Received: 19 January 2017 | Accepted: 7 February 2017

DOI: 10.1002/jcp.25847

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

NSI-189, a small molecule with neurogenic properties, exerts behavioral, and neurostructural benefits in stroke rats

Naoki Tajiri[1] | David M. Quach[2] | Yuji Kaneko[1] | Stephanie Wu[2] | David Lee[2] | Tina Lam[2] | Ken L. Hayama[2] | Thomas G. Hazel[2] | Karl Johe[2] | Michael C. Wu[3] | Cesar V. Borlongan[1]

[1] Department of Neurosurgery and Brain Repair, Center of Excellence for Aging and Brain Repair, University South Florida College of Medicine, Tampa, Florida

[2] Neuralstem, Inc., Rockville, Maryland

[3] Neurodigitech, LLC., San Diego, California

Correspondence
Cesar V. Borlongan, Ph.D., Professor and Vice-Chairman for Research, Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, Tampa, FL.
Email: cborlong@health.usf.edu

Funding information
National Institutes of Health, National Institute of Neurological Disorders and Stroke, Grant numbers: 1R01NS071956, 1R01NS090962, 1R21NS089851; Department of Defense, Grant number: W81XWH-11-1-0634; James and Esther King Foundation for Biomedical Research Program

Enhancing neurogenesis may be a powerful stroke therapy. Here, we tested in a rat model of ischemic stroke the beneficial effects of NSI-189, an orally active, new molecular entity (mol. wt. 366) with enhanced neurogenic activity, and indicated as an anti-depressant drug in a clinical trial (Fava et al., 2015, Molecular Psychiatry, DOI: 10.1038/mp.2015.178) and being tested in a Phase 2 efficacy trial (ClinicalTrials.gov, 2016, ClinicalTrials.gov Identifier: NCT02695472) for treatment of major depression. Oral administration of NSI-189 in adult Sprague–Dawley rats starting at 6 hr after middle cerebral artery occlusion, and daily thereafter over the next 12 weeks resulted in significant amelioration of stroke-induced motor and neurological deficits, which was maintained up to 24 weeks post-stroke. Histopathological assessment of stroke brains from NSI-189-treated animals revealed significant increments in neurite outgrowth as evidenced by MAP2 immunoreactivity that was prominently detected in the hippocampus and partially in the cortex. These results suggest NSI-189 actively stimulated remodeling of the stroke brain. Parallel in vitro studies further probed this remodeling process and demonstrated that oxygen glucose deprivation and reperfusion (OGD/R) initiated typical cell death processes, which were reversed by NSI-189 treatment characterized by significant attenuation of OGD/R-mediated hippocampal cell death and increased Ki67 and MAP2 expression, coupled with upregulation of neurogenic factors such as BDNF and SCF. These findings support the use of oral NSI-189 as a therapeutic agent well beyond the initial 6-hr time window to accelerate and enhance the overall functional improvement in the initial 6 months post stroke.

KEYWORDS
behavioral recovery, cerebral ischemia, neurite outgrowth, neurogenesis, pharmacotherapy, trophic factors

1 | INTRODUCTION

Impaired neurogenesis (Acosta et al., 2013; Esposito, Hayakawa, Maki, Arai, & Lo, 2015; Kokaia & Darsalia, 2011; Sun et al., 2013, 2016; Xia et al., 2006; Zhang et al., 2012) accompanies many neurological disorders, including stroke. Enhancement of host neurogenesis may serve as a novel therapy for stroke, which remains a significant unmet clinical need with efficacy of tissue plasminogen activator or tPA limited to 4.5 hr. Until recently, the non-regenerative capability of the adult damaged brain was an accepted scientific dogma. However, accumulating evidence over the last decade indicates that neurogenesis occur during adulthood, in that neurons and astrocytes can be generated from isolated cells of the adult mammalian CNS (Reynolds & Weiss, 1992). Largely based on this phenomenon of neurogenesis,

several laboratory studies have examined stem cell therapy for treating various diseases in the CNS, including stroke, and neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease (Borlongan, 2011; Borlongan, Glover, Tajiri, Kaneko, & Freeman, 2011). Stem cell therapy, however, remains as an experimental treatment. While brain injury has been shown to trigger transient and limited neurogenesis, this endogenous protective mechanism is not capable of reversing the cell death cascade in the CNS. It is, however, recognized that strategies designed to enhance the endogenous neurogenesis are potentially beneficial for treating brain disorders (Borlongan et al., 2011; Borlongan, 2011; Hess & Borlongan, 2008a; Yasuhara et al., 2006). 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 (Hess & Borlongan, 2008b; Picard-Riera, Nait-Oumesmar, & Baron-Van Evercooren, 2004).

The present study focused on the latter one, which incorporates a natural way of protecting, and possibly repairing the damaged brain and correcting the behavioral impairments. In this study, we tested in a stroke model the therapeutic effects of NSI-189, a small molecule with enhanced neurogenic activity, which is already in clinical trial for treatment of major depression and prevention against suicide (ClinicalTrials.gov, 2016; Fava et al., 2015). While the brain exerts self-repair acutely, over time the stroke-induced cascade of cell death events outweighs the endogenous regenerative mechanisms (Acosta et al., 2013; Fava et al., 2015; Kokaia & Darsalia, 2011; Sun et al., 2013, 2016; Zhang et al., 2012). Thus, finding a therapeutic strategy to stimulate the brain to mount a prolonged and stable reparative machinery during the secondary cell death progression after stroke will likely afford beneficial effects. Although NSI-189 has been reported to show neurogenic effects (Fava et al., 2015), the exact mechanisms mediating this observed neurogenesis remains not well understood. To this end, we posit that a therapeutic approach against stroke may be achieved by rendering the brain to maintain its reparative capacity via enhancement of host neurogenesis that likely entails growth factor upregulation and neurite outgrowth enhancement. The proposed study elucidated the biological effects of orally administered small molecule NSI-189, on promoting neurogenesis, and exerting functional benefits in a stroke model.

2 | METHODS

This study was designed to evaluate potential therapeutic value of treatment with a novel neurogenic compound, NSI-189. Phosphate, hereafter referred to as NSI-189 (supplied by Neuralstem Inc., Germantown, MD) in an animal model of adult ischemic stroke. A summary of timeline and procedures is provided for clarity of experimental design (Table 1). Drug treatment was initiated at 6 hr after stroke and daily thereafter for 12 weeks, with functional readouts of behavioral and histological deficits conducted during the subsequent 12- or 24-week period post-treatment. We characterized motor and neurological performance at baseline (prior to stroke), then at 1, 3, and 7 days after stroke and at weekly/monthly intervals thereafter. Following completion of behavioral testing at 12 weeks or 24 weeks post-treatment, animals were randomly euthanized by transcardial perfusion and brains harvested to histologically characterize the extent of cerebral ischemia and to reveal the host tissue endogenous repair mechanism (i.e., neurogenesis). Investigators who were involved in drug treatments, behavioral testing, and post-mortem analyzes were blind to the treatment conditions.

2.1 | Stroke surgery

A total of 60 adult Sprague–Dawley, male rats (weighing around 250 g at beginning of the study) received experimental stroke surgery using the middle cerebral artery occlusion (MCAo) model. All surgical procedures will be conducted under aseptic conditions. The animals were anesthetized with 1–2% isoflurane in nitrous oxide/oxygen (69%/30%) using a face mask and checked for pain reflexes. Under deep anesthesia, animals underwent the MCAo surgery. The MCAo suture technique involved insertion of a filament through the carotid artery to reach the junction of the MCA, thus blocking the blood flow from the common carotid artery, as well as from the circle of Willis. The right common carotid artery was identified and isolated through a ventral midline cervical incision. The suture size was 4–0, made of sterile, non-absorbable suture (Ethicon, Inc., Somerville, NJ), with the diameter of the suture tip tapered to 24–26-gauge size using a rubber cement. About 15–17 mm of the filament was inserted from the junction of the external and internal carotid arteries to block the MCA. The right MCA was occluded for 1 hr. A heating pad and a rectal thermometer allowed maintenance of body temperature within normal limits (37 ± 0.3°C). To determine successful occlusion and reperfusion, a laser Doppler probe was placed at the distal end of the MCA and revealed at least 80% reduction in regional cerebral blood flow. To further ensure similar degree of stroke insults, physiological parameters including PaO_2, $PaCO_2$, and plasma pH measurements were monitored, and we found no significant differences in our stroke animals. Based on laser Doppler readouts and behavioral tests after MCAs, a total of 48 animals were enrolled in this study.

2.2 | Drug treatment

All drug treatments were conducted under aseptic conditions. Test article was NSI-189 H_3PO_4 (mol. wt. 464.50), with lot number DAJ-F-40(2). Purity was 99.8% and stability was determined as stable

TABLE 1 Treatment conditions

Group	Size	Test article	Timing (first dose)	Survival time post stroke
1	24	Vehicle	6 hr post-stroke, daily by oral gavage for 12 wks	A: *N* = 12 @ 12 wks C: *N* = 12 @ 24 wks
2	24	NSI-189	6 hr post-stroke, daily by oral gavage for 12 wks	B: *N* = 12 @ 24 wks D: *N* = 12 @ 24 wks

under storage condition (−10 to −30°C). The administration dose of 30 mg/kg was based on the weight of the API (active pharmaceutical ingredient) base without the weight of phosphate salt. For vehicle, 0.03N HCl in deionized water was used. Following MCAo, animals were randomly assigned to receive oral NSI-189 ($n = 24$) or vehicle ($n = 24$), starting at 6 hr after stroke, and daily for the next 12 weeks.

2.3 | Motor and neurological tests

All investigators testing the animals were blinded to the treatment condition. Animals will be subjected to elevated body swing test (EBST) and neurological exam. EBST involved handling the animal by its tail and recording the direction of the swings. The test apparatus consisted of a clear Plexiglas box (40 × 40 × 35.5 cm). The animal was gently picked up at the base of the tail, and elevated by the tail until the animal's nose is at a height of 2 inches (5 cm) above the surface. The direction of the swing, either left or right, was counted once the animals head moves sideways approximately 10° from the midline position of the body. After a single swing, the animal was placed back in the Plexiglas box and allowed to move freely for 30 s prior to retesting. These steps were repeated 20 times for each animal. About 1 hr after the EBST, the neurological exam was conducted. Neurologic score for each rat was obtained using three tests which include (1) forelimb retraction, which measured the ability of the animal to replace the forelimb after it was displaced laterally by 2–3 cm, graded from 0 (immediate replacement) to 3 (replacement after several seconds or no replacement); (2) beam walking ability, graded 0 for a rat that readily traversed a 2.4-cm-wide, 80-cm-long beam to 3 for a rat unable to stay on the beam for 10 s; and (3) bilateral forepaw grasp, which measured 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 three tests, which were done over a period of about 15 min on each assessment day, were added to give a mean neurologic deficit score (maximum possible score, 9 points divided by 3 tests = 3). Animals were subjected to both tests at baseline (prior to stroke), then at 1, 3, 7 days after stroke and at weekly intervals post-treatment.

2.4 | Histology

All post-mortem histology was performed by Neurodigitech. At scheduled intervals post-stroke (either 12 weeks or 24 weeks), rats were randomly euthanized ($n = 12$ per treatment), perfused by transcardial perfusion with 4% paraformaldehyde. The brains were dissected, post-fixed for overnight in 4% paraformaldehyde, then subsequently immersed in 30% sucrose until immunohistochemical processing. Brain section preparations were designed to identify stroke-induced cerebral infarction and NSI-189-induced neurogenic effects. All brains were embedded in gelatin blocks and sectioned on a freezing sliding microtome at 40-µm. The sections were washed in PBS 5 × 10 min to remove the antigen preservative solution. Endogenous peroxidase was blocked using 3% H_2O_2 for 15 min. The sections were then incubated in 1% Triton-X100 for 30 min, and then blocked with 5% Normal Horse Serum (NHS) for 1 hr. The sections were incubated with the primary antibody, Ki67 (1:2000, Cat. #: ab16667, Abcam, CA) or MAP2 (1:2000, Cat. #: AB5622, Millipore, MA) overnight. The sections were rinsed 5 × 5 min in PBS prior to pre-incubation in 5% NHS for 1 hr, then incubated with the secondary antibody which corresponded to the respective host of the primary antibody (Donkey α Rabbit, 1:2000, Cat. #: 711-066-152, Jackson ImmunoResearch Laboratories, Inc, PA) for 90 min. After rinsing in PBS (5 × 5 min), the sections were incubated with peroxidase-conjugated streptavidin (1:5000, Cat. #: 016-030-084, Jackson ImmunoResearch Laboratories) with 1% NHS. The sections were washed 5 × 5 min in PBS after the streptavidin, prior to developing with DAB (3,3′-Diaminobenzidine Tetrahydrochloride Hydrate, Cat #: 1001306853, Sigma–Aldrich, St. Louis, MO) and Nickel chloride. After DAB processing, the sections were rinsed, mounted, and air-dried overnight. The slides were dehydrated, and coverslipped with DPX Mounting Medium (Cat #: 13512, Electron Microscopy Sciences, Hatfield, PA).

The slides were imaged and reviewed under Nikon bright-field microscope (Nikon, Tokyo, Japan). The regions of interest (ROIs), including the cerebral cortex, the hippocampal subfields (CA1 + CA2, CA3, and DG) were contoured rostro-caudally and then the immunoreactivity of the MAP2 density was measured by Nikon NIS-Element software and Ki67+ cells counted rostro-caudally along the subgranular zone (SGZ) by Image-Pro Premier (v10.10), respectively. It was noted that all IDs were coded and blinded to the analysts during the course of the study. After completion, all of the quantitative data were extracted and transferred for the statistical analysis (Prism, GraphPad®, La Jolla, CA). The data values between groups were compared using ANOVA with Tukey's post-hoc tests ($p \leq 0.05$).

2.5 | Cell culture experiments

Primary rat neurons (hippocampus) were obtained from BrainBits (E18 rat cortex; Springfield, IL). According to the protocol, cells (4×10^4 cells/well) were suspended in 200 µl Neural Medium (NbActive 4, BrainBit) containing 2 mM L-glutamine and 2% B27 in the absence of antibiotics and grown in Poly-L-Lysine-coated 96-well (354516, BD Biosciences, Franklin Lakes, NJ) at 37°C in humidified atmosphere containing 5% carbon dioxide in 40% of the neuron and 60% astrocyte cell population (determined immunocytochemically using vesicular glutamate trranspoter-1). After 5 days in culture (approximately cell confluence of 70%), randomly selected cell wells supplemented with NSI-189 (1:1000 dilution) or vehicle, then exposed to an in vitro stroke paradigm of oxygen glucose deprivation and reperfusion (OGD/R). The cells were initially exposed to OGD/R medium (glucose-free Dulbecco's Modified Eagle Medium, Gibco, Life Technologies, Thermo Fisher Waltham, MA), then placed in an anaerobic chamber (Plas-Labs, Inc., Lansing, MI) containing 95% nitrogen and 5% carbon dioxide for 15 min at 37°C, and finally the chamber was sealed and incubated for 90 min at 37°C (hypoxic-ischemic condition). Control cells were incubated in same buffer containing 5 mM glucose at 37°C in a regular CO_2 (5%) incubator (normoxic condition). OGD/R was terminated by adding 5 mM glucose to medium and cell cultures re-introduced to the regular CO_2 incubator (normoxic condition) at 37°C for 2 hr, of

which period represented a model of "reperfusion." Measurement of cell viability was performed by both fluorescent live/dead cell assay. A two-color fluorescence cell viability assay was performed using Calcein-AM (L3224, Invitrogen, Waltham, MA), which was retained within live cells, including an intense uniform green fluorescence and ethidium homodimer (EthD-1) which bound to the nuclei of damaged cells (bright red fluorescence). After 2 hr reperfusion, the cells were incubated with 2 μM Calcein-AM and 4 μM EthD-1 for 45 min at room temperature in darkness according to the manufacturer's instructions. Green fluorescence of the live cells was measured by the Gemini EX florescence plate reader (Molecular Device, Sunnyvale, CA), excitation at 485 nm and emission at 538 nm. In addition, trypan blue (15250-061, Gibco, Life Technologies) exclusion method was conducted and mean viable cell counts were calculated in four randomly selected areas (1 mm^2, $n = 10$) to reveal the cell viability. To precisely calibrate the cell viability, the values were standardized form florescence intensity and trypan blue data. For immunocytochemistry analysis, cells (8×10^4 cell/well) in 400 μl Neural medium containing 2 mM L-glutamine and 2% B27 in the absence of antibiotics and grown in Poly-L-Lysine 8-chamber (354632, BD Biosciences) were fixed in 4% paraformaldehyde for 20 min at room temperature after OGD/R or non-OGD/R treatment. Fixed cells were blocked with 5% normal goat serum (Millipore, CA) and 0.2% Triton X-100 (Amresco, OH) in PBS for 30 min and incubated with antibodies against Ki67 (rabbit monoclonal antibody, 1:400; Thermo Scientific, CA) or mouse monoclonal anti-Map2 (Sigma–Aldrich) for 1 hr. After PBS rinses, the cells were incubated for 30 min with secondary antibodies conjugated to Cy3 (goat anti-rabbit IgG, 1:1000; Jackson ImmunoResearch) or an Alexa fluor 488-labelled goat anti-mouse antibody (Invitrogen Thermo Scientific, CA) followed by 5 min in 4′,6-diamidino-2-phenylindole (DAPI, 1:10000 in PBS; Sigma–Aldrich) to stain the nuclei. Finally, cells were washed five times for 10 min in PBST and three times for 5 min in PBS, and subsequently embedded with mounting medium. Immunofluorescent images were visualized using Zeiss Axio Imager Z1 (Zeiss, Thornwood, NY). MAP2- and Ki67-positive cells were counted and normalized to total DAPI-positive cell numbers. Control experiments were performed with the omission of the primary antibodies yielding negative results. Finally, to reveal whether NSI-189 induced secretion of neurogenic factors in cultured hippocampal cells which might have facilitated cell proliferation and neurogenesis, we measured from the conditioned media (harvested from NSI-189 supplemented and standard growth medium) trophic factors such as SCF, brain derived-neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF) which have been implicated as critical secretory factors associated with stemness properties (Lladó, Haenggeli, Maragakis, Snyder, & Rothstein, 2004; Sieber-Blum, 1998). The levels of BDNF, GDNF, VEGF, and SCF were determined using ELISA kits according to the protocols of the manufacturer (BDNF and GDNF from Promega; human VEGF and human SCF from R & D Systems). The CM of the cells was analyzed by interpolation from the standard curves assayed on individual plates. These ELISA systems can detect a minimum of 7.8, 15.6, 31.2, and 31.2 pg/ml BDNF, GDNF, VEGF, and SCF, respectively. Next, to validate the dominant trophic factors detected in the CM of NSI-189-treated cells, the combination of the CM and anti-BDNF antibody (rabbit polyclonal IgG, 10 ng/ml; Abcam) and anti-SCF antibody (rabbit polyclonal IgG, 10 ng/ml; Abcam) was used. In addition, BDNF- and SCF-removed CM by immunoprecipitation (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was also performed to eliminate possible effects of BDNF- and SCF-antibody complex, which paralleled the anti-BDNF and anti-SCF results (data not shown).

3 | RESULTS

3.1 | NSI-189 promotes behavioral recovery in stroke

ANOVA revealed treatment and interaction effects (treatment and time) (p's < 0.0001). Pairwise comparisons revealed that oral treatment with NSI-189 starting at 6 hr and continued daily over the 12 weeks post-stroke promoted robust behavioral effects at acute stage, which remained stable over chronic period even when NSI-189 was already terminated (p's < 0.05). At baseline, all animals exhibited normal biased swing activity and neurological performance as revealed by EBST (Fig. 1A) and neurological examination (Fig. 1B). At day 1 around 1 hr after MCAo, all animals enrolled in this study displayed significant biased swing activity and impairment in neurological performance. Following drug treatment, stroke animals that received NSI-189 displayed significant amelioration of stroke-induced motor and neurological deficits as early as day 3 post-stroke compared to stroke animals that received vehicle alone ($p < 0.05$). Moreover, the NSI-189-treated stroke animals continued to improve over time compared to vehicle-treated stroke animals, not only during the 12 weeks of drug treatment, but even during the next 12 weeks (week 13 to week 24) after NSI-189 withdrawal (p's < 0.05). The observed behavioral recovery in NSI-189-treated stroke animals was noted in all monthly test time points, and in both EBST and neurological examination.

3.2 | NSI-189 enhances cell proliferation and neurogenesis in stroke

Histopathological examination of stroke brains from both NSI-189 and vehicle-treated stroke rats at either 12 weeks and 24 weeks post-stroke showed that cerebral infarction as revealed by neuronal marker MAP2 expression in the cortex and striatum did not significantly differ between groups indicating absence of NSI-189 effects in reducing the primary stroke insult on the brain (p's > 0.05). Moreover, the neurogenic niche subventricular zone (SVZ), which lies in proximity to the primary stroke insulted striatal region, and the remote hippocampal area, specifically the neurogenic subgranular zone (SGZ), exhibited comparable levels of Ki67 between NSI-189 and vehicle-treated stroke animals (p's > 0.05) (Fig. 2). In contrast, examination of secondary cell death in the peri-infarct cortex revealed apparent upregulation of cell proliferation and neurogenesis as evidenced by increased Ki67 (Fig. 2) and MAP2 (Fig. 3) staining,

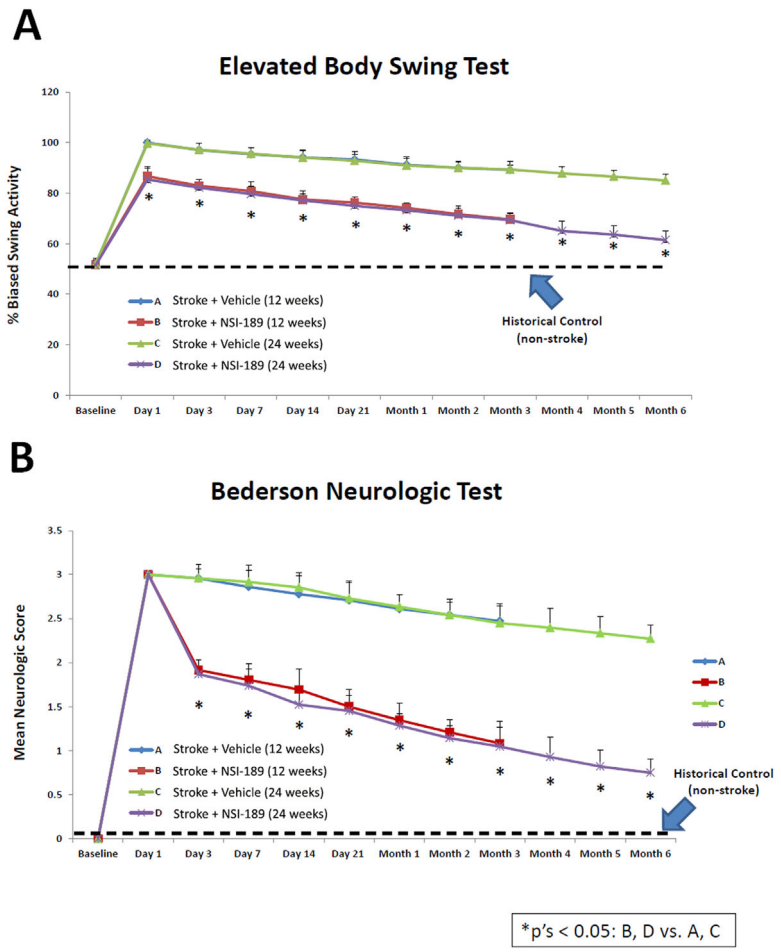

FIGURE 1 NSI-189 promotes behavioral recovery in stroke. Stroke animals that received NSI-189 displayed significant amelioration of stroke-induced motor and neurological deficits as early as day 3 post-stroke, and continued to improve over the 24 weeks survival compared to vehicle-treated stroke animals. The observed behavioral recovery in NSI-189-treated stroke animals was noted in all monthly test time points, and in both EBST (A) and neurological examination (B)

respectively, in NSI-189-treated stroke animals compared to vehicle-treated stroke animals. NSI-189-treated stroke animals displayed significant increments in MAP2 density compared to vehicle-treated stroke animals, which were more pronounced in the hippocampus than the cortex, in that the amplified MAP2 density in the cerebral cortex was only detected at the 12-week period, whereas such increased MAP2 density was found in the hippocampus for both 12- and 24-week time points (p's < 0.05). Altogether, these data suggest an active remodeling of the stroke brain, characterized by cell proliferation and neuronal maturation preferentially in the cortex and hippocampal areas, respectively, undergoing secondary cell death as opposed to the striatal areas proximal to the primary stroke insulted region.

3.3 | NSI-189 upregulates neurogenic factors in tandem with neurogenesis

In an effort to understand the mechanism of action mediating the observed in vivo therapeutic effects of NSI-189 in stroke, we conducted in vitro studies in order to further probe this remodeling process and demonstrated that oxygen glucose deprivation (OGD) initiated typical cell death processes accompanied by minimum Ki67 expression and decreased MAP2 expression, which were reversed by NSI-189 treatment characterized by significant attenuation of OGD-mediated hippocampal cell death and increased Ki67 and MAP2 expression (p's < 0.05) (Fig. 4). ELISA demonstrated that VEGF, GDNF, BDNF, and SCF levels were upregulated in the CM of NSI-treated hippocampal cells compared to standard growth media (p's < 0.05), with BDNF and SCF much more robustly increased than VEGF and GDNF (p's < 0.05). Antibody blocking treatments with combined antibody-BDNF and antibody-SCF suppressed the NSI-189-mediated rescue of cell viability against OGD (p > 0.05), further implicating the role of neurogenic factors in tandem with neurite outgrowth enhancement (Fig. 5).

4 | DISCUSSION

The present findings support the use of oral NSI-189 at a wider therapeutic window of 6 hr post-stroke in enhancing host neurogenesis, possibly acting via upregulation of neurogenic factors, towards functional recovery in stroke. Both sets of in vivo and in

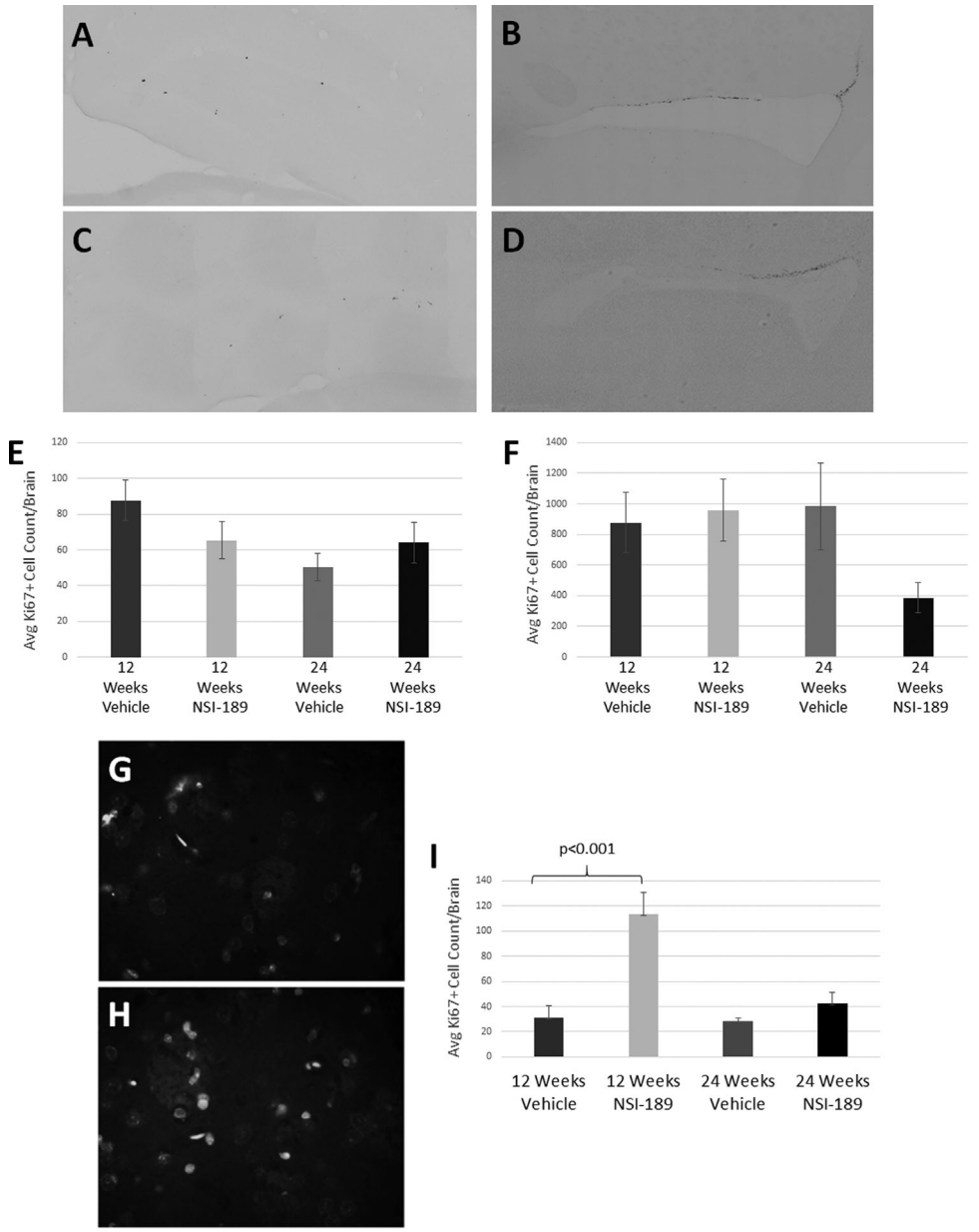

FIGURE 2 NSI-189 enhances cell proliferation in peri-infarct cortex. Comparison of the Ki67 immuno-positive cell counts in the SGZ (A and C), SVZ (B and D) and cerebral cortex (G and H) after 12-week treatment with either vehicle (12 Weeks Vehicle; A, B and G) or NSI-189 (12 Weeks NSI-189; C, D and H) and after additional 12 weeks of treatment-free observational period of vehicle (24 Weeks Vehicle) or NSI-189 (24 Weeks NSI-189). Quantifications of Ki67 expression in SGZ (E), SVZ (F), and cortex (I) are shown in bar graphs. Representative images were taken from 12 weeks of survival period. Ki67 expression was significantly increased in the peri-infarct cortex of NSI-treated animals at 12 weeks, but did not differ with vehicle-treated animals in SGZ or SVZ at either survival period

vitro experiments indicated that targeting the secondary cell death of impaired neurogenesis may confer robust benefits against stroke.

The early behavioral effects of NSI-189 in MCAo stroke animals, starting at day 3 after drug initiation, highlight the robust effects of this small molecule, likely owing in part to its ability to penetrate the blood brain barrier (BBB) even before the stroke-induced BBB breakdown. Equally notable is the continued behavioral improvement and maintained behavioral recovery over time (up to 24 weeks), even when NSI-189 was terminated at 12 weeks post-stroke. This sustained functional improvement suggests that the drug likely initiated a host brain repair mechanism that was able to continue the tissue remodeling process of the stroke brain in the absence of daily NSI-189 treatment.

That the brain remodels itself after stroke has been documented (Hermann et al., 2014; Tajiri et al., 2012; Watson et al., 2015; Zhang & Chopp, 2015), but this host repair mechanism is not sufficient to account for sustained relevant stroke recovery (Doeppner et al., 2009; Hess & Borlongan, 2008a; Hu et al., 2013; Kuge et al., 2009). Exogenous treatment, either pharmacologically or via stem cell transplantation, designed to enhance host brain regenerative process is needed to achieve clinically relevant functional outcomes in stroke (Kaneko et al., 2012; Matsukawa et al., 2009; Peña & Borlongan, 2015;

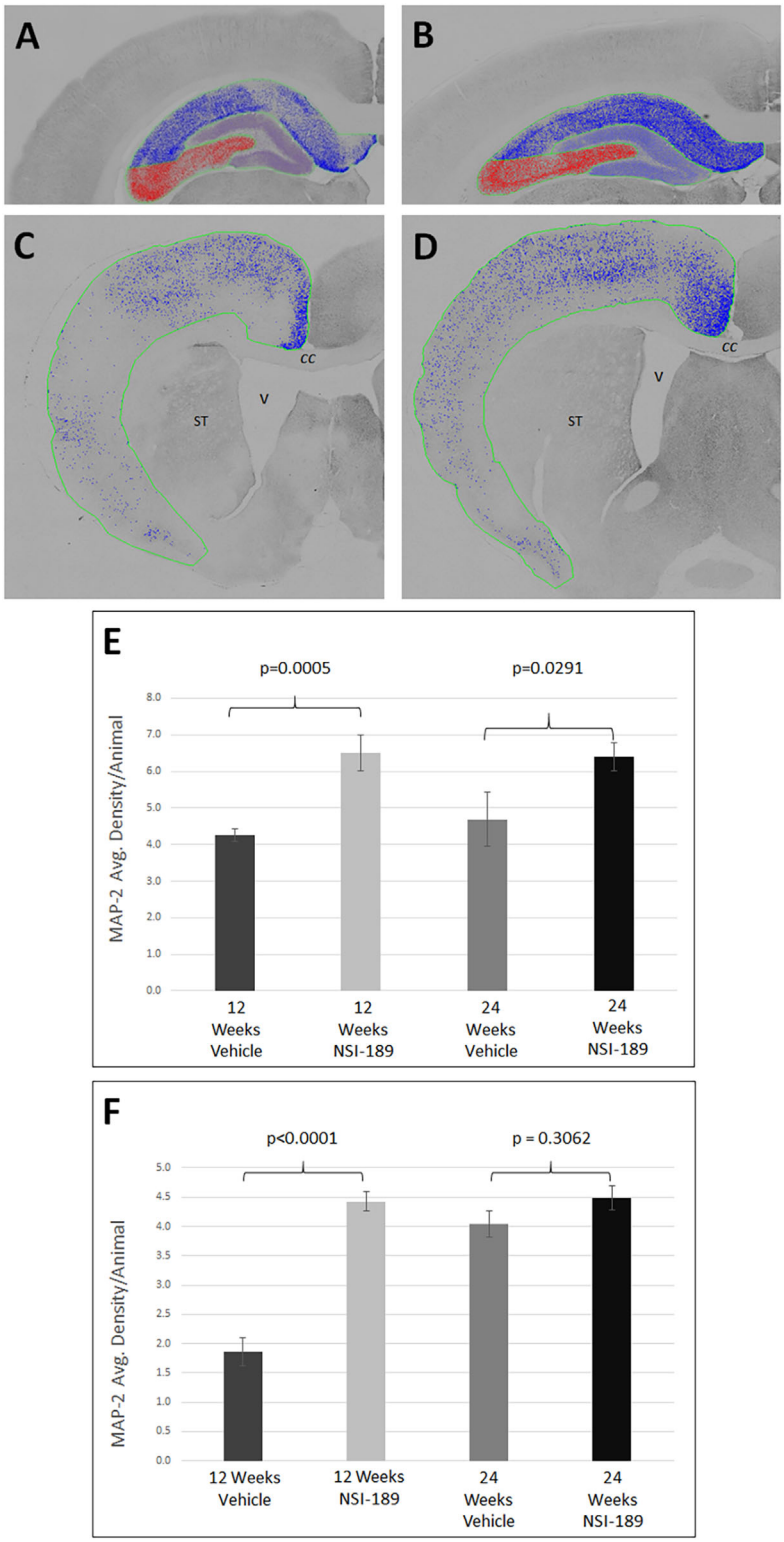

FIGURE 3 NSI-189 enhances neurogenesis in stroke. Histopathological examination of secondary cell death in the peri-infarct cortex (A) and the remote hippocampal area (B) revealed increased MAP2 immunoreactivity in NSI-189-treated stroke animals compared to vehicle-treated stroke animals (*p's < 0.05). These results suggest that NSI-189 promoted an active remodeling of the stroke brain. Quantification of MAP-2-immunopositive staining densities in the hippocampus and the cerebral cortex of NSI-189- and vehicle-treated animals. Brain sections after MAP-2-immuno reactivity, developed by HRP-DAB reaction, were microscopically scanned and converted into digital images. The regions of interest to quantify were manually lassoed, and individual pixels and their optical densities were automatically captured by a software. (A and B) Representative examples of ipsilateral hippocampal subfields demarcated to quantify the densities within CA1 (deep blue), CA3 (red), and dentate gyrus (DG, light blue), from an animal administered with 12 weeks of vehicle (A) or with 12 weeks of NSI-189. (C and D) Representative examples of ipsilateral brain section demarcated to quantify the densities within cortex (deep blue), from an animal administered with 12 weeks of vehicle (C) or with 12 weeks of NSI-189 (D). ST, striatum; V, ventricle; cc, corpus callosum. Bar graphs correspond to quantification of hippocampus (E) and cerebral cortex (F) showing significant increments in MAP2 density, especially at 12 weeks of NSI-189 treatment

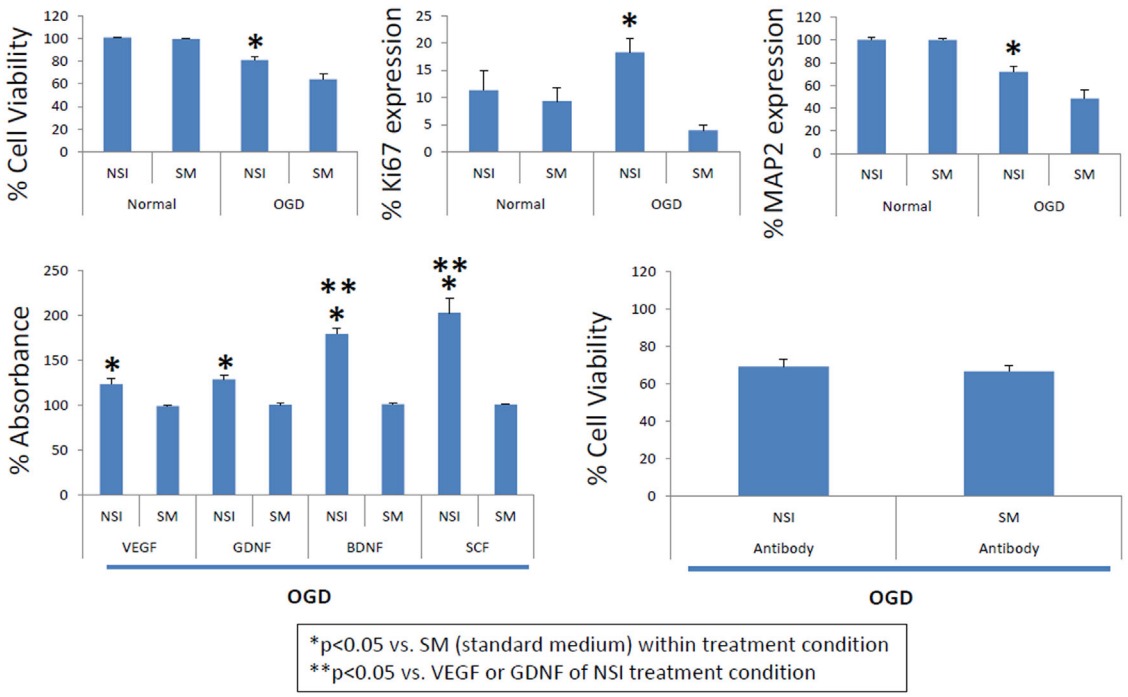

FIGURE 4 NSI-189 upregulates neurogenic factors in tandem with neurogenesis. Parallel in vitro studies that exposed primary rat hippocampal neurons to the oxygen glucose deprivation (OGD) insult model revealed reduced cell death viability, Ki67 expression and decreased MAP2 expression under standard media (SM) condition, which were significantly attenuated by NSI-189 (NSI) treatment (p's < 0.05). Growth factors were upregulated in the CM of NSI-treated hippocampal cells compared to SM-grown cells (*p's < 0.05), in particular BDNF and SCF (**p's < 0.05). Combined antibody-BDNF and antibody-SCF suppressed the NSI-189-mediated rescue of cell viability against OGD (p > 0.05)

Tajiri et al., 2012; Wang et al., 2014; Yasuhara et al., 2010). The present data demonstrated that NSI-189 augmented endogenous repair mechanism by increasing cell proliferation and neuronal lineage commitment in discreet neurogenic niches, as well as non-neurogenic brain regions. In particular, active remodeling of the stroke brain is shown in the present study to preferentially occur in the hippocampal areas and peri-infarct cortex undergoing secondary cell death as opposed to the striatal areas proximal to the primary stroke insulted region. Indeed, stroke appears to awaken quiescent stem cells in the brain, in both neurogeneic and non-neurogenic niches (Lindvall & Kokaia, 2015; Péron & Berninger, 2015), and stimulation of these cells with neurogenic molecules, such as NSI-189, may facilitate host brain neurogenesis (Kreuzberg et al., 2010; Li, Yu, Mohamad, Genetta, & Wei, 2010; Osman, Porritt, Nilsson, & Kuhn, 2011). Our observation that enhanced neurogenic activity was more prominent in the SGZ, remote from the principally stroke infarcted area, suggests that obliterating the secondary cell death as opposed to the principal stroke insult, may be more amenable to treatments targeting host repair mechanisms.

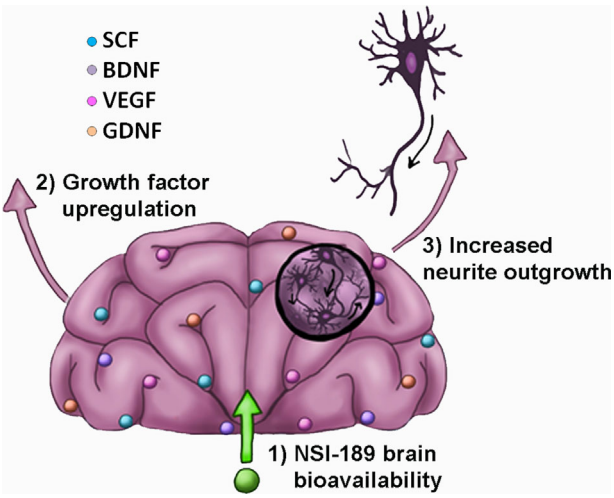

FIGURE 5 NSI-189's mechanism of action. The entry to the brain of the small molecule, NSI-189, is prerequisite to realizing therapeutic benefits in stroke (1), likely by upregulating growth factors (2), and increasing neurite outgrowth (3)

Our cell culture studies revealed that in concert with enhanced neurogenesis, NSI-189 also upregulated neurogenic factors. NSI-189 treatment reversed the OGD-triggered cell death in cultured hippocampal cells, coincident with increased Ki67 expression and MAP2 density, likely corresponding to an enhanced neurogenic activity. While the composition of our cell population represented 40% neurons and 60% astrocytes, the phenotypic expression of these cells may be considered as immature because the timing of cell harvest corresponds to gestation age day 16. Moreover, the hippocampus being the targeted donor brain region contains one of the most active neurogenic niches, the SGZ. Accordingly, it is highly likely that due to the young age and potent neurogenic tissue source of our neural cultures, there was a small subset of immature and proliferative cells that are Ki67 positive cells whose proliferative activity was enhanced by NSI-189. In tandem with such active neurogenesis, elevated

neurogenic factors most prominently BDNF and SCF were detected in the CM from NSI-treated hippocampal cells. Moreover, antibodies directed against BDNF and SCF blocked the NSI-189-mediated rescue of cell viability against OGD. Similar reports have demonstrated cross-talk between secreted factors (e.g., apoptosis, inflammation, oxidative stress) (; Brill et al., 2009; Chapman et al., 2015; Lu, Jones, Snyder, & Tuszynski, 2003; Zhang et al., 2015) and neurogenesis following brain insults, including stroke (Kernie and Parent, 2010; Yamashita et al., 2006). We (Hara et al., 2007; Morimoto et al., 2011; Tajiri et al., 2013), and others, have shown the important contribution of neurogenic factors in stroke brain remodeling (Jablonska et al., 2016; Rosell et al., 2013; Seo et al., 2014; Venna, Xu, Doran, Patrizz, & McCullough, 2014). Altogether, these results allude to host neurogenesis and its secreted neurogenic factors as key therapeutic targets of NSI-189 in affording behavioral and neurostructural benefits in stroke.

The recent entry of NSI-189 in the clinic as a treatment for major depression (ClinicalTrials.gov, 2016; Fava et al., 2015), coupled with the present laboratory data demonstrating the therapeutic efficacy of this small molecule in an animal model of stroke, warrants a careful consideration of its application in ischemic stroke patients. Until now, the molecular mechanisms mediating the neurogenic effects of NSI-189 remain underexplored. The observed growth factor elevation and neurite outgrowth enhancement provide insights into optimizing the therapeutic benefits of NSI-189 in neurological disorders.

ACKNOWLEDGMENT

The authors thank Connor Stonesifer and Sydney Corey for technical assistance and figure reproduction for this manuscript.

CONFLICTS OF INTEREST

The content is solely the responsibility of the authors and does not necessarily represent the official views of the sponsors.

REFERENCES

Acosta, S. A., Tajiri, N., Shinozuka, K., Ishikawa, H., Grimmig, B., Diamond, D., . . . Borlongan, C. V. (2013). Long-term upregulation of inflammation and suppression of cell proliferation in the brain of adult rats exposed to traumatic brain injury using the controlled cortical impact model. *PLoS ONE*, *8*, e53376.

Borlongan, C. V., Glover, L. E., Tajiri, N., Kaneko, Y., & Freeman, T. B. (2011). The great migration of bone marrow-derived stem cells toward the ischemic brain: Therapeutic implications for stroke and other neurological disorders. *Progress in Neurobiology*, *95*, 213–228.

Borlongan, C. V. (2011). Bone marrow stem cell mobilization in stroke: A 'bonehead' may be good after all! *Leukemia*, *25*, 1674–1686.

Brill, M. S., Ninkovic, J., Winpenny, E., Hodge, R. D., Ozen, I., Yang, R., . . . Götz, M. (2009). Adult generation of glutamatergic olfactory bulb interneurons. *Nature Neuroscience*, *12*, 1524–1533.

Chapman, K. Z., Ge, R., Monni, E., Tatarishvili, J., Ahlenius, H., Arvidsson, A., . . . Kokaia, Z. (2015). Inflammation without neuronal death triggers striatal neurogenesis comparable to stroke. *Neurobiology of Disease*, *83*, 1–15.

ClinicalTrials.gov Identifier: NCT02695472, Last verified: August, 2016.

Doeppner, T. R., Dietz, G. P., El Aanbouri, M., Gerber, J., Witte, O. W., Bähr, M., & Weise, J. (2009). TAT-Bcl-x(L) improves survival of neuronal precursor cells in the lesioned striatum after focal cerebral ischemia. *Neurobiology of Disease*, *34*, 87–94.

Esposito, E., Hayakawa, K., Maki, T., Arai, K., & Lo, E. H. (2015). Effects of postconditioning on neurogenesis and angiogenesis during the recovery phase after focal cerebral ischemia. *Stroke*, *46*, 2691–2694.

Fava, M., Johe, K., Ereshefsky, L., Gertsik, L. G., English, B. A., Bilello, J. A., . . . Freeman, M. P. (2015). A Phase 1B, randomized, double blind, placebo controlled, multiple-dose escalation study of NSI-189 phosphate, a neurogenic compound, in depressed patients. *Molecular Psychiatry*, *10*, 1372–1380. https://doi.org/10.1038/mp.2015.178

Hara, K., Matsukawa, N., Yasuhara, T., Xu, L., Yu, G., Maki, M., . . . Borlongan, C. V. (2007). Transplantation of post-mitotic human neuroteratocarcinoma-overexpressing Nurr1 cells provides therapeutic benefits in experimental stroke: In vitro evidence of expedited neuronal differentiation and GDNF secretion. *Journal of Neuroscience Research*, *85*, 1240–1251.

Hermann, D. M., Peruzzotti-Jametti, L., Schlechter, J., Bernstock, J. D., Doeppner, T. R., & Pluchino, S. (2014). Neural precursor cells in the ischemic brain—integration, cellular crosstalk, and consequences for stroke recovery. *Frontiers in Cellular Neuroscience*, *8*, 291.

Hess, D. C., & Borlongan, C. V. (2008a). Stem cells and neurological diseases. *Cell Proliferation*, *41*, 94–114.

Hess, D. C., & Borlongan, C. V. (2008b). Cell-based therapy in ischemic stroke. *Expert Review of Neurotherapeutics*, *8*, 1193–1201.

Hu, X., Zhang, F., Leak, R. K., Zhang, W., Iwai, M., Stetler, R. A., . . . Chen, J. (2013). Transgenic overproduction of omega-3 polyunsaturated fatty acids provides neuroprotection and enhances endogenous neurogenesis after stroke. *Current Molecular Medicine*, *13*, 1465–1473.

Jablonska, A., Drela, K., Wojcik-Stanaszek, L., Janowski, M., Zalewska, T., & Lukomska, B. (2016). Short-lived human umbilical cord-blood-derived neural stem cells influence the endogenous secretome and increase the number of endogenous neural progenitors in a rat model of lacunar stroke. *Molecular Neurobiology*, *53*, 6413–6425.

Kaneko, Y., Cortes, L., Sanberg, C., Acosta, S., Bickford, P. C., & Borlongan, C. V. (2012). Dietary supplementations as neuroprotective therapies: Focus on NT-020 diet benefits in a rat model of stroke. *International Journal of Molecular Sciences*, *13*, 7424–7444.

Kernie, S. G., & Parent, J. M. (2010). Forebrain neurogenesis after focal Ischemic and traumatic brain injury. *Neurobiology of Disease*, *37*, 267–274.

Kokaia, Z., & Darsalia, V. (2011). Neural stem cell-based therapy for ischemic stroke. *Translational Stroke Research*, *2*, 272–278.

Kreuzberg, M., Kanov, E., Timofeev, O., Schwaninger, M., Monyer, H., & Khodosevich, K. (2010). Increased subventricular zone-derived cortical neurogenesis after ischemic lesion. *Experimental Neurology*, *226*, 90–99.

Kuge, A., Takemura, S., Kokubo, Y., Sato, S., Goto, K., & Kayama, T. (2009). Temporal profile of neurogenesis in the subventricular zone, dentate gyrus and cerebral cortex following transient focal cerebral ischemia. *Neurological Research*, *31*, 969–976.

Li, Y., Yu, S. P., Mohamad, O., Genetta, T., & Wei, L. (2010). Sublethal transient global ischemia stimulates migration of neuroblasts and neurogenesis in mice. *Translational Stroke Research*, *1*, 184–196.

Lindvall, O., & Kokaia, Z. (2015). Neurogenesis following stroke affecting the adult brain. *Cold Spring Harbor Perspectives in Biology*, *7*, a019034.

Lladó, J., Haenggeli, C., Maragakis, N. J., Snyder, E. Y., & Rothstein, J. D. (2004). Neural stem cells protect against glutamate-induced

excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. *Molecular and Cellular Neurosciences*, *27*, 322–331.

Lu, P., Jones, L. L., Snyder, E. Y., & Tuszynski, M. H. (2003). Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Experimental Neurology*, *181*, 115–129.

Matsukawa, N., Yasuhara, T., Hara, K., Xu, L., Maki, M., Yu, G., … Borlongan, C. V. (2009). Therapeutic targets and limits of minocycline neuroprotection in experimental ischemic stroke. *BMC Neuroscience*, *10*, 126.

Morimoto, T., Yasuhara, T., Kameda, M., Baba, T., Kuramoto, S., Kondo, A., … Date, I. (2011). Striatal stimulation nurtures endogenous neurogenesis and angiogenesis in chronic-phase ischemic stroke rats. *Cell Transplantation*, *20*, 1049–1064.

Osman, A. M., Porritt, M. J., Nilsson, M., & Kuhn, H. G. (2011). Long-term stimulation of neural progenitor cell migration after cortical ischemia in mice. *Stroke*, *42*, 3559–3565.

Péron, S., & Berninger, B. (2015). Reawakening the sleeping beauty in the adult brain: Neurogenesis from parenchymal glia. *Current Opinion in Genetics and Development*, *34*, 46–53.

Peña, I. D., & Borlongan, C. V. (2015). Translating G-CSF as an adjunct therapy to stem cell transplantation for stroke. *Translational Stroke Researchs*, *6*, 421–429.

Picard-Riera, N., Nait-Oumesmar, B., & Baron-Van Evercooren, A. (2004). Endogenous adult neural stem cells: Limits and potential to repair the injured central nervous system. *Journal of Neuroscience Research*, *76*, 223–231.

Reynolds, B. A., & Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, *255*, 1707–1710.

Rosell, A., Morancho, A., Navarro-Sobrino, M., Martínez-Saez, E., Hernández-Guillamon, M., Lope-Piedrafita, S., … Montaner, J. (2013). Factors secreted by endothelial progenitor cells enhance neurorepair responses after cerebral ischemia in mice. *PLoS ONE*, *8*, e73244.

Seo, J. H., Maki, T., Maeda, M., Miyamoto, N., Liang, A. C., Hayakawa, K., … Arai, K. (2014). Oligodendrocyte precursor cells support blood-brain barrier integrity via TGF-β signaling. *PLoS ONE*, *9*, e103174.

Sieber-Blum, M. (1998). Growth factor synergism and antagonism in early neural crest development. *Biochemistry and Cell Biology*, *76*, 1039–1050.

Sun, C., Sun, H., Wu, S., Lee, C. C., Akamatsu, Y., Wang, R. K., … Liu, J. (2013). Conditional ablation of neuroprogenitor cells in adult mice impedes recovery of poststroke cognitive function and reduces synaptic connectivity in the perforant pathway. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*, 17314–17325.

Sun, X., Zhou, Z., Liu, T., Zhao, M., Zhao, S., Xiao, T., … Zhao, C. (2016). Fluoxetine enhances neurogenesis in aged rats with cortical infarcts, but this is not reflected in a behavioral recovery. *Journal of Molecular Neuroscience*, *58*, 233–242.

Tajiri, N., Acosta, S., Glover, L. E., Bickford, P. C., Jacotte Simancas, A., Yasuhara, T., … Borlongan, C. V. (2012). Intravenous grafts of amniotic fluid-derived stem cells induce endogenous cell proliferation and attenuate behavioral deficits in ischemic stroke rats. *PLoS ONE*, *7*, e43779.

Tajiri, N., Kaneko, Y., Shinozuka, K., Ishikawa, H., Yankee, E., McGrogan, M., … Borlongan, C. V. (2013). Stem cell recruitment of newly formed host cells via a successful seduction? Filling the gap between neurogenic niche and injured brain site. *PLoS ONE*, *8*, e74857.

Venna, V. R., Xu, Y., Doran, S. J., Patrizz, A., & McCullough, L. D. (2014). Social interaction plays a critical role in neurogenesis and recovery after stroke. *Translational Psychiatry*, *4*, e351.

Wang, L. L., Chen, D., Lee, J., Gu, X., Alaaeddine, G., Li, J., … Yu, S. P. (2014). Mobilization of endogenous bone marrow derived endothelial progenitor cells and therapeutic potential of parathyroid hormone after ischemic stroke in mice. *PLoS ONE*, *9*, e87284.

Watson, N., Ji, X., Yasuhara, T., Date, I., Kaneko, Y., Tajiri, N., & Borlongan, C. V. (2015). No pain, no gain: Lack of exercise obstructs neurogenesis. *Cell Transplantation*, *24*, 591–597.

Xia, C. F., Yin, H., Yao, Y. Y., Borlongan, C. V., Chao, L., & Chao, J. (2006). Kallikrein protects against ischemic stroke by inhibiting apoptosis and inflammation and promoting angiogenesis and neurogenesis. *Human Gene Therapy*, *17*, 206–219.

Yamashita, T., Ninomiya, M., Hernández Acosta, P., García-Verdugo, J. M., Sunabori, T., Sakaguchi, M., … Sawamoto, K. (2006). Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*, 6627–6636.

Yasuhara, T., Matsukawa, N., Hara, K., Yu, G., Xu, L., Maki, M., … Borlongan, C. V. (2006). Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*, 12497–12511.

Yasuhara, T., Hara, K., Maki, M., Xu, L., Yu, G., Ali, M. M., … Borlongan, C. V. (2010). Mannitol facilitates neurotrophic factor up-regulation and behavioural recovery in neonatal hypoxic ischaemic rats with human umbilical cord blood grafts. *Journal of Cellular and Molecular Medicine*, *14*, 914–921.

Zhang, Z. G., & Chopp, M. (2015). Promoting brain remodeling to aid in stroke recovery. *Trends in Molecular Medicine*, *21*, 543–548.

Zhang, Y., Chopp, M., Mahmood, A., Meng, Y., Qu, C., & Xiong, Y. (2012). Impact of inhibition of erythropoietin treatment-mediated neurogenesis in the dentate gyrus of the hippocampus on restoration of spatial learning after traumatic brain injury. *Experimental Neurology*, *235*, 336–344.

Zhang, W., Wang, H., Zhang, H., Leak, R. K., Shi, Y., Hu, X., … Chen, J. (2015). Dietary supplementation with omega-3 polyunsaturated fatty acids robustly promotes neurovascular restorative dynamics and improves neurological functions after stroke. *Experimental Neurology*, *272*, 170–180.

How to cite this article: Tajiri N, Quach DM, Kaneko Y, et al. NSI-189, a small molecule with neurogenic properties, exerts behavioral, and neurostructural benefits in stroke rats. *J Cell Physiol*. 2017;232:2731–2740. https://doi.org/10.1002/jcp.25847