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Delayed Treatment of 6-Bromoindirubin-3′-oxime Stimulates Neurogenesis and Functional Recovery after Focal Ischemic Stroke in Mice

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Abstract

Glycogen synthase kinase 3β (GSK3β) was originally identified as a regulator for glycogen metabolism and is now an important therapeutic target for a variety of brain disorders including neurodegenerative diseases due to its pivotal role in cellular metabolism, proliferation and differentiation. In the development of stroke therapies focusing on tissue repair and functional recovery, promoting neurogenesis is a main approach in regenerative medicine. In the present investigation, we explored the effects of a GSK3β specific inhibitor, 6-Bromoindirubin-3′-oxime (BIO), on regenerative activities of neuroblasts in the subventricular zone (SVZ) and functional recovery after focal cerebral ischemia. Adult C57/BL mice were subjected to occlusion of distal branches of middle cerebral artery (MCA) supplying the sensorimotor barrel cortex. Three days later, BIO (8.5 μg/kg, i.p.) was administered every 2 days until sacrificed at 14 or 21 days after stroke. The BIO treatment significantly increased generation of neuroblasts labeled with BrdU and BrdU/doublecortin (DCX) in the SVZ. Comparing to vehicle controls, increased number of neuroblasts migrated to the peri-infarct region where they differentiate into mature neurons. Along with the elevated BDNF expression at the peri-infarct area, the number of newly formed neurons was significantly increased. BIO treatment significantly enhanced sensorimotor functional recovery after the focal ischemia. It is suggested that the GSK3 signaling may be a potential therapeutic target for regenerative treatment after ischemic stroke.

Keywords
Cerebral ischemia; GSK3β inhibitor; 6-Bromoindirubin-3′-oxime (BIO); regeneration; functional recovery; Wnt
Introduction

Stroke possesses serious threat on human life and health. Neuroprotective treatments of stroke have so far failed in clinical translation. Recent demonstrations on the existence of neurogenesis in the adult brain have implicated a potential regenerative therapy after stroke. Neurogenesis persists in the adult mammalian brain mainly in two restricted areas, the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus of the hippocampus (Woodgett, 1990). Newly generated neural stem cells and neuroblasts at the SVZ normally migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS), where they can differentiate into interneurons (Lois et al., 1996). Neurogenesis may also contribute to tissue repair and recovery after brain injuries (Scafidi et al., 2009). Previous findings have shown that, in the ischemic brain cell proliferation in the SVZ is augmented and, in response to the chemoattractant cues, newly generated cells can migrate to the ischemic cortex and other sub-cortical regions via the striatum route (Arvidsson et al., 2002). Hypoxia and ischemia also may stimulate neuronal differentiation and neurite outgrowth (Wang et al., 2015). However, newly formed neural progenitor cells in the ischemic brain have limited contribution to brain repair after injury due to significant cell death, inadequate migration/homing ability and insufficient regenerative activity of these cells. To overcome this dilemma, treatments with exogenous factors have been explored to promote the endogenous regenerative mechanism.

Glycogen synthase kinase-3β (GSK3β) is widely expressed in all tissues with particularly abundant levels in the brain (Woodgett, 1990). A great number of studies indicates that GSK3β acts as a key regulator in neural development, including neuroblast generation/migration, neuronal polarization, axon growth/guidance, and synaptic plasticity (Hartwig et al., 2014; Hur and Zhou, 2010; Jaeger et al., 2013; Zhou and Snider, 2005). Inhibition of GSK3β decreases phosphorylation of β-catenin, preventing its degradation by the ubiquitin-mediated pathway (Komiya and Habas, 2008). The stabilized β-catenin acts on the nucleus by activating T-cell factor/lymphoid enhancer factor–mediated transcription of target genes that elicits a variety of effects on cells, which may be a mechanism underlying neuroprotective effect against in vitro model of ischemia in human neural progenitor cells (Skardelly et al., 2011).

Based on previous investigations that a GSK3 inhibitor could promote adult hippocampal neurogenesis under normal conditions (Morales-Garcia et al., 2012), we tested the hypothesis that inhibiting GSK3β using a small molecule inhibitor 6-bromoindirubin-30-oxime (BIO) could promote neurogenic activity and functional recovery after focal cerebral ischemia stroke. The present investigation shows novel evidence that delayed BIO treatment 3 days after stroke exhibits regenerative and functional benefits after ischemic stroke in adult mice.

Materials and methods

Focal cortex ischemic stroke model of mice

All experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Adult male C57BL/6 mice (National
Cancer Institute, NCI, Bethesda, MD) weighing 24 to 28g and in the age range of 8 to 10 weeks old were used in this study. The focal cortex ischemic stroke was induced as previously described (Whitaker et al., 2007). In brief, mice were anesthetized with intraperitoneal (i.p.) injection of 4% chloral hydrate (100 mg/kg). The animal’s head was held in a non-invasive holder between the palate and the bridge of the nose, without interfering with breathing. A 10 mm incision was made midway between the right eye and ear. The underlying muscle was separated, and a 4-mm diameter circle was incised in the skull with a dental drill with a sterile round 1.5-mm bit to the inner layer of cranium; then the encircled bone was avulsed with a dental tool. The transparent dura was kept intact. The cranial window was rinsed with sterile saline and closed with a sterile glass coverslip. By videomicroscopy the barrel cortex can be localized by intrinsic optical signals (IOS) during whisker stimulation (Wei et al., 1995). After correlating the IOS signal with the pattern of surface vessels, 3 distal branches of the middle cerebral artery (MCA) enclosing the sensorimotor/barrel cortex were permanently ligated with a #10-0 sterile monofilament sutures (Surgical Specialties CO., Reading, PA, USA) through the dura. This procedure was accompanied by 7-min bilateral common carotid artery (CCA) ligation to induce focal ischemia. The combined occlusions of the MCA and CCA, which also supply the anterior and posterior cerebral arteries, ensure infarct formation in the somatosensory cortex 1 day later. The wound was closed with sterile 6-O sutures. In sham surgical controls, all of these steps were identical and the sutures were placed but not tied shut. Monofilament sterile 6-O sutures with triangular section needles were used for closing the skin over the cranial window. Body temperature was monitored using rectal probe and maintained at 37.0°C using a temperature control unit and heating pads (Cole-Parmer Instrument Co, Chicago, IL) during surgery and the recovery period.

Experimental design

Mice were randomly divided into ischemic stroke-vehicle group and stroke plus BIO treatment group. Three days after ischemia, BIO 8.5 μg/kg or equivalent volume of DMSO was administered intraperitoneally every 2 days until sacrificed at 14 or 21 days after stroke. To label proliferating cells, 5-bromo-20-deoxyuridine (BrdU) (50 mg/kg/day, i.p.) (Sigma, St Louis, MO) was administered to all animals beginning on day 3 after ischemia until the day of sacrifice.

Although BIO is an established inhibitor of GSK-3β (Meijer et al., 2003), there is limited information about its effective dosages in vivo. High doses of 50 mg/kg BIO was tested and showed certain cytotoxic effect (Kehn-Hall et al., 2012). BIO was also given at 0.2 mg/kg for repeated intraperitoneal (i.p.) administrations (Wang et al., 2009). We selected a low dosage of 8.5 μg/kg BIO based on its effect of reducing the downstream molecule phosphorylated GSK-3β (see Fig. 1A).

Western blot analysis

Fresh brain tissue was isolated under a microscope from the peri-infarct area defined as the region within 500 μm from the edge of the infarct area (Ahn et al., 2014). Western blot analysis was performed to analyze protein expression in penumbra following previous procedures (Manzerra et al., 2001). In brief, brain tissue was lysed with modified
radioimmunoprecipitation assay buffer (50 mmol/L HEPES, pH 7.3, 0.1% sodium deoxycholate, 150 mmol/L NaCl, 1mmol/L ethylenediaminetetraacetic acid, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF), and protease inhibitor cocktail (Sigma) with continuous manual homogenization. After 30 min, lysate was spun at 17,000 rpm for 15 min at 4°C and supernatant was collected. Protein concentration was determined using the Bicinchoninic Acid Assay (Sigma). Equal amounts of protein (30 μg) were electrophoresed on 6% to 20% sodium dodecyl sulfate-polyacrylamide gradient gel (SDS-PAGE) in a Hoefer Mini-Gel system (Amersham Biosciences, Piscataway, NJ) and transferred in the Hoefer Transfer Tank (Amersham Biosciences) to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA). Membranes were blocked with 5% BSA in Tris buffered saline (TBS, 20 mmol/L Tris, 137 mmol/l NaCl and 0.1% tween-20) at room temperature for 1 hr and incubated overnight at 4°C with primary antibodies against brain-derived neurotrophic factor (BDNF) (1:500, Santa Cruz, sc546), GAP-43(1:500, AB5220; Millipore), β-catenin (1:1000, R&D), β-tubulin (1:2500, No.2146S; Cell Signalling, Danvers, MA) was used as the protein loading control. After 3 washes with TBST, blots were incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibodies (Promega, Madison, WI) for 2 hrs at room temperature. Finally, membranes were washed with TBST and the signal was developed by the addition of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) solution (Sigma), quantified, and analyzed by the imaging software ImageJ (NIH, Bethesda, MD). The level of protein expression was normalized to β-tubulin controls.

**Immunohistochemical staining and cell counting**

Preparation of fresh frozen brain sections was performed following previous procedures (Li et al., 2008; Wei et al., 2012). Coronal fresh frozen sections of 10-μm thick were sliced using a cryostat Vibratome (Ultratrace 5000; St. Louis, MO, USA). After the slides were completely dried on warming plate, slices were fixed in 10% buffered formalin phosphate for 10 min, followed by treatments in a −20°C ethanol/acetic acid (2:1) solution for 12 min, in 0.2% Triton-100 for 5 min and washed by PBS three times between each step. Slides were blocked in 1% gelatin from cold water fish (Sigma, St. Louis, MO, USA), diluted in PBS at room temperature for 1 h, and subsequently incubated with primary antibodies diluted in PBS overnight at 4°C. The primary antibodies were mouse anti-NeuN (1:400, Millipore) and anti-doublecortin (rabbit anti-DCX, 1:50, Santa Cruz Biotechnology). After rinsing with PBS, brain sections were then treated with Alexa Fluor 488-conjugated anti-mouse IgG and anti-rabbit IgG (1:200; Life Technologies) secondary antibodies for 2 hrs at room temperature. To double stain DCX with BrdU, animals were transcardially perfused with warm saline and buffered 10% formalin (VWR, PA, USA) before sacrificed. Slides were washed with −20°C pre-cooled methanol before incubated in 2N HCl at 37°C for 40 min, followed by 0.2% Trition-X 100 incubation for 40 min. The BrdU staining procedure was performed with rat anti-BrdU (1:600, AbD Serotec) and Cy3-conjugated goat antirat IgG (Jackson ImmunoResearch, West Grove, PA, USA) as the primary and secondary antibodies according to our established technique (Li et al., 2008). Vectashield mounting media for fluorescence (Vector Laboratory, Burlingame, CA) was used to cover-slip slides in preparation for microscopy and imaging analysis. Fluorescence was visualized by microscopy (BX61; Olympus, Tokyo, Japan).
Cell counting was performed following a modification of the principles of design-based stereology. Systematic random sampling was employed to ensure accurate and non-redundant cell counting (Schmitz and Hof, 2005). Every section under analysis was at least 100 μm away from the next to ensure the same cells are not counted twice. For each animal, six sections spanning the entire region of interest were selected for cell counting. The optical dissector method was used to avoid oversampling errors. The z-position of a cell/neuron in a given section was confirmed in 3-D images and counted. Migration distance (μm) was evaluated from the SVZ to the furthest co-labeled DCX and BrdU-positive cell, determined by Z-stack imaging. Counting was performed on 6 randomly selected non-overlapping fields per section. Sections from different animals represent the same area in the anterior-posterior direction.

Evaluation of neurological function deficits: adhesive removal test

The adhesive removal test, a sensitive method to assess sensorimotor deficits in focal cerebral ischemic mice, was performed according to the procedure described before (Bouet et al., 2009; Zhang et al., 2000). In brief, a piece of adhesive tape was placed on each (right and left) forepaw of awaken animals. After returning the animals to their home cage, the time-to-contact (latency) and the time-to-remove (removal) the tape were recorded. The latency of time-to-contact was counted right after placing the dot on the paw until the animal showed reaction by licking or touching using the other paw. The removal time was counted from the attachment of the sticky dot until the dot was actually removed. Animals were trained for three days (1–2 trials per day) before stroke induction until the mice could take the adhesive tape off their paws within 12 sec to get a basal level of performance. Animals were tested before stroke and 7, 14 and 21 days after stroke by an investigator who was blinded to the experimental groups. The mean time (averaged from 5 trials) required to detect (time-to-contact) and remove (time-to-remove) the adhesive tape from the left forepaw was recorded. All tests were conducted during day time.

Statistical analysis

Student’s two-tailed t test was used for comparison of two experimental groups. The non-parametric Mann-Whitney test was conducted to examine the differences in Western blot results. Repeated Measures analysis was applied for functional tests as described in the text. The SPSS Statistics software (IBM Corporation, Armonk, New York) was used in the statistical analysis. Results are expressed as mean ± SEM. P<0.05 was considered significant for all comparisons.

Results

Low dose of BIO was sufficient to regulate the GSK3β pathway signaling in the normal and stroke brain

In the normal adult mouse brain, Western blotting showed that the low dosage of 8.5 μg/kg (i.p.) induced a significant effect of reducing the downstream signaling molecule phosphorylated GSK-3β (Fig. 1A). We selected to use 8.5 μg/kg BIO in the following experiments.
A focal cerebral ischemic stroke was induced in adult C57BL/6 mice by permanent occlusion of the distal branches of the right middle cerebral artery (MCA) plus 7-min occlusion of the common carotid arteries (CCAs), targeting the sensorimotor cortex (Wang et al., 2014). Animals were randomly divided into sham control, stroke plus vehicle (0.2% DMSO), and BIO (8.5 μg/kg, i.p.) groups. Treatment was started 3 days after stroke followed by the same injections every two days. Fourteen days after stroke, Western blot analysis showed increasing effects on β-catenin, GAP-43, and BDNF expressions compared to sham control and vehicle treated stroke controls (Fig. 1B).

**BIO stimulates cell proliferation and the number of neuroblasts in the SVZ**

This study aimed to test the delayed treatment of BIO for enhanced regenerative mechanisms and long-term functional recovery after focal ischemic stroke in adult mice. The GSK3β inhibitor BIO (8.5 μg/kg, i.p.) or vehicle control was applied 3 days after stroke and once every two days as described above. The therapeutic window of 3 days after stroke was selected for the main goal of increasing regenerative activities in the post-ischemic brain.

To label proliferating cells during the chronic phase after stroke, BrdU (50 mg/kg/day, i.p.) was administered from day 3 after stroke. Compared to vehicle controls, BrdU-positive cells in the ipsilateral hemisphere significantly increased in the BIO treatment group 14 days after stroke (Fig. 2). To examine neurogenesis more specifically, we determined the number of cells in the SVZ region expressing doublecortin (DCX), an intermediate filament protein expressed in neuronal precursor cells and immature neurons (Couillard-Despres et al., 2005). DCX is also used for labeling migrating neuroblasts in the brain (Brown et al., 2003). Compared to stroke vehicle controls, the BIO treatment significantly increased BrdU/DCX double positive cells in the SVZ. The ratio of BrdU/DCX double positive cells to BrdU positive cells, on the other hand, was unchanged (Fig. 2), implying that BIO generally enhanced cell proliferation in the SVZ.

**BIO promotes migration of neuroblasts towards the peri-infarct region**

An increased number of neuroblasts in the SVZ could be a result from either enhanced proliferation or failure of cell migration out of SVZ. Since directed migration of neuroblasts from the SVZ to ischemic lesion sites is a critical step for tissue repair after stroke, migration of neuroblasts from the SVZ was examined in the post-stroke brain. Fourteen days after stroke, we observed that more neuroblasts were observed along the migration route from the SVZ to the ischemic cortex, and these cells moved longer distance than that in stroke control mice (Fig. 2). Consistent to this enforced migration activity, a greater number of BrdU/DCX double positive cells reached the peri-infarct region 21 days after stroke in BIO-treated animals than that in stroke control mice (Fig. 3).

**BIO enhanced newly formed neurons in the peri-infarct region**

To determine neuronal differentiation in the peri-infarct region where they are needed for tissue repair, NeuN staining for mature neurons and the proliferation marker BrdU were co-stained 21 days after stroke. In the peri-infarct region from mice received BIO, there were
significantly more BrdU/NeuN co-stained cells compared with the stroke-vehicle group (Fig. 3), suggesting enhanced neurogenesis by BIO treatment.

**BIO attenuated ischemia induced neurological deficits**

The adhesive-removal test was used to assess sensorimotor deficits in mice 7 to 21 days after focal ischemia (Lubjuhn et al., 2009). The time needed for stroke animals to feel (time-to-detect) and remove (time-to-feel and remove) the sticky dot from the left paw was measured at different times before and after stroke. Repeated measures analysis showed that there was significant difference between the two treatment groups on both the time-to-detect and time-to-remove outcomes (Table 1). Furthermore, there is significant difference in the treatment (BIO or vehicle) influences on temporal changes in removal time (p=0.029). Specifically, the removal time in the BIO-treated group decreased consistently after the BIO treatment while the vehicle-control group didn’t show such consistent decrease in the removal time (Table 1 and Fig. 4). Thus, stroke induced functional impairment was significantly ameliorated in mice received BIO treatment.

**Discussion**

The present study was designed to evaluate relatively long-term effects of delayed treatment using the GSK3β inhibitor BIO on endogenous neurogenesis and behavior recovery after focal ischemic stroke in adult mice. Different from many neuroprotective treatments, we tested the wide therapeutic window of 3 days after stroke based on the goal of promoting regenerative activities during the recovery period after stroke. Our data demonstrate that the delayed BIO treatment showed marked promoting effects on cell proliferation, migration to the ischemic cortex and neuronal differentiation in the peri-infarct region. The functional outcomes of stroke tested in the adhesive-removal test were significantly improved by the BIO treatment.

Although a few papers reported that inhibition of GSK3β enhanced cognitive recovery after stroke mediated by transforming growth factor-β-activated kinase (TAK1), and a COX-2 inhibitor showed neuroprotection by phosphorylation of Akt and GSK3β (Venna et al., 2015; Ye et al., 2012), our study appears the first demonstration of regenerative benefits of delayed inhibition of the GSK3β signaling after ischemic stroke. Most previous neuroprotective investigations administered neuroprotectants before or acutely after the onset of stroke, which may at least partly explain the difficulties of translating these treatments to clinical settings. Similarly, GSK-3 inhibitors were previously tested in stroke models administrated before or within 3 hrs after stroke and showed neuroprotective effect of reducing the brain infarct formation (Chuang et al., 2011; Koh et al., 2008). We took the more clinically feasible approach of initiating the BIO treatment as late as 3 days after stroke, aiming specifically at promoting regenerative mechanisms, tissue repair and functional recovery after stroke. This much delayed time point obviously demonstrates a high clinical potential for the treatment of ischemic stroke.

BIO has been shown to reduce GSK3β kinase activity and signals in the canonical Wnt pathway by causing an increase in β-catenin levels (Meijer et al., 2003). Both in vitro and in vivo studies have demonstrated that the Wnt/β-catenin pathway regulates the proliferation
and differentiation of neural progenitor cells (Hirabayashi and Gotoh, 2005; Polakis, 2000; Skardelly et al., 2011). The β-catenin signaling promotes proliferation of progenitor cells in the adult mouse SVZ, and an inhibitor of GSK3β promotes the proliferation of Mash1 cells (Adachi et al., 2007). Using several diverse inhibitor of GSK-3 as pharmacological tools, Morales-Garcia and his colleagues showed that inhibition of GSK-3 induces proliferation of neural stem cells (Morales-Garcia et al., 2012). Although the proliferation of progenitor cells increased at the SVZ after focal cortical ischemia (Ohab et al., 2006), it is not enough for the purpose of tissue repair in the ischemic brain. Thus, the development of a chronic therapy for increased endogenous regenerative activities is much needed in a regenerative treatment of ischemic stroke. It is possible, however, an earlier neuroprotective treatment using BIO or other protective drugs (hours and/or 1–2 days after the onset of stroke) combined with the delayed BIO treatment may exhibit even more comprehensive beneficial effects after stroke. This possibility of proving both neuroprotection and neuro-regeneration using the same and/or different drugs could be an effective combinational therapeutic approach.

Neurogenesis after focal ischemic stroke comprises at least three processes: proliferation, migration and differentiation/survival (Zhang et al., 2005). In addition to proliferation, GSK3β has shown to be involved in the motility of various cells by regulating actin rearrangement and microtubule turnover (Lapid et al., 2013). Phosphorylation of proteins by GSK3 is involved in the modulation of microtubules and neurofilament stabilization, which affect the cytoskeleton. In the present study, the pool of progenitor cells identified as BrdU-positive cells in the SVZ was significantly increased in BIO-treated animals compared to stroke/vehicle controls. Based on the numbers of newly born cells in the ipsilateral SVZ and the ischemic cortex, we conclude that the increased progenitor cells in the SVZ were not the results of reduced migration of these cells out of the SVZ. Rather, the increased BrdU/DCX double positive cells in cell counting assays and the migration distance all suggest that BIO treatment results in significant increases not only in cell proliferation but also directed migration from the ipsilateral SVZ to the ischemic cortex. Since the ratio of neuroblast cell generation shows no significant difference between the two groups, we also assume that BIO may exert proliferating effect on glial lineage cells.

BIO treatment increased the number of newly formed neurons showed as BrdU/NeuN co-stained cells in the peri-infarct area 21 days after stroke. These are presumably mature neurons differentiated from migrated neuroblasts. Inhibition of GSK3β favors an increase in unphosphorylated β-catenin levels, allowing interaction with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors and, as a consequence, promoting the expression of cell survival genes (van Noort and Clevers, 2002). Wnt signaling pathway directly induces BDNF expression (Yi et al., 2012), which is a well-known neurotrophin mediating a wide variety effects of neuroprotection and regeneration after injuries. These events should play important roles in the observed greater numbers of neuroblasts and differentiated neurons after BIO treatment. It was also shown that BIO mediated Wnt activation is functionally reversible, withdrawal of the compound leads to differentiation in both HESCs and MESCs (Sato et al., 2004). The fluctuation of the GSK3β level in the peri-infarct area caused by the repeated injections in our experimental protocol may also stimulate neuroblast differentiate.
As one of few master switch kinases that regulate many aspects of cell functions, GSK3β involved in multiple signaling pathway in vivo. The present investigation provides new evidence that inhibiting GSK3β during the sub-acute and chronic phases after ischemic stroke can stimulate post-stroke neurogenesis, neuroblast migration to the ischemic cortex, neuronal differentiation and functional recovery after ischemic stroke. It is suggested that GSK3β inhibitors may be explored as a novel regenerative therapy with a wide therapeutic window after focal ischemic stroke.

Acknowledgments
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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BIO</td>
<td>6-Bromoindirubin-3′-oxime</td>
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<tr>
<td>CCA</td>
<td>common carotid artery</td>
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<td>i.p</td>
<td>intraperitoneal</td>
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<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
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<td>DCX</td>
<td>doublecortin</td>
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<td>BDNF</td>
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<td>SVZ</td>
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<td>MCA</td>
<td>middle cerebral artery</td>
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References


### Highlights

- GSK3β inhibition by BIO 3 days after stroke increased neuroblasts proliferation in the SVZ.
- BIO treatment promoted neuroblasts migration from SVZ to the ischemic cortex.
- BIO treatment enhanced neuronal differentiation of neuroblasts.
- BIO treatment increased trophic factor expression.
- BIO treatment improved functional recovery after stroke.
Figure 1. BIO regulation of GSK-3β and gene expression in the normal and post-ischemic brain
Western blotting was performed to measure GSK-3β and related genes in the normal and post-stroke brains of the mouse. A. In the normal mouse brain, BIO at the low dosage of 8.5 μg/kg (i.p.) caused significant inhibition of phosphorylation of GSK-3β 12 hrs later. The bar graph is a quantified summary showing significant reduction of p-GSK-3β from normal controls (n=5; ** P<0.01 by Student t test). B. Adult mice were subjected to focal ischemia of the right sensorimotor cortex and 3 days later randomly divided into two groups that received vehicle or BIO injection followed by the same injections every two days. Fourteen days after stroke, Western blot was performed to assess the protein expression of several GSK3β-related factors including β-catenin, BDNF and GAP43 in the ischemic peri-infarct...
region. C – E. Quantified analysis of each factor. Gray intensity was normalized against β-actin and quantified using ImageJ software. The non-parametric Mann-Whitney test was conducted to examine the differences in gene expression levels between treated and sham and the difference between vehicle and sham. The results show that the BIO-treated group were marginally different from the sham control for GAP-43 (p=0.1) and BDNF (p=0.1) while vehicle was not different from sham control for these measurements. For beta-catenin, there is moderate evidence that the BIO-treated group is different from sham control (p=0.2), the result is probably due to the limited sample size. N=6 each group.
Figure 2. BIO stimulates cell proliferation in the post-ischemic brain

To label proliferating cells during sub-acute and chronic phases, daily BrdU injection was started from day 3 after stroke. Immunohistochemical staining of BrdU and DCX was performed 14 days after stroke. A and B. Fluorescent images show BrdU (red) and DCX (green) double staining in the SVZ (★) and the adjacent corpus callosum region as well as the peri-infarct region (#). C. The bar graph shows the total proliferating (BrdU positive) cells and proliferating DCX-positive neuroblasts calculated from cell count assays on 6 brain sections/mouse (20-μm thickness, 100 μm apart crossing affected brain regions the ipsilateral hemisphere). BIO treatment increased both BrdU positive cells as well as BrdU/DCX double positive neuroblasts in the post-ischemic brain. D. The number of BrdU/DCX double positive cells in the SVZ area. E. The distance of neuroblasts migrated.
from the SVZ towards the ischemic cortex. BIO treatment showed a significant effect of promoting the directed migration of neuroblasts. F. The ratio of BrdU/DCX double positive cells to BrdU positive cells was unchanged. N=8, each group, * p<0.05 vs. controls.
Figure 3. BIO promotes neurogenesis in the peri-infarct cortex 21 days after stroke
The effect of neurogenesis was assessed using immunostaining of BrdU (red) and NeuN (green) staining in the brain sections 21 days after stroke. A and B. BrdU (A) and NeuN (B) staining in the peri-infarct region. C. The 3-D image of A and B shows the overlap if the BrdU and NeuN labeled cells, indicating newly generated neurons in the peri-infarct cortex. D. The bar graph shows quantified data of the BrdU/NeuN double positive cells in the peri-infarct area. As a result from increased cell migration, more new newly formed neurons were seen in the peri-infarct region of BIO-treated mice. N=5, each group, * p<0.05 vs. controls.
Figure 4. Functional recovery after focal cortex ischemia
Long-term sensory-motor functional recovery was evaluated with adhesive removal test at 7, 14 days and 21 days after stroke. A. BIO treatment decreased the time needed for detecting adhesive tape on the left paw at all time points tested (7 to 21 days after stroke), suggesting improved functional deficit associated with the right sensorimotor cortex ischemia. N=9 animals per group, * p<0.05. B. Along with the recovered sensory function, BIO treatment showed significant improvement in the time needed for removing the sticky dot from the left paw. 14 days and 21 days after stroke, the removal time for BIO treated animals was much shorter compared to vehicle controls N=8–9, * p<0.05 vs. controls. See Table 1 for factors and parameters in the repeated measures ANOVA analysis.
Table 1

Results of Deviance tests in mixed effect model for the statistical analysis of the adhesive removal test

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<tr>
<td>P value</td>
<td>0.002</td>
<td>&lt;0.0001</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Repeated measures ANOVA was performed to model the latency and removal longitudinally and assess the effect of treatment, time and their interaction effect. Specifically, linear mixed model is applied where each mouse was treated as an experimental unit in the mixed model. The fixed effects in the models include 1) time, 2) treatment group, 3) time and treatment group interaction. The fixed effects are tested using the Deviance test in the mixed model.