

## Dietary Supplementation Exerts Neuroprotective Effects in Ischemic Stroke Model

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### ABSTRACT

This study examined whether dietary supplementation can be used to protect against ischemic stroke. Two groups of adult male Sprague-Dawley rats initially received NT-020, a proprietary formulation of blueberry, green tea, Vitamin D3, and carnosine ( $n = 8$ ), or vehicle ( $n = 7$ ). Dosing for NT-020 and vehicle consisted of daily oral administration (using a gavage) over a 2-week period. On day 14 following the last drug treatment, all animals underwent the stroke surgery using the transient 1-hour suture occlusion of middle cerebral artery (MCAo). To reveal the functional effects of NT-020, animals were subjected to established behavioral tests just prior to stroke surgery and again on day 14 post-stroke. ANOVA revealed significant treatment effects ( $p < 0.05$ ), characterized by reductions of 11.8% and 24.4% in motor asymmetry and neurologic dysfunction, respectively, in NT-020-treated stroke animals compared to vehicle-treated stroke animals. Evaluation of cerebral infarction revealed a significant 75% decrement in mean glial scar area in the ischemic striatum of NT-020-treated stroke animals compared to that of vehicle-treated stroke animals ( $p < 0.0005$ ). Quantitative analysis of subventricular zone's cell proliferative activity revealed at least a one-fold increase in the number of BrdU-positive cells in the NT-020-treated stroke brains compared to vehicle-treated stroke brains ( $p < 0.0005$ ). Similarly, quantitative analysis of BrdU labeling in the ischemic striatal penumbra revealed at least a three-fold increase in the number of BrdU-positive cells in the NT-020-treated stroke brains compared to vehicle-treated stroke brains ( $p < 0.0001$ ). In addition, widespread double labeling of cells with BrdU and doublecortin was detected in NT-020-treated stroke brains (intact side 17% and ischemic side 75%), which was significantly higher than those seen in vehicle-treated stroke brains (intact side 5% and ischemic side 13%) ( $p < 0.05$ ). In contrast, only a small number of cells in NT-020-treated stroke brains double labeled with BrdU and GFAP (intact side 1% and ischemic side 2%), which was significantly lower than those vehicle-treated stroke brains (intact side 18% and ischemic side 35%) ( $p < 0.0001$ ). Endogenous neurogenic factors were also significantly upregulated in the ischemic brains of NT-020-treated stroke animals. These data demonstrate the remarkable neuroprotective effects of NT-020 when given prior to stroke, possibly acting via its neurogenic potential.

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## INTRODUCTION

**S**TROKE IS THE THIRD LEADING CAUSE OF DEATH and the leading cause for disability in the United States with approximately two out of every 1000 adult Americans experiencing their first stroke in any given year. The current therapy for stroke is limited to tissue plasminogen activator (tPA). The numbers of affected individuals, the costs necessary to facilitate their care and rehabilitation, coupled with the lack of therapies, warrant significant research efforts to alleviate the suffering of stroke victims.

Accumulating evidence over the past decade indicates that neurogenesis occurs in the adult mammalian CNS.<sup>1</sup> Regenerative medicine has emerged as a new scientific field advancing stem cell therapy for treating brain disorders, with emphasis on either transplanting exogenous stem cells or amplifying endogenous stem cells via neurogenesis.<sup>2,3</sup> In this study, we explored how diet supplementation affects neurogenesis in adult stroke rats. Relevant diet manipulation paradigms have been examined in the recent past, including diet restriction and fasting, which have been reported to influence neurogenesis.<sup>4-9</sup> In particular, caloric restriction has been shown to increase neurogenesis in CNS neurogenic sites, which coincided with amelioration of stroke-induced behavioral and histological deficits.<sup>5,10,11</sup> Careful examination of the diet restriction regimen reveals that the overarching principle of such neurogenesis-enhancing strategy is to eliminate the unwanted calories. We posit here that an equally robust neuroprotective approach might be achieved by pursuing the reverse paradigm of increasing the nutritive diet that should also render a therapeutically potent neurogenesis. Hence, we put forward the unifying concept of diet manipulation that encompasses either eliminating the detrimental calories or enhancing the nutritive diet in order to achieve neuroprotection. Our hypothesis of neurogenesis-induced neuroprotection complements the concept suppression of oxidative stress as an equally efficacious benefit rendered by a nutritive diet including blueberries,<sup>12,13</sup> green tea,<sup>14,15</sup> vitamin D3,<sup>16,17</sup> and carnosine,<sup>18,19</sup> considering that these plant extracts are rich in potent antioxidants and chelating polyphenol

compounds.<sup>20</sup> The proposed study will elucidate the biological effects of supplementation of nutritive diet extracts/compounds, referred to herein as NT-020, on promoting neurogenesis and exerting functional benefits in a stroke model.

## EXPERIMENTAL RESEARCH DESIGN AND METHODS

### *Subjects*

Adult male Sprague-Dawley rats (Harlan, Madison, WI) weighing about 250 g served as subjects. Animals had free access to food and water before and after surgical procedures. All experimental procedures followed the Augusta NIH guidelines for the use of animals in research. Surgical procedures were conducted under aseptic conditions, and every effort was made to minimize animal suffering and to reduce the number of animals used.

### *Drug dosing*

Animals were randomly assigned to initially receive NT-020 ( $n = 8$ ) or vehicle ( $n = 7$ ). In using NT-020, the highest dosages of the components determined to promote the greatest amount of cell proliferation in the *in vitro* cell studies<sup>21</sup> were adjusted accordingly for *in vivo* administration. NT-020 is a proprietary formulation of blueberry, green tea, Vitamin D3, and carnosine based upon previously published research.<sup>17,21</sup> However, in this previous study<sup>21</sup> a higher level of Vitamin D3 was included in the formulation. The present dosing for NT-020 and vehicle consisted of daily oral administration (using a gavage) over a 2-week pre-stroke period. As indicated by Wang and colleagues,<sup>22</sup> such a dosing regimen of pre-stroke diet supplementation is within the therapeutic range to promote neuroprotection; however the amount of individual ingredients used in the present study is 20 times less than that in the previous studies.<sup>21,22</sup>

### *Stroke surgery*

On day 14, following the last drug treatment, all animals underwent stroke surgery using the

transient 1-hour suture occlusion of middle cerebral artery (MCAo). This MCAo stroke model followed the procedures described in our previous studies.<sup>23–26</sup>

### *Behavioral testing*

To reveal the functional effects of NT-020, animals were subjected to established behavioral tests just prior to stroke surgery and again on day 14 post-stroke. Behavioral tests included Bederson test and elevated body swing test (EBST), which are both sensitive to stroke-induced motor and neurological deficits respectively. The *Bederson test* is conducted following the procedures previously described.<sup>23,24</sup> Neurologic score for each rat is obtained using 4 tests which include: (1) observation of spontaneous ipsilateral circling, graded from 0 (no circling) to 3 (continuous circling); (2) contralateral hindlimb retraction, which measures the ability of the animal to replace the hindlimb after it is displaced laterally by 2 to 3 cm, graded from 0 (immediate replacement) to 3 (replacement after minutes or no replacement); (3) beam walking ability, graded 0 for a rat that readily traverses a 2.4-cm-wide, 80-cm-long beam to 3 for a rat unable to stay on the beam for 10 s; and (4) bilateral forepaw grasp, which measures the ability to hold onto a 2-mm-diameter steel rod, graded 0 for a rat with normal forepaw grasping behavior to 3 for a rat unable to grasp with the forepaws. The scores from all 4 tests are added and the average calculated to give a neurologic deficit score (maximum possible score, 3).

The *EBST* involves handling the animal by its tail and recording the direction of the swings. The animal is gently picked up at the base of the tail and elevated until the animal's nose is at a height of 2 in (5 cm) above the surface. The direction of the swing, either left or right, is counted once the animal's head moves sideways approximately 10 degrees from the midline position of the body. After a single swing, the animal is placed back in the Plexiglas box and allowed to move freely for 30 s prior to retesting. These steps are repeated 20 times for each animal. Normally, intact rats display a 50% swing bias, that is, the same number of swings to the left and to the right.

A 75% swing bias would indicate 15 swings in one direction and 5 in the other during 20 trials. We have previously utilized the EBST, and noted that MCAo stroke animals display >75% bias swing activity as early as the day of stroke surgery (i.e., after recovery from anesthesia), and such motor asymmetry is stable for up to 6 months.<sup>27–29</sup> All tests were conducted by two investigators blinded to the treatment condition.

### *Cerebral infarction analysis*

Following the behavioral testing at day 14 post-stroke, all animals were euthanized for evaluation of cerebral infarction using the glial fibrillary acidic protein (GFAP) immunostaining.<sup>30</sup> This routine GFAP assay reveals the extent of glial scar, which closely approximates the cerebral damage in stroke. Although triphenyltetrazolium chloride (TTC) is more routinely used to reveal cerebral infarction, such TTC assay is only sensitive when performed within 7 days post-stroke. Using an NIH imaging system, the glial scar was calculated by capturing images using AxioPhot (Carl Zeiss, Thornwood, NY) at 1.6-fold magnification. The damaged area was selected according to the morphology of the cells based on glial infiltration, which clearly delineated the ischemic core from the ischemic penumbra.<sup>30</sup> The mean area of damage of 5–6 sections per coronal slice was calculated using the following formula:  $C = D/(A - B)$  to reveal the total infarct area per brain (see Fig. 2A).

### *Cell proliferation assay*

To determine whether the NT-020-induced cell proliferation seen *in vitro* also occurs *in vivo*, which could serve as the mechanistic explanation for the observed behavioral and histological protection, we examined the BrdU staining in discrete regions of the stroke brain. Alternate brain sections obtained from the same NT-020- or vehicle-treated stroke animals above were processed for BrdU immunostaining (50 mg/kg, i.p. every 8 h during days 10–14 post-stroke; Sigma, St. Louis, MO) to reveal cell proliferation. Analyses of BrdU labeling were focused at the neurogenic subventricular zone (SVZ) and the non-neurogenic striatum. The ra-

tionale for initially focusing on SVZ is to reveal that NT-020 could indeed increase cell proliferation in a neurogenic site. Although an increase cell proliferation in SVZ could be used as a reasonable index to support a mechanism for NT-020-mediated neuroprotection, we further tested this hypothesis by subsequently analyzing BrdU labeling in the ischemic striatum to reveal whether newly formed cells from the SVZ are able to migrate towards the site of injury. This migration potential would reveal to us stronger evidence of NT-020's therapeutic efficacy as such data demonstrate the compound's potential to not only increase cell proliferation, but also to facilitate proper homing of newly formed cells to the appropriate injured brain site.

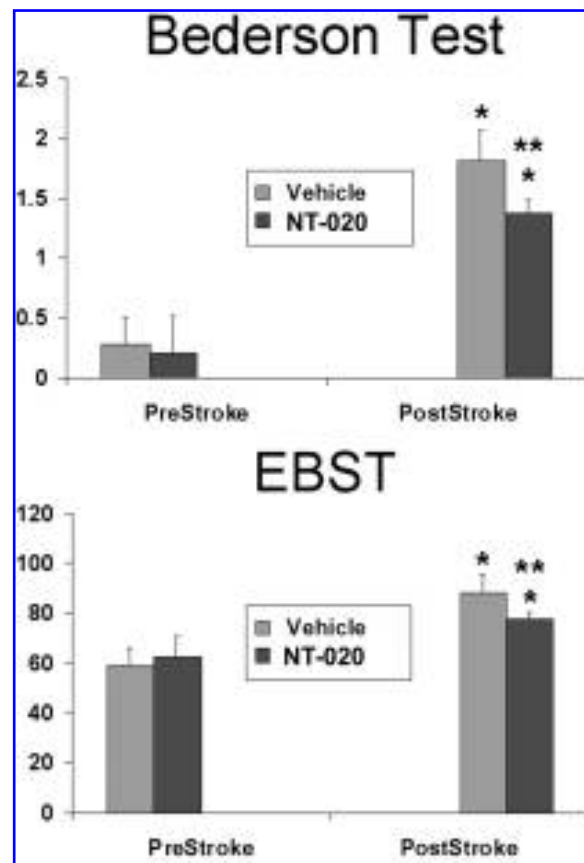
#### Neuronal differentiation assay

In the same vein of determining functionality of cell proliferation and migration, we further pursued characterizing whether NT-020 enhancement of newly formed cells similarly leads to increased expression of neuronal phenotypes. We find such neural differentiation to be a much more stringent marker than merely enhanced cell proliferation and migration, which would bolster our hypothesized mechanism of neurogenesis underlying NT-020 neuroprotection. BrdU-labeled brain sections (i.e., ischemic striatal penumbra), used for cell proliferation/migration studies above, were double labeled with the neuronal marker doublecortin or glial marker GFAP.

#### ELISA of neurogenic factors upregulated by NT-020

Accumulating evidence implicates trophic factors, such as stem cell factor (SCF), brain-derived glial cell line-derived neurotrophic factor (GDNF), neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF), as critical neurogenic factors.<sup>31-33</sup> Thus, we measured these molecules as possible neurotrophic factors upregulated with NT-020 treatment. Equal amounts of cortical and striatal tissue homogenates were collected for ELISA. Tissue punches from the ischemic striatum and cortex, as well as the contralateral intact striatum and cortex, were used as brain sampling sites. To control for intra-rat variability, we used the rat brain

matrix to accurately target the same striatum and cortex along the anteroposterior axis (i.e., the third slice of 2 mm block). Thereafter, a 2 mm diameter tissue punch (using a calibrated tissue puncher; Harris Micro-Punch, Redding, CA) was made immediately below the corpus callosum (corresponding to the striatum) and a second 2 mm punch was made immediately above the corpus callosum (corresponding to the cortex). This was done bilaterally, giving four sampling sites per animal. The levels of SCF, GDNF, BDNF, and VEGF were determined using ELISA kits according to the manufacturer's protocols (BDNF and GDNF, Promega, Madison, WI; VEGF and SCF, R&D Systems, Inc., Minneapolis, MN). These ELISA systems can detect a minimum of 31.2, 15.6, 7.8, and 31.2 pg/mL of SCF,



**FIG. 1.** Neurologic (Bederson test) and motor (EBST) evaluations prior to stroke surgery (pre-stroke) revealed no detectable behavioral deficits between groups. At 14 days after stroke (post-stroke), while both groups exhibited motor and neurologic deficits (vs. pre-stroke,  $*p < 0.05$ ), the NT-020-treated rats exhibited significantly less motor and neurologic deficits than the vehicle-treated rats did ( $**p < 0.05$ ).

GDNF, BDNF, and VEGF, respectively. Triplicate runs for each assay were performed.

## RESULTS

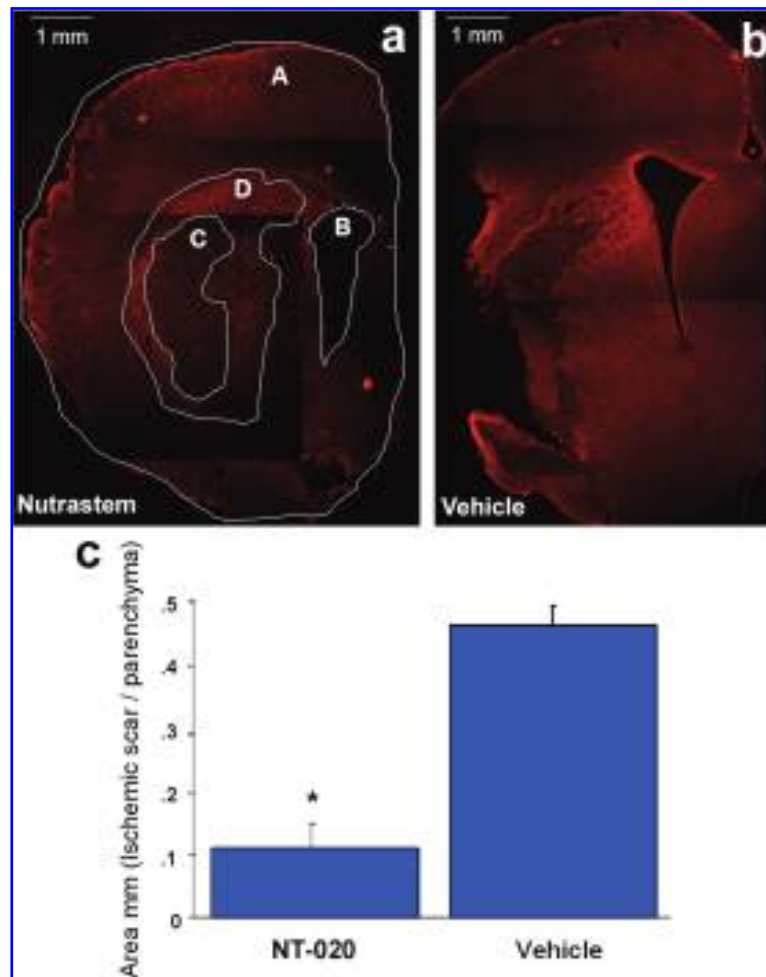
### *NT-020 lessens stroke-induced behavioral deficits*

ANOVA revealed significant treatment effects in both Bederson ( $F_{3,26} = 81.65$ ,  $p < 0.0001$ ) and EBST ( $F_{3,26} = 29.26$ ,  $p < 0.0001$ ) (Fig. 1). Pair-wise comparisons between treatment groups using Fisher's PLSD post-hoc  $t$  tests revealed NT-020- and vehicle-treated groups displayed no detectable behavioral impairments at pre-stroke testing, but both exhibited neurologic and motor deficits at post-stroke testing (compared to pre-stroke,  $p <$

0.05). However, NT-020-treated stroke animals showed significantly less abnormalities in both neurologic and motor tests compared to vehicle-treated stroke animals (at post-stroke testing,  $p < 0.05$ ). Reductions of 24.4% and 11.8% in Bederson neurologic dysfunction and EBST motor asymmetry, respectively, were detected in NT-020-treated stroke animals compared to vehicle-treated stroke animals. These results indicate that pre-stroke treatment with NT-020 significantly ameliorated stroke-induced neurologic and motor abnormalities.

### *NT-020 reduces stroke-induced cerebral infarcts*

Student  $t$  test revealed that NT-020 reduced the glial scar/ischemic area damage in the striatum (Fig. 2a) compared to vehicle treat-



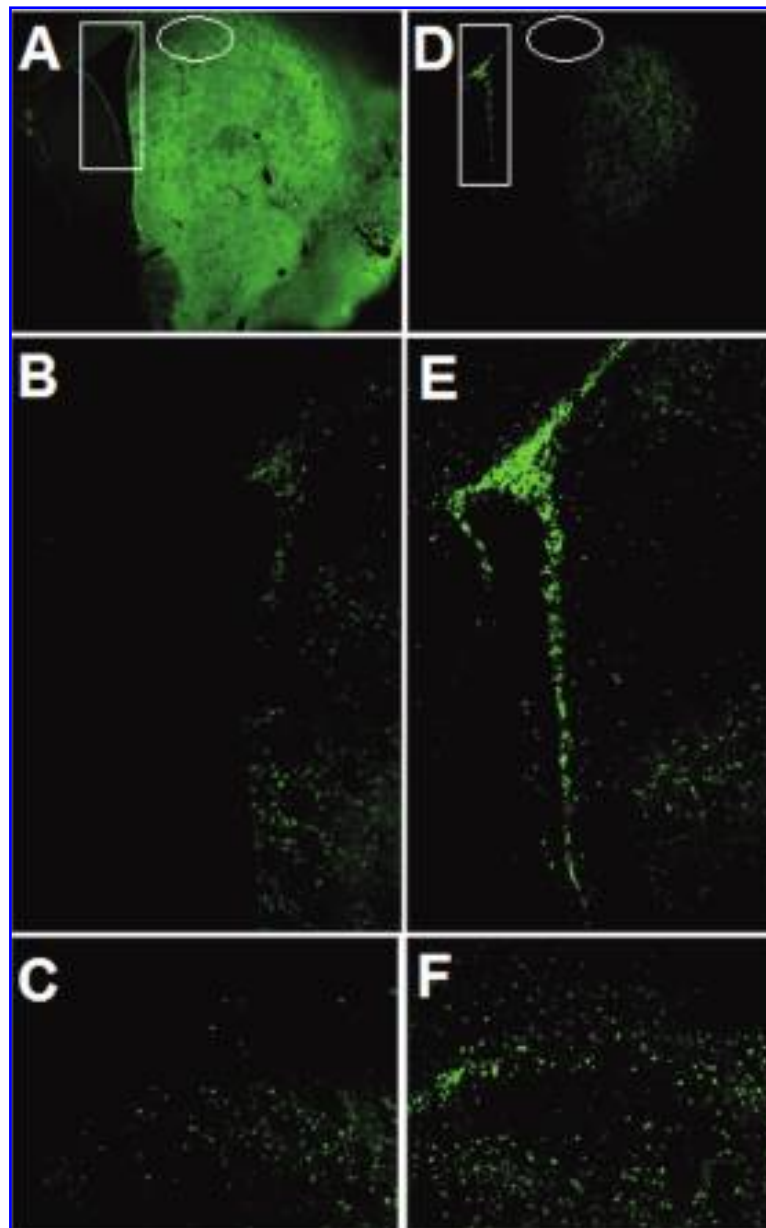
**FIG. 2.** GFAP immunostaining was used to reveal glial scar. (a) Calculation of glial scar area using the formula  $C = D/(A - B)$ , with total hemisphere area (A), lateral ventricle (B), glial scar (C), and penumbra (D). NT-020 treatment (a) significantly reduced the glial scar compared to vehicle treatment (b), as quantified in c (\* $p < 0.0005$ ).

ment (Fig. 2b). A significant 75% decrement in mean glial scar area was observed in the ischemic striatum of NT-020-treated stroke animals compared to that of vehicle-treated stroke animals ( $p < 0.0005$ ) (Fig. 2c). These histological results parallel the behavioral performance of NT-020-treated stroke animals, suggesting that a reduction in the extent of cerebral infarction

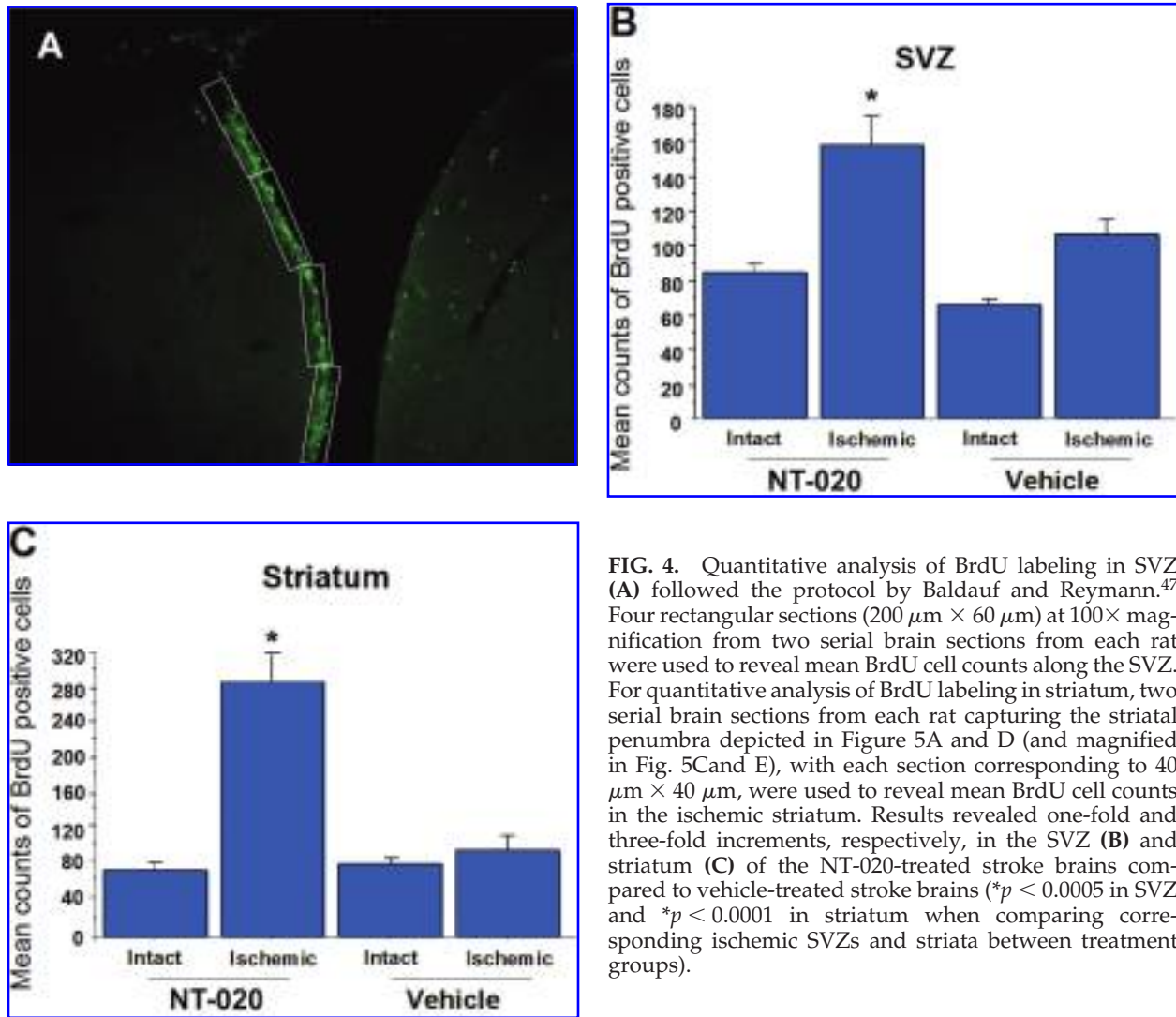
translated to attenuation of stroke-induced functional deficits.

*NT-020 promotes endogenous birth of new neurons*

Immunofluorescence microscopy revealed NT-020 (Fig. 3D-F) enhanced cell proliferation



**FIG. 3.** BrdU labeling of brains from vehicle-(A-C) and NT-020-treated (D-F) stroke animals. NT-020 increased the number of BrdU-positive proliferating cells in SVZ (D in rectangle, magnified in E) and ischemic striatum (D in circle, magnified in F) compared to vehicle treatment (SVZ, A in rectangle, magnified in B; striatum, A in circle, magnified in C).



**FIG. 4.** Quantitative analysis of BrdU labeling in SVZ (A) followed the protocol by Baldauf and Reymann.<sup>47</sup> Four rectangular sections ( $200\ \mu\text{m} \times 60\ \mu\text{m}$ ) at  $100\times$  magnification from two serial brain sections from each rat were used to reveal mean BrdU cell counts along the SVZ. For quantitative analysis of BrdU labeling in striatum, two serial brain sections from each rat capturing the striatal penumbra depicted in Figure 5A and D (and magnified in Fig. 5C and E), with each section corresponding to  $40\ \mu\text{m} \times 40\ \mu\text{m}$ , were used to reveal mean BrdU cell counts in the ischemic striatum. Results revealed one-fold and three-fold increments, respectively, in the SVZ (B) and striatum (C) of the NT-020-treated stroke brains compared to vehicle-treated stroke brains ( $*p < 0.0005$  in SVZ and  $*p < 0.0001$  in striatum when comparing corresponding ischemic SVZs and striata between treatment groups).

in SVZ and ischemic striatum characterized by significantly increased BrdU labeling in these brain sites compared to that of vehicle-treated stroke brains (Fig. 3A-C). Quantitative analysis of SVZ's cell proliferative activity (Fig. 4A) revealed significant treatment effects ( $F_{3,16} = 8.03$ ,  $p < 0.0001$ ), with at least a one-fold increment in the number of BrdU-positive cells in the NT-020-treated stroke brains compared to that of vehicle-treated stroke brains ( $p < 0.0005$ ) (Fig. 3A, B, D, and E, with quantitative data shown in Fig. 4B). Similarly, quantitative analysis of BrdU labeling in the ischemic striatal penumbra revealed significant treatment effects ( $F_{3,16} = 11.84$ ,  $p < 0.0001$ ), with at least a 3-fold increase in the number of BrdU-posi-

tive cells in the NT-020-treated stroke brains compared to vehicle-treated stroke brains ( $p < 0.0001$ ) (Fig. 3A, C, D, and F, with quantitative data shown in Fig. 4C). In contrast, when evaluation of BrdU labeling targeted the ischemic core (instead of the penumbra, Fig. 3A and D), there was a massive proliferation of BrdU-positive cells in the vehicle-treated stroke brains compared to NT-020-treated stroke brains. This obvious increment in BrdU core is not surprising as it has been established that BrdU labels infiltrating reactive microglia, as well as degenerating or dead cells, which are abundant in the necrotic core.<sup>28,34-36</sup> Since NT-020-treated stroke brains have smaller ischemic core, it is expected that BrdU labeling in this region is

less than that of the vehicle-treated stroke brains. Accordingly, examination of cell proliferation in the ischemic core presents as an artifact and may not truly reflect the neuroprotective BrdU labeling index. With this in mind, we limited the evaluation of cell proliferation to SVZ and ischemic penumbra. Both these sets of data indicate that NT-020 increases cell proliferation in the neurogenic SVZ and also facilitates the migration of several of these newly formed cells towards the ischemic striatal penumbra.

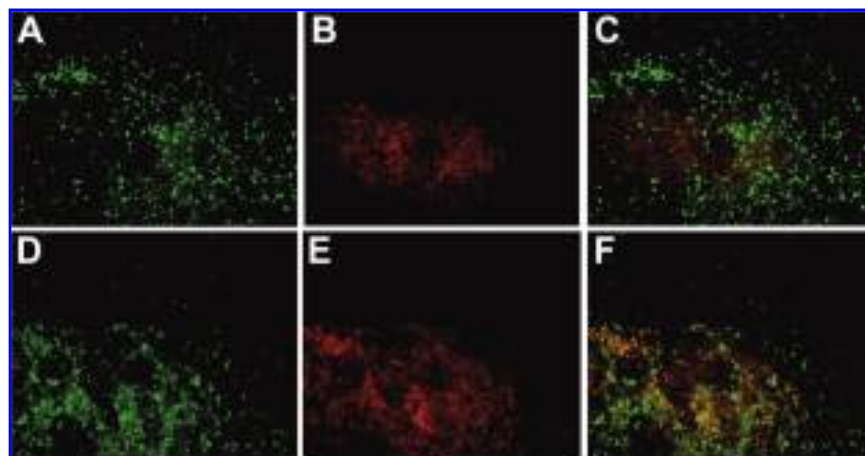
*NT-020 facilitates neuronal phenotype expression of newly formed cells*

Immunofluorescence microscopy revealed widespread double labeling of cells with BrdU and doublecortin in NT-020-treated stroke brains, whereas only a few cells double labeled with both BrdU and doublecortin in vehicle-treated stroke brains (Fig. 5). Quantitative analysis revealed about 17% and 75% double-labeling of BrdU and doublecortin in respective intact and ischemic side of NT-020-treated stroke brains, which were significantly higher than those seen in the intact (5%) and ischemic side (13%) of vehicle-treated stroke brains ( $*p < 0.05$ ,  $**p < 0.0001$ ) (Fig. 6, 7A). In contrast, only a few cells in NT-020-treated stroke brains double labeled with BrdU and GFAP, while many cells in vehicle-treated stroke brains double labeled

with BrdU and GFAP (Fig. 6). Quantitative analysis revealed about 1% and 2% double labeling of BrdU and GFAP in respective intact and ischemic side of NT-020-treated stroke brains, which were significantly lower than those seen in the intact (18%) and ischemic side (35%) of vehicle-treated stroke brains ( $**p < 0.0001$ ) (Fig. 7B). These data indicate that NT-020 induces neural differentiation, with increased tendency towards neuronal over glial lineage.

*NT-020 attenuates stroke-induced alterations in neurogenic factors*

ELISA of brain tissue homogenates revealed that the levels of SCF, GDNF, BDNF, and VEGF in the ischemic cortex and striatum of vehicle-treated stroke animals were significantly reduced to 40-50% of the corresponding intact brain regions. In contrast, the levels of these same trophic factors in the ischemic cortex and striatum of NT-020-treated stroke animals were maintained to 70-80% of the corresponding intact brain regions. Single ANOVAs for each trophic factor assay revealed significant differences between the treatment groups ( $p < 0.0001$ ), and post-hoc tests further confirmed the neurogenic effects of NT-020 on the ischemic cortex and striatum compared to vehicle treatment ( $*p < 0.05$  vs. corresponding brain region from vehicle-treated stroke animals) (Fig. 8).



**FIG. 5.** NT-020 enhanced neuronal differentiation of newly formed cells in the ischemic striatum as revealed by a high number of BrdU (D) and doublecortin (E) double-labeled cells (F) compared to vehicle treatment (A, BrdU; B, doublecortin; C, merged).



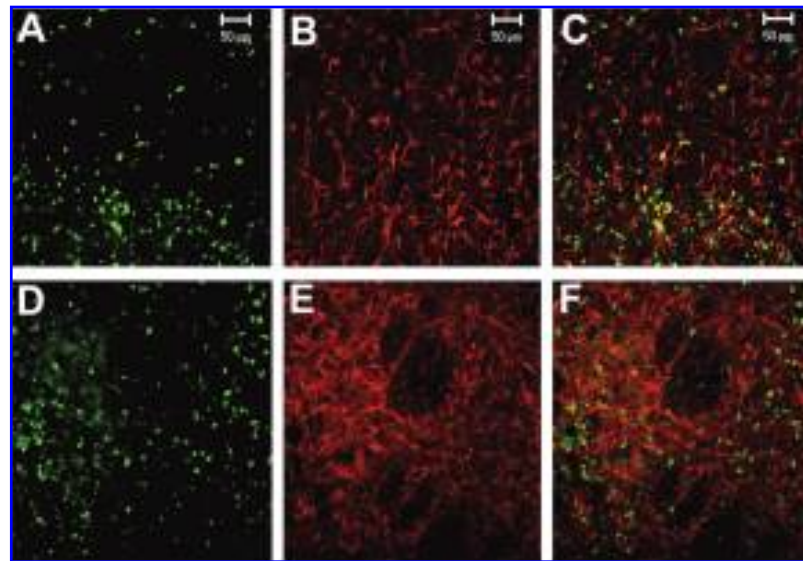


FIG. 6. NT-020 did not enhance differentiation of newly formed cells into glial lineage in the ischemic striatum, as revealed by a few cells with BrdU (D) and GFAP (E) double labeling (F). In contrast, there were several cells that double labeled with BrdU and GFAP in the vehicle-treated ischemic striatum (A, BrdU; B, GFAP; C, merged).

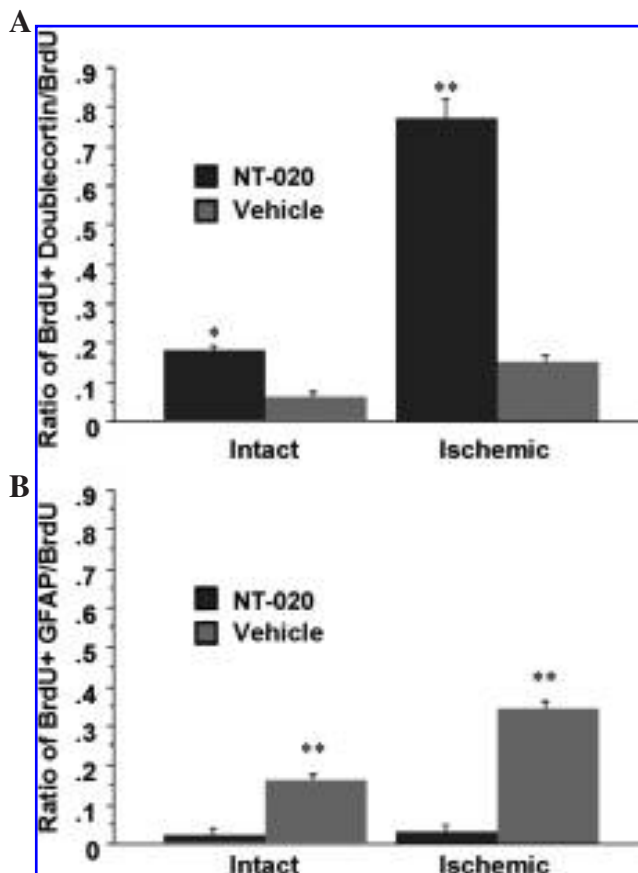


FIG. 7. (A) Quantitative analysis revealed significantly higher double labeling of BrdU and doublecortin in NT-020 than vehicle-treated stroke brains ( $*p < 0.05$ ,  $**p < 0.0001$ ). (B) In contrast, there was significantly lower BrdU and GFAP double labeling in NT-020 than vehicle-treated stroke brains ( $**p < 0.0001$ ).

## DISCUSSION

The present study demonstrates the robust neuroprotective effects of NT-020 when given prior to stroke. The observed amelioration of stroke-induced behavioral deficits by NT-020 was accompanied by increased cell proliferation and neuronal differentiation of newly formed cells in the stroke brain.

### *Diet supplementation in ischemic injury*

Wang and colleagues<sup>22</sup> recently demonstrated the neuroprotective effects of diet supplementation in focal ischemic brain. In this study, they fed adult male Sprague-Dawley rats with equal amounts of diets (blueberry, spinach, or spirulina) or with control diet over a 4-week period, and thereafter subjected the animals to stroke surgery involving transient ligation of the middle cerebral artery (MCA). This MCA stroke model produces consistent cerebral infarction predominantly to the cortex. Behavioral and histological assays at 24 and 48 h revealed that stroke animals that received blueberry-, spinach-, or spirulina-enriched diets displayed significant increase in locomotor activity that coincided with reduction in the volume of infarction in the cerebral cortex. Analyses of physiological parameters revealed no differences in blood biochemistry, blood

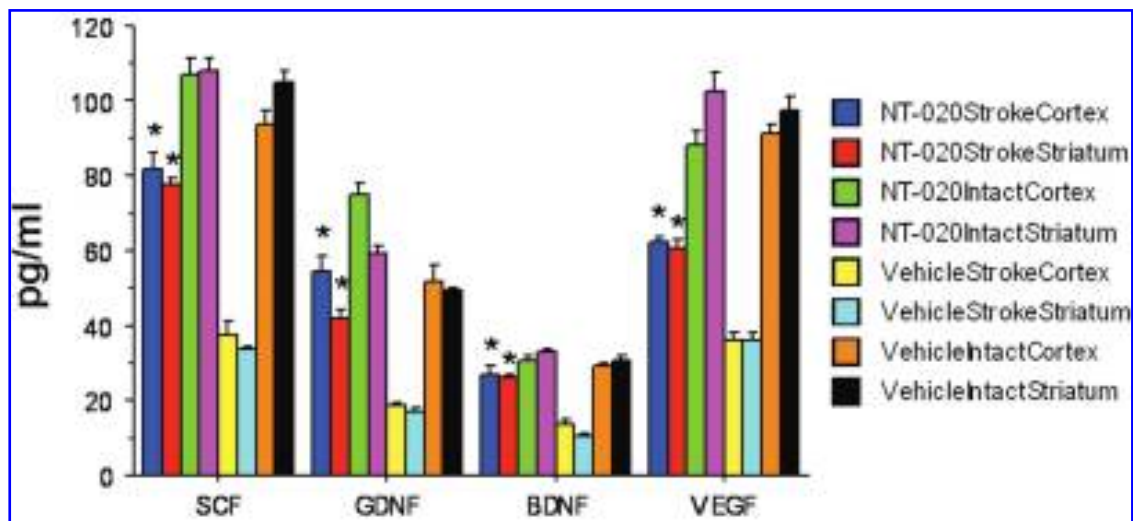


FIG. 8. ELISA revealed NT-020 treatment maintained 70-80% levels of trophic factors in the ischemic cortex and striatum (\* $p < 0.05$  vs. corresponding brain region from vehicle-treated stroke animals).

CO<sub>2</sub>, and electrolyte levels across groups, suggesting that changes in physiological functions did not contribute to the observed neuroprotection. In addition, stroke animals treated with blueberry, spinach, or spirulina exhibited significantly lower caspase-3 activity in the ischemic hemisphere. These data demonstrate that diet supplementation with blueberry, spinach, or spirulina attenuated stroke-induced cerebral infarction and apoptosis and general locomotor impairments, and parallel similar neuroprotective effects of diet supplementation seen in other age-related disorders, such as Alzheimer's disease<sup>37</sup> and Parkinson's disease.<sup>38</sup> However, until now the exact mechanism underlying such neuroprotection remained to be determined.

#### *Diet supplementation and neurogenesis*

Here, we demonstrate that NT-020 facilitated endogenous neurogenesis in the stroke brain. The mechanism underlying such enhanced neurogenesis likely involves NT-020 upregulating a myriad of endogenous neurogenic factors, including SCF, BDNF, GDNF and VEGF, which in turn stimulate the birth of new neurons in the ischemic brain. Previous studies have implicated neurogenesis as a potent neuroprotective pathway triggered by diet

supplementation. For example, short-term supplementation with blueberry (one of the components of NT-020) has been shown to increase hippocampal neurogenesis,<sup>39</sup> which could account for the improvement of cognitive function of aged animals treated with this diet.<sup>37</sup>

The reverse diet manipulation paradigm involving caloric restriction and intermittent fasting has also been found to protect against stroke.<sup>5,10,11</sup> In these studies, rodent maintained on a caloric restriction diet or intermittently fasted developed an increased resistance of brain cells to experimental models of stroke. Postulated mechanisms underlying the neuroprotection produced by restricted diet intake include decreased oxidative stress and increased cellular stress resistance, which singly or in combination can promote a "preconditioning" state, priming the brain to withstand a stroke insult. Additionally, upregulation of neurotrophic factor (i.e., increased BDNF levels) signaling pathways has been observed.<sup>11</sup> Furthermore, these authors noted that diet restriction can stimulate neurogenesis and enhance synaptic plasticity.<sup>10,40</sup> Clearly, the studies by Mattson and colleagues<sup>5,10,11,40</sup> indicate that a regimen of reduced food intake exerts neuroprotection against stroke.

These past reports and our current data, when taken together, support our general hypothesis that diet manipulation, either by restricting high caloric intake or via supplementation of nutritive diet extracts/compounds, stands as an efficacious stroke adjunct therapy. The present study provided direct evidence that neurogenesis, implicated as a mechanism underlying neuroprotective effects of diet restriction,<sup>40</sup> similarly plays a major role in the functional benefits of diet supplementation. In view of the pathological manifestation of stroke, at least in this MCAo model, characterized by extensive neuronal loss accompanied by increased glial cell activation, it appears that neuronal replacement will be more advantageous than glial cell replenishment. The robust neuronal differentiation at 2 weeks post-stroke is equally beneficial since a rapid cell death cascade proceeds after the stroke onset. Thus, the preferential neuronal differentiation by newly formed cells in the ischemic brain provides a solid mechanistic pathway by which NT-020 renders neuroprotection in stroke.

#### *Nutritive diet and oxidative stress*

An equally important pathway for neuroprotection by nutritive diet is the suppression of oxidative stress. Indeed, a bulk of laboratory evidence implicates the robust antioxidative and chelating polyphenol effects<sup>20</sup> of blueberries,<sup>12,13</sup> green tea,<sup>14,15</sup> vitamin D3,<sup>16,17</sup> and carnosine.<sup>18,19</sup> These antioxidant features of nutritive extracts, taken together with the observed neurogenic potential, make these compounds appealing as a class of neuroprotective agents with multiple targets for arresting cell death cascades. However, an ongoing controversy is the ability of these compounds to traverse the blood brain barrier (BBB) and afford their effects in the CNS. In the diseased brain, such as the present stroke, the BBB is shown to be compromised,<sup>41,42</sup> which could facilitate the entry of these compounds to the brain. Alternatively, the development of small molecule derivatives of these nutritive compounds that could easily permeate across the BBB should improve the CNS bioavailability and therapeutic benefits of NT-020.

#### *Towards a multi-agent nutritive diet for stroke*

Recent studies have demonstrated that some of the agents we used in the present study are neuroprotective against stroke<sup>22,43–46</sup> and that blueberry has been shown to increase neurogenesis.<sup>39</sup> Based on these recent publications under the major umbrella theme of plant extracts (specifically blueberry) and neuroprotection, the present study at first glance appears lacking in novelty. Despite these recent publications with overlapping observations to our study, the reports to date on nutritive agents implicating their neuroprotective effects against stroke have used a single agent therapy. Accordingly, for such single agent treatment regimen to render a therapeutic outcome, the dosage of each agent is obviously higher than the present multi-agent combination regimen. We wish to advance the notion that this agent dosage is critical for practical purposes, especially when translating this potential treatment to the clinic. In order to achieve neuroprotection against stroke with a single agent treatment regimen, one must load the patient with high doses of each agent. However, in order for the multi-agent combination regimen to afford neuroprotection, the present study proposes a modest and tolerable agent dosage (i.e., at least 20 times less of the dosage required for the single agent regimen<sup>21,22</sup>). Equipped with this new translational medicine information, the present results provide a critical advance in the scientific and clinical arena by demonstrating the advantage of multi-agent over single agent regimen. In addition, a careful examination of the published study on the role of blueberry on neurogenesis<sup>39</sup> reveals that blueberry treatment (again as a single agent) in aged animals enhanced hippocampal neuronal plasticity, which correlates with improvements in spatial memory. Although redundancies and overlapping pathologic conditions exist in “aging” and “stroke” cell death pathways, investigations on the specific human disorder (in our case, stroke) is necessary to either support or detract from the proposed “neurogenic” potential of blueberry. Thus, the present study provides the first direct evidence of neurogenic potential of nutritive diet (blueberry included)

in stroke animals, which parallels the neuroprotective mechanism of such compounds seen in aged animals.

## CONCLUSIONS

The present results highlight the functional benefits of diet supplementation in enhancing neurogenesis in the adult stroke brain. The improved outcome in stroke animals exposed to diet supplementation complements the existing reverse paradigm of diet restriction<sup>5,10,11,40</sup> as a neuroprotective strategy. Furthermore, we identified that endogenous neurogenesis closely accompanies diet supplementation as seen with diet restriction, thereby advancing the concept of enhancing the neurogenic potential of the injured brain as a powerful strategy in promoting neuroprotection against stroke.

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