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Michka Sharpe April 18, 2012

Expanding the Window of Opportunity: Intransal Delivery of Wnt 3a and Whisker Stimulation in the Ischemic Stroke Mouse Model

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Abstract

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By Michka Sharpe

Each year, one out of six individuals will suffer a stroke. Despite extensive basic and clinical investigations, stroke remains to be the leading threat to human life and health in the US and worldwide. Up to now, there are very few effective treatments for acute stroke patients. Novel neuroprotective therapy and regenerative therapy are urgently needed for clinical applications. The purpose of this investigation was to test a combination therapy of a canonical Wnt signaling protein involved in neurodevelopment, Wnt 3a and whisker stimulation in the enhancement of neurogenesis and angiogenesis following focal ischemic stroke in Nestin-GFP mice. In an ischemic barrel cortex stroke mouse model induced by ligations of distal branches of the middle cerebral artery (MCA) in Nestin-GFP mice, Wnt 3a was administrated intranasally to stimulate the Wnt signaling pathway while peripheral whisker stimulation was applied as a central specific rehabilitation treatment after stroke. The combination therapy showed increased expression of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) in the penumbra region 14 days after stroke. Co-labeling of 5-Bromo-2-deoxyuridine (BrdU) and NeuN positive cells in the penumbra demonstrated augmented neurogenesis, while increased co-labeling of BrdU and Collagen IV suggested increased angiogenesis in these mice. Wnt 3a paired with whisker stimulation also increased local blood flow compared to the saline control. In addition, the SDF-1 receptor CXCR4 expression increased when Wnt 3a was paired with peripheral stimulation, suggesting enhanced sensitivity to chemoattractant directed migration that may promote homing of neuroblasts from the subventricular zone to the infarct site. Cellular migration was confirmed with positive Doublecortin (DCX) and BrdU co-labeling. These data

support that the combination therapy targeting the Wnt pathway paired with peripheral signals may be beneficial for long-term tissue repair and functional recovery after ischemic stroke.

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Terminology and Abbreviations

MCAO: Middle cerebral artery occlusion

BrdU: 5-Bromo-2-deoxyuridine is a thymidine analogue that labels proliferating cells.

NeuN: Neuronal nuclear antigen. Protein expressed by mature neurons.

BDNF: Brain derived neurotrophic factor is a member of the neurotrophin family of proteins important in neuronal survival, development and function.

VEGF: Vascular endothelial growth factor. Protein involved in formation of new vessels.

DCX: Doublecortin. Microtubule associated protein expressed by developing neurons.

CXCR4: Chemokine receptor for stromal derived cell factor-1 (SDF-1) with chemotactic activity.

Fzr: Frizzled G-protein coupled receptor involved in Wnt signaling.

Dvl: Dishevlled phosphoprotein involved in Wnt signaling

APC: Adenomatous Polypsis coli. Protein that is a part of the Wnt signaling destruction complex.

GSK3-β: Protein that is part of the Wnt signaling destruction complex.

CKI: casein kinase I. Protein that is part of the Wnt signaling destruction complex.

β-catenin: Wnt signaling effector protein that transcribes Wnt genes.

XAV939: Wnt signaling inhibitor

SVZ: subventricular zone. Stem cell niche of the lateral ventricle.

SGZ: subgranular zone. Stem cell niche of the dentate gyrus in the hippocampus.

Collagen IV: type of collagen found with the extracellular matrix.

CBF: cerebral blood flow

Wnt 3a: canonical Wnt signaling protein involved in neuro-development

Abstract

Each year, one out of six individuals will suffer a stroke. Despite extensive basic and clinical investigations, stroke remains to be the leading threat to human life and health in the US and worldwide. Up to now, there are very few effective treatments for acute stroke patients. Novel neuroprotective therapy and regenerative therapy are urgently needed for clinical applications. The purpose of this investigation was to test a combination therapy of a canonical Wnt signaling protein involved in neurodevelopment, Wnt 3a and whisker stimulation in the enhancement of neurogenesis and angiogenesis following focal ischemic stroke in Nestin-GFP mice. In an ischemic barrel cortex stroke mouse model induced by ligations of distal branches of the middle cerebral artery (MCA) in Nestin-GFP mice, Wnt 3a was administrated intranasally to stimulate the Wnt signaling pathway while peripheral whisker stimulation was applied as a specific rehabilitation treatment after stroke. The combination therapy showed increased a trend towards increased expression of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) in the penumbra region 14 days after stroke. Co-labeling of 5-Bromo-2-deoxyuridine (BrdU) and NeuN positive cells in the penumbra demonstrated augmented neurogenesis, while increased co-labeling of BrdU and Collagen IV suggested increased angiogenesis in these mice. Wnt 3a paired with whisker stimulation also increased local blood flow compared to the saline control. In addition, the stromal derived stem cell factor (SDF-1) receptor CXCR4 expression increased when Wnt 3a was paired with peripheral stimulation, suggesting enhanced sensitivity to chemoattractant directed migration that may promote homing of neuroblasts from the subventricular zone to the infarct site. Cellular migration was confirmed with positive Doublecortin (DCX) and BrdU co-labeling. These data support that the combination

therapy targeting the Wnt pathway paired with increased peripheral signals may have translational benefits for long-term tissue repair and functional recovery after ischemic stroke in humans.

Introduction

"Time is brain" (Saver, 2006). For every minute an ischemic stroke is untreated, an average of 1.9 million neurons, 13.8 billion synapses and 7 miles of axonal fibers are lost (Saver, 2006). Stroke is defined as the occlusion of a cerebral vessel or the spontaneous rupture of an intracranial artery by hemorrhage into the brain parenchyma or subarachnoid space (Walker and Marx, 1981; Seta et al. 1992; Garcia 1984). The fourth leading cause of death in the United States, stroke occurs in over 175,000 Americans each year (Heart Disease and Stroke Statistics-2012 Update). Globally, one in six individuals will suffer from stroke each year (Centers for Disease Control-2005 Statistical Report). There are three distinct types of stroke: hemorrhagic, ischemic and transient ischemic attacks that occur as a result of the rupture, vascular occlusion usually from the heart and transient vascular occlusion in which stroke symptoms improve before tissue damage respectively frequently in the neck vessels (Seta et al., 1992). As many as 88% of all strokes that occur each year are ischemic (Whitaker et al., 2007). Stroke is one of the leading causes of long term functional disability (Rui et al., 2011; Biernaskie et al., 2001; Wolf et al., 2012) Up to six months following ischemic stroke, some patients experience aphasia (19%), depressive symptoms (35%) and dependence on care giving (26%) (American Heart Association Updated Statistics-2008). Hemiparesis affects as many as 50% of patients after stroke (American Heart Association Updated Statistics- 2008). Functional recovery of impaired limbs is a major initiative employed for patients with motor asymmetries after stroke (Rui et. al, 2011; Biernaskie et al. 2001). Mechanical manipulation of a weak limb while constraining the stronger limb offers long-term therapeutic benefits for patients. Therapies involving the use of robotic arms or virtual simulation

rehabilitative techniques aim to enhance plastic changes in affected brain regions through forced usage of the affected limb (Rui et al. 2011; Biernaskie et al. 2001; Celinder and Peoples, 2012). However, there still remains a limited combined therapeutic treatment for patients aimed at both mitigating the physical damage caused by ischemic stroke and treatment for long term functional recovery.

The Ischemic Cascade: Pathophysiology of Ischemic Stroke

Brain function is dependent on continuous blood flow supplying glucose and oxygen (Iadecola and Anrather, 2011). Ischemic stroke is caused by the depletion of oxygen to the brain, precipitating a destructive cascade of events that manifest in behavioral and molecular neural deficits (Graham and Hickey, 2002; Iadecola and Anrather, 2011). Immediately following occlusion of a vessel, loss of oxygen to the occluded site activates necrotic and apoptotic pathways that destroy brain tissue (Choi et al., 1996). Within minutes of occlusion at the ischemic core, inadequate glucose and oxygen supplies to neurons cause changes in metabolic rates that decrease the production of ATP necessary to supply the metabolic demand of neurons (Watters and O'Connor, 2011; Choi et al., 1996; Iadecola and Anrather, 2011). Consequently, Na^+/K^+ pumps are dysregulated, causing a passive diffusion of Na^+ into the cell and the ionic imbalance results in swelling until cell membranes and organelles rupture (Harvey et al. 2011; Watters and O'Connor, 2011; Kanekar et al. 2011). Peri-infarct depolarizations (PID) increase the levels of glutamate to excitotoxic levels and contribute to acute cell death (Heiss, 2011; Back, 1998). Activation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole proprianate acid (AMPA) receptors by glutamate saturation causes the levels of intracellular Ca^{2+}

to build up to toxic levels (Watters and O'Connor, 2011; Kanekar et al. 2011; Iadecola et al. 2011). Tissue damage after stroke increases the generation of reactive free radicals that in turn activate apoptotic and necrotic pathways (Kaneker et al. 2011; Iadecola and Anrather, 2011). Cell death also activates both innate and adaptive inflammatory response that perpetuates cell death (Iadecola and Anrather, 2011). Infiltration of macrophages, T cells and dendritic cells have been shown to persist in the brains of patients for years and may be contributing factors in post ischemic disabilities such as dementia (Iadecola and Anrather, 2011). Previous studies describe tumor necrosis factor-α to be increased to neurotoxic levels following stroke (Watters and O'Connor, 2011). Ultimately, stroke induced pathology causes a destruction cascade of metabolic dysfunction, excitotoxicity, swelling, inflammation and cell death of regions in and around the site of occlusion.

The Ischemic Penumbra as a Therapeutic Target Region in Stroke

The ischemic penumbra is defined as the region of the brain adjacent to the infarct core with the potential for functional recovery under the condition that blood flow be restored within a sufficient time window (Heiss, 2011) This definition is not complete. Time largely determines the extent to which post ischemic damage is irreversible. It is also important to note that unlike the infarct core, protein synthesis within the penumbra continues to occur (Ramos Cabrer, 2011; Heiss, 2011). Stress proteins such as heat shock protein 70 mediate neuronal survival by mollifying protein misfolding, inflammation, apoptosis and necrosis and have been shown to be upregulated within the penumbra (Giffard and Yenari, 2004; Ramos Cabrer, 2011; Heiss, 2011). Apoptosis within the penumbra demonstrates partial retention of organized cell death compared to

necrosis in the infarct core (Ramos Cabrer, 2011; Heiss, 2011). A less severe reduction in blood flow and a predominately apoptotic or delayed death of cells within the penumbra together expand the window of opportunity for post-ischemic therapeutic intervention. The ischemic penumbra remains the target region in current stroke therapies for the opportunity to reverse damage based on partial maintenance of metabolic processes and hypoperfusion of oxygen (Heiss, 2011).

Current Stroke Treatment

Treatment of ischemic stroke is largely restricted by time. Therapeutic targets have been aimed at both neuroprotection of the brain preceding an ischemic attack as well as in treatment following an ischemic attack. Largely, the treatments available for stroke prevention include aspirin, anticoagulants, thrombolytic surgery to remove atherosclerotic plaques and other causes of embolic for blood flow restoration and maintenance of normal blood pressure and cholesterol. Previous investigations focusing on attenuating Ca2+ influx and intracellular Ca2+ accumulation by NMDA receptor antagonists and many other blockers showed effective neuroprotection in experimental stroke animals however all of them so far failed clinical trials due to a variety of reasons such as narrow therapeutic window, unbearable side effects, patient selection, and differences between animal models and clinical human situation. A number of other strategies have also been extensively explored. For example, TNF-α was targeted for a potential neuroprotective effect (Watters and O'Connor (2011) and over expressing glutamate transporter 1(GLT-1) was tested to reduces glutamate accumulation therefore attenuate excitotoxicity (Harvey et. al., 2011). These approaches, however, still remain to be verified by clinical and pre-clinical studies. Tissue plasminogen activator (tPA) treament is currently the only FDA approved

treatment for acute ischemic stroke but is restricted to a three-hour treatment time window (Su et al. 2008). tPA treatment is also limited by its ability to increase cerebrovascular permeability and thus increase the risk of hemorrhage (Su et al. 2008). Therefore, further exploration of both neuroprotective and therapeutic treatments is warranted.

Focal Ischemia of Barrel Cortex Stroke and Peripheral Whisker Stimulation

About 40% of clinical strokes are relatively small and affect only part of the cortex (AHA update 2012). To investigate the pathology, mechanism and treatment of small strokes, a focal ischemia specifically targeting the barrel cortex was developed first in rats in 1995 by Wei and Woolsey and later extended to mice (Wei et al., 1995; Majid et al., 2000). This barrel cortex stroke model is now an established model for studying morphological and functional changes after a focal ischemic stroke. Whisker vibrassae of the mouse project to the barrels on the contralateral side of the brain where each whisker projects to its own barrel (Li and Crair, 2011). Stimulation to the whiskers of the mouse can therefore be used to study sensorimotor plasticity in stroke models. Whitaker et al. (2007) describe physical activity to restore blood flow back to the penumbra using peripheral stimulation as a therapy post stroke. In accordance with Whitaker et al. (2007), it was found that peripheral stimulation within two hours of stroke exhibited neuroprotective effects as well as a restoration of cortical function through evoked firing potentials of cortical neurons in brain regions damaged by stroke (Lay et al., 2011).

We have previously described the use of peripheral whisker stimulation to enhance angiogenesis *in vivo* through an up-regulation of neurogenic and angiogenic trophic factors in the ischemic

penumbra (Whitaker et al., 2007). Similarly, peripheral stimulation to the mouse barrel cortex has demonstrated neurogenesis and cell migration around the ischemic core as well as increased local cerebral blood flow (Wen-Lei et al., 2008). Increased expression of brain-derived neurotrophic factors, MAP kinases, angiogenic factors VEGF, EPO, EPOR, and other growth factors are the likely mediators involved in stimulating endogenous regenerative activities underlying thalamocortical plasticity after stroke (Vallés et al., 2011;Whitaker et al., 2007; Wen-Lei et al. 2011;Li et al., 2008). Therefore, a plausible future direction would be to use whisker stimulation as a physical therapy or rehabilitation method to enhance post-stroke regeneration and tissue repair.

Wnt 3a and Canonical Wnt Signalling

Wnt signaling has shown to be important in cell proliferation, organogenesis and survival (Kühl M., 2010). Previously, reports have shown int-1 to be highly expressed in mouse embryos between 8 and 22 days of development and was seen primarily in the hind-brain regions and spinal cord (Rijsewijk et al., 1987). Dnt-1, found in *Drosophila melanogaster* is homologus to the int-1 gene in the mouse and was found to be associated with the wingless phenotype in *D. melanogaster* (Rijsewijk et al., 1987). Wnt, stemming from the wingless *D. melanogaster* phenotype and the int-1 gene, is an oncogene with expression restricted to development (Rijsewijk et al., 1987). Wnt is highly conserved across species and has canonical and noncanonical signaling pathways (Davidson et al., 2007; Kühl 2010). In particular, the canonical pathway involves a β-catenin effector protein. When a Wnt protein binds to its serpentine seven pass transmembrane Frizzled receptor (Fzr) and a low-density lipoprotein receptor, LRP 5/6, Both

the LRP 5/6 and the Fzr receptor associate with a protein, Dishevelled (Dvl). Interactions of Dvl with the LRP 5/6 and Fzr transduce a signal to trigger the disassembly of the destruction complex- adenomatous polypsis coli (APC) tumor suppressor, Axin and the kinases GSK3-β and CKI, allowing β-catenin to translocate to the nucleus and transcribe various Wnt target genes. However, when Wnt proteins are not bound to their Frz receptors, the destruction complex is allowed to assemble and phosphorylate the amino terminal end of β-catenin, tagging it for ubiquitination and degradation in the proteasome (Reya and Clevers, 2005). In the human genome, there are about 20 Wnt genes that also play important roles in the signaling cascades associated with development and cellular proliferation (Reya and Clevers, 2005; Nusse, 2008). Wnt 3a is one of these genes.

Wnt 3a has been found to be an important player in the development and vascularization of the Blood Brain Barrier (BBB) in embryonic and postnatal development (Liebner et al. 2008). Haslinger et al. (2010) show that Wnt3a enhances the migration of trophoblasts *in vitro,* and has implications in the maintenance of blood flow to the placenta as well as placental vascularization. Muroyama et al. (2003) demonstrate that Wnt 3a enhances differentiation of neuronal stem cells into neurons and astrocytes *in vitro* via growth factor like activity (Muroyama et al., 2004; Willert et al. 2003; Ohira, 2011). Previous reports by David et al. (2010) illustrate by an outgrowth of neurites *in vitro,* the ability of Wnt 3a to stably enhance proliferation and neurogenesis of spinal cord neuron precursors. Studies also show that Wnt3a also regulates the survival and proliferation of human embryonic stem cells (hESC) *in vitro* (Davidson et al., 2007). Wnt signaling is an important mediator in development of the central nervous system (CNS) (Yu et al., 2005).

The Adult Brain and Neurogenesis

In 1928, Ramon y Cajal stated that "in adult centres, the nerve paths are something fixed, ended immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree" (Quiñones-Hinojosa and Chaichana, 2007). It was not until the 1960s that the notion of the adult brain being incapable of generating new neurons; neurogenesis was challenged. Since then, two main stem cell niches have been identified within the adult brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. Both niches continuously maintain and generate neural progenitors within the adult brain (Quiñones-Hinojosa and Chaichana, 2007; Alvarez Buylla A and Lim D, 2004; Kojima et al., 2010). Found within the walls of the lateral ventricles of the forebrain, the SVZ is the largest stem cell niche in the adult brain (Quiñones-Hinojosa and Chaichana, 2007). Three distinct cell types A, B and C form the architechture of the SVZ (Quiñones-Hinojosa and Chaichana, 2007; Alvarez Buylla A and Lim D, 2004; Kojima et al., 2010). B cells are of astrocytic lineage and serve to maintain the stem cell microenvironment. C cells are rapidly diving cells derived from B cells and A cells are neuroblasts that migrate to the olfactory bulb to differentiate into neurons (Quiñones-Hinojosa and Chaichana, 2007; Alvarez Buylla A and Lim D, 2004; Kojima et al., 2010).

Within the SVZ, neural progenitors migrate long distances via the rostral migratory stream (RMS) to the olfactory bulb (OB) to differentiate into interneurons (Toledo et al. 2008; Li W et al., 2008; Kishimoto, 2011; Quiñones-Hinojosa and Chaichana, 2007; Alvarez Buylla A and Lim D, 2004; Kojima et al., 2010). During an ischemic stroke, neural progenitor pathways are diverted to the

site of the infarct where neuronal differentiation is increased to replace dead or damaged neurons (Zhang et al., 2007; Kishimoto et al., 2011; Kojima et al., 2010; Zhang et al. 2004; Ohira, 2011).

Our previous data have demonstrated that Wnt 3a increases *de novo* neurogenesis in the penumbra following focal ischemic stroke at 14 and 21 days respectively. Similarly, Wnt 3a was found to enhance migration of neuronal stem cells in the subventricular zone (SVZ) 14 days after focal ischemic stroke. Increased proliferation and migration of neurons to the penumbra after ischemic stroke suggest that Wnt 3a interacts with the SVZ neural progenitor niche and regulates *in vivo* proliferation of adult neural stem cells and increases their migration to the peri-infarct region. Increased expression of brain-derived neurotrophic factor 14 days post stroke suggest that Wnt 3a mediates increased neuronal growth and mitigates neuronal depletion via neurotrophic regulation (Zhang et al., 2011; Ferrer et al., 2001). A more parsimonious explanation is that increased expression of the Wnt effector protein β-catenin at 14 days strengthens pre-existing neuronal networks and increases neurogenesis within the penumbra.

In addition, the proliferative and migratory effects of Wnt 3a demonstrated *in vitro* make it a probable candidate in stroke therapy (Davidson et al., 2007;Haslinger et al., 2010; David et al., 2010). However, there may be synergistic effects using Wnt 3a together with whisker stimulation in improving these effects more than either therapy by itself. Therefore, a plausible future direction would be to explore the effect of Wnt3a in tandem with whisker stimulation for the further enhancement of *de novo* neurogenesis by capitalizing on the neuroprotective and proliferative effects of both therapies for long-term functional recovery and repair after ischemic stroke.

Our aim is to determine if Wnt 3a together with peripheral stimulation will further enhance neurogenesis and angiogenesis *in vivo* following focal ischemic stroke. We hypothesize that Wnt 3a and whisker stimulation together will further enhance *de novo* neurogenesis and angiogenesis after ischemic stroke. We also predict that by blocking canonical Wnt signaling, there will be decreased neurogenesis after ischemic stroke.

Materials and Methods

Focal Ischemia in Animals

12 -16 week old male Nestin GFP mice (C57/BL6 origin) weighing 25-45 grams were anesthetized with 2% isofluorane (i.p). Middle cerebral artery occlusion (MCAO) surgeries were performed as previously described by Wei et al. (1995). The middle cerebral artery (MCA) supplying the right whisker barrel cortex was permanently ligated (10-0 suture Surgical Specialties CO, Reading, PA, USA) and both common carotid arteries (CCA) were temporarily ligated for 7 minutes. During surgery, body temperature was maintained at 37^{o}C. Animals were given rodent diet chow and water *ad libitum.* Mice were housed in standard cages (30 x 20 x 1 cm^{3}) with a maximum of five per cage. All animal experiments and surgical procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Wnt 3a Intransal Delivery

One day after surgery, Wnt 3a injections (50ng/25μL) were given intranasally to the mice once a day for a total of seven days. Saline (25μL) was also given intranasally to the control mice once a day for a total of seven days.

BrdU Injections

5-bromo-2'-deoxyuridine (BrdU) injections, labelling cellular proliferation, were administered at 50mg/kg intraperitoneally (i.p) once per day starting one day post stroke and ending on the day the day of sacrifice.

Whisker Stimulation

Starting one day after ischemic stroke, whiskers ipisilateral to the side of stroke were trimmed, leaving normal whisker length on the contralateral side to enhance stimulation to the right barrel cortex as previously described by Whitaker et al. (2007) with some modifications. Whisker stimulation was conducted once per day in the rostro-caudal direction at a rate of 140 strokes per minute until sacrificed. Whisker stimulation is performed to enhance afferent signals to the mouse somatosensory cortex (Whitaker et al., 2007). Stimulating whiskers to the contralateral side of stroke sends afferent signals to the affected side of the mouse brain. Trimmed whiskers have been shown to significantly enhance this effect by reducing afferent signals to the stronger ipsilateral side and increasing afferent signals to the affected side of the brain (Whitaker et al., 2007).

Measurement of Local Cerebral Blood Flow (CBF)

Animals were anesthetized with 4% chloral hydrate solution (400mg/kg). The ischemic window over the right coronal suture above the middle cerebral artery (MCA) of each mouse was opened to laser Doppler scanner to measure local cerebral bloodflow. Photons from the laser interact with red blood cells flowing through the MCA, causing a Doppler shift that reflects both average velocity and concentration of moving blood cells (Fabricus and Lauritzen, 1996). The mean

Doppler shift was quantified for each animal using LDPI Win 2 software (Perimed AB, Stolkholm Sweden).

Coronal Sectioning and Immunohistochemistry

Sacrificed mouse brains were frozen in Optimal Cutting Temperature Compound (Sakura Finetek) and stored at -80^{o}C. 10μm coronal brain sections, each separated by 90μm per slide were cut and mounted on slides in preparation for staining. Each brain was cut into a total of 26 sections to collect the subventricular zone (SVZ). All brain sections were counted blindly. Brain sections were fixed in 10% buffered formalin then washed with 10% phosphate buffered saline (PBS) three times. Fixed brain slices were then incubated in -20^{o}C methanol for 10 minutes and then washed three times with PBS. Brain sections were air dried and then re-hydrated in PBS before incubation in 0.02% Triton X for 15 minutes. Slides were blocked in 1% fish gel for 1 hour. Newly proliferating cells (BrdU, Serotec), neuronal nuclei (NeuN, Millipore), CXCR4 chemokine receptor expressesion (CXCR4, R&D) and collateral vessels (Collagen IV, Millipore) were incubated overnight at 4^{o}C. Brain slices were washed with PBS and incubated with donkey anti-rat cy3 (Jackson Immunologicals), donkey anti-mouse cy5 (Jackson Immunologicals) and donkey anti rabbit 488 (Invitrogen) secondary antibodies for 2 hours. To measure neuroblast migration (DCX, Santa Cruz Biotechnology), brain tissues were fixed in 10% buffered formalin and methanol fixed. Brain sections were air dried and incubated in 0.2% Triton X-100 for 5 minutes before incubating in 1% fish gel for 1 hour. DCX anti goat (1:500) and BrdU anti rat (1:200) antibodies were incubated overnight 4^{o}C. Secondary antibodies donkey anti goat Alexafluor 488 (1:200) and donkey anti rat (1:200) were incubated for 2 hours. All slides were dried and prolong anti-fade (Molecular Probes, CA) to preserve fluorescence for imaging.

Western Blot Analysis

The penumbra region was defined as the 500μm of tissue bordering the core infarct region (Ohab et al., 2006). The whole brain was dissected fresh into 1mm-thick coronal sections and the penumbra region was dissected and flash frozen on dry ice 14 days after stroke. The tissues were homogenized in lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 100 mMNaF, 1% Triton, leupeptin, aprotinin, and pepstatin), spun down by centrifuge at 13,000 rpm for 15 min to remove the insoluble fraction and the supernatant that contained the protein was collected. Each sample was assayed for protein concentration with the Bicinchoninic Acid Assay (Sigma, St Louis, MO). Samples were diluted with a loading buffer to a standardized concentration of 1μg/μl for each sample. 30μg of each sample was run on a SDS-polyacrylamide gel through electrophoresis in a Hoefer Mini-Gel system (Amersham Bio- sciences, Piscataway, NJ) and transferred in the Hoefer Transfer Tank (Amersham Biosciences, Piscataway, NJ) onto a PVDF membrane. The membrane was blocked in a 5% evaporated milk solution for one hour followed by incubation of primary VEGF antibody (Millipore 05.443, 1:1000), Wnt 3a primary antibody (R&D Rat 1:1000), β-catenin primary antibody (R&D Mouse 1:1000) and BDNF primary antibody (Santa Cruz Biotechnology Rabbit 1:500) at 4˚C overnight. After washing with Tris-buffered saline with 0.1%Tween 20 (TBST) and Tris-buffered saline (TBS), an alkaline phosphatase-conjugated secondary antibody (anti-mouse) was incubated at room temperature for 1 hour. The membrane was washed with TBST and TBS and BCIP/NBT was applied to the active side of the membrane to visualize the protein bands. β-actin was used as a loading control for each sample (Sigma, St. Louis MO).

XAV939 Wnt 3a Antagonist

In order to determine the importance of canonical Wnt signaling in neurogenesis, an XAV939 Wnt 3a antagonist was used to stabilize the axin destruction complex and prevent β-catenin from translocating to the nucleus. A reduction of β-catenin is indicative of a reduction in Wnt 3a signaling activity. XAV939 was dissolved in DMSO to final concentrations of 1μM and 0.5μM. One day following stroke C57/B6 mice were given intranasal deliveries of 25 μL saline or 50ng/25μL Wnt 3a followed by 1μM XAV939. On day 2 through 14, mice were given 0.5μM XAV939 to maintain levels of XAV939 and inhibit Wnt 3a activity. Wnt 3a and saline intranasal deliveries were given for 7 days. There was no mortality from XAV939 treatments.

Stereological Cell Counting

For systematic random sampling, every third slide per animal across the region of interest was counted. Six fields per brain section were counted blindly under 20X and 40X magnification in confocal images.

Statistical Analyses

Student's T-tests were performed when comparing single samples. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. Changes were identified as significant if p values were less than 0.05 and are indicated by *. Mean values were reported with standard error of the mean (SEM).

Results

Combined treatment of Wnt 3a and whisker stimulation shows trend towards increased BDNF expression in the post-stroke brain

As a quantitative measure for differentiation of neuroblasts into neurons as well as an indicator of neuronal repair, we measured the expression of BDNF in the penumbra at 14 days after stroke. Wnt 3a and β-catenin expression were also quantified. Tissue within the peri-infarct region was collected after sacrifice and levels of BDNF protein expression were analyzed. Compared to saline, Wnt 3a and stimulation only control groups, Wnt 3a+stimulation demonstrated a trend towards increased expression of the neurotrophic factor BDNF 14 days after stroke. Similarly, stimulation combined with Wnt 3a showed a trend in increased expression of Wnt 3a and β-catenin than in expression with Wnt 3a treatment only (Figure 1A-B).

Combined treatment of Wnt 3a and whisker stimulation enhances *de novo* neurogenesis in the post-stroke brain

We hypothesized that Wnt 3a paired with whisker stimulation would further enhance *de novo* neurogenesis. Immunostaining for neuronal nuclei (NeuN) colabelled with newly synthesized cells (BrdU) revealed a significant increase in neural progenitors in the penumbra region 14 days after stroke when Wnt 3a is paired with stimulation compared to saline, Wnt 3a and stimulation control groups (Figure 2-3).

Combined treatment of Wnt 3a and whisker stimulation increases expression of VEGF in the post-stroke brain

Next, we sought to quantify the expression of the angiogenic protein VEGF in the penumbra 14 days after ischemic stroke. Tissue within the peri-infarct region was collected and protein was isolated and analyzed. VEGF expression is increased when Wnt 3a is paired with whisker stimulation compared to the Wnt 3a and saline groups. Surprisingly, Wnt 3a +Whisker stimulation did not exhibit a further increase in VEGF expression compared to stimulation 14 days post stroke. As demonstrated previously, stimulation alone significantly increased VEGF expression compared to the other groups (Figure 4A-B).

Combined treatment of Wnt 3a and whisker stimulation enhances angiogenesis in the post-stroke brain

In order to determine the extent of new endothelial vascularization, colabeled BrdU and Collagen IV immunostaining were used to quantify total newly synthesized endothelial cells in the penumbra following focal ischemic stroke. Wnt 3a+ whisker stimulation showed a significant increase in newly synthesized vessels in the penumbra 14 days after stroke. Interestingly, Wnt 3a alone was not sufficient to significantly increase angiogenesis 14 days following ischemic stroke. As previously demonstrated (Whitaker et al., 2007), stimulation alone was sufficient to increase angiogenesis 14 days following stroke (Figure 5).

Effect of Wnt3a treatment and whisker stimulation on cerebral blood flow

To determine the effects of Wnt 3a paired with whisker stimulation on cerebral blood flow, we used a Doppler laser to measure the mean perfusion of six measurements above the occluded branch of the middle cerebral artery (MCA). Animals were divided into four groups: 50ng/25µL intranasal delivery of Wnt 3a, 25µL intransal delivery of saline, intranasal delivery of Wnt 3a and whisker stimulation and stimulation only to the whiskers contralateral to the stroke infarct for 140 strokes per minute once per day. Focal ischemic stroke was induced by transiently ligating both common carotid arteries (CCA) and permanently ligating the middle cerebral artery (MCA) supplying the right whisker barrel cortex. Cerebral blood flow is represented as a percentage of the baseline blood flow. When both the CCA and MCA are occluded, blood flow is reduced up to about 80% of the baseline blood flow, inducing an ischemic stroke. 14 days after stroke, Wnt 3a paired with whisker stimulation significantly increases CBF compared to the saline control and is restored to a level slightly but not significantly higher than baseline (Figure 6-7).

Migration of neuroblasts from the subventricular zone to the penumbra region in the post-stroke brain

In order to determine if progenitors are migrating out of their SVZ stem cell niche and towards the infarct, we used immunostaining for colabeled migrating cells (DCX) with newly synthesized cells (BrdU) using perfused brain sections. We were able to demonstrate the presence of migrating cells out of the SVZ and towards the penumbra at 14 days (Figure 8A).

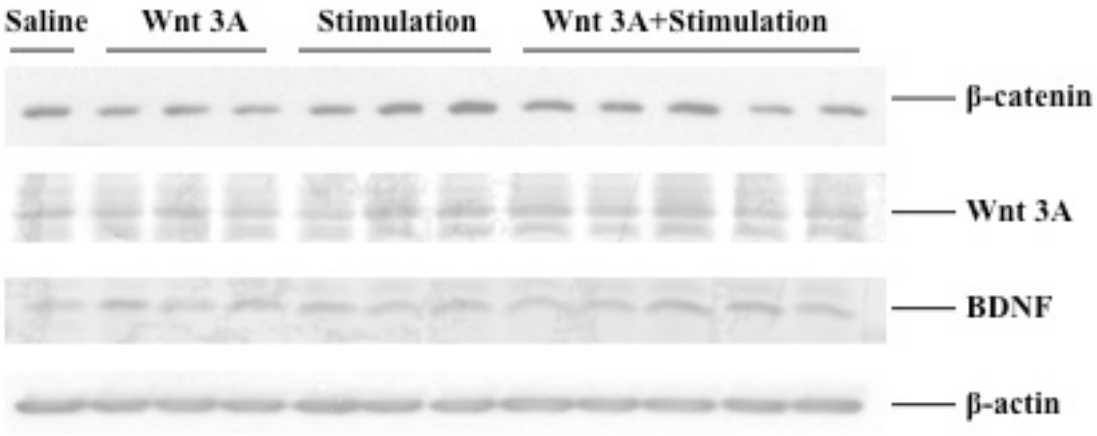
Combined treatment of Wnt 3a and whisker stimulation increases CXCR4 expression

Migration of neural and endothelial progenitors in addition to moving to along a structural pathway to the penumbra, is guided by chemotactic factors (Yu et al. 2012). Immunolabeling was used to qualitatively assess expression of the CXCR4 chemokine receptor, known to be involved in cellular migration. CXCR4 expression was increased when Wnt 3a was paired with whisker stimulation 14 days after stroke. However, expression of CXCR4 in the stimulation group was not very different from Wnt 3a+stimulation group, indicating that stimulation alone is sufficient to express high levels of CXCR4 (Figure 8B).

Blocking the Wnt signaling attenuates *de novo* neurogenesis in the post-stroke brain

In order to determine the extent to which canonical Wnt signaling is important in *de novo* neurogenesis after ischemic stroke, we used a potent Wnt blocker, XAV939 to attenuate canonical Wnt signaling through stabilization of the Axin protein within the destruction complex, increasing the phosphorylation and subsequent degradation of β-catenin. Intranasal delivery of 0.1 μM XAV939 was given in conjunction with 50ng/25mL Wnt 3a. Intranasal deliveries of Wnt 3a were given for one week after stroke. On days 2 through 14, 0.05 μM XAV939 was delivered intranasally. Immunostaining for newly synthesized cells (BrdU) and neuronal nuceli (NeuN) was used to quantifiy colabeled NeuN and BrdU cells in the penumbra 14 days after stroke. XAV939 severely attenuated *de novo* neurogenesis *in vivo* 14 days after stroke. Although there are BrdU positive cells, there is reduced colabeling of NeuN and BrdU compared to the Wnt 3a and saline controls (Figure 9-11A/B).

A.



B.

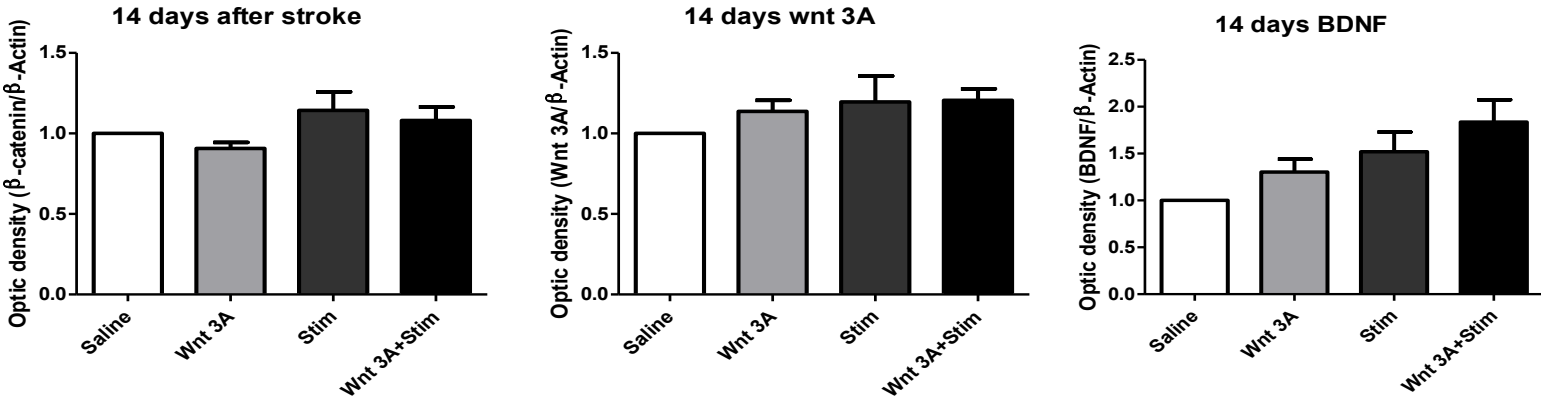


Figure 1. Trend towards increased BDNF expression with combination therapy of Wnt 3a and whisker stimulation. A. Expression of β-catenin, Wnt 3a, BDNF relative to β-actin. B. Quantification of protein expression demonstrates a trend towards increased BDNF expression as well as β-catenin and Wnt 3A proteins when stimulation is combined with exogenous Wnt 3A (p=ns).

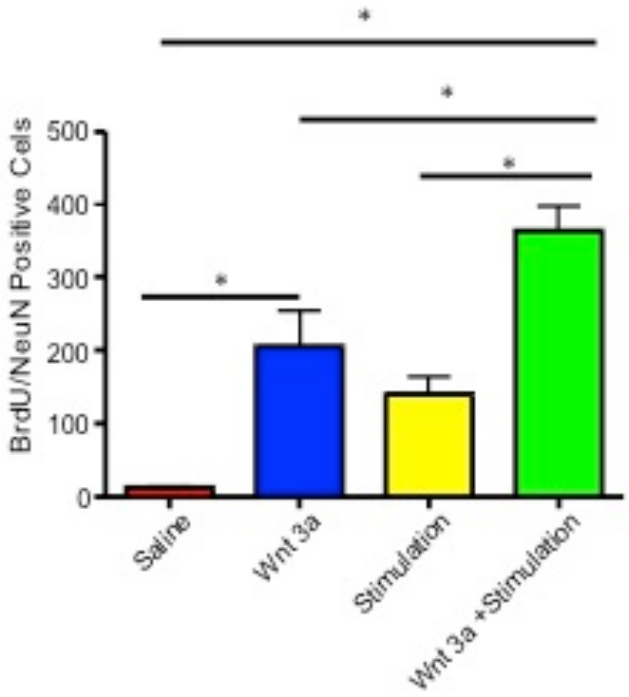


Figure 2. Combination therapy further enhances neurogenesis in the penumbra 14 days after stroke. A. Immuno-staining of NeuN and BrdU in the penumbra 14 days after stroke. B. Co-labeling of NeuN and BrdU were quantified. Wnt 3+whisker stimulation further enhanced neurogenesis 14 days after stroke compared to control groups (n= 4-7 per group, *$p<0.05$)

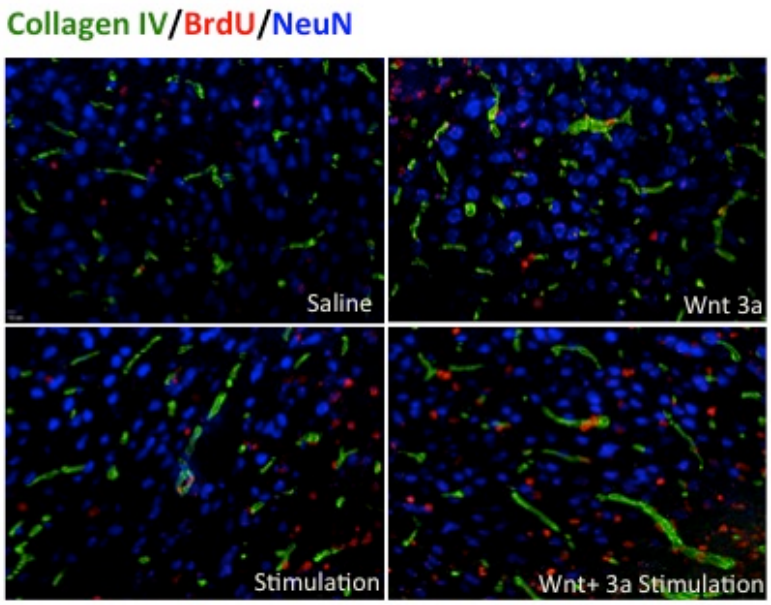
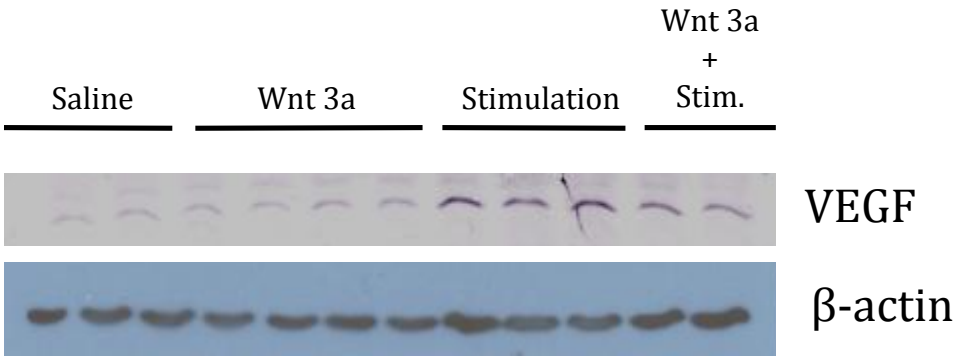


Figure 3. Immunohistochemistry for vessels (Collagen IV), neuronal nuclei (NeuN) and newly proliferating cells (BrdU) in the penumbra 14 days after ischemic stroke (n= 3-7 per group).

A.



B.

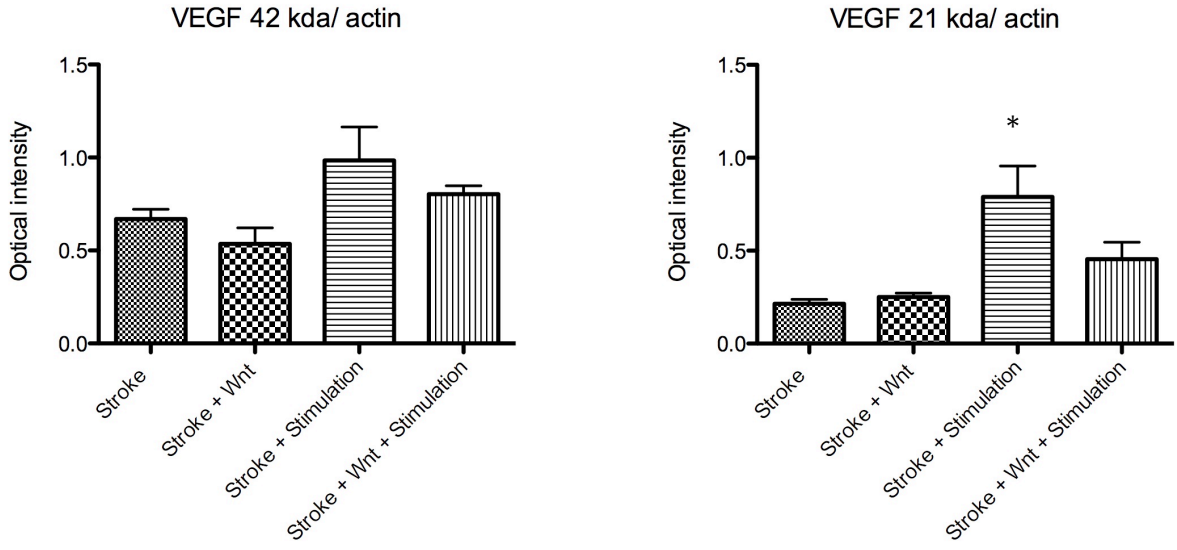


Figure 4. Increased expression of VEGF when stimulation is paired with Wnt 3a. A. VEGF is visible as two bands, 42 kds and 21 kda (representative 21kda band shown). B. Quantification of VEGF demonstrates a further enhancement of VEGF with a combination of Wnt 3a and whisker stimulation (n=3-4, *$p<0.05$).

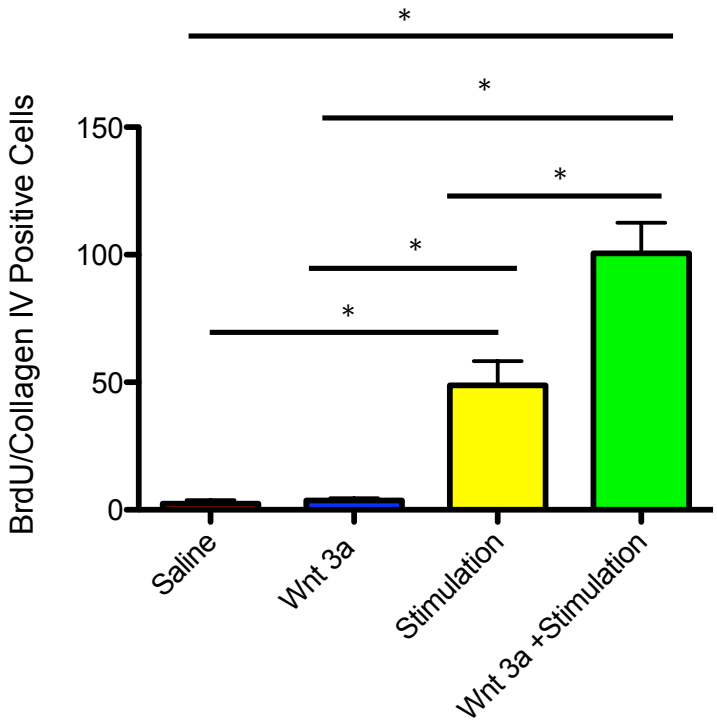
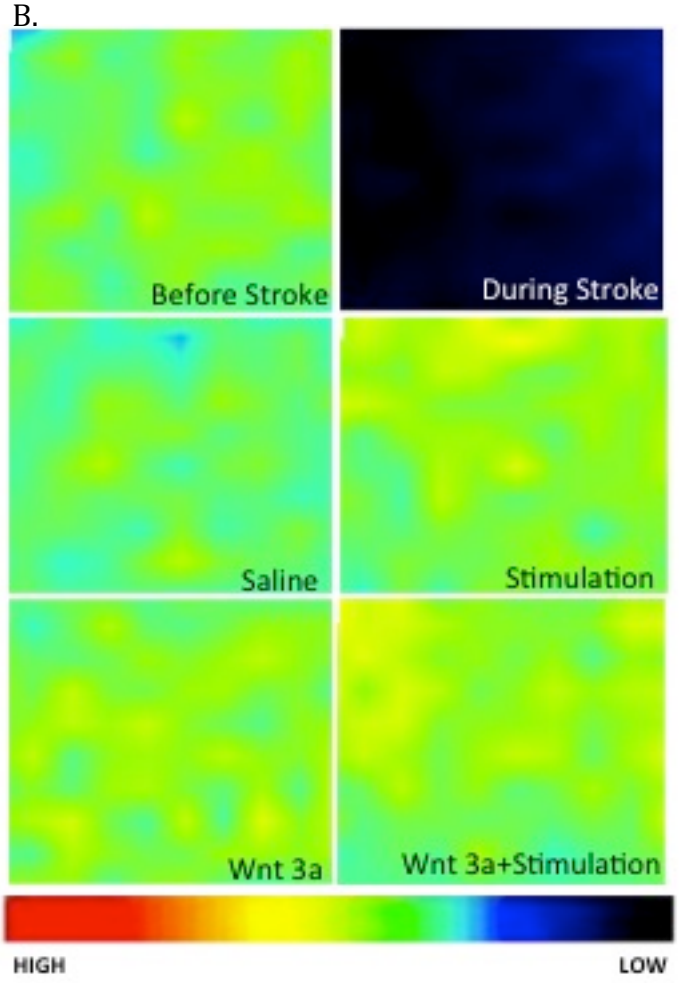


Figure 5. Combination treatment of Wnt 3a +Whisker Stimulation further enhances angiogenesis 14 days after ischemic stroke. A. Immuno-staining for co-labeled vessels (Collagen IV) and newly synthesized cells (BrdU). B. Quantification of co-labeled Collagen IV positive and BrdU positive vessels (n=3-5 per group. *$p<0.05$).

A.

B.



C.

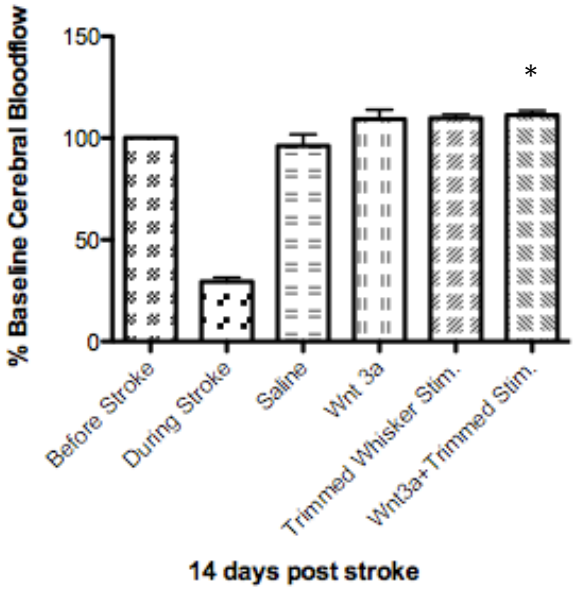
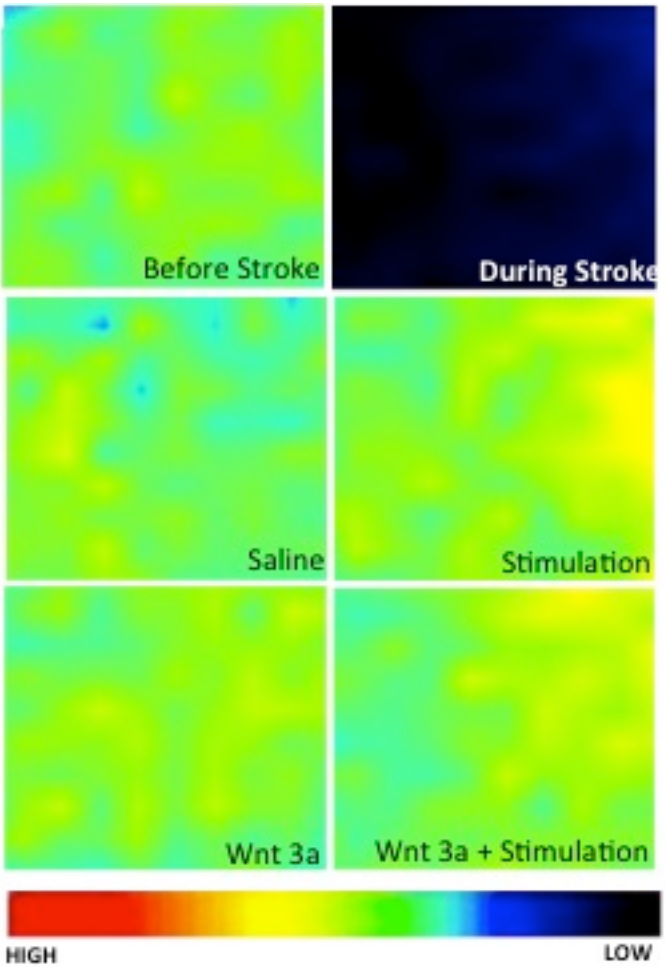


Figure 6. Wnt 3a + Stimulation enhances blood flow to the penumbra 14 days after ischemic stroke. A. Laser doppler measures the perfusion of blood above the Middle cerebral artery of the mouse. B. Laser Doppler images of local cerebral blood flow 14 days after stroke. C. Quantification of blood perfusion as a baseline percentage of cerebral blood flow (n=3-6, *$p<0.05$).

A.

B.



C.

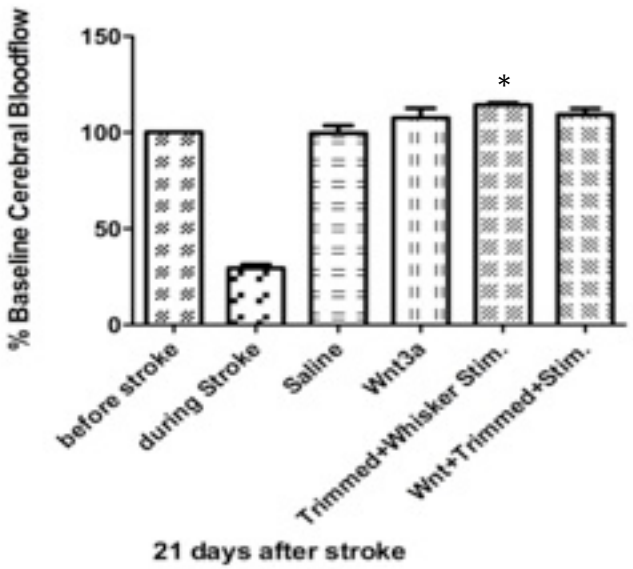
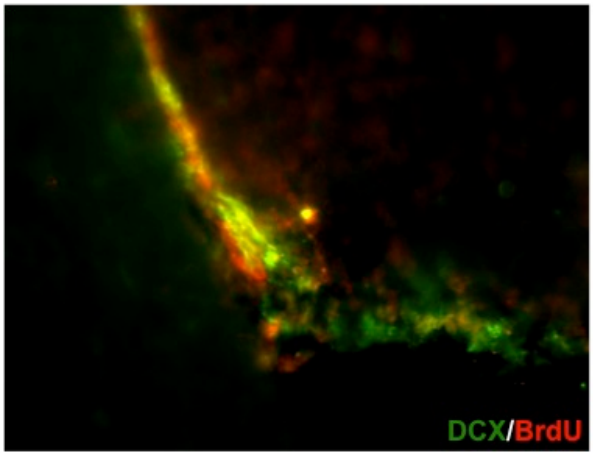


Figure 7. Stimulation and not Wnt 3a + Stimulation enhances cerebral blood flow 21 days after stroke. A. Laser Doppler measures the perfusion of blood above the Middle cerebral artery of each mouse. B. Laser Doppler images of local cerebral blood flow 14 days after stroke. C. Quantification of blood perfusion as a baseline percentage of cerebral blood flow (n=3-7, *$p<0.05$).

A.



B.

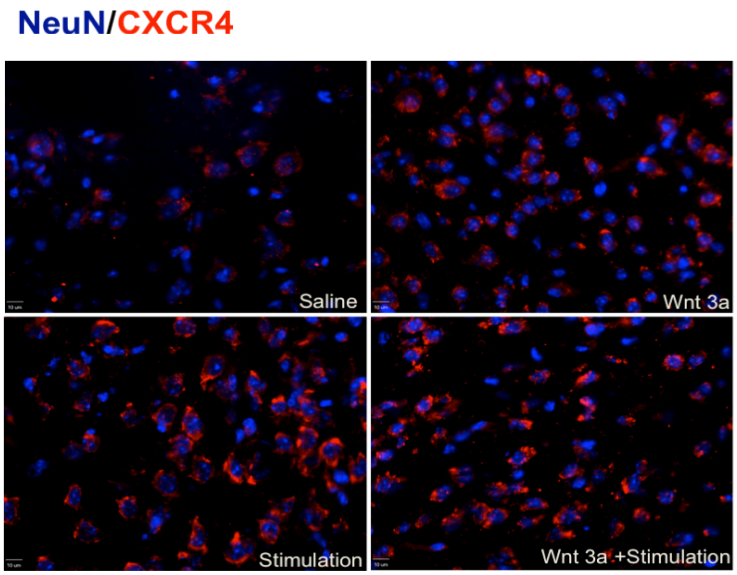


Figure 8A. Wnt 3a + Whisker Stimulation demonstrates cell migration towards the penumbra region 14 days after ischemic stroke. Mouse brains were perfused and cut into 30 µm floating sections. Representative immunohistochemistry staining for migrating cells (DCX) and newly proliferating cells (BrdU) 14 days after stroke. Results are reported from three independent experiments (n=3). Images were taken at 40X magnification. **Figure 8B. Wnt 3a +Whisker Stimulation has increased CXCR4 expression compared to Wnt 3a and Saline groups but not Stimulation only group.** Mouse brains were sectioned at 10 µm and then Immunohistochemistry was performed for CXCR4 and NeuN at 20X to determine qualitatively the expression of CXCR4 (Results are reported from three independent experiments).

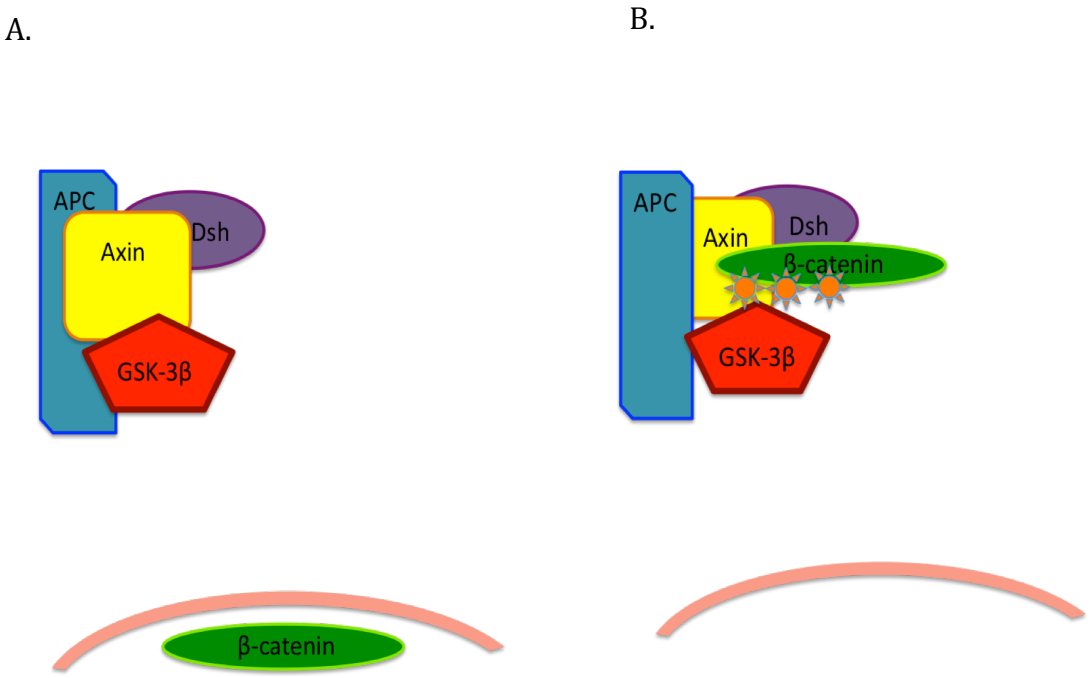


Figure 9. XAV939 Mechanism of Action as described by *Karlberg et al. (2010)*

A. When Wnt 3a is bound to its Frizzled receptor, the constituents of the destruction complex disassemble, allowing β-catenin to translocate to the nucleus and transcribe Wnt target genes. B. XAV939 stabilizes Axin within the Wnt signaling destruction complex and increases phosphorylation and subsequent degradation of Wnt signaling effector protein β-catenin.

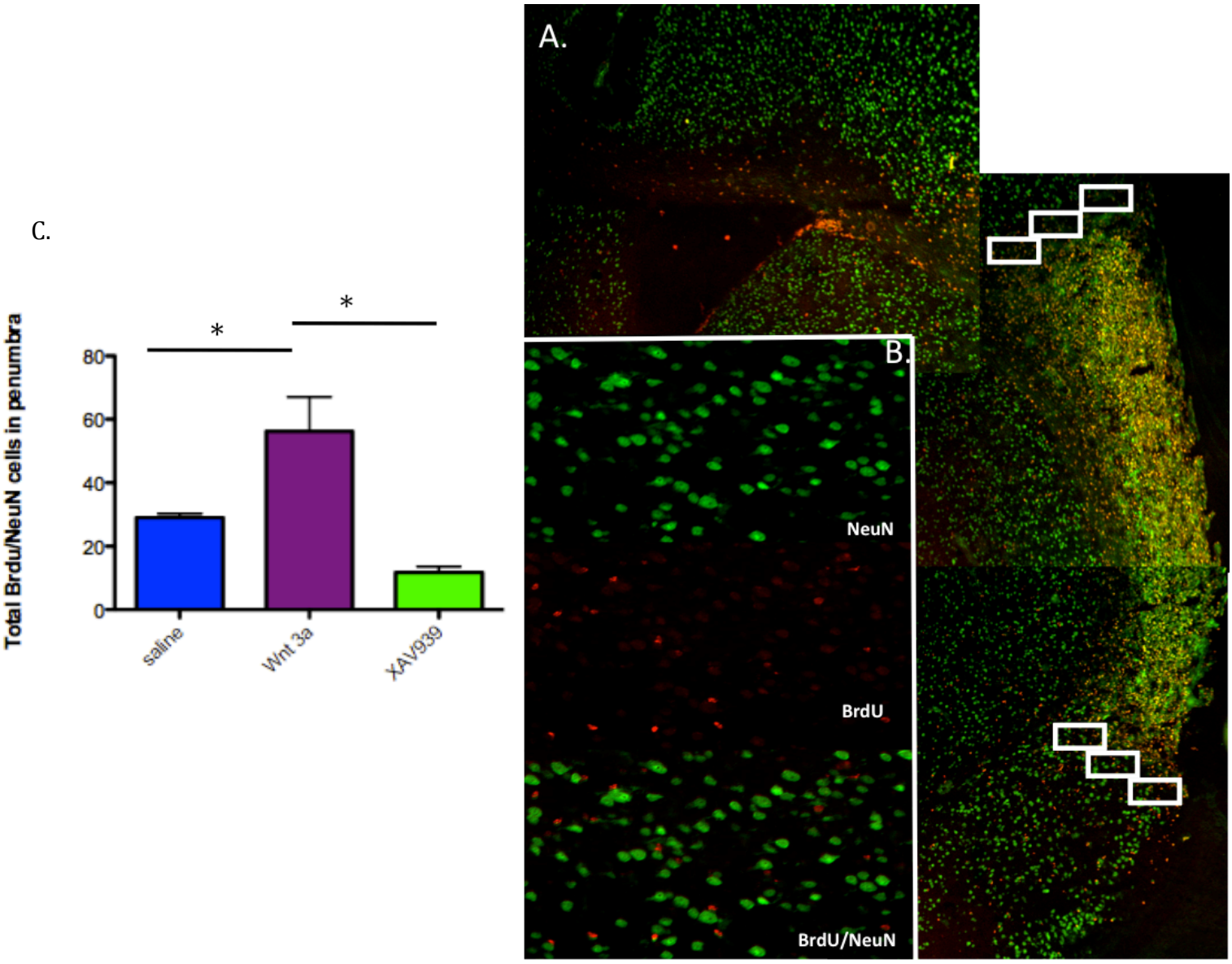


Figure 10A. Ischemic stroke in saline control C57/B6 mouse. Immunohistochemistry of neuronal nuclei (NeuN) in green and newly synthesized cells (BrdU) in red. Ischemic core is located on the right side of each mouse. White boxes demonstrate the images taken in the peri-infarct region per mouse (Total of 6 images per brain section and 8 sections per animal).

Figure 10B. XAV939 Wnt antagonist truncates *de novo* neurogenesis 14 days following ischemic stroke. Representative image of an XAV939 animal. XAV939 attenuates neurogenesis *in vivo* compared to Wnt 3a and saline groups. Each animal was given intranasally at 0.1μM one day after stroke and 0.05μM until sacrifice. C. Quantification of immunohistochemical staining BrdU and NeuN co-labeling (n=4 per group, *$p<0.05$) All values are recorded as SEM.

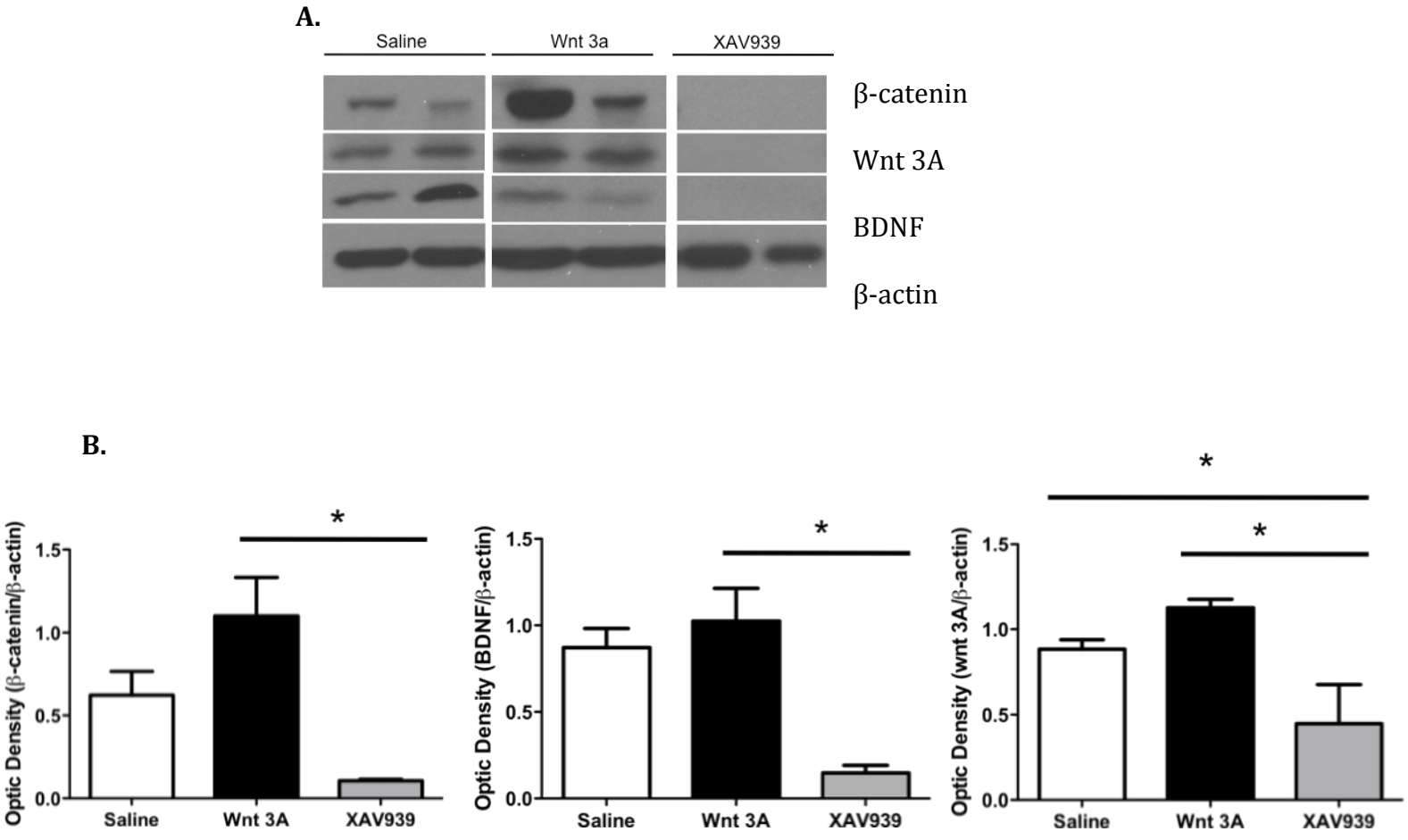


Figure 11. XAV939 blocks Wnt 3a activity *in vivo*

A. XAV939 attenuates expression of β-catenin, Wnt 3a and BDNF compared to Saline and Wnt 3a controls. B. Quantification of protein expression demonstrates XAV939 to block Wnt signaling activity (n= 3-6, *p< 0.05).

Discussion

We have previously demonstrated that Wnt 3a *in vivo* increases *de novo* neurogenesis 14 days after ischemic stroke (Taylor et al., submitted). Here, we demonstrate that whisker stimulation in tandem with exogenous Wnt 3a further enhances endogenous proliferation of neural progenitors *in vivo* 14 days after ischemic stroke. Immunohistochemistry for BrdU and NeuN colabelling confirmed a further increase in neurogenesis in the penumbra region 14 days after stroke. We have demonstrated previously that Wnt 3a mobilizes neural progenitors in the penumbra after focal ischemic stroke. Previous studies describe Wnt signaling to be important in mediating hematopoietic stem cell self-renewal (Nusse, 2008; Staal and Clevers, 2005; Willert et al. 2003) as well as increasing differentiation of human neural progenitor cells *in vitro* (Hübner et al. 2010). In the case of the adult brain, Wnt involvement in the stem cell niche is largely restricted to the subventricular zone (SVZ) of the lateral ventricle and the subgeniculate zone (SGZ) of the hippocampal dentate gyrus (Nusse, 2008; Alvarez-Buylla and Lim, 2004). Previous studies report that Wnt 3a is required for both hippocampal development and neurogenesis (Lie et al. 2005; Lee et al. 2000) These data point towards Wnt 3a as an important signal in the maintenance and proliferation of stem cells, and in the case of the adult brain, within the SVZ and SGZ stem cell niches.

Using a Wnt blocker XAV939 to antagonize Wnt signaling through stabilization of the axin protein (Karlberg et al. 2010; Huang et al. 2009) we were able to demonstrate a marked decrease in neurogenesis 14 days after ischemic stroke. Other studies have also demonstrated that blocking Wnt signaling robustly attenuates hippocampal neurogenesis (Lie et al. 2005) and

decreases *in vitro* stem cell numbers (Piccin and Morshead, 2011), demonstrating Wnt proteins to be integral signaling components in maintaining the SVZ and SGZ stem cell niches respectively (Willert et al., 2003; Nusse, 2008; Piccin and Morshead, 2011). Within these niches, stem cells undergo asymmetric cell division to maintain the stem cell pool and symmetric division to generate stem cell of a restricted lineage (Piccin and Morshead, 2011). In normal physiological conditions, stem cells within the stem cell niches migrate to the olfactory bulb and differentiate into interneurons (Piccin and Morshead, 2011; Lois and Alvarez-Buylla, 1994). In the case of insult, such as ischemic stroke, progenitors are diverted to the site of injury (Kojima T et al., 2010; Li et al., 2008). β-catenin signaling cells have also been demonstrated to localize within the site of injury after traumatic brain injury (White et al., 2010). Piccin and Morshead (2011) show that in normal conditions of asymmetric cellular division, Wnt signaling is not observed. After stroke however, there is a shift from asymmetric to symmetric division in which Wnt signaling is markedly increased. These studies show Wnt signaling proteins as important stem cell factors that promote mobilization of neural stem cells and maintenance of the stem cell pool in response to cerebral insult (Piccine and Morshead, 2011; Nusse, 2008; Lie et al., 2005).

We have also previously demonstrated that whisker stimulation alone increases neurogenesis up to 30 days after focal ischemic stroke and angiogenesis in the penumbra up to 14 days after ischemic stroke (Li et al., 2008; Whitaker et al., 2007). Both studies show that upregulation of the angiogenic factors vascular endothelial factor (VEGF), basic fibroblast growth factor (bFGF), Tie-1 and Ang-2 enhanced vascularization within the penumbra 14 days after stroke (Li et al., 2008; Whitaker et al., 2007). Increased endothelial cell and neuronal cell proliferation by stimulation to the mouse barrel cortex has also shown to increase cerebral blood flow to the

penumbra, bringing with it trophic factors such as VEGF and BDNF (Li et al., 2008; Whitaker et al., 2007). Genome wide analyses show that sensory input to the rat barrel cortex increases activation of MAP kinsases, PDGF signaling molecules, neurotrophic factors and genes involved in blood vessel formation such as Cyr61 and Apold1 (Vallés et al., 2011). Blocking VEGFR2 has been shown to attenuate collateral formation after stroke (Wen-Li et al., 2011).

With increased vascularization after ischemic insult, it has also been demonstrated that there is increased diversion of neural progenitors from the migratory stream originally directed towards the olfactory bulb to the site of the infarct (Yamashita T et al. 2006; Kojima T et al., 2010;Li et al., 2008). Such migration of progenitors from the stem cell niche to the penumbra following ischemic stroke has been shown to occur through movement of progenitor cell bodies along blood vessels that form a structural scaffolding matrix (Yamashita T et al. 2006; Kojima T et al., 2010;Li et al., 2008). Movement out of the SVZ stem cell niche occurs not only along a vascular trellis, the migration of these stem cells is also directed in a chemotactic manner (Kojima et al., 2010; Yu et al., 2012; Li et al., 2008; Cui et al., 2009). The chemotaxic molecule stromal derived stem cell factor 1 (SDF-1) and its chemokine receptor CXCR4 have been shown to be up regulated within the penumbra after stroke and enhance the guided migration of progenitors from the SVZ stem cell niche to the penumbra (Hill et al., 2004; Yu et al., 2012; Cui et al., 2009; Li et al., 2008). We have previously demonstrated that whisker stimulation increases migration of neural progenitors to the penumbra following ischemic stroke through increased expression of SDF-1 (Li et al., 2008; Whitaker et al., 2007).

In this study, we explored the potential therapeutic benefits of combining the neurogenic effects of Wnt 3a as well as the increased neurogenic, angiogenic and migratory effects of whisker stimulation *in vivo*. We have demonstrated that there is increased neurogenesis when Wnt 3a is combined with whisker stimulation compared to Wnt 3a or stimulation alone as illustrated by increased colabeling of NeuN and BrdU positive cells. Increased protein expression of BDNF, β-catenin and Wnt 3a in the penumbra was shown when Wnt 3a was paired with stimulation group and was greater than Wnt 3a alone. However, stimulation alone was sufficient to increase β-catenin and Wnt 3a expression to levels slightly higher than the combination of Wnt 3a and whisker stimulation. Similarly, we have demonstrated a further increase in cerebral blood to the penumbra with Wnt 3a in tandem with whisker stimulation. However, we see an overall, but not significant increase in cerebral blood flow to the penumbra 21 days after ischemic stroke when Wnt 3a is paired with stimulation.

To our knowledge, we are the first to report the relationship of Wnt 3a to angiogenesis in a stroke model. We have demonstrated a significant enhancement in angiogenesis when simulation is paired with Wnt 3a. Surprisingly, there is very little angiogenesis in Wnt 3a only treated animals. Western blot analysis also demonstrates a significantly lower expression of VEGF in the Wnt 3a group but is enhanced when paired with stimulation. Studies on the role of Wnt signaling in endothelial proliferation are vastly restricted to its role is vascular development. Wnt proteins have been widely demonstrated to increase angiogenesis during development (Franco et al., 2009; Wang et al., 2006; Samarzija et al., 2009***).*** However, in addition to activators of canonical Wnt signaling, negative regulators of β-catenin such as Dickkopf and Naked Cuticle proteins are shown to be secreted by endothelial progenitors, regulating vessel formation and

Wnt signaling (Wang et al., 2006; Franco et al., 2009; Dejana et al., 2010). Supporting the low expression of VEGF in our Wnt 3a group, it has been shown that signaling of Wnt 3a in vessel formation and cellular migration occurs independently of VEGF expression (Samarzija et al. 2010).

Alternatively, Wnt 3a has been shown to promote expression of genes that aid in matrix remodeling (Nakase et al., 2006; Chew et al., 2010; Samarija et al., 2009; Goodwin and D'Amore, 2002; Wang et al., 2006; Franco et al., 2009; Hall-Glenn et al., 2012; Chen et al., 2007). In ischemic conditions, increased levels of a gap junction protein, connexin-43 have been shown maintain cell-cell interactions after ischemic insult (Chew et al. 2010; Goodwin and D'Amore, 2002) Wnt 3a has been shown to increase expression of such proteins (Chew et al. 2010; Goodwin and D'Amore, 2002). Similarly, Wnt 3a has been shown to up-regulate connective tissue growth factor (CCN2), a fibrotic protein that mediates wound healing and fibrogenesis through cell adhesion, migration and endothelial basement membrane assembly (Hall-Glenn, 2012; Chen et al., 2007). Fibronectin, another protein important in maintaining the integrity and stability of the extracellular matrix and surrounding vessels has also been shown to increase with Wnt signaling (Wang et al., 2006). Similarly, Wnt 3a is also shown to up regulate Claudin-3, a tight junction protein important in maintaining Blood Brain Barrier (BBB) integrity (Franco et al., 2009). Therefore, it may be that after an ischemic insult, Wnt 3a, rather than mobilizing endothelial progenitors and directly increasing angiogenesis, increases reparative proteins associated with maintenance of cell-cell interactions, matrix remodeling, and repair of the BBB. In addition to an overall increase in angiogenesis when stimulation is paired with Wnt 3a, we see an increase in β-catenin expression, the effector protein in canonical signaling, which

would further enhance the expression of the aforementioned proteins involved in repair. Based on our previous studies, it may be that increased blood flow to the penumbra, increased expression of VEGF paired and an increase in proteins involved in the repair of existing vessels, we see a synergistic coupling of Wnt 3a and whisker stimulation to further enhance angiogenesis *in vivo*.

We then sought to determine if and how neural progenitors were migrating to the peri-infarct region after stroke. DCX/BrdU staining confirmed the presence of migrating progenitors out the SVZ stem cell niche and toward the penumbra 14 days after stroke. Wnt 3a paired with stimulation further increases the migration of neural progenitors out of their stem cell microenvironment. Next, we explored the chemotactic relationship to cell migration and the extent to which neural progenitors are diverted from their journey to differentiation in the olfactory bulb to the site of stroke by looking at expression of the chemokine receptor, CXCR4 (Yu et al., 2012; Alvarez-Buyla et al., 2004; Kojima et al., 2010; Cui et al., 2009; Hill et al., 2004). Our data show that qualitatively, there is a higher expression of CXCR4 in the penumbra when treated with Wnt 3a compared to the saline control. However, we demonstrate that when stimulation is paired with Wnt 3a, there is a qualitative increase in CXCR4 expression in the penumbra than in Wnt 3a only animals and suggests increased chemotactic homing of neural progenitors to the infarct site following stroke.

We propose based on our results in an *in vivo* model of focal ischemic stroke, a potential mechanism for the synergistic effect of combining Wnt 3a and whisker stimulation. Following ischemic stroke, Wnt 3a mobilizes neural progenitors by enhancing symmetric division of stem

cells within the SVZ stem cell niche. Stimulation enhances this effect by enhancing the levels of both Wnt 3a and its downstream effector β-catenin. Remodeling of the extracellular matrix, maintenance of cell-cell interaction and upregulation of gap and tight junction proteins by Wnt 3a restore the vascular network that progenitors can migrate along towards the site of stroke. Together with increased levels of VEGF expression by whisker stimulation, Wnt 3a induced expression of fibrotic proteins work to repair and maintain the vascular milieu, increasing endothelial progenitor proliferation. An increase in blood flow aids in both neurogenesis and angiogenesis by bringing trophic factors to the penumbra. Therefore, based on the data presented, Wnt 3a functions to repair the damaged extracellular network that leads to the peri-infarct region for migrating neural and endothelial progenitors as well as is directly involved with the mobilization of neural progenitors out of the SVZ.

Conclusion

For patients that survive a stroke, only 10% have a full recovery, meaning that the 90% majority will have some level of function impairment (Xie et al., 2007). However, less than one-third of stroke survivors will receive outpatient rehabilitation and approximately one-half of those who need in-patient rehabilitation will not receive it (Xie et al., 2007; O'Brien, 2010). Taken together, our data show that combination therapy using Wnt3a in tandem with whisker stimulation further enhances *de novo* neurogenesis and angiogenesis after stroke. Further behavioral studies are warranted to determine the effects of combinational therapy on long-term functional recovery after stroke and corroborate our angiogenesis and neurogenesis results. Similarly, increasing the number saline animals is needed to determine significance of BDNF expression in the penumbra after ischemic stroke. In future studies, a more in depth analysis of our proposed mechanism for Wnt 3a and whisker stimulation is needed to determine exactly how combination therapies work together. We show in this study the importance of using physical therapy with drug therapy to enhance neurogenesis and angiogenesis that together, may further mitigate long-term functional impairments following ischemic stroke and increase long term functional recovery outcomes.

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