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REACTIVE ASTROCYTES AND THEIR CONVERSION TO FUNCTIONAL NEURONS IN
ISCHEMIC STROKE MOUSE MODEL

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ABSTRACT

Previous research has shown that the expression of the transcriptional factor, NeuroD1, can efficiently reprogram reactive astrocytes into functional neurons in an Alzheimer's disease mouse model (Guo et al., 2014). In this study, a stroke mouse model was used to investigate if NeuroD1 could convert reactive astrocytes of the glial scar into neurons. This investigation is important because it has the potential to produce an effective therapy for strokes, improving the lasting deficits caused by the injury. NeuroD1 was injected into the cortex of ischemic stroke mice, using an adeno-associated virus carrier. We then measured the expression levels of cellular markers such as NeuN, a neuronal marker, GFAP, an astrocyte marker, and CSPG and LCN2, which are reactive glial cell markers. We found that over time, the number of NeuN positive cells increases in mice that have had NeuroD1 injections, compared to the control group. We also found that GFAP, CSPG, and LCN2, which are generally upregulated in reactive cells, decrease in expression levels in NeuroD1 mice compared to the control. This suggests that NeuroD1 is able to create new neurons, while also decrease the signaling of reactive astrocytes in ischemic stroke mice.

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Chapter 1

Introduction

Glial Scar Formation after Brain Injury

The Central Nervous System (CNS) is comprised of two major cell types: neurons and glial cells. While neurons function to transmit electrical and chemical signals throughout the brain and body, glial cells (astrocytes, microglia, oligodendrocytes, etc.) support the neurons, both structurally and physiologically (Fitch and Silver, 2009). One important type of glial cell is astrocytes, which function as the housekeeping cells of the CNS. Astrocytes control ion and water homeostasis, regulate neurotransmitters, help form the blood brain barrier, release neurotrophic factors, and remove metabolite and waste products found in the brain (Takano et al., 2008).

After CNS damage from trauma, ischemia, or disease, the cells in the area of the insult begin to die, and inflammatory and immune cells are recruited to the area. A matrices of fibrin and collagen form over the injury to serve as a scaffold for leukocyte immune cells so that they may monitor for pathogens, remove debris, and send signals to other cells involved in the wound repair process (Bush et al., 1999). Microglia, which are immune cells specific to the brain, and NG2 cells also migrate to the damage. Astrocytes, on the other hand, swell in response to injury, become reactive and proliferate. An astrocyte scar forms around a central lesion core of non-neuronal tissue (Burda and Sofroniew, 2014). The scar forms a border between the injury core and the surrounding healthy neuronal tissue. It also serves as a protective barrier to restrict

inflammatory cells and cytotoxins to the core. Previous research has shown that although the scar formation initially has a protective function, the reactive glial cells that form the scar also secrete inhibitory factors, like CSPG and LCN2, which prevent neuronal growth in the injured area (Guo et al., 2014). Because neurons are generally not regenerative cells, and the scar prevents axon growth into the injured area, much of the tissue damage remains long after the injury. New research has shown that delivery of axon specific growth factors into the glial scar may actually allow axons to grow through the injury. In these studies, there is less axonal growth when the glial scar has been completely removed, showing that astrocytes of the scar might aid in axon formation if the correct growth factors are provided (Anderson et al., 2016). These factors still only allow for axon regrowth, and do not create new neurons. Without implantation of axonal growth factors, however, the glial scar remains an obstacle for new growth.

Ischemic Stroke

One type of brain injury that causes glial scarring is ischemic stroke. During an ischemic stroke there is a sudden obstruction of a blood vessel by an embolism, which results in an immediate decrease or loss in blood circulation to an area of the brain (Lakhan et al., 2009). This loss of blood also results in loss of oxygen and glucose to that area of cerebral tissue. The embolism may be due to a blood clot (thrombus), fat globule, bubble of gas, or a piece of foreign material (Muir et al., 2007). Within seconds of the blockage, a cascade is activated which leads to disintegration of cell membranes and neuronal death at the core of the injury. The loss of blood also leads to excitotoxicity, oxidative damage, inflammation and impairments in the blood-brain barrier (Hossmann et al., 1996). These factors aggravate the injury, causing it to spread

from the initial site and trigger more cell death. The amount of damage, and the size of the glial scar that forms around the injury, is dependent upon the severity and duration of the ischemia (Dirnagl et al., 1999). Strokes are currently the third leading cause of death in industrialized countries and the most frequent cause of permanent disability in adults across the world (Lo et al., 2003). Persistent deficits caused by ischemic stroke are thought to be due to inflammation and the inability of mature axons to regenerate through the glial scar formed at the injury core (Yiu and He, 2006). Creating a technology to reverse the glial scar would provide significant therapeutic benefits for people who have suffered from these injuries by reducing inflammation and creating new neuronal connections through the previously injured tissue. A method to generate new neurons after stroke in the damaged area has the potential to reduce the otherwise permanent disability that strokes can cause. Reversing the glial scar may also reduce the number of deaths due to stroke complications, such as increased pressure on the brain from swelling (Johnston et al., 1998), by decreasing the inflammatory signaling of the reactive astrocytes.

Healthy vs. Reactive Astrocytes

When astrocytes become reactive there are many neurochemical markers that become upregulated compared to non-reactive astrocytes. One such marker is glial fibrillary acid protein, or GFAP, an intermediate filament protein which reveals an astrocytes cytoskeletal structure (Middeldorp and Hol, 2011). It is a commonly used astrocyte marker, and is known to become upregulated when astrocytes become reactive (Ridet et al., 1997). Previous studies have shown that in response to injury, GFAP immunostaining shows that astrocytes have thicker and longer cellular processes and an increased number of processes leaving the soma (Wilhelmsson et al.,

2006). Another pathological protein marker found in reactive glial cells is Lipocalin-2 (LCN2). LCN2 is secreted by astrocytes in the glial scar to cause neuroinflammation in the injured area (Suk et al., 2016) and limit bacterial growth by sequestering iron sidephores (Zamanian et al., 2012). LCN2 is released in response to injury, and is generally not found in healthy tissue. Bi et al. (2013) reported that reactive astrocytes secrete LCN2 to promote neuron apoptosis. Genomic studies have also revealed that LCN2 gene expression is greatly induced in reactive astrocytes after stroke (Zamanian et al. 2012). Lastly, chondroitin sulfate proteoglycans (CSPG) is also a marker of reactive glial cells. CSPG is an inhibitory molecule, mainly secreted by reactive astrocytes, which prevents the growth of new axons through the injured area (Soleman et al., 2013). These factors contribute to the inability of the brain to regenerate and heal an injury back into normal, healthy tissue.

NeuroD1: Converting Astrocytes to Functional Neurons

Although researchers now understand the glial scar pathway and the molecules involved in forming it, previous attempts to regenerate the lost neural tissue have been relatively unsuccessful. Replacement therapies using embryonic stem cells or induced pluripotent stem cells to create new neurons in the injured or diseased area face various obstacles, such as, immunorejection, tumorigenesis, and cell differentiation uncertainty (Lee et al., 2013). In an effort to avoid these complications, our lab has created a new approach, which involves reprogramming endogenous brain cells, such as astrocytes, into functional neurons. This method is referred to as in vivo reprogramming, and involves prompting a tissue's ability for self repair through induced cell proliferation or conversion of a resident cell type into the desired type

(Heinrich et al., 2015). To do so, we created a retrovirus, which encodes a transcription factor, NeuroD1 (ND1), to reprogram cells infected with the virus to become neurons. The transcription factor has been shown to successfully convert astrocytes and NG2 cells into functional neurons in an Alzheimer's disease (AD) mouse model (Guo et al., 2014). In this experiment, a retrovirus is used to carry the transcription factor, because it is known to only target and express its carried gene in currently dividing cells (Miller et al., 1990), such as the reactive astrocytes that form the glial scar. This method will not deplete the injured tissue of astrocytes, but target the actively proliferating, reactive cells. Because neurons found in the surrounding tissue are not dividing, the virus does not infect healthy neurons. ND1 was chosen because of its normal role in embryonic brain development and adult neurogenesis (Cho and Tsai, 2004; Gao et al., 2009), which is to initiate neuronal differentiation and maintain the nervous system over the lifespan. When the retrovirus containing ND1 was injected into the cortex of AD mice, the infected cells began to show NeuN marker over time, a cellular marker known to be found in the soma of neurons. This evidence, as well as electrophysiology results, shows that astrocytes can be successfully converted to functional neurons in an AD model (Guo et al., 2014).

To further apply this research, we decided to test the technology in a focal stroke mouse model. In this experiment, an adeno-associated virus (AAV) was used to carry the ND1 transcription factor and a fluorescent gene, GFP. AAV was used because it is easily manipulated to infect a specific cell type, and it has a higher infection efficiency than the previously used retrovirus (Gao et al., 2002). I hypothesize that, similarly to the AD model, new neurons (NeuN positive) will be able to be generated from astrocytes by injecting AAV-ND1 into the injury core of the stroke model. This means that a higher number of NeuN positive cells will be seen in the ND1 group when compared to the AAV-GFP control group. To assess the process of conversion

over time, we also tested to see if the NeuN positive number of cells differed at different time points. I hypothesize that the number of NeuN positive cells will increase over time, and that the ratio of NeuN and GFP double positive cells in AAV-ND1 will increase over time, while in the AAV-GFP group, the ratio will remain low.

In conjuncture with the previous findings on GFAP, LCN2, and CSPG, I hypothesize that in response to ischemic stroke in a mouse model, these markers will all be upregulated compared to the brain of a healthy mouse. In addition, I hypothesize that in response to injection with ND1, GFAP, LCN2, and CSPG will have decreased expression when compared with the stroke model.

Chapter 2

Materials and Methods

AAV Plasmid Construction and Virus Production

The hGFAP promoter was removed from a pDRIVE-hGFAP plasmid and inserted into pAAV-MCS. Next, the Cre gene was obtained from hGFAP-Cre and inserted into the pAAV-MCS as well to construct a pAAV-hGFP-Cre vector. From this, the control and experimental plasmids were created. The cDNAs coding NeuroD1 and GFP were obtained through PCR using retroviral constructs. The NeuroD1 gene was fused with the P2A-GFP gene to code for fluoresce and subcloned into the plasmid to create the full pAAV-FLEX-NeuroD1-P2A-GFP plasmid. P2A was used to mediate protein cleavage (Szymczak et al., 2004). FLEX refers to the flip-excision switch, which is used to achieve stable Cre-dependent transgene inversion (Atasoy et al., 2008). For the control group GFP was inserted alone to create a PAAV-FLEX-GFP-P2A-GFP plasmid. Recombinant AAV9 was then produced in 293AAV cells. Viruses were prepared by Dr. Pei.

Stroke and Viral Injection

All experiments were conducted using wild-type FVB/NJ mice from the Jackson Laboratory. To induce a focal cortical stroke, an incision was made near the midline and a hole was drilled into the skull at the motor cortex. Endothelin-1 (ET-1) was dissolved in phosphate buffered saline (PBS) to a dilution of 2 ug/ul. A volume of 0.5 of this solution was injected into the cortex, which simulates an ischemic stroke. At different time points following stroke, virus injection followed with a similar procedure using the constructed AAV viral plasmid, rather than ET-1. Surgeries were done by Yuchen Chen and Ningxin Ma.

Immunocytochemistry to Analyze Cell Marker Expression

The animals were first anesthetized with 2.5% Avertin and then perfused with artificial cerebral spinal fluid. The brains were removed, trimmed, and placed in a 4% paraformaldehyde PFA solution overnight at 4 C for fixation. Each brain was then sliced into 40 mm sections by a vibrotome (Leica). The slices chosen for immunostaining were washed 3 times, 5 minutes each, with 1X PBS. The brain slices were then permeabilized in 2% Triton x-100 in PBS for a half an hour, followed by incubation in blocking buffer for one hour. The blocking buffer is comprised of 2.5% normal goat serum, 2.5% normal donkey serum, and 0.1% Triton x-100 in PBS. After blocking, the primary antibodies were added in a blocking buffer solution overnight, and were incubated at 4 C. Each primary antibody used was specific to a different cellular marker of interest and had a specific dilution. Chicken GFAP was used in a dilution of 1:400 ul blocking buffer, chicken GFP was used in a dilution of 1:400 ul blocking buffer, rabbit NeuN was used in a dilution of 1:1000 ul blocking buffer, goat LCN2 was used in a dilution of 1:400 ul blocking buffer and mouse CSPG was used in a dilution of 1:400 ul blocking buffer. Following primary antibody incubation, the slices were washed with 0.05% Triton x-100 in PBS. The samples were then incubated for one hour with secondary antibodies, prepared in a 1:800 dilution with the same blocking buffer. These secondary antibodies stained different fluorescence, such as Alexa Fluor 488, 647 and cy3. The last step was a 0.3% Triton x-100 PBS wash 3 times, at 10 minutes each. The samples were then mounted onto glass slides using antifade mounting solution with DAPI (Invitrogen).

Quantification

For each experiment all samples were incubated in the same dilution of primary and secondary antibodies. Images were taken for each sample to be used for analysis with a confocal

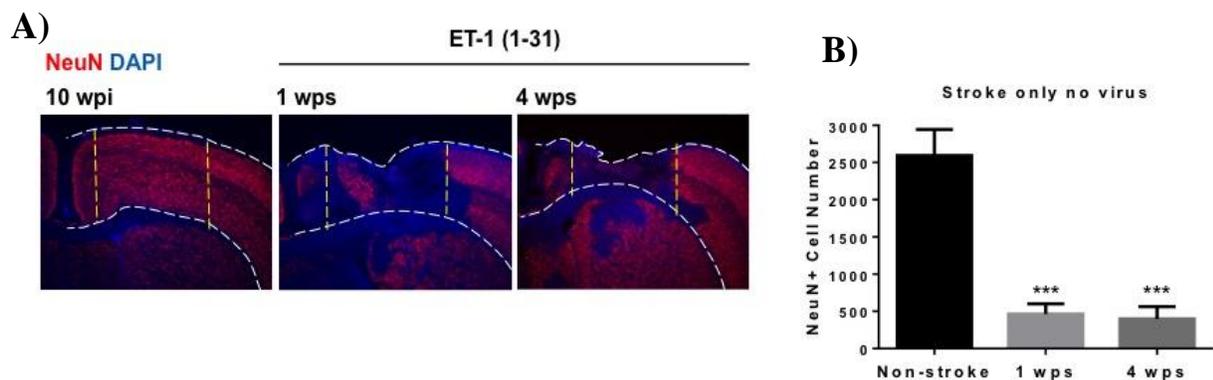
(Zeiss LSM800 or Olympus FV1000) and Keyence microscope. For quantification, images were taken of the cortex, at areas close to the stroke core. For each type of quantification, the display parameters were kept constant across all images to ensure that they had comparable levels of intensity. During quantification, Yuchen Chen coded the images, so that the quantification was blind to the group. For NeuN quantification tile images were taken using the confocal microscope. To begin quantifying the number of NeuN+ cells in the cortex of the control and ND1 mice, I measured from the midline to 2500 μm away. Starting at 500 μm from the midline, 250 μm intervals were measured, to create eight intervals. Each cell expressing NeuN signal was counted in each interval to the lower boundary of the cortex using ImageJ. For LCN2 and CSPG quantification, 40x images were taken using the confocal microscope. For each sample an image of healthy cortex brain tissue was taken to use as a control. Image J was also used to quantify the mean intensity and covered area of LCN2 and CSPG signaling. For normalization purposes, images were taken of the healthy cortical area for both groups. The parameters for intensity and mean covered area were set using the image of the healthy tissue for each sample, and then were kept constant for all other quantification done of that sample. Lastly, 40x images were also used to quantify the ratio of NeuN+/GFP cells and GFAP+/GFP cells. For both ratios, the same mice samples were used for quantification to ensure that the number of GFP positive cells was similar. The number of GFP positive cells was first counted for each sample. I then counted the number of NeuN positive or GFAP positive cells that were colocalized with GFP/RFP positive cells, to create a ratio over the total number of GFP positive cells for that sample.

Chapter 3

Results

NeuroD1 Mediated Increase in NeuN+ Cell Number

Our goal was to investigate whether in vivo cell conversion can achieve physical and functional brain repair after a focal stroke in mice. To do so, we first needed to establish a focal stroke model, which produces consistent and prolonged neuronal loss. To induce a focal stroke, Yuchen Chen and Ningzin Ma injected a vasoconstrictor peptide, endothelin-1 (ET-1), into the motor cortex of mice to produce an ischemic injury (Horie et al., 2008). Ischemia caused by ET-1 induces neuronal loss (Baron et al., 2013), which can be seen by a decrease in NeuN positive cells. As shown in Figure 1, a non-stroke mouse has over 2,500 neurons in the cortex, whereas a mouse one week after stroke (no viral injection) has only about 500 neurons. Figure 1 also shows that four weeks after stroke the neuron number remains low at around 500 neurons, meaning that



the neurons do not regenerate over that time. This data validates ET-1 injection as an effective ischemic stroke model that causes neuronal loss.

Figure 1. Ischemic Stroke Causes a Decrease in NeuN+ Cells at One and Four Weeks Post Stroke. A) Immunostaining of NeuN and DAPI signaling in a non stroke mouse, one week post stroke, induced through ET-1 injection, and four weeks post stroke. B) Neurons were quantified using ImageJ for non stroke, one week post stroke, and four week post stroke mice.

In order to see if ND1 has the potential to improve physical and functional deficits after ischemic stroke by increasing the number of neurons in the injury core, the ND1 transcription factor was injected into the cortex of ischemic stroke mice through the AAV virus. To see if ND1 was able to convert cells of the glial scar into new neurons, I immunostained for the neuronal marker, NeuN, in both control (stroke with AAV-GFP) mice, and ND1 stroke mice. I then quantified the number of neurons in the cortices of mice at 7 days post infection (dpi), 17 dpi, 40 dpi, and 60 dpi to see how the neuronal number changes over time. For each time point, and for both the control and ND1 group, three different mice samples were used to find an average number of neurons at each point. Neurons were quantified within the cortex boundaries in 250 um intervals from 500 um to 2500 um in order to obtain a more precise neuronal value. Figure 2A shows representative images of the neuronal level at each time point for both control and ND1 mice. The cortex boundaries are marked with a white dashed line to show the region of quantification. These images show that for the control mice, the size of the cortex, as well as the neuronal level within the cortex, is considerably smaller at each time point than its ND1 counterpart. Figure 2B shows the average number of neurons of all samples quantified for both groups at each time point. At 7dpi the control and ND1 mice had a similar number of NeuN positive cells, at about 600. At the next time point, the difference between the two groups increased: the ND1 neuronal average was around 1600, while the control group remained low around 550. The value of neurons at 42 dpi and 60 dpi remained low, at about 600. The ND1 group, however, experienced a significant increase in NeuN positive cells at 60 dpi, with an average of over 1,900 neurons. This data shows that ND1 injection into the cortex is able to increase the number of neurons after ischemic stroke in mice models. Although the number of neurons at 60 dpi is slightly lower than the value for non stroke mice, it is a significant

improvement compared to the stroke control model, which shows that ND1 has therapeutic potential.

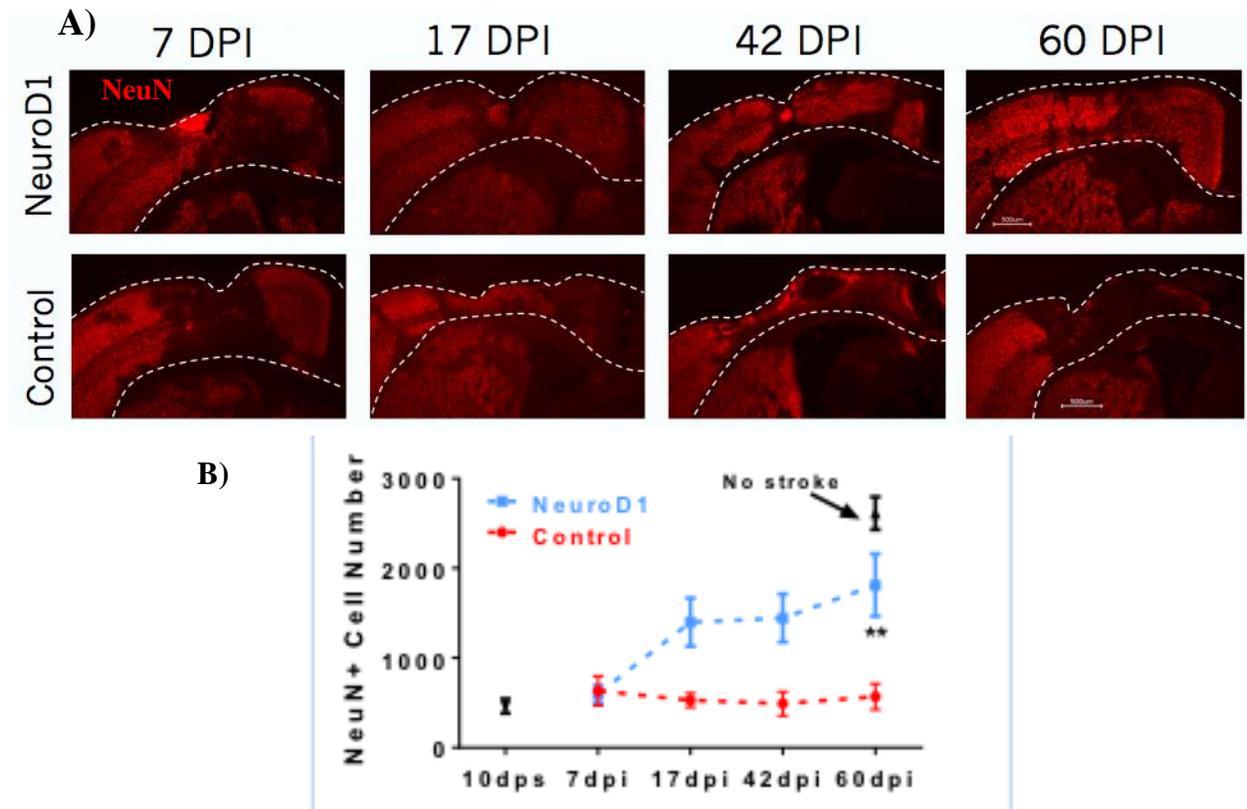


Figure 2. NeuroD1 Mediated Increase in NeuN+ Cells Compared to Control Mice. A) Immunostaining of NeuN in NeuroD1 and control (non stroke) mice at 7 DPI, 17 DPI, 42 DPI, and 60 DPI. B) Compares the number of NeuN+ cells for non stroke, control, and ND1 mice at 7 dpi, 17 dpi, 42 dpi, and 60 dpi.

NeuroD1 mediated decrease in reactive astrocyte signals

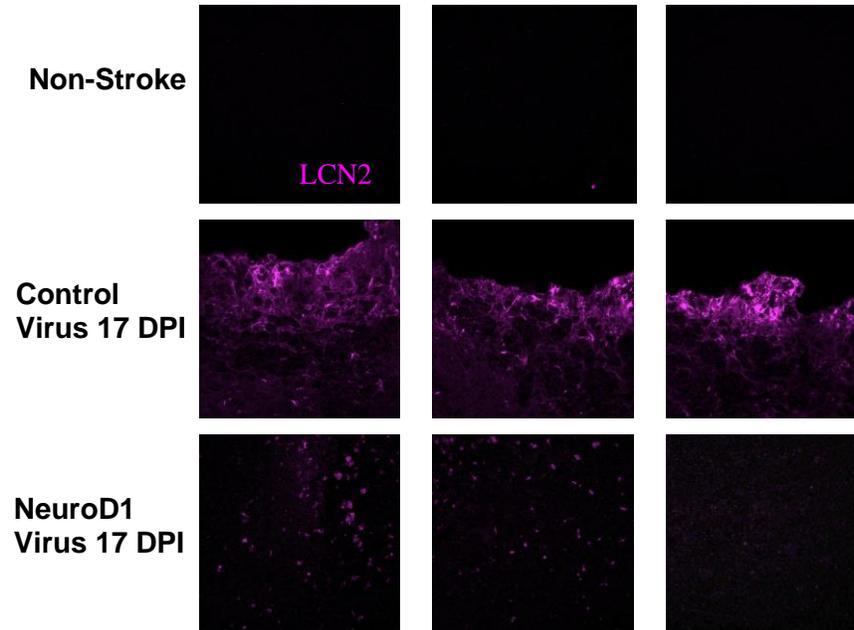
One of the hallmarks of ischemic stroke is the formation of a glial scar, compromised mainly of reactive astrocytes (Huang et al., 2014). The glial scar is a physical and chemical barrier of axonal regrowth through the injury (Silver et al., 2015). Reactive astrocytes also secrete proteins such as CSPG, which inhibits axon regeneration (Soleman et al., 2013) and LCN2, which causes neuroinflammation (Suk et al., 2016). In order to help new axons grow through the injury and establish new neuronal connections, we believe that the astrocytes of the glial scar

must be made less reactive. To see if ND1 injection into ischemic cortices helps to reduce glial scarring in the mouse model, we used immunostaining to quantify the reactive astrocyte markers LCN2 and CSPG. These proteins are generally upregulated in the stroke control model compared to the wild type (non stroke) mice, which provide evidence that glial scarring has occurred. We quantified the intensity and mean covered area of LCN2 and CSPG for wild type, control and ND1 mice. We also co-immunostained LCN2 with GFAP and CSPG with GFAP to see if their expressions are systematically downregulated in ND1 injected mice.

Representative images that were used for LCN2 quantification are shown in Figure 3A. This figure shows LCN2 signaling in non stroke, stroke-control, and ND1 mice at 17 dpi. In non stroke mice, there is almost no LCN2 signaling, while the images show that in the stroke-control, LCN2 is significantly upregulated. This shows that reactive astrocytes in the glial scar are producing strong LCN2 signals. While there is still some LCN2 signaling in ND1 mice, as shown in the third row of Figure 3A, the intensity of the immunostaining is much weaker compared to the stroke-control, showing that the signaling is reduced. An example of LCN2 quantification of non stroke, stroke control, and ND1 images is shown in Figure 3B. For LCN2, three wild type mice were quantified. Together they had a mean intensity of 2.52, and a mean covered area of 0.636%. Four control stroke mice were quantified; they had a mean intensity of 9.68 and a mean covered area of 11.87%. Lastly, four ND1 mice were quantified, which had a mean intensity of 1.68 and a mean covered area of 0.8%. These values can be seen in Figure 3C, which shows that there is a significant difference in both the mean intensity and covered area for the control and ND1 mice. This data shows that ND1 significantly reduces LCN2 signaling. Figure 5A also shows that in stroke-control mice, LCN2 and GFAP expression is high and that there is a strong colocalization of signals, while in the ND1 group, the expression of both signals

decreased. GFAP is a known astrocyte marker, which is upregulated in reactive cells. Strong colocalization of GFAP and LCN2 show that the astrocytes are reactive and are producing LCN2. The decrease in expression of both signals may indicate that astrocytes become less reactive with ND1 injection, and release less LCN2.

Images representing typical CSPG images of non stroke, stroke-control and ND1 mice that were used for signal quantification are shown in Figure 4A. Similarly to LCN2, there is almost no CSPG signaling for non stroke mice, which shows that non reactive astrocytes do not produce the molecule. On the other hand, there is significant CSPG signaling in the images showing stroke-control mice, showing that the reactive astrocytes in the glial scar produce CSPG. Compared to the stroke-control, the ND1 group shows much weaker CSPG signaling. An example of CSPG quantification for the three groups is shown in Figure 4B. Three wild type mice were quantified for the CSPG protein. These had a mean intensity of 0.59 and a mean covered area of 0.78%. Six control stroke mice were quantified, which had a mean intensity of 6.76 and a mean covered area of 20.6%. Lastly, four ND1 mice were quantified for CSPG. They had a mean intensity of 1.93 and a mean covered area of 6.02%. These values can be seen in Figure 4C, which shows that there is a significant difference for both mean intensity and covered area percent for the control and ND1 mice. Figure 5B shows that for the control mice, CSPG and GFAP expression is high and colocalized, and again, there is much less expression of both signals in the ND1 group. This evidence shows that ND1 conversion does significantly reduce CSPG signaling, in addition to LCN2. Together, this data may provide evidence that ND1 decreases the reactivity of astrocytes in the glial scar because LCN2 and CSPG signals are significantly reduced.



Mean Intensity	Non-stroke	Control (151999)	NeuroD1
Healthy area		3.9	2.691
Area 1	1.879	9.576	4.046
Area 2	2.006	9.43	4.516
Area 3	1.409	8.776	1.43
Covered area %	Non-stroke	Control (151999)	NeuroD1
Healthy area		1.039	0.257
Area 1	0.321	10.574	2.494
Area 2	0.281	10.391	3.938
Area 3	0.178	11.235	0.178

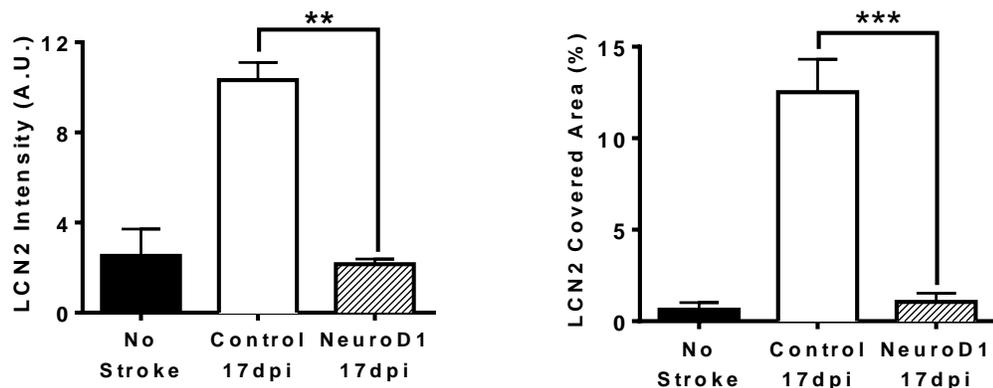
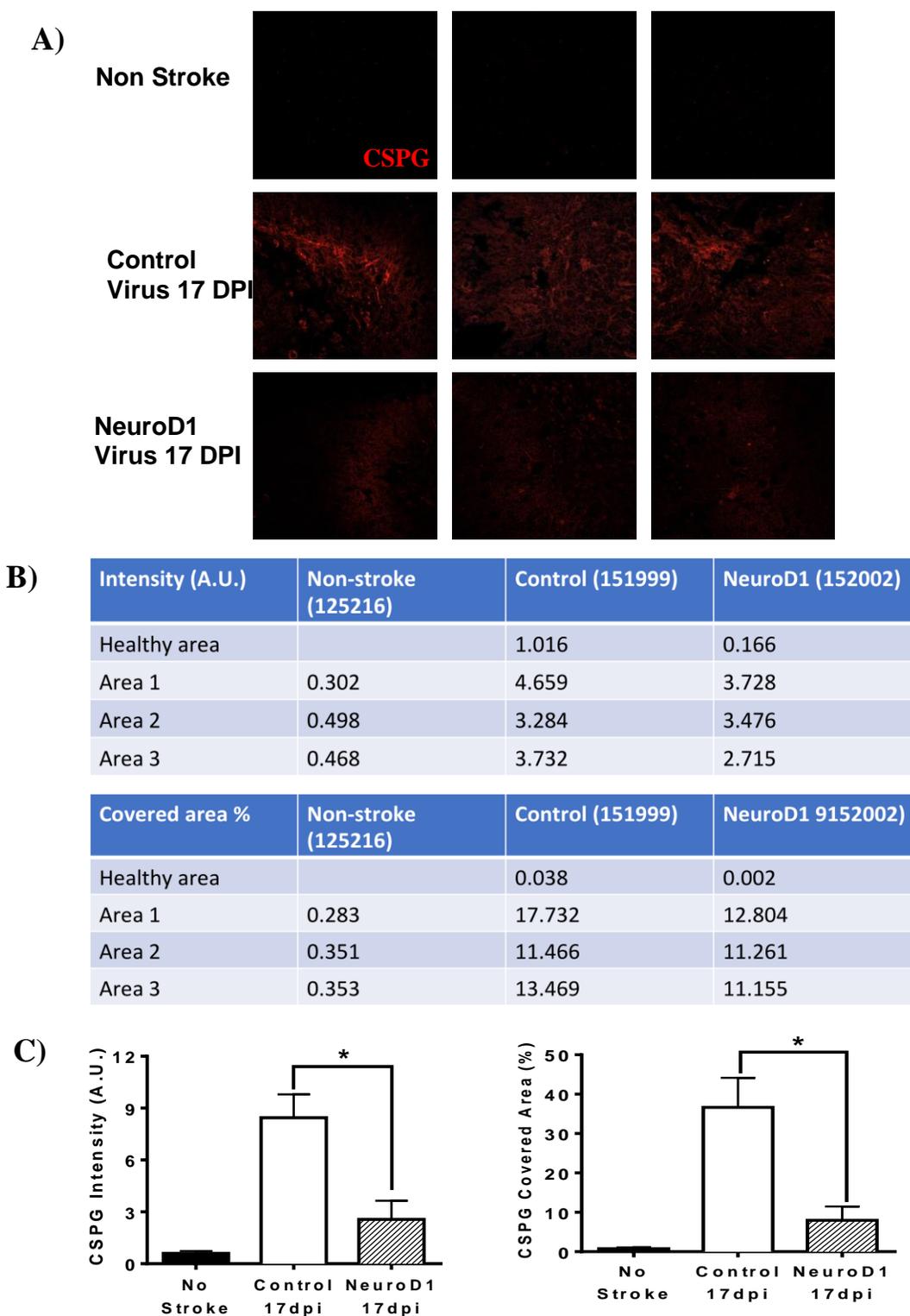


Figure 3. NeuroD1 Mediated Decrease in LCN2 Signaling. A) 40x images of LCN2 immunostaining in wild type, control and ND1 mice. B) Example quantification of the mean intensity and covered area percent for LCN2 signaling done using ImageJ. C) The mean covered area and intensity of LCN2 at 17 dpi for non stroke, control, and ND1 mice.



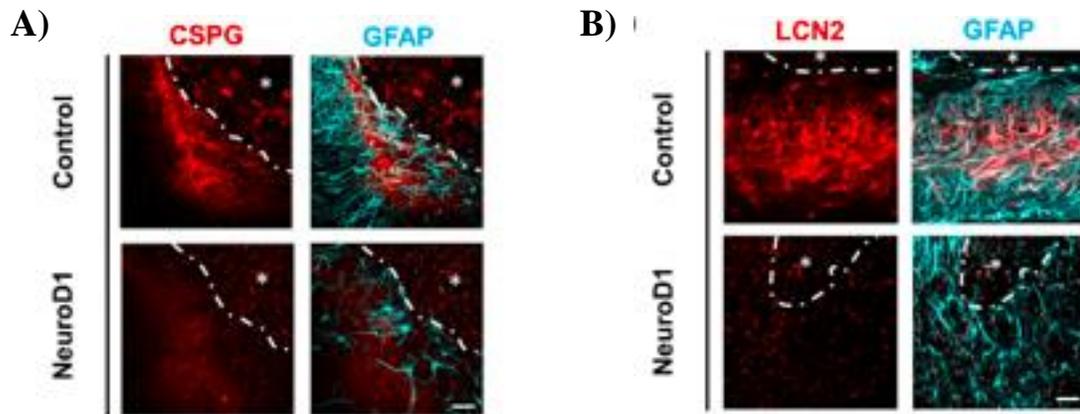


Figure 5. NeuroD1 Mediated Reduction in Reactive Gliosis Through CSPG, GFAP and LCN2 Immunostaining. A) 40x images of CSPG and GFAP colocalization in control and NeuroD1 mice. B) 40x images of LCN2 and GFAP colocalization in control and NeuroD1 mice.

NeuroD1 increase NeuN+ signaling and decreases GFAP signaling

When ND1 is injected into the cortex of ischemic stroke mice models, it is injected as AAV-ND1-P2A-GFP, so that the cells that are infected with ND1 will also express green fluorescent protein. The goal of the virus is to target reactive, proliferating, cells in the glial scar, such as astrocytes. In a typical glial scar, reactive astrocytes show morphological changes, such as an elongation and increase in number of cellular processes. This process can be seen through GFAP immunostaining, which stains the processes of astrocytes. To see if cells infected with the AAV-ND1-GFP virus are astrocytes specifically, we co-immunostained GFAP, and GFP, which should only stain cells expressing the fluorescent protein. To see if cells infected with the AAV-ND1-GFP virus become neurons over time, we also co-immunostained NeuN, the neuron marker, with GFP. To analyze this process over time, we quantified the number of GFAP⁺/GFP⁺ cells over the total number of GFP⁺ cells for both control and ND1 mice to see what percent express GFAP. This will allow us to see if ND1 injections help to reduce the number of GFP cells which are reactive astrocytes. We also quantified the number of NeuN⁺/GFP⁺ cells over the total

number of GFP+ at 4 dpi, 7 dpi, and 17 dpi to see if the ratio increases over time. If the ratio of NeuN+/GFP+ cells increases over time, it will provide evidence that cells that are infected with GFP become neurons.

An example of the 40x images used to quantify GFAP and GFP at 4 dpi, 7 dpi, and 17 dpi for both the control and ND1 mice are shown in Figure 6A. At each time point there is an image which displays the number of GFP+ cells and an image which shows the amount GFP and GFAP colocalization. All GFP+ cells were first counted, followed by cells expressing both signals. These values were used to create a ratio, the average of which is shown in Figure 6B. As shown in the graph, the percent of GFAP+/GFP+ cells decreases for the ND1 group from 60% expression at 4 dpi to about 30% at 17 dpi. On the other hand, the percent of GFAP+/GFP+ cells for the control group was 75% at 4dpi, and decreased very minimally at 17 dpi. ND1 expressing cells gradually lose astrocyte properties and gain neuronal properties. It might also signify that ND1 overexpression decreases GFAP expression, indicating a decrease in reactivity of astrocytes.

Examples of the 40x images used to quantify NeuN and GFP at 4 dpi, 7dpi, and 17 dpi for both the stroke-control and ND1 mice are shown in Figure 7A. At each time point there is an image showing GFP expression only, and another showing GFP and NeuN colocalization. Similarly to the GFAP quantification, a ratio was created of NeuN+/GFP+ cells to the total GFP+ cells. The average value at each time point is shown in Figure 7B. For the control group, the percent of NeuN+/GFP+ cells was 5% at 4 dpi, showing that most neurons do not express the virus. At 17 dpi the percent of NeuN+/GFP+ cells only increased to 15%. For the ND1 group, the percent of NeuN+/GFP+ cells at 4 dpi was 15%, but increased to 78% by 17 dpi. Together, the graphs in Figure 6B and 7B show that as the percent of GFAP+ cells decreases, the percent

of NeuN+ cells increases. In the beginning of the experiment, the majority of GFP+ cells for the ND1 group were shown to be astrocytes because of their colocalization with GFAP, but as time progressed, the majority of GFP+ cells for the ND1 group were shown to be astrocytes because of their localization with NeuN. This data indicates that astrocyte to neuron conversion is occurring.

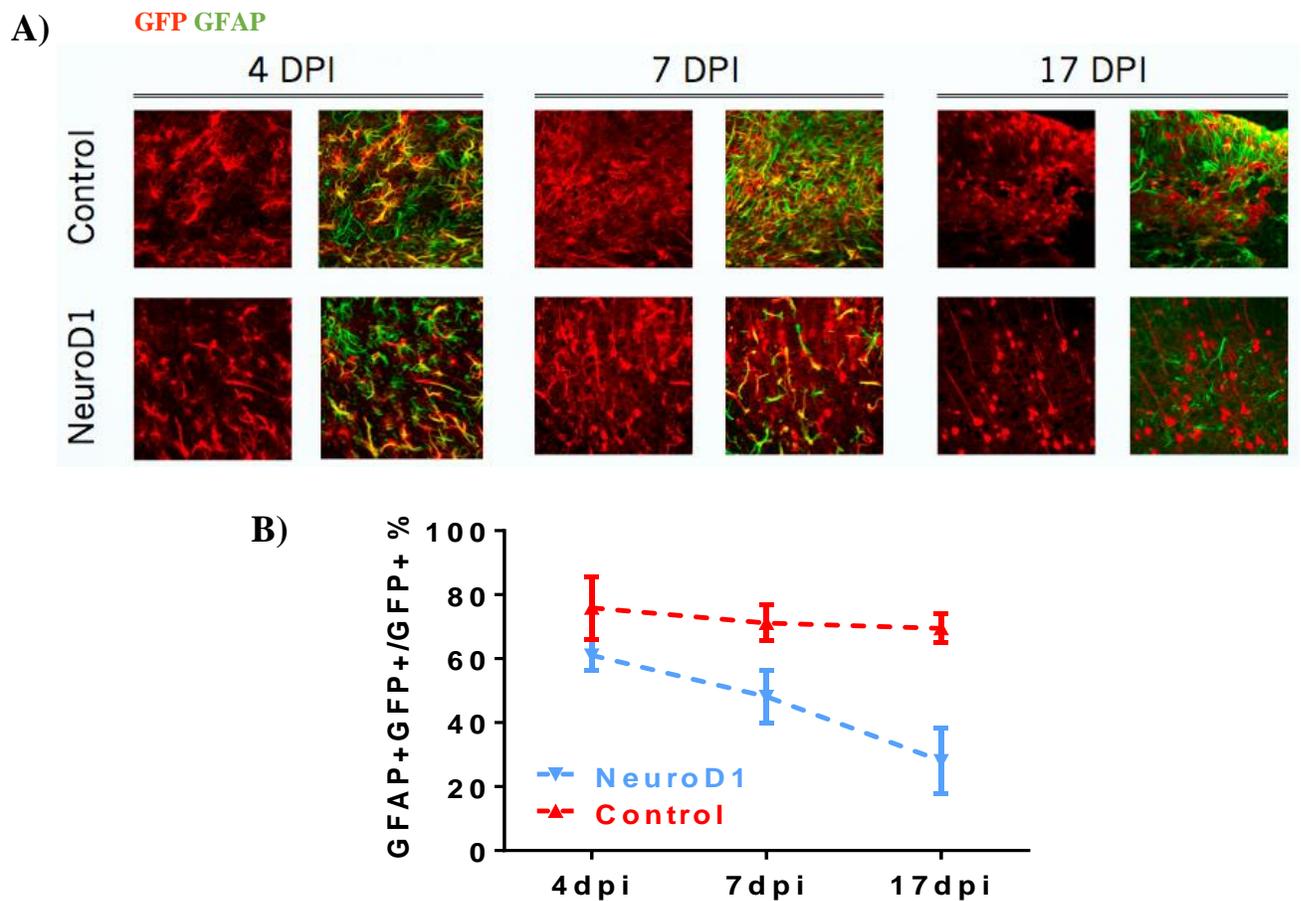


Figure 6. ND1 Mediated Decrease in the Percent of GFAP+/GFP+ Cells. A) 40x Images of immunostaining of GFAP and GFP, showing colocalization, for control and ND1 mice at 4 dpi, 7 dpi, and 17 dpi. B) The percent of GFAP+/GFP+ over the total number of GFP+ cells for ND1 and control mice at 4 dpi, 7 dpi, and 17 dpi.

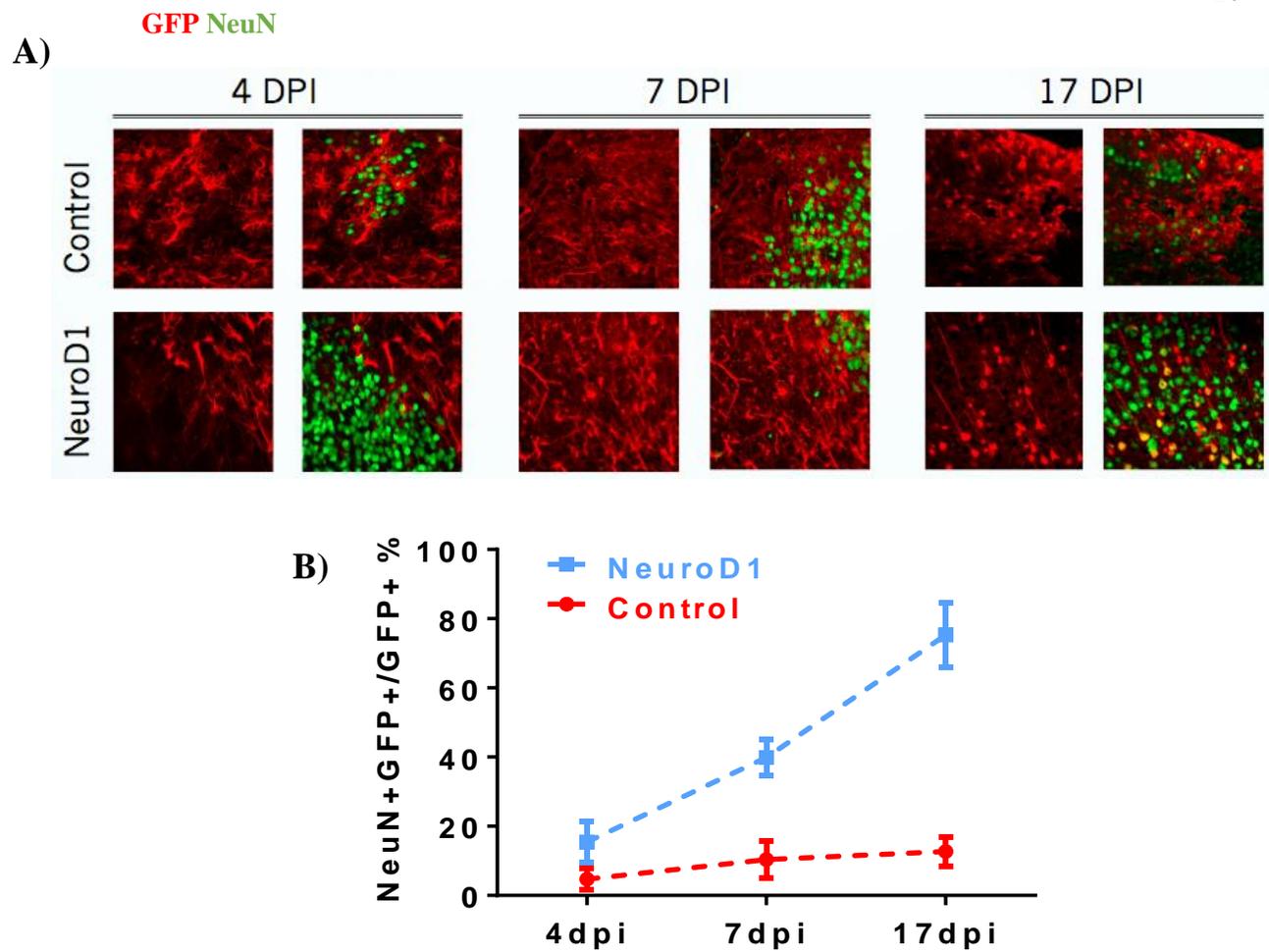


Figure 7. ND1 Mediated Increase in the Percent of NeuN+/GFP+ Cells. A) 40x Images of immunostaining of NeuN and GFP, showing colocalization, for control and ND1 mice at 4 dpi, 7 dpi, and 17 dpi. B) The percent of NeuN+/GFP+ over the total number of GFP+ cells for ND1 and control mice at 4 dpi, 7 dpi, and 17 dpi.

Chapter 4

Discussion

Our results indicate that injection of ND1, carried by an AAV virus, into the cortex of ischemic stroke mouse models can efficiently and effectively reprogram mouse astrocytes of glial scars into neurons *in vivo*. The glial scar cells also become less reactive, as shown by a decrease in LCN2, CSPG, and GFAP signaling, which reduces inflammation in the brain and pave the way for neuronal growth through the injured core. The astrocyte converted neurons are immunopositive for the neuronal marker, NeuN, can survive for at least 2 months in the cortex, and are shown to be functional because they can produce repetitive action potentials and spontaneous synaptic events.

Using NeuroD1 as a Method of Regeneration

Healthy brain function occurs when there is an appropriate balance of neurons to their surrounding support cells, known as glia. After ischemic stroke, like many other brain injuries, neurons die in the injury core, some immediately and some gradually over time do to secondary processes caused by the brain's attempt to seal off the injury (Baron et al., 2014). During this time, the neighboring glial cells become activated and start to proliferate, forming a glial scar. This results in a neuron to glia ratio that is not appropriate for healthy function. The glial scar also causes inflammation, and the cells of the scar secrete factors that inhibit neuronal growth, like CSPG and LCN2, causing potentially permanent damage (Huang et al., 2014). Our goal is to use NeuroD1 mediated *in vivo* cell conversion to restore a healthy cell ratio in the injured area by creating new neurons from astrocytes in the glial scar, and to reduce the reactivity of the cells in the scar. This method has several advantages over other methods, like stem cell therapy, which

attempt to generate new neurons after stroke. By using endogenous cells, rather than external cells that must be implanted, you avoid immunorejection by the host organism. Another advantage is that our method does not pose a risk for creating tumors, while the implantation of stem cells does because they may stimulate uncontrollable cell proliferation (Herberts et al., 2011). Because our method converts preexisting cells, rather than stimulate cell division, this risk is minimized. Our method also has a much higher efficiency rate of creating new neurons than stem cell therapies, and has a large reservoir of cells that can be used. By reducing reactivity of the cells in the glial scar, rather than just implanting new neurons, you are also creating a better environment for the neurons to successfully function.

NeuroD1 Mediated Regeneration of Neurons Through Cell Conversion

One of the obstacles that ischemic stroke patients face during recovery is a loss of neurons at the injury core. Neurons do not regenerate naturally in the injured area, leading to long term functional deficits (Garcia et al., 2016). By creating a method to increase the number of functional neurons in the area, there is hope to see functional improvement after a stroke. Through ND1 mediated astrocyte conversion we were able to increase the number of neurons in the cortex of ischemic stroke mice. Our evidence shows that cells expressing the AAV-ND1-GFP virus decrease in GFAP signaling over time, and increase in NeuN, signaling. This signifies that originally, the majority of cells expressing GFP (the virus) are astrocytes, and that over time, they become neurons. We also compared the number NeuN positive cells in the control (stroke) group and the ND1 group at four different time points (4, 17, 42, and 60 dpi) and found that the number of NeuN+ cells increases over time for the ND1 group, and remains relatively constant at around 500 neurons for the control group. By 60 dpi, the number of NeuN+ cells is almost to that

or a no stroke model. This shows that ND1 is able to significantly increase the number of neurons in the cortex of stroke mice. Whole cell patch clamp electrophysiology was also done to show that the converted neurons are functional. At 60 dpi, the neurons were able to evoke repetitive action potentials and have spontaneous synaptic events. This suggests that the converted neurons are able to integrate into the organism's brain circuit and establish new connections with the surrounding neurons.

Impact of NeuroD1 on the Glial Scar

Injecting AAV-ND1-GFP into the cortex of ischemic stroke mice helps to reduce the reactivity of astrocytes in the glial scar. Reactive astrocytes undergo morphological changes, such as hypertrophy and an increase in cellular process. This causes an increase in the protein GFAP, which can be found in the processes of astrocytes. ND1 improves astrocyte morphology so that cells look much less reactive. Evidence of this is shown through GFAP immunostaining, which levels are significantly decreased in ND1 injected mice. Previous research has shown that reactive astrocytes also show a significant upregulation of CSPG and LCN2 in the stroke core. CSPG is known to inhibit axonal regeneration through the injury core (Yiu and He, 2006), while LCN2 is known to cause neuroinflammation (Shashidharamurthy et al., 2014). Our findings were consistent with this; immunostaining of both CSPG and LCN2 showed that they had a significantly greater intensity and mean covered area than non stroke mice. In ND1 treated mice, levels of CSPG and LCN2 were significantly reduced compared to the stroke only mice. The reduction of CSPG and LCN2 in the ND1 group suggests that ND1 mediated glial conversion helps to alleviate glial inhibitory signaling, showing further evidence for reduced reactivity of

astrocytes. The finding that glial scars can be made less reactive after ischemic stroke represents great potential for tissue therapy and functional restoration.

Therapeutic potential and Future of NeuroD1

We have shown that NeuroD1 mediated in vivo conversion of astrocytes into neurons increases the number of neurons in the ischemic stroke injury core, creating a healthier neuron to glial cell ratio. Dr. Zheng Wu also performed electrophysiology to test if the ND1-mediated astrocyte converted neurons are functional in the ischemic stroke injured area. He found that they are able to produce repetitive action potentials, as well as spontaneous excitatory and inhibitory post synaptic currents. This evidence shows that the converted neurons are able to establish synaptic connections with surrounding neurons in the cortex. Lastly, we have shown that ND1 is effective in reducing the reactivity of astrocytes in the glial scar. Together, this evidence shows that NeuroD1 mediated therapy has the potential to be used to treat ischemic strokes in other organisms.

To further show that NeuroD1 creates functional neurons, we are doing behavioral testing in control (stroke) and ND1 mice. Ischemic stroke mice show many behavioral deficits during simple tests. Mice that have been injected with the ND1 virus, however, perform better in behavioral tests than the control group. This functional rescue of behavioral deficits further supports the use of ND1 as a therapeutic treatment for strokes and other brain injuries.

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