mediated Wnt2 translation is intriguing. Cap-dependent translation is inhibited during apoptosis and only a few proteins essential for cell survival or apoptosis can be translated through a cap-independent mechanism [23]. DAP5 plays a key role in the protein translation of apoptotic cells which requires the IRES structure in the 5UTR of target protein to recruit ribosome [26, 38]. Our data demonstrated that the translation of Wnt2 during apoptosis was dependent on DAP5, and the mutation of the IRES sequence in the 5UTR of Wnt2 abolished the translation of Wnt2. The high homology of Wnt2 5UTR among mouse, macaca mulatta and human suggested that this apoptosis-induced Wnt2 up-regulation may be a conserved mechanism. The functional recovery by over-expressing β-catenin suggested that amplifying this apoptosis-activated Wnt signaling may be utilized for treating cerebral ischemia.

**Conclusion**

In cerebral cortex, apoptotic neurons up-regulate and release Wnt2 protein, which activates Wnt/β-catenin signaling and induces dedifferentiation in reactive astrocytes. Over-expressing Wnt2 or β-catenin can enhance the dedifferentiation response of astrocytes and promote neurogenesis. The up-regulation Wnt2 protein by apoptotic neurons is achieved by IRES-mediated protein translation. Our data revealed a novel "SOS" mechanism for astrocyte dedifferentiation and indicated that Wnt/β-catenin signaling might be targeted for treating ischemic stroke in the future.

**Supplementary information:**

Supplementary information includes sequences of shRNA targets, primary information for realtime RT-PCR, and information of primary antibodies.

**Abbreviations**

AICM: Conditioned medium from apoptosis-inhibited neurons; ANCM: Conditioned medium from apoptotic neurons; CNS: Central nerve system; CC3: cleaved caspase-3; DAP5: Death associated protein 5; dnTCF-4: dominant-negative form of TCF-4; EbC: Lentiviral vectors of stabilized β-catenin; IGF: insulin-like growth factor; IRES: Internal ribosome entry site; Lenti-Wnt2: lentivirus expressing Wnt2; NNCM: Conditioned medium from normal cultured neurons; NSCs: Neural stem cells; OGD, oxygen-glucose deprivation; Retro-GFP: Retrovirus expressing GFP; SVZ: Subventricular zone; sEPSCs: spontaneous excitatory post-synaptic currents; XIAP: X-linked inhibitor of apoptosis; WT: wild type; 5UTR: 5'-untranslated region.

**Declarations**

**Ethics approval and consent to participate:**

All the animals were treated in strict accordance with Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines, and this study was formally reviewed and approved by the
Ethics Committee of Xijing hospital (KY20202003).

**Consent for publication:** Not applicable.

**Availability of supporting data:**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

**Competing interests:** The authors declare that they have no competing interests.

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**Authors’ contributions:**

Y.W., Y.-Q.D. and S. W. designed experiments, supervised the project, interpreted data, provided financial support and wrote the paper. H.F. performed most of the morphological studies, in vitro experiment and all Western-blottings. J.Y. contributed to mouse ischemic model establishment, Wnt signaling activation, effects of caspase-3 knockout and Wnt2 siRNA. J.X., B. G. and W.W. contributed to electrophysiological study. Y.H. and J. W. contributed to monkey ischemia model. W.L. contributed to human sample experiments. Y.H. contributed to photothrombosis model. J.D. contributed to molecular mechanism. L. S. and Y. Z. contributed to immunohistochemistry. W.D. and C.Z. contributed to manuscript preparation. Y.W., Y.-Q.D. and S. W. All authors read and approved the final manuscript.

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**References**
1. Escartin C, Guillemaud O, Carrillo-de Sauvage MA: *Questions and (some) answers on reactive astrocytes.* *Glia* 2019, 67:2221-2247.

2. Adams KL, Gallo V: *The diversity and disparity of the glial scar.* *Nature neuroscience* 2018, 21:9-15.

3. Gotz M, Sirko S, Beckers J, Irmler M: *Reactive astrocytes as neural stem or progenitor cells: In vivo lineage, in vitro potential, and Genome-wide expression analysis.* *Glia* 2015, 63:1452-1468.

4. Shimada IS, LeComte MD, Granger JC, Quinlan NJ, Spees JL: *Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke.* *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2012, 32:7926-7940.

5. Robel S, Berninger B, Gotz M: *The stem cell potential of glia: lessons from reactive gliosis.* *Nature reviews Neuroscience* 2011, 12:88-104.

6. Torper O, Gotz M: *Brain repair from intrinsic cell sources: Turning reactive glia into neurons.* *Progress in brain research* 2017, 230:69-97.

7. Magnusson JP, Goritz C, Tatarishvili J, Dias DO, Smith EM, Lindvall O, Kokaia Z, Frisen J: *A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse.* *Science* 2014, 346:237-241.

8. Sirko S, Behrendt G, Johansson PA, Tripathi P, Costa M, Bek S, Heinrich C, Tiedt S, Colak D, Dichgans M, et al: *Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog.* [corrected]. *Cell stem cell* 2013, 12:426-439.

9. Guo Z, Zhang L, Wu Z, Chen Y, Wang F, Chen G: *In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model.* *Cell stem cell* 2014, 14:188-202.

10. Niu W, Zang T, Zou Y, Fang S, Smith DK, Bachoo R, Zhang CL: *In vivo reprogramming of astrocytes to neuroblasts in the adult brain.* *Nature cell biology* 2013, 15:1164-1175.

11. Wang YZ, Plane JM, Jiang P, Zhou CJ, Deng W: *Concise review: Quiescent and active states of endogenous adult neural stem cells: identification and characterization.* *Stem cells* 2011, 29:907-912.

12. Kalamakis G, Brune D, Ravichandran S, Bolz J, Fan W, Ziebell F, Stiehl T, Catala-Martinez F, Kupke J, Zhao S, et al: *Quiescence Modulates Stem Cell Maintenance and Regenerative Capacity in the Aging Brain.* *Cell* 2019, 176:1407-1419 e1414.

13. Baskin YK, Dietrich WD, Green EJ: *Two effective behavioral tasks for evaluating sensorimotor dysfunction following traumatic brain injury in mice.* *Journal of neuroscience methods* 2003, 129:87-93.

14. Sweetnam D, Holmes A, Tennant KA, Zamani A, Walle M, Jones P, Wong C, Brown CE: *Diabetes impairs cortical plasticity and functional recovery following ischemic stroke.* *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2012, 32:5132-5143.

15. DasGupta R, Fuchs E: *Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation.* *Development* 1999, 126:4557-4568.
16. Liu Y, Wang X, Lu CC, Kerman R, Steward O, Xu XM, Zou Y: Repulsive Wnt signaling inhibits axon regeneration after CNS injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2008, **28**:8376-8382.

17. Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang Y, Nathans J, Tessier-Lavigne M, Zou Y: Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 2003, **302**:1984-1988.

18. Liang H, Zhao H, Gleichman A, Machnicki M, Telang S, Tang S, Rshtouni M, Ruddell J, Carmichael ST: Region-specific and activity-dependent regulation of SVZ neurogenesis and recovery after stroke. *Proceedings of the National Academy of Sciences of the United States of America* 2019, **116**:13621-13630.

19. Kernie SG, Parent JM: Forebrain neurogenesis after focal ischemic and traumatic brain injury. *Neurobiology of disease* 2010, **37**:267-274.

20. Todd KL, Baker KL, Eastman MB, Kolling FW, Trausch AG, Nelson CE, Conover JC: EphA4 Regulates Neuroblast and Astrocyte Organization in a Neurogenic Niche. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2017, **37**:3331-3341.

21. Fuerer C, Nusse R: Lentiviral vectors to probe and manipulate the Wnt signaling pathway. *PloS one* 2010, **5**:e9370.

22. Huttner HB, Bergmann O, Salehpour M, Racz A, Tatarishvili J, Lindgren E, Csonka T, Csiba L, Hortobagyi T, Mehes G, et al: The age and genomic integrity of neurons after cortical stroke in humans. *Nature neuroscience* 2014, **17**:801-803.

23. Holcik M, Sonenberg N: Translational control in stress and apoptosis. *Nature reviews Molecular cell biology* 2005, **6**:318-327.

24. Giraud S, Greco A, Brink M, Diaz JJ, Delafontaine P: Translation initiation of the insulin-like growth factor I receptor mRNA is mediated by an internal ribosome entry site. *The Journal of biological chemistry* 2001, **276**:5668-5675.

25. Spriggs KA, Bushell M, Mitchell SA, Willis AE: Internal ribosome entry segment-mediated translation during apoptosis: the role of IRES-trans-acting factors. *Cell death and differentiation* 2005, **12**:585-591.

26. Liberman N, Gandin V, Svitkin YV, David M, Virgili G, Jaramillo M, Holcik M, Nagar B, Kimchi A, Sonenberg N: DAP5 associates with elf2beta and elf4Al to promote Internal Ribosome Entry Site driven translation. *Nucleic acids research* 2015, **43**:3764-3775.

27. Henis-Korenblit S, Shani G, Sines T, Marash L, Shohat G, Kimchi A: The caspase-cleaved DAP5 protein supports internal ribosome entry site-mediated translation of death proteins. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**:5400-5405.

28. Faiz M, Sachewsky N, Gascon S, Bang KW, Morshead CM, Nagy A: Adult Neural Stem Cells from the Subventricular Zone Give Rise to Reactive Astrocytes in the Cortex after Stroke. *Cell stem cell* 2015, **17**:624-634.
29. Benner EJ, Luciano D, Jo R, Abdi K, Paez-Gonzalez P, Sheng H, Warner DS, Liu C, Eroglu C, Kuo CT: Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4. *Nature* 2013, **497**:369-373.

30. Bastakoty D, Young PP: Wnt/beta-catenin pathway in tissue injury: roles in pathology and therapeutic opportunities for regeneration. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2016, **30**:3271-3284.

31. Chera S, Ghila L, Dobretz K, Wenger Y, Bauer C, Buzgariu W, Martinou JC, Galliot B: Apoptotic cells provide an unexpected source of Wnt3 signaling to drive hydra head regeneration. *Developmental cell* 2009, **17**:279-289.

32. Li F, Huang Q, Chen J, Peng Y, Roop DR, Bedford JS, Li CY: Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. *Science signaling* 2010, **3**:ra13.

33. Huang Q, Li F, Liu X, Li W, Shi W, Liu FF, O’Sullivan B, He Z, Peng Y, Tan AC, et al: Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. *Nature medicine* 2011, **17**:860-866.

34. Goessling W, North TE, Loewer S, Lord AM, Lee S, Stoick-Cooper CL, Weidinger G, Puder M, Daley GQ, Moon RT, Zon LI: Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* 2009, **136**:1136-1147.

35. Magavi SS, Leavitt BR, Macklis JD: Induction of neurogenesis in the neocortex of adult mice. *Nature* 2000, **405**:951-955.

36. Diaz F, McKeahan N, Kang W, Hebert JM: Apoptosis of glutamatergic neurons fails to trigger a neurogenic response in the adult neocortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2013, **33**:6278-6284.

37. Askew K, Li K, Olmos-Alonso A, Garcia-Moreno F, Liang Y, Richardson P, Tipton T, Chapman MA, Riecken K, Beccari S, et al: Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain. *Cell reports* 2017, **18**:391-405.

38. Marash L, Kimchi A: DAP5 and IRES-mediated translation during programmed cell death. *Cell death and differentiation* 2005, **12**:554-562.

**Figures**
Figure 1

Dedifferentiation and activation of Wnt/β-catenin signaling in reactive astrocytes after cerebral ischemia

(a) Western-blotting and quantification of Nestin and Sox2 in ischemic cortex at different time points. *PNestin-1dpi=0.031, *PNestin-3dpi=0.033, **PNestin-7dpi=0.0026, *PSox2-1dpi=0.029, **PSox2-3dpi=0.0031, *PSox2-7dpi=0.028, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, n = 3 mice per time points. (b,c) Triple-immunostaining of Nestin/Sox2/GFAP in contralateral (con) and

Figure 1
ipsilateral (ipsi) side of ischemic cortex at 5 dpi. (d) Quantification of Nestin/GFAP- and Sox2/GFAP-positive cells in the zone expanded 200 μm from lesion center. (e) Western-blotting of Axin2 and β-gal in ischemic cortex at different time points post injury. Notice the up-regulation of Axin2 and β-gal from one day after ischemia. *P_{Axin2-1dpi}=0.019, **P_{Axin2-3dpi}=0.0024, *P_{Axin2-7dpi}=0.012, *P_{Axin2-14dpi}=0.035, *P_{βgal-1dpi}=0.017, *P_{βgal-3dpi}=0.021, *P_{βgal-7dpi}=0.018, *P_{βgal-14dpi}=0.027, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, n = 3 mice per time points. (f) Representative images of triple-immunostaining of GFAP/Nestin/β-gal and GFAP/Sox2/β-gal in Topgal mice at 5 dpi. Bar = 10 μm. Notice the induction of β-gal in Nestin- and Sox2-positive astrocytes in the injured cortex (arrowheads).

**Figure 2**

Up-regulation Wnt2 by apoptotic neurons ant its involvement in Wnt/β-catenin signaling activation. (a, b) Western-blotting of Wnt2 at different time point in ischemic cortex. Notice that Wnt2 protein increases quickly after ischemia. *P=0.026(1h), 0.028 (2h), 0.024 (4h), 0.048(8h), 0.043(12h). P values were calculated using Student’s t test comparing the ischemic groups with the normal control. N = 4 per group. (c) Combination of TUNEL staining and Wnt2 immunostaining at 24 hpi. Wnt2high immunoreactivity overlapped well with TUNEL-staining (arrowheads). Dashed line indicates lesion border and asterisk.
indicates lesion area. Bar = 30 μm. (d) Western-blotting of Wnt2 in conditioned medium of normal cultured (NNCM), apoptotic (ANCM), Wnt2 shRNA pretreated apoptotic (shRNA ANCM) wild type and caspase-3/- neurons. Apoptotic neurons increase Wnt2 release into the culture medium, and this increase is compromised by caspase-3 ablation and Wnt2 shRNA. (e) Western-blotting of cytoplasmic and nuclear β-catenin in astrocytes treated by NNCM, ANCM, AICM or shRNA ANCM. ANCM greatly increases the nuclear level of β-catenin. LMNB1 was used as loading control. (f) Double-immunostaining of GFAP/β-gal in Topgal mice pretreated with Wnt2 shRNA. (g) Western-blotting of β-gal in ischemic cortex of Topgal mice treated with Wnt2 shRNA or control. Notice the less induction of β-gal in Wnt2 shRNA pretreated cortex. Arrows point to representative double-positive cells. Bar = 50 μm.

Figure 3
Effects of Wnt2 knockdown and Wnt signaling inhibition on astrocyte dedifferentiation and cortical neurogenesis. (a) Representative images of Nestin/GFAP double-immunostaining in the bilateral cortex with two ischemic injures infected by lentivirus expressing control shRNA (right) and Wnt2 shRNA (left), respectively. Bar = 200 μm. (b) Western-blotting of Nestin and Sox2 in Wnt2 shRNA treated mice. Wnt2 shRNA dramatically suppressed the expression of Nestin and Sox2 without obviously affecting the expression of GFAP. (c) Double-immunostaining and quantification of DCX/BrdU in ischemic cortices treated by control shRNA and Wnt2 shRNA, respectively. Arrowheads point to double-positive cells. Arrows point to magnified cells. Bar = 20 μm. **P=0.0087, two-tailed Student’s t-test, n = 4 mice per group. (d) Western-blotting of β-gal and Nestin in ischemic cortex of Topgal mice infected by lentivirus expressing luciferase (pLenti-Luci as control) and dnTCF-4. Notice that dnTCF-4 reduces the expression of β-gal and Nestin in the ischemic cortex. (e) Double-immunostaining and quantification of DCX/BrdU in in ischemic cortex of infected by pLenti-Luci and dnTCF-4. N = 3 mice per group. ***P<0.001, two-tailed Student’s t test. Bar =50 μm.
Figure 4

Effects of Wnt2 over-expression on astrocyte dedifferentiation and cortical neurogenesis. (a) Western-blotting of Wnt2 in ischemic cortex infected by pLenti-Luci and Wnt2. (b) Western-blotting of Nestin and double-immunostaining of Nestin/Sox2 in ischemic cortex infected by pLenti-Luci and Wnt2. Bar = 50 μm. (c) Representative images of Nestin/GFAP double-immunostaining in the bilateral cortice with two ischemic injures infected by pLenti-Luci (left) and Wnt2 (right) at 14 dpi, respectively. Bar = 200 μm. Bar =
50 µm in enlarged view. (d) Double-immunostaining and quantification of DCX/BrdU in ischemic cortex infected by pLenti-Luci and Wnt2. N = 3 mice per group. **P = 0.0075, two-tailed Student’s t-test. Bar = 50 µm. (e) Double-immunostaining and quantification of NeuN/BrdU in ischemic cortices infected by lentivirus expressing luciferase (control) or Wnt2. S1 and S2 are two representative sections showing one NeuN/BrdU-positive cell in each section. Arrows point to double-stained cells. Bar = 30 µm, and 5 µm in insert. ***P < 0.001, two-tailed Student’s t-test, n = 4 mice per group.

Figure 5
Requirement of caspase-3 for Wnt2 activation and neurogenesis in ischemic cortex. (a) Combination of TUNEL staining and Wnt2 immunostaining (left panels), and Western-blotting of Wnt2 in the ischemic cortex of WT and caspase-3-/- mice at 24 hpi (right panels). Expression of Wnt2 in the injured WT cortex is significantly increased, but remains unchanged in caspase-3-/- cortex. Dashed lines indicate lesion border. Bar = 30 μm. **P=0.0019, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, n = 3-4 mice per group. (b) Representative images of β-gal/Nestin double immunostaining (left panels), and Western-blotting of β-gal in the ischemic cortex of Topgal mice with or without caspase-3 mutation at 5 dpi (right panels). The expression of β-gal in the ischemic cortex of Topgal:caspase-3+/+ mice is greatly enhanced, whereas it shows no obvious changes in Topgal:caspase-3-/- mice. Bar = 30 μm. **P=0.0015. one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, n = 3-4 mice per group. (c) Double staining of BrdU with Nestin, and Western-blotting of Nestin in the ischemic WT and caspase-3-/- cortex. Notice the lower level of Nestin in the ischemic caspase-3-/- cortex. **P=0.0012, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, n = 3-4 mice per group. (d) Double staining and quantification of BrdU/DCX. The numbers of BrdU/DCX-positive cells is significantly decreased in the caspase-3-/- cortex. Arrows in A-C point to double-positive cells and in D point to magnified cells. Arrowheads in D point to double-positive cells. Bars = 30 μm. **P=0.0072 (left panel), **P=0.0021 (right panel), two-tailed Student’s t-test, n = 3-4 per group.
Figure 6

Restoration of astrocyte dedifferentiation and promotion of neurogenesis in caspase-3-deficient cortex by stabilized β-catenin. (a) Western-blotting of Nestin and immune-staining of Nestin/GFAP in ischemic caspase-3/- cortex infected by lentivirus expressing luciferase (pLenti-Luci as control) or EbC. (b) Immuno-staining of Nestin/Sox2 in ischemic caspase-3/- cortex infected by pLenti-Luci or EbC. (c, d) Representative images and quantification of DCX/BrdU double immunostaining in ischemic caspase-3/- cortex infected by pLenti-Luci or EbC. EbC significantly increases the number of Nestin/Sox2- and BrdU/DCX-double positive cells. Arrowheads point to double-positive cells and arrows point to magnified cells. Bar = 30 μm. **P=0.0063, two-tailed Student’s t-test, n = 3 mice per group. (e, f) Double
immunostaining and quantification of NeuN/BrdU-positive cells in ischemic caspase-3-deficient cortex infected with control virus or EbC. There is approximately one BrdU/NeuN-positive cell on average per section of EbC treated cortex. S1, section 1. S2, section 2. Bars = 30 μm in (E), 5μm in (e'). Arrows point to BrdU/NeuN-positive cells. ***P<0.001, two-tailed Student's t-test, n = 3 per group.

Figure 7

Promotion of functional recovery of caspase-3-deficient mice by stabilized β-catenin (a) Experimental design and triple-staining of Retro-GFP/NeuN/Biocytin in patch-clamped cells. Bar = 5 μm. (b) Typical action potentials of recorded cells and typical EPSCs of the spike-firing cells. 9 (out of the 23) patched Retro-GFP-positive cells fire action potentials. Notice the spontaneous synaptic activities. (c) Sliding test. Stabilized β-catenin in Caspase-3-/- mice significantly reduces sliding incidence of the contralateral forelimb in the glass cylinder from 3w post-injury (n=6). *P<0.05. (d) Footfault test. Stabilized β-catenin reduces footfault incidence of the contralateral forelimb on a horizontally placed ladder from 3w post-injury (n=6). * P <0.05.
Figure 8

Expression of Wnt2 in ischemic cortex of macaca mulatta and human cortex. (a, b) Double-immunostaining of Wnt2/NeuN, Wnt2/CC3 in control and injured cortex of macaca mulatta 24h after ischemia. Bar = 30 μm. (c, d) Double-immunostaining of Wnt2/NeuN, Wnt2/CC3 in human cortical autopsy tissue (control and 24 hpi). Bar = 30 μm. Notice the higher Wnt2-immunoreactivity in CC3-positive cells in both macaca mulatta and human cortex. Arrows point to representative double-positive cells.
Figure 9

IRES-mediated Wnt2 protein translation. (a) Real time RT-PCR of Wnt2 in OGD treated neurons. (b) Western-blotting of Wnt2 in apoptotic neurons treated with PD169316. (c) Western-blotting and quantificatio of Wnt2 in apoptotic neurons treated with CHX. Notice that OGD does not induce Wnt2 mRNA, and that CHX blocks OGD-induced Wnt2 protein up-regulation. *P con vs OGD = 0.011, *P OGD VS OGD+CHX = 0.031, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, N = 3 batches of cells. (d) Sequence alignment of mouse, macaca mulatta and human Wnt2-5UTR. (e) Secondary structure of mouse Wnt2-5UTR. Blue frames in (d) and (e) show the largest stem-loop and the
corresponding sequence. The sequence underlined by black dots (in d) was replaced by the sequence below highlighted with yellow color to make Wnt2-5UTR mutant construct in (e). (f-d) Bistronic luciferase reporter assay of XIAP-5UTR, Wnt2-5UTR, and Wnt2-5UTR mutant under control and OGD condition. **PXIAP = 0.0022, **PWnt2 = 0.0019, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, N = 3 batches of cells. Notice that Wnt2-5UTR initiates translation and mutation at the largest loop abolishes the OGD-induced translation.

Figure 10
Involvement of DAP5 in Wnt activation and astrocyte dedifferentiation. (a) Western-blotting of DAP5 in the contralateral (Contra) and ipsilateral (Ipsi) cortex at 24h post ischemia. (b) IRES activity of Wnt2 5UTR with or without DAP5 shRNA under OGD. DAP5 shRNA effectively blocked the OGD-induced IRES activity. **P= 0.0052, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, N = 3 batches of cells. (c) Western-blotting of Wnt2 in the contralateral and ipsilateral cortex infected by lentivirus expressing DAP5 shRNA or control shRNA. *P = 0.039, one-way ANOVA followed by Bonferroni’s post hoc comparisons tests, N = 3 mice per group. (d) Double-immunostaining and quantification of GFAP/β-gal in ischemic cortex treated by control shRNA and DAP5 shRNA. Notice the attenuated expression of β-gal in DAP5 shRNA treated cortex. (e) Double-immunostaining and quantification of GFAP/Nestin in ischemic cortex treated by control shRNA and DAP5 shRNA. Ischemia-induced Nestin was reduced in DAP5 shRNA treated cortex. (f) Double-immunostaining of DCX/BrdU in ischemic cortex treated by control shRNA and DAP5 shRNA. Notice the decreased number of DCX/BrdU-positive cells in DAP5 shRNA treated cortex. Arrowheads point to double-positive cells. Arrows points to magnified cells. Bar = 30 μm. **P = 0.0063, two-tailed Student’s t-test, n = 3 mice per group.

**Supplementary Files**

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