2019

Effect of Manipulation of Notch Signaling Pathway on Neural Stem Cell Proliferation in the Hippocampus Following Traumatic Brain Injury

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Hippocampus Following Traumatic Brain Injury

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

at Virginia Commonwealth University

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April 22, 2019

Acknowledgement

It has been a great honor to participate in this valuable research at Virginia Commonwealth University. I like to give special thanks to my advisor Dr. Dong Sun, who took me into her lab, training and mentoring me to completion of my thesis. I also like to thank my thesis committee members Dr. Raymond J. Colello, who is also a graduate program director and facilitated my transition to VCU's graduate program, and Dr. Kirsty J. Dixon for enduring through the completion of my thesis.

I also thank my lab members Andrew Rolfe, who generated all animals for the study, Jyoti Lodtha & Mili Patel for their assistance in the lab.

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Abbreviations

ABC	Avidin-Biotin Complex
AD	Alzheimer's disease
bFGF	Basic Fibroblast Growth Factor
BrdU	5-Bromo-2-deoxyuridine
CCI	Controlled Cortical Impact Injury
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
CT	Contralateral
DAB	3,3'-Diaminobenzadine-tetra-hydrochloride
DCX	Doublecortin
DG	Dentate Gyrus
ED	Emergency Department
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
FPI	Fluid Percussion Injury
GCL	Granular Cell Layer
GZ	Granular Zone
IGF	Insulin-like Growth Factor
I.P.	Intraperitoneal
IP	Ipsilateral
IPC	Intermediate progenitor cell

LFPI   Lateral Fluid Percussion Injury

NPC   Neural Progenitor Cells

NSC   Neural Stem Cells

PBS   Phosphate-Buffered Saline

RGL   Radial Glia-like Cell

RMS   Rostral Migratory Stream

SADHD   Secondary Attention-deficit/Hyperactivity Disorder

SGZ   Subgranular Zone

SSC   Saline Sodium Citrate

SVZ   Subventricular Zone

TAC   Transit Amplifying Cell

TBI   Traumatic Brain Injury

VEGF   Vascular Endothelial Growth Factor

WDIAI   Weight Drop Impact Acceleration Injury

Abstract

Effect of Manipulation of Notch Signaling Pathway on Neural Stem Cell Proliferation in the Hippocampus Following Traumatic Brain Injury

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The Notch signaling pathway is known as a core signaling system in maintaining neural stem cells (NSCs) in embryonic development and adulthood including cell proliferation, maturation, and cell fate decision. Proliferation of NSCs persists throughout lifespan in neurogenic niches and is often upregulated following neurological insults including traumatic brain injury (TBI). Therefore, NSCs are viewed as the brain's endogenous source for repair and regeneration. We speculate Notch signaling pathway is also involved in injury-induced cell proliferation in the neurogenic niche following TBI.

  TBI, which is a leading cause of death and disability, has been a huge burden to our society. Many efforts have been made in attempt to treat and manage TBI. In this study, we examined the involvement of Notch signaling pathway in injury induced NSC proliferation in the

neurogenic niche, by administering exogenous Notch ligands including, Notch agonist or antagonist.

Adult rats were intraventricularly infused with Notch1 receptor agonists (anti-Notch1 antibody at the dose of 0.5, 2 or 4µg/ml), Notch1 receptor antagonist (recombinant Jagged1 fusion protein at the dose of 25, 50 or 100µg/ml) or vehicle for 7 days following TBI. 5-bromo-2-deoxyuridine (BrdU) was administered single daily via intraperitoneal injection to label proliferating cells for 7 days post injury. The animals were sacrificed on the 7th day at 2 hours after the last BrdU injection. Sequential vibratome sliced coronal brain sections were processed for proliferation marker BrdU, Ki67 or immature neuronal marker DCX staining. BrdU, Ki67 or DCX-labeled cells in the dentate gyrus of the hippocampus were quantified using unbiased stereological method. We found TBI in the form of moderate lateral fluid percussion injury (LFPI) induced cell proliferation was further augmented by 7-day infusion of Notch agonist (Notch1-2µg/ml) as shown by BrdU and Ki67 labeling. Further, 7-day infusion of Notch antagonist (Jagged1-50µg/ml) post-injury greatly reduced the number of BrdU+ cells. However, ambiguous dose related responses were also observed where 7-day infusion of higher dose of Notch agonist (Notch1-4µg/ml) resulted in reduced cell proliferation. No major changes in the numbers of newly generated neurons were observed across the animals, except a slight reduction in Notch agonist (Notch1-2µg/ml) and Notch antagonist (Jagged1-50µg/ml) infused animals as shown by DCX labeling.

Infusion of Notch agonist or antagonist affects NSC proliferation following TBI suggesting the involvement of Notch signaling pathway in regulating post-TBI NSC proliferation in the neurogenic niche. For the unexpected opposite results of higher dosing of Notch 1

agonist, the presence of other Notch receptors regulating NSC in the neurogenic niche should

be considered. Future studies involving selective manipulation of these Notch receptors and

their downstream effectors would clear some results.

Chapter 1 - Introduction and Background

Epidemiology

Traumatic brain injury (TBI) affects millions of people globally each year. According to the Centers for Disease Control and Prevention (CDC), in United States alone, 2.8 million TBI related deaths, hospitalization, and emergency department visits occurred in 2013. Approximately 2.5 million emergency department (ED) visits were TBI-related, approximately 282,000 TBI-related hospitalizations, and approximately 56,000 resulted in deaths (Taylor et al., 2017). These deaths do not include number of individuals who did not seek medical care after sustaining TBI, which might account for another one fourth of all persons who sustain a TBI (Coronado et al., 2011).

The most common cause of TBI-related to ED visits and hospitalization includes fall, being struck by an object, and motor vehicle crashes, the latter being the leading cause of TBI-related deaths. The highest rate of TBI related ED visits were occurred in young children age of 0 to 4 and in adult age of 75 or higher primarily caused by fall, accounting for 17.9% increase in number of TBI-related ED visits from 2007 to 2013 (Taylor et al., 2017). Although, the rate and number of TBI-related ED visits, hospitalization, and deaths involving motor vehicle crashes have decreased due to efforts for auto accident prevention and safety regulations (Taylor et al., 2017). The frequency of brain injury yet remains higher than of any other diseases, such as breast cancer, AIDS, Parkinson's disease and multiple sclerosis (Prins et al., 2013).

Many TBI survivors develop some form of disability lasting months to life. There are currently about 5.3 million people living in United States with TBI related disability (Chauhan,

2014), costing approximately $77 billion in average each year (Faul et al.,2010). Currently TBI is

a best-known epigenetic risk factor for later development of neurodegenerative diseases and

dementia, people sustaining TBI are approximately 4 times more likely to develop dementia at a

later stage than people without TBI (Chauhan, 2014). Nevertheless, aftermath of TBI is more of

a disease process which is associated with immediate and long-term sensorimotor, physical and

cognitive impairment.

Annually, much more efforts are provided for therapy and rehabilitation of individuals

suffering long-term TBI related deficits. However, to this date no effective cure for TBI has been

found mainly due to the complexity nature of TBI. Recent studies have discovered possibility of

natural recovery response following TBI (Gao et al., 2009), suggesting occurrence of innate

response mechanism for repair and regeneration within the brain (Sun, 2014).

Mechanism and Biomechanics of TBI

TBI is caused by a physical force applied on the head that leaves an impact, penetration,

or rapid movement of the brain within the skull which results in altered mental state (Prins et

al., 2013). The damage to the brain can vary from structural to biochemical levels potentially

leading to cognitive and behavioral dysfunction depending on how the injury was induced.

Most traumatic events that cause mechanical insults to the brain can be classified as an impact

and non-impact injury. Impact injury occurs when immediate force contacts the skull causing

deformation and brain tissue damage (Bauer et al., 2015). Impulse (non-impact) injury occurs

when a force such as blast waves or rapid acceleration causes sudden and rapid head

movements causing brain tissue damage without causing deformation to skull (Bauer et al., 2015, Prins et., al 2013).

TBI can be classified into primary event and secondary event. Primary event is due to mechanical tissue damage involving immediate neuronal damage from axonal shearing, often completed within seconds of impact (Sun, 2014, Yarham and Absalom, 2008). TBI can be further broken down into focal and diffuse injury depending on the location of the tissue damage. Focal injury occurs in specific area of the brain with a mechanical force vector delivered to a reduced intracranial region, often results in contusion, subdural, and epidural hematoma. Diffuse injury is more scattered and is not linked to specific focus of destructive tissue damage. Rather it shows widely distributed structural damages scattered along neuronal or vascular components, involving diffuse neuronal damage, neuronal perturbation or disconnection (McGinn et al., 2016).

However, the secondary event can happen from minutes to days from primary impact and consists of a complex cascade of ischemia, excitotoxicity, and metabolic failure which results in further cell death and dysfunction (Galgano et al., 2017, McIntoshi 1996). Because of the complexity of effect, secondary injury is often considered more devastating (Reilly, 1997).

Pathology of TBI

TBI of varying levels of severity has been associated with neural and cognitive changes that usually persist chronically years after the initial injury, which are often associated with damage to white matter integrity (Kraus et al., 2007, Hayes et al., 2015, Miller et al., 2016). Clinical studies suggest that hippocampus, which plays a key role in memory formation and

cognitive function, is particularly vulnerable to secondary insults (Sun, 2014). Electrophysiologic

studies show TBI can result in changes in hippocampal circuitry which affect excitatory and

inhibitory synaptic transmission, causing hippocampal dysfunction (Reeves et al., 1997). These

excitatory and inhibitory changes in the brain is believed to be associated with altered

glutamatergic and GABAergic function following posttraumatic episode (Cohen et al., 2007).

Hippocampal injury is often associated with cognitive impairments such as memory loss,

decreased rate of information processing, and cognitive rigidity, and often manifested into

behavioral deficits such as lack of impulse control, increased agitation, and mood lability

(Hilton, 1994). These deficits are the hallmark of brain trauma, which is commonly observed

among TBI patients regardless of their age (Panwar et al., 2018). The cognitive and behavioral

impairment can have a devastating effect on social behavior and integration into a normal

lifestyle.

The behavioral changes can cause development of various psychiatric disorders

(Castriotta et al., 2007). Studies show that mood disorders are often developed from cognitive

deficit and impaired emotional processing cause by TBI. Depressive disorders are most common

mood disorders, including mania, hypomania, and mixed mood states. Mood disorders are

frequent psychiatric complications of TBI that overlap with prominent anxiety, substance

misuse, impulsivity and aggression. (Jorge and Arciniegas, 2014). Individuals sustaining TBI are

susceptible to developing substance use disorder due to the changes in molecular mechanisms

in mesolimbic system occurred by TBI (Merkel et al., 2017). Also, high prevalence of sleep

disorders occurs among subjects with TBI, including obstructive sleep apnea, posttraumatic

hypersomnia, narcolepsy, and periodic limb movements in sleep due to disrupted neurologic

signals in the brain (Castriotta, 2007). Other novel psychiatric disorders can trigger onset of TBI, such as personality changes, secondary attention-deficit/hyperactivity disorder (SADHD) as well as other disruptive disorders, and internalizing disorders that are common to children complicating child function and affecting family members post-TBI (Max, 2014)

Surviving TBI patients are also susceptible to long-term neurological disorders such as dementia and Alzheimer's disease. People sustaining TBI are approximately 4 times more likely to develop dementia at a later stage than people without TBI (Chauhan, 2014). A mild TBI can put individual with greater risk for Alzheimer's disease due to a greater neurodegeneration and reduced memory performance caused by injury, especially the individuals who are predisposed with genetic risk for Alzheimer's disease (Hayes et al., 2017). Despite the detrimental effects that follows TBI including structural deformation, neuronal damage, synaptic disruption, and changes in molecular mechanisms, heightened levels of cell proliferation and neurogenesis have been observed, this is believed to be in response to brain trauma or insults, which suggests that the brain may possess the inherent potential to restore populations of damaged or destroyed neurons (Sun, 2014).

Adult Neurogenesis

Mature mammalian brain has two discrete neurogenic niches for endogenous neurogenesis, the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle. Neurogenesis in the neurogenic niches are believed to persist throughout life (Braun and Jessberger, 2014, Gage, 2002, Lois and Alvarez-

Buylla A,1993). Adult neurogenesis is known as a sequential process, which require cell

proliferation and cell fate decision of neural stem cells (NSCs) into transit amplifying cells

(TACs), surviving cells eventually leading to rise of neuroblasts that could potentially migrate

and integrate into functional network (Gage, 2000, Kreigstein, and Alvarez-Buylla, 2009).

During proliferation, NSCs can divide either symmetrically or asymmetrically. Symmetric

NSC division can be proliferative or differentiative, one NSC can divide into two identical NSCs

or two neuroprogenitor cells (NPCs) which can differentiate into either glial or neuronal cell

lineage. Asymmetric division involves one NSC dividing into one NSC and one NPC (Kreigstein,

and Alvarez-Buylla, 2009). The NPCs that have taken neuronal cell lineage differentiate into new

neurons. Neuroblasts arise from the ventral SVZ and migrate along the Rostral migratory

stream (RMS) to the olfactory bulb and differentiate into functional olfactory interneurons

(Wang et al., 2011, Lim and Alvarez-Buylla, 2016). NPCs from the DG migrate laterally into the

granule cell layer and exhibit properties of fully integrated mature dentate granule neurons

(Kempermanm and Gage, 2000).

In the DG, adult born granule neurons pass through series of developmental stages

before becoming fully functional neurons. Type 1 cells also known as radial glia-like cells (RGLs),

can generate proliferating intermediate progenitor cells (IPCs) also known as type 2 cells, these

type 2 cells can give rise to neuroblasts which subsequently differentiate into immature

neurons, then mature granule neurons (Figure 1.1. a. Aimone et al., 2014). During the

maturation process, dendrites of the new neurons extend into molecular zone while the axon

extends into CA3 region led by both intrinsic and extrinsic factors (Aimone et al., 2014), at

approximately 10-11 days following generation (Zhao et al., 2006). Dendritic arborization and

axonal projections take place between 2-3 weeks after birth and begin to approach

physiologically and anatomically mature neuron by 4-8 weeks after birth (Aimone et al., 2014).

About 10 weeks post proliferation, the surviving new cells become dentate granule

neurons (Sun et al., 2007). Mature adult born granule neurons become functionally

incorporated into circuitry of the hippocampus, receiving synaptic inputs, fire action potentials,

and establish synapses to hilus and CA3 cells. (Gonçalves et al., 2016). Studies show that

anatomical integration of adult born DG into neuronal circuitry follows a precise sequence of

connectivity (silent -> slow GABA -> glutamate -> fast GABA) that resembles formation of

developing hippocampus (Espósito et al., 2005). Which suggests that adult hippocampus

maintains the same development rule for neuronal integration through adulthood, ensuring the

functional and structural integrity of the newly formed adult hippocampus circuitry.

This is consistent with recent findings in adult neurogenesis in DG, which could

contribute to normal hippocampal functions such as memory formation, pattern integration,

temporal separation, or encoding familiar environments (Aimone et al., 2014). The same can be

true for injury-induced neurogenesis in DG of adult hippocampus. A study in a rat TBI model

reported the observation that restoration of cognitive recovery of Morris Water Maze (MWM)

performance was within the similar timeframe (56-60 days post injury) as the anatomical

integration of newborn DG neurons into hippocampus was observed (Sun et al., 2007). Further

study found that the inhibition of injury induced cell proliferation in DG of rat hippocampus

completely abolished the innate cognitive recovery of MWM performance (Sun et al., 2015).

These observations suggest the involvement of adult born DG granule neurons in both

physiological or pathological conditions in brain's innate response to maintain normal cognitive

function or promote repair/regeneration.
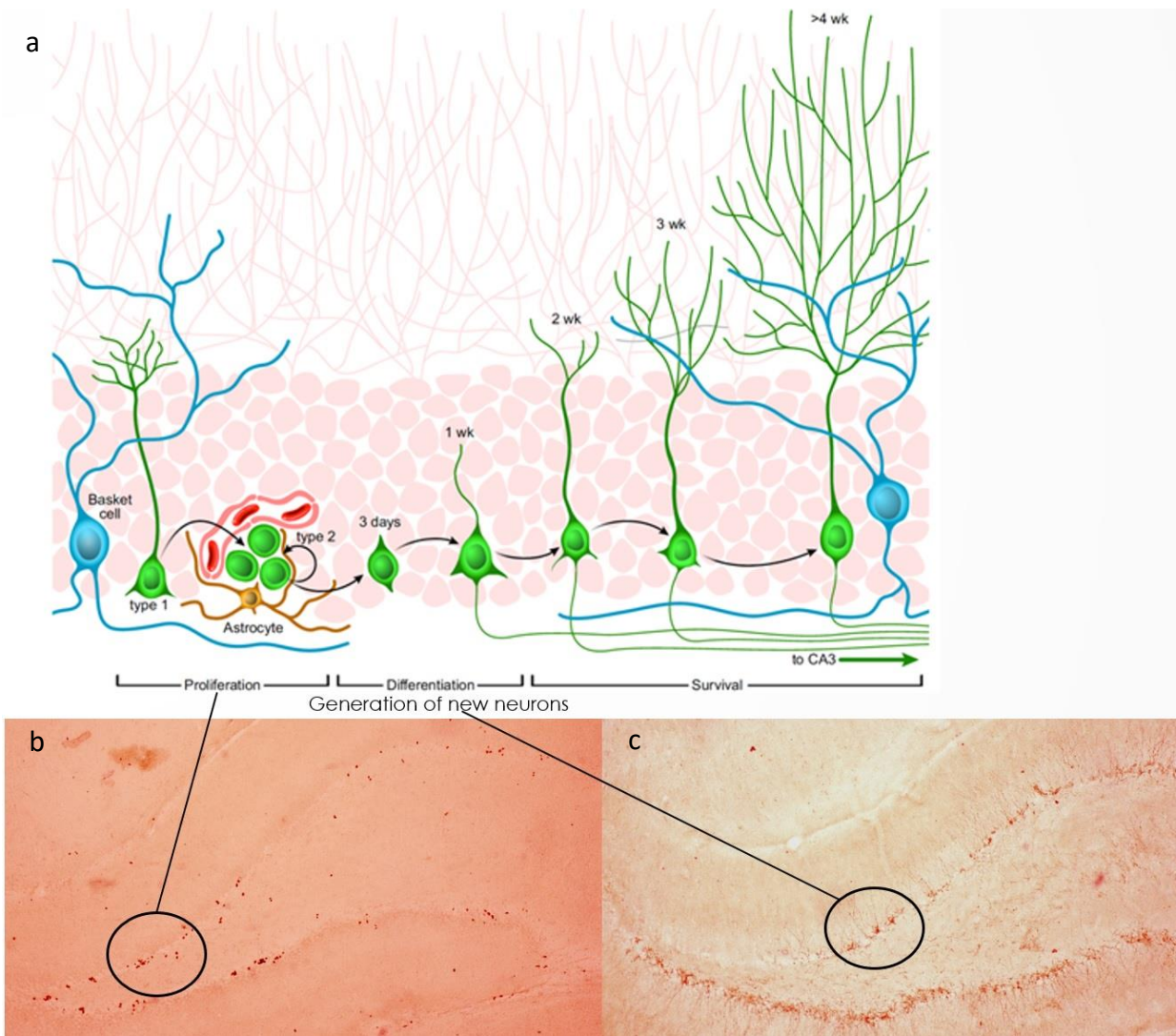
Figure 1.1. Neurogenesis in adult hippocampus. (a) Image extracted from Aimone et al., 2014.

Developmental stages of adult born granular neuron in DG of hippocampus. Proliferation occurs

at subgranular zone, differentiation occurs 3 – 7 days, migration and maturation into granule

cell layer by 2-3 weeks, before functionally integrated into hippocampal circuitry by 4-8 weeks.

(b) Image of rodent DG displaying cell proliferation (BrdU-positive cells in box) in SGZ of DG. **(c)**

Image of rodent DG displaying newly generated adult born DG neurons (DCX-positive neurons

in box) in the granule cell layer.

TBI-induced Neurogenesis in TBI Animal Models

Over the past three decades various animal models have been developed to replicate human TBI with the goal to better understand the underlying pathophysiology and in attempt to discover potential treatments for TBI. With studies supporting the similarity of active neurogenic regions (SVZ and hippocampus) between human and mammalian brains (Eriksson, 1998), various animal models have been developed in TBI studies using, dogs, cats, sheep, swine, and mouse. Although larger animals are closer in size and physiology to human, rodents are often selected for TBI models, mainly due to their modest costs, small size and, standardized outcome measurements (Xiong et al., 2013). Among various TBI models, the most frequently used are fluid percussion injury (FPI) and controlled cortical impact injury (CCI). Other injury models such as weight drop impact acceleration injury (WDIAI) and Blast injury model are also used in TBI studies. . FPI uses fluid pressure pulse caused from pendulum impact to the intact dura through a craniotomy and produces a focal cortical contusion in brain. The percussion produces brief displacement and deformation of brain tissue, replicating clinical intracranial hemorrhage, brain swelling, and gray matter damage (Xiong et al., 2013). Lately FPI model has been modified to lateral fluid percussion injury model (LFPI), which creates not only focal cortical contusion, but it also transmits traumatic injury into subcortical structures (Galgano et al., 2017). The injury creates progressive degenerative cascades that persist in selectively vulnerable brain regions, including ipsilateral hippocampus, thalamus medial septum, striatum and amygdala (Hicks et al., 1996), the results usually associated with neurological and cognitive deficits, such as difficulties in memory and movement commonly seen in TBI patients (Hamm, 2001). CCI model uses a pneumatic or electromagnetic impact

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device to drive an impactor onto the exposed intact dura. CCI provides a more controlled injury in terms of velocity, force, time and depth of injury as compared to FPI model (Xiong et al., 2013). CCI is often used to replicate clinical cortical injury, axonal injury, and subcortical injury in the thalamus and hippocampus (Galgano et al., 2017).

As the result of various TBI models, injury-induced cell proliferation was observed in DG and SVZ of animals that received FPI injury (Chirumamilla et al., 2002, Sun et al., 2005, Rice, 2003), CCI injury (Gao et al., 2009), also WDIAI (Villasana et al., 2014) and blast (acceleration-impact) injury model (Bye et al., 2011). This cell proliferation response is known to be particularly common in TBI-stimulated endogenous response in both DG and SVZ, and the cell increases is rather transient (Sun, 2016). The injury induced (FPI) proliferated cells peak at 2nd day post injury and were only observable during the first week in SGZ of DG (Figure 1.2. a Sun et al., 2005). Unlike proliferation, different injury models reported varying results of neuronal generation post-TBI. It is known to arise from variation of injury models such as intensity of the injury, tissue harvest time and processing method, markers used for quantification (Sun, 2016), even age of the animals used could produce different results due to age related neuronal/glial cell fate decision in neuronal maturation process (Figure 1.2. b Sun et al., 2005).

Although exact mechanisms for TBI-induced cell proliferation and neurogenesis is unclear, many studies have reported possible involvement of growth factors in neuro-regenerative process, such as basic fibroblast growth factor (bFGF), Insulin-like growth factor (IGF), epidermal growth factor (EGF), or vascular endothelial growth factor (VEGF) (Lee and Agoston, 2010, O'Kusky, 2012, Sun et al., 2009,2010, Thau-Zuchman et al., 2010). Similarly, modulatory attempts have been made to unveil underlying mechanisms for cell proliferation

and neurogenesis via exogenous administration of growth factors for means to enhance natural neurogenerative processes. Studies reported post-TBI animals infused with recombinant VEGF increased cell proliferation in SVG along with enhanced neuroprotective effects such as neurogenesis and angiogenesis (Thau-Zuchman et al., 2010) and survivability of newly generated neurons (Lee and Agoston, 2010). Other pharmacological treatment such as Statin has been found effective in promoting neurogenesis and cognitive function in TBI animals (Lu et al., 2007). Intraventricular infusion of growth factors such as bFGF (Sun et al., 2010) and EGF in TBI induced animals had a significant enhancement in cell proliferation in the hippocampus and SVZ, while showing a drastic improvement in cognitive functional recovery of the injured adult animals (Figure. 1.3. Sun et al., 2009).

While the enhancement of cell proliferation and neurogenesis involving cognitive functional recovery has been observed in many adult TBI-animal models (Sun et al., 2009, Sun et al.,2010, Sun et al., 2016), the molecular mechanism responsible for maintaining cell proliferation, differentiation, and restoration of neuronal function remains unknown. However, emerging evidences suggest the possible involvement of Notch signaling pathway as a key mechanism in regulating adult NSC proliferation and neurogenesis in neurogenic niches such as SVZ and DG of hippocampus. Notch signaling pathway functions as regulatory signaling system during embryonic development in maintenance of NSC proliferation and cell fate decision in neurogenic regions (Artavanis-Tsakonas et al., 1999). Studies also confirmed the presence of Notch signaling pathway in postnatal and adult brain (Stump, 2002, Traiffort and Ferent, 2015). Studies also reported observation of transient increase in Notch signaling activity (Tatsumi, 2010) along with recovery of cognitive function (Zhang, 2014) in adult brain post-injury,

suggesting possible involvement of Notch signaling pathway in brain's innate response to

promote repair and regeneration post-TBI.
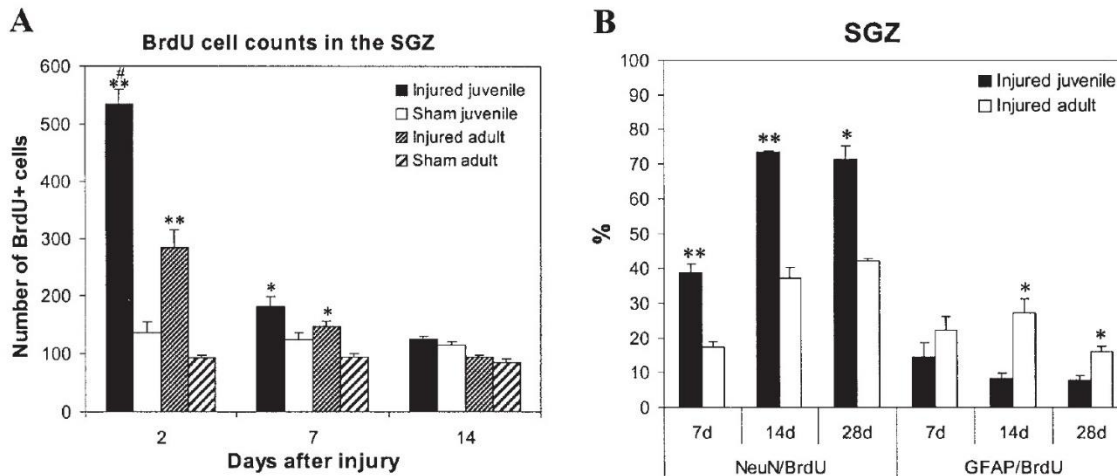
Figure 1.2. Cell proliferation and Neurogenesis in SGZ of DG post-TBI. Image extracted from Sun et al., 2005. **(A)** Number of BrdU-positive cells in SGZ of DG at 2nd, 7th, and 14th day post injury. For both Injured juvenile and injured adult rats, Cell proliferation peaks at 2nd day and gradually declines by 14th day (**$p < 0.01$). Significant difference in numbers of BrdU-positive cells are still observed by 7th day (*$p < 0.05$). **(B)** Percentage of BrdU-positive cells co-localized with neuronal (NeuN) and glial (GFAP) marker. Generation of neurons observed in SGZ of DG at 7th day, 14th day, 28th day of injury. (Animals received 3 doses of BrdU (50 mg/kg body weight) I.P injection at 2h intervals on 2nd, 7th or 14th day post FPI injury. Sacrificed 24h after last injection)
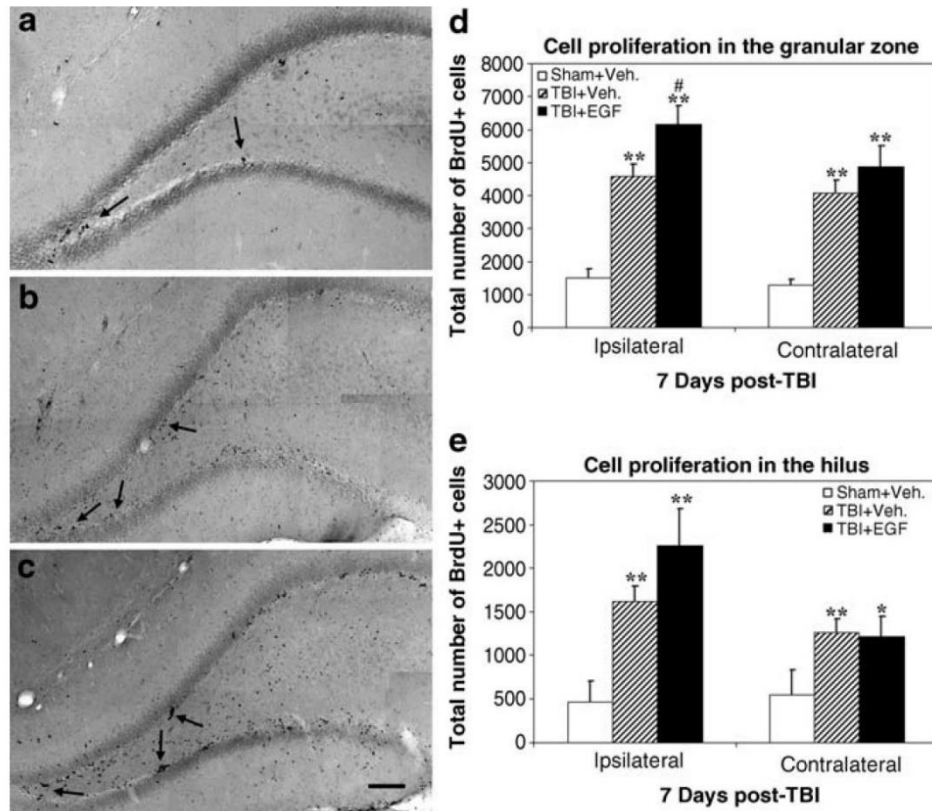
Figure 1.3. Effect of 7-day EGF infusion on cell proliferation in DG, 7 days post injury. Image extracted from Sun et al., 2009. Comparison of cell patterns and proliferation between Sham animals that received 7-day infusion of vehicle, injured animals that received 7-day infusion of vehicle, and injured animals that received 7-day infusion of EGF. **(a)** Coronal section DG of rat Hippocampus, Sham + Vehicle animal displaying lower numbers of BrdU-positive cells along in granular cell layer (GSL). **(b)** TBI + Vehicle animal displaying significantly increased numbers of BrdU-positive cells predominately localized in GSL. **(c)** TBI + EGF animal displaying enhanced number of BrdU-positive cells localized in GSL and scattered through hilus. **(d)** In granular zone (GZ), Significant increase in number of BrdU-positive cells in TBI + Vehicle compared to Sham + Vehicle (**$p < 0.01$), further significant increase in number of BrdU-positive cells in TBI + EGF

(**$p < 0.01$) compared to Sham + Vehicle. **(e)** Similar findings are seen in the hilus. (all animals

received daily single IP. injections of 5-bromo-2-deoxyuridine (BrdU; 50mg=kg) for 5

consecutive days.)

Notch Signaling Pathway

Notch gene was first discovered in 1913 by Thomas Hunt Morgan, while studying a

strain of *drosophila melanogaster* which were involved in partial loss of function that resulted

notches in their wing blades (Morgan and Bridges, 1916). However, not much of the gene has

been known until recently. Studies show that proteins involved in Notch signaling pathway are

highly conserved and heavily involved with many developmental roles including maintenance,

proliferation, and differentiation of NSCs (Artavanis-Tsakonas et al., 1999). Since adult

neurogenesis occur throughout life, and the Notch pathway proteins are expressed in germinal

zone of embryonic and adult brain (Stump, 2002), it is highly supportive that Notch signaling

might be involved in regulating maintenance of postnatal NSCs, which has been confirmed by

recent transgenic mouse study (Imayoshi et al., 2010). Moreover, Notch signaling pathway is

also known to be responsible for regulating neurogenesis in neurogenic niches during

embryonic development and adulthood (Zhang et al., 2015).

In mammals, *Notch* gene transcribes a highly evolutionary conserved large

transmembrane protein that acts as receptor for DSL (Delta, Serrate, Lag-2) family of ligands

(Xiao et al, 2009). There are 4 known heterodimeric receptors resulting from proteolytic

cleavage (S1) form Golgi-network (Logeat et al., 1998), referred to as Notch1, Notch2, Notch3,

and Notch4. The proteolytic cleavages by furin-like protease are thought to contribute to net

signal activity by facilitating exocytosis of Notch (Lake et al., 2009), which are expressed on cell

surface with one extracellular region, a single-pass transmembrane, and a small intracellular

region (Artavanis-Tsakonas et al., 1999). The extracellular domain of Notch receptor contains

various number of EGF like repeats and this is where ligands are known to interact with the

receptor (Xiao et al, 2009). In postnatal brain, Notch1 is localized in subventricular and ventricular germinal zones, whereas Notch2 and Notch3 are more highly localized in Ventricular zones. Notch1 and Notch3 are expressed along the inner aspect of the dentate gyrus, and Notch2 is expressed in the external granular cell layer (Irvin et al., 2001). All Notch receptors binds to all DSL ligands at different affinity, and the unbound receptors are constantly internalized to be either recycled or broken-down (McGill et al., 2009). Mammals also possess highly conserved five Notch ligands, the *drosophila* Serrate homologs Jagged 1 and Jagged 2, and the *drosophila* Delta homologs Delta-like1, Delta-like3, and Delta-llike4. All Notch ligands are single-pass transmembrane polypeptides and includes EGF-like repeats, with a highly conserved DSL domain which is known to be necessary for ligand binding onto EGF-like repeats expressed on Notch receptor. (Figure 1.4, Chiba, 2006). Because of membrane bound nature of both Notch receptors and ligands, Notch signaling is usually involved with direct cell to cell interaction known as canonical Notch signaling pathway.

The canonical Notch pathway functions as a core signaling system during embryonic development, as well as regulation of tissue homeostasis and stem cell maintenance in the adult (Artavanis-Tsakonas et al., 1999, D'souza et al., 2010, Gridley, 1997). Defects in the expression of *Notch* pathway proteins or disruption in Notch signaling can result in severe, often lethal developmental abnormalities. Mutation in Notch1 is known to be responsible for various cancers such as chronic lymphocytic leukemia (Vavrova E et al., 2017), and T-cell acute lymphoblastic leukaemia (Sanchez-Martin and Ferrando, 2017) also haploinsufficiency of Notch is associated with aortic disease (Garg et al., 2005). Mutation in Notch2 causes severe progressive bone loss such as Hajdu-Cheney syndrome which can lead to facial anomalies and

development of osteoporosis (Isidor et al., 2011, Simpson et al., 2011), and haploinsufficiency

of either Jagged 1 or Notch2 is associated with Alagille syndrome (McDaniell et al., 2006).

Defects in Notch3 is thought to be responsible for intractable chronic pain such as fibromyalgia

and neuropathic pain (Rusanescu and Mao, 2014), and is also related to development of facial

anomalies and meningocele related neurologic dysfunction in human (Gripp et al., 2015).

Notch4 has been identified as a candidate susceptibility receptor for schizophrenia, however

the studies have been inconclusive.

In canonical Notch signaling pathway, Notch ligands interacts with Notch receptors in

two models, *cis*-inhibition and *trans*-activation (Figure.1.5. a, D'Souza et al., 2010). *Cis*-inhibition

involves, inhibition of binding between the Notch ligand on the signaling cell and the Notch

receptor on the signal receiving cell which is already bound to Notch ligand on the same cell.

Cis-inhibition model is a poorly understood and highly controversial. However, a competition

between *trans*- and *cis*- ligand binding to Notch receptor is one of the explanations for the

ability of Notch ligand to activate or inactivate Notch signaling. Which is consistent with recent

findings that Notch DSL ligand such as Jagged1 possess binding site for both *trans*- and *cis*-

interactions with Notch receptor (Cordle et al., 2008).

In *trans*-activation model, Notch ligand from signaling cell binds onto unoccupied Notch

receptor expressed on the signal receiving cell (Figure.1.5. b, D'Souza et al., 2010). Upon ligand

binding, 1) Notch receptor at the cell surface is ubiquitylated by DTX4 (E3 ubiquitin ligase)

leading to internalization of Notch1 extracellular domain (NECD) by the ligand-expressing cell

and the 2) internalization of Notch1 intracellular domain (NICD) which consist the membrane

anchored fragment of Notch receptor and DTX4 by the Notch receptor-expressing cell in the form of bilateral endocytosis (Chastagner et al., 2017).

Internalized NECD is recycled by the Notch-ligand expressing cell and re-expressed on the cell surface, this recycling is required to acquire affinity for receptor in some Notch ligands (Heuss et al., 2008). A Disintegrin And Metalloproteinase (ADAM) cleaves (also known as S2 cleavage) ligand-receptor complex and forms product that is necessary for the formation of Notch Intracellular Domain (NICD) (Chastagner et al., 2017). γ-Secretase cleaves (also known as S3 cleavage) and releases NICD which translocated into nucleus following cleavage, however the process and proteins involved in NICD nuclear translocation is still unclear.

Upon translocation into the nucleus, NICD binds to a transcription factor, DNA binding protein CSL (CBF1 in humans, Suppressor of hairless in *drosophilia*, LAG in *C. elegans*). CSL, which is encoded by RBP-J gene in mammals, is believed to plays a central role in transducing Notch signals into changes in genes expression (Borggrefe and Oswald, 2009). CSL, by a default is a repressor protein preventing gene transcription, however NICD binding displaces co-repressor proteins and histone deacetylase (HDAc) to convert DNA-bound CSL into an activator. Meanwhile, NICD with CSL interaction creates an interface that is recognized by co-activator MAML (Matermind-like protein 1-3) and gets recruited to form a complex. This tertiary complex containing CSL, NICD, MAML is essential for up-regulation of Notch targets (Kovall, 2008). The CSL/NICD/MAML co-activator then can recruit histone acetyltransferase p300 (HAc) (Oswald, 2001) and initiates transcription of Notch target genes such as Hes (hairy enhancer of split family members) and Hey (Hes-related with YRPW motif). (Figure. 1.6, Xiao et al., 2009).

In Hes family members, Hes1, and Hes5 are known to regulate cell proliferation and neuronal differentiation in the nervous system (Kageyama et al., 2007). Hes1 expression is known to promote cell proliferation by repressing neuronal transcription factors and inhibiting subsequent neurogenesis (Kageyama et al., 2007). Studies have shown Hes1 expression as a result of downstream target of Notch signaling, represented as a negative regulator for adult hippocampal neurogenesis post-TBI (Zhang et al, 2014), and in the same way Hes1 expression improved spatial-learning and memory capacity of adult mice post-TBI (Zhang et al, 2014). Also, Hes1 protein works as a transcription repressor for its own expression. Upon induction, Hes1 protein directly binds to its promotor region of the gene and inhibit its transcription. Meanwhile short-lived Hes1 mRNA and Hes1 proteins disappear rapidly (Kageyama et al., 2007). Thus, Hes1 expression autonomously oscillates (2-3h per period) by a negative feedback, this oscillation is very important for cell proliferation and differentiation for neural stem cells, because the steady expression inhibits the proliferation and activates neuronal differentiation (Baek et al., 2006).

Numerous studies have been conducted to modulate downstream effects of Notch pathway by inhibiting or activating Notch signaling. Notch pathway inhibition by administration of γ-gamma secretase inhibitor delayed G1/S-phase transition and committed NSC to neurogenesis (Borghese et al., 2010). In contrast, inhibition of γ-gamma secretase facilitated the differentiation of human-induced pluripotent stem cells into neural stem cells. (Chen et al., 2014). Antibody-mediated Notch-ligand receptor binding inhibition downregulated transcription factors of the Notch target gene such as HES5 in both mouse and human neural stem cells, leading into neuronal cell fate. (Falk et al., 2012). Notch antisense transgenic mice

with reduced Notch levels exhibited impaired long-term potentiation (LTP) leading to long-term depression (LTD), however activation of Notch signal pathway by introducing Notch ligand corrected the defect in LTP of Notch antisense transgenic mice (Wang et al., 2004).

These studies collectively suggest the   cell proliferation and differentiation of NSCs via inhibition or activation of either Notch receptor or its downstream effectors. However, there are evidences of other signaling activities that intersects with Notch signaling pathway, at the level of Notch ligand expression (Hurlbut, 2007). These include VEGF, tumor necrosis factor alpha (TNFα), fibroblast Growth Factor (FGF), interlukin-6 (IL6), platelet derived growth factors (PDGF) and other factors resulting in upregulation or downregulation of Notch DSL ligands (D'Souza et al., Table 1.1). Suggesting manipulation of Notch signaling pathway could be far more complicated in vivo.
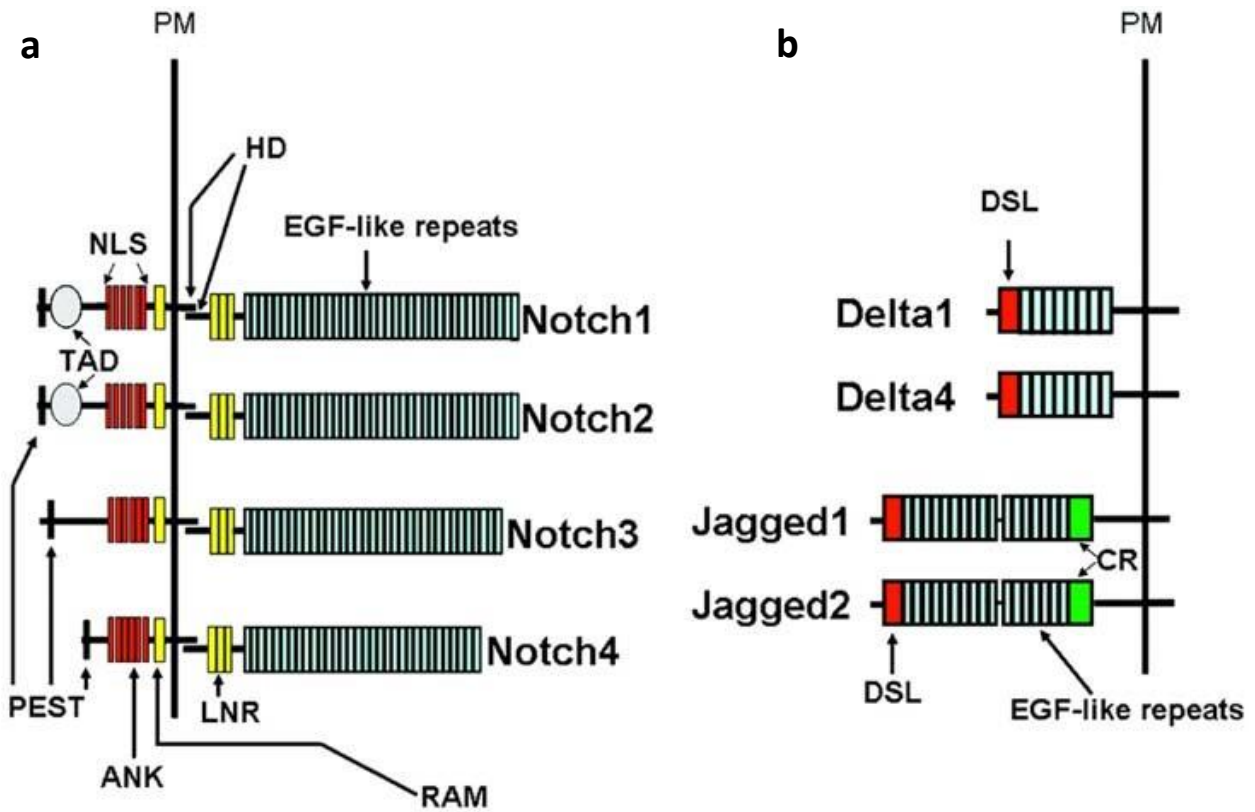
Figure 1.4. Protein structure of Notch receptor and their ligands. Image extracted from Chiba et al., 2006. **(a)** Notch receptors (1-4) expressing highly conserved, various EGF-like repeats. **(b)** Notch ligands, *drosophila* Serrate homolog Jagged1 and Jagged 2, *drosophila* Delta homolog Delta-like 1 and delta-like 4 (Delta-like 3 is excluded in picture) in mammals, expressing highly conserved EGF-like repeats and DSL domain. DSL is a binding site for Notch. LNR includes inhibitory function against cleavage. RAM associates with CSL complex. ANK associates with proteins to form complex. PEST regulates half-life of Notch proteins.

(Abbreviations: ANK, ankyrin repeat; CR, cysteine-rich repeat; DSL, Delta-Serrate-Lag2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; LNR, Lin-Notch repeat; NLS, nuclear localization signal; PEST, PEST domain; PM, plasma membrane; RAM, ram domain; TAD, transactivation domain.)
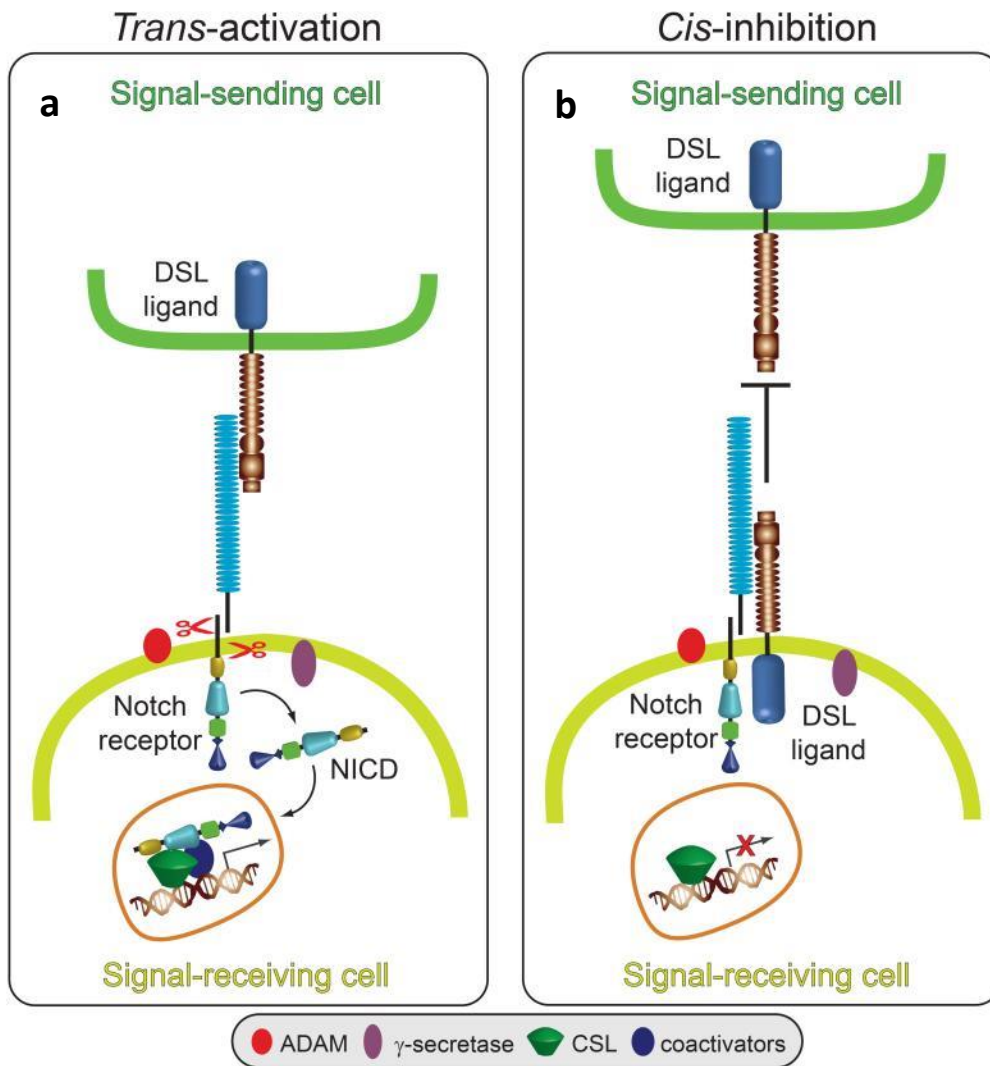
Figure 1.5. Models for Notch DSL Ligand binding in Notch signaling. Image extracted from

D'Souza et al., 2010. **(a)** *trans*-activation: Notch ligand expressed on signal sending cell binds to

Notch receptor expressed on the surface of signal receiving cell leading to cleavage

internalization of NICD, activating Notch. **(b)** *cis*-inhibition: Notch ligand binding to Notch

receptor expressed on the same signal-receiving cell prevents the binding of other Notch

ligands from signal-sending cell, blocking Notch signaling. (ADAM: A Disintegrin And

Metalloprotease; NICD: Notch Intracellular Domain; CSL: CBF1, Suppressor of Hairless, Lag-1;

coactivators such as Mastermind-like proteins (MAMLs).
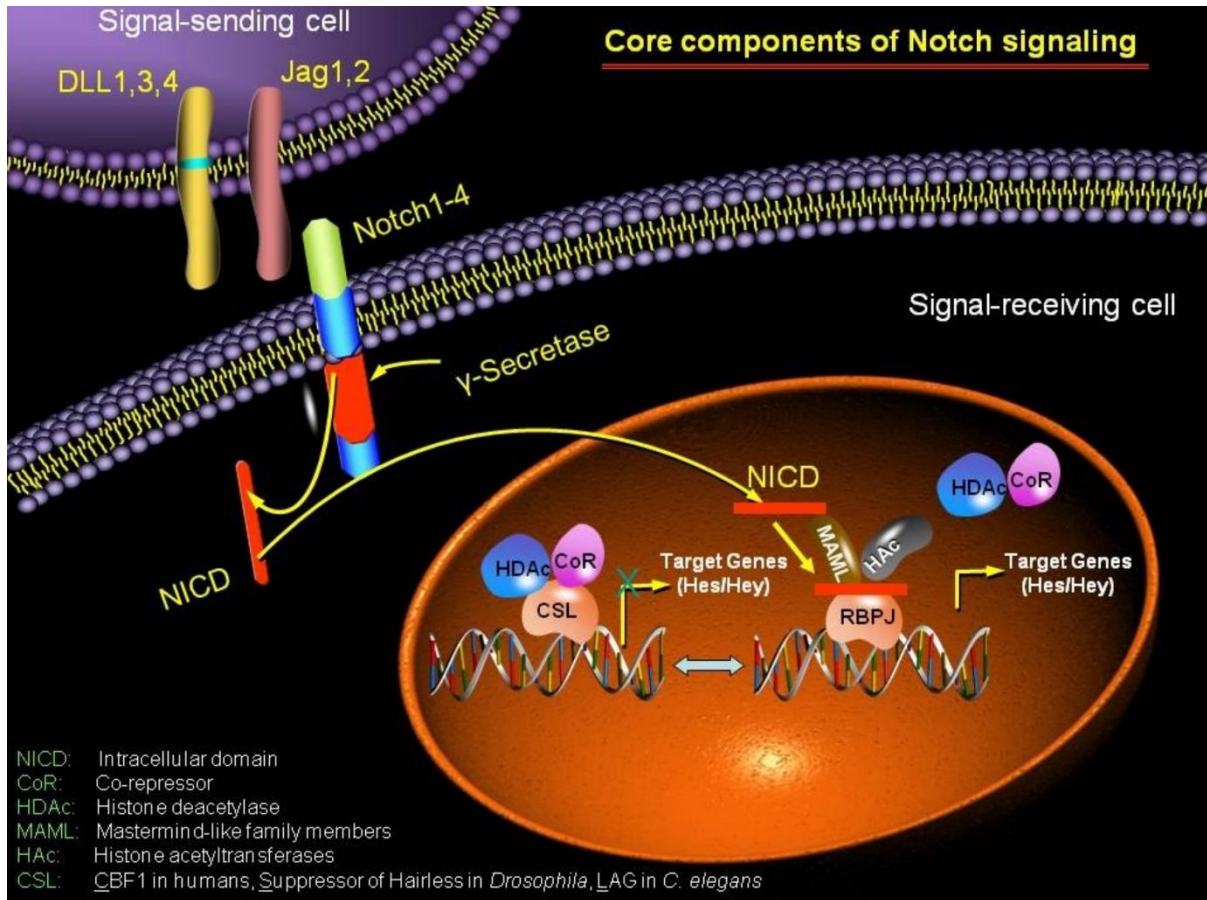
Figure 1.6. Canonical Notch signaling pathway. Image extracted form Xiao et al., 2009. Schematic

Notch signaling presentation in mammals demonstrating Notch signaling pathway upon ligand (Jagged

1,2, Delta-like 1,3,4,) binding on Notch receptors (Notch 1-4). Ligand-Notch receptor complex is cleaved

by enzymes such as ADAM and γ-Secretase. Upon release, NICD translocate into nucleus forming

transcription complex (NICD-RBPJ-MAMl) which initiates gene transcription of Notch target genes such

as Hes and Hey, leading cell to proliferative state, preventing neurogenesis. The CSL (RBPJ in human)

remains inactive, bound to co-repressor in absence of Notch signaling.

Effector of DSL ligand expression	DSL ligand	Effect on ligand expression: Upregulation (+) Downregulation (−)	Cell type	Biological effect
VEGF[a]	Dll4[b]	+	Endothelial	Inhibition of angiogenic sprouting; arterial specification
TNFα[c]	Jagged1	+	Endothelial	Promotion of angiogenic sprouting
FGF[d]	Dll1[b]	+	Neural stem cells	Maintenance of spinal cord stem cells
LPS[e]	Dll4[b]	+	Dendritic cells	CD4^{+} Th1[k] polarization
LPS[e] / PGE2[f]	Jagged1	+	Dendritic cells	CD4^{+} Th2[k] polarization
IL6[g]	Jagged1	+	Mammary epithelial cells	Proliferation and invasion
Hedgehog	Jagged1	+	Mesenchymal cells	Limb development
VEGF[a] + FGF2[d]	Dll1[b]	+	Endothelial cells	Postnatal Arteriogenesis
Wnt	Jagged1	+	Hair follicle precortex	Hair follicle differentiation
Wnt	Jagged1	+	Intestinal epithelial cells	Proliferation (tumorigenesis)
Wnt	Dll1[b]	+	Presomitic mesoderm	Somitogenesis
DER[h] and/or Heartless	Drosophila Delta	+	Embryonic mesoderm	Specification of muscle and heart progenitors, photo-receptor and non-neuronal cone cells
TGFβ[i]	Jagged1	+	Epithelial cells	Epithelial - mesenchymal transformation
FGF1[d] / FGF2[d]	Dll1[b]	-	Neuroepithelium	Maintenance of neuroepithelial precursors
PDGF[i] / angiotensin II	Jagged1	-	Vascular smooth muscle cells	Growth retardation
LPS[e]	Jagged1	-	Bone-marrow mesenchymal stem cells	Proliferation of CD4^{+} T cells

Table 1.1. Cellular factors that regulate DSL ligand expression. Image extracted from D'Souza et al., 2010. Various signaling pathways intersecting with Notch pathway via upregulation/downregulation of Notch DSL ligands inducing different cell type specific effects.

a Vascular Endothelial Growth Factor, *b* Dll: Delta-like, *c* Tumor Necrosis Factor α, *d* FGF: Fibroblast Growth Factor, *e* Lipopolysaccharide, *f* Prostaglandin E2, *g* Interleukin 6, *h* Drosophila Epidermal Growth Factor Receptor, *I* Transforming Growth Factor β, *j* Platelet-derived Growth Factor, *k* Th: T helper cell

Hypothesis

TBI is usually associated with upregulated cell proliferation activity in neurogenic niche and this endogenous cell response is viewed as brain's innate ability to promote repair. Since Notch signaling pathway is known key regulator in maintenance of NSC proliferation in the neurogenic regions, we hypothesize that Notch signaling pathway is responsible for injury-induced cell proliferation in the neurogenic regions. To test this hypothesis, in this study, we examined the involvement of Notch signaling pathway in injury induced neural stem cell proliferation in the DG of the hippocampus by administering exogenous Notch agonist or Notch antagonist with intraventricular infusion immediately following TBI.

In canonical Notch signaling pathway, Notch ligand-receptor binding interaction activates Notch signaling downstream effectors, leading to NSC to maintain proliferative state and hinders neuronal differentiation. Therefore, we expect to see enhanced cell proliferation in Notch agonists administered animals and reduced cell proliferation in Notch antagonists administered animals post TBI. We also expect to see the subsequent hinderance in the neurogenesis in Notch agonist administered animals and enhanced neurogenesis in Notch antagonist administered animals.

Chapter 2- Materials and Methods

Experimental Animals

Sprague-Dawley rats were selected for the study. Three-month-old male rats weighing approximately 300g were used for all groups, rats were purchased from Harlan Inc, Indiana. Rats were housed at the animal facility in Virginia Commonwealth University and adequate food and water were provided as needed, the animals were kept in 12-hour day/night cycles at a room temperature. Proper maintenance and care procedures were followed as approved by institution of animal care and use committee (IACUC) and in accordance to the guide for care and use of laboratory animals provided by Department of health and human services.

Experimental Setup

A total of 36 animals were included in the study, all of which received similar care under identical conditions. The animals were randomly selected and divided into total of nine groups with n=4 for each group: sham, TBI only, TBI-vehicle, TBI - Notch1 agonist-0.5mg/ml, TBI - Notch1 agonist-2mg/ml, TBI - Notch1 agonist-4mg/ml, TBI-Notch1 antagonist-25ug/ml, TBI-Notch1 antagonist-50ug/ml, TBI-Notch1 antagonist-100ug/ml. All animals received seven consecutives single daily BrdU I.P. injections at the dose of 50mg/kg, the last injection was given at two hours before the animal was sacrificed. All animals were sacrificed at 7 days post injury.

Surgical Procedures

All rats including sham received the same surgical procedures. All instruments which contact the surgery area were pre-sterilized following aseptic procedures. The rat was first anesthetized in acrylic glass chamber with 5% isoflurane and intubated and ventilated with 2% isoflurane gas mixture (30% O2 and 70% N2) and fixed onto a stereotaxic frame with continuous anesthesia during the surgery, and a warming pad was inserted underneath the animal to maintain body temperature. The respiration and pulse rate, and the temperature were constantly monitored during the entire procedure. First, betadine was applied to the shaved head to sterilize the surgical site and the animal received midline incision to expose the skull, hemostat was used to retract the skin to prepare for craniotomy. All rats received 4.9 mm craniotomy on the parietal bone between lambda and bregma sutures over the left hemisphere using trephine and dental Dremel tool which had a small dental drill bit attached at the tip. Luer-lock syringe hub made from 20-gauge 1½ inch needle was affixed to the craniotomy site and was sealed with cyanoacrylate, and dental acrylic was also used to further secure the hub to the skull. The rate of anesthesia administration was off at this point, the animal was brought back to consciousness by showing paw reflexes before receiving injury. All rats except sham received LFPI as described below. After injury, the animal was returned to surgical table, the Lure-lock fitting and the acrylic seal was removed. After the righting time was recorded to assess injury severity, the animal was then re-anesthetized with 2% isoflurane gas mixture to prepare for intraventricular infusion. An Alzet brain infusion cannula (Brain Infusion Kit II; DURECT, Cupertino, CA) was implanted into the ipsilateral posterior lateral ventricle (coordinates: AP + 0,8mm, lateral 1.4 mm, 3.5 mm under pial surface). The cannula was then

connected to a mini osmotic pump (Model 1007D) containing vehicle, Notch 1 agonist or

antagonist at different concentration, which was subcutaneously placed around posterior neck.

The incision site was sutured using 5.0 polyamide sutures, anesthetic was off, lidocaine

hydrochloride jelly and antibiotic ointment was applied to keep the animal form infection.

Intubation tubing was removed, and the animal was left to regain consciousness by showing

normal breathing patterns. Rats were transferred to a warm cage lined with sterile surgical

drapes and observed there for three hours before returning to the housing facility.

Lateral Fluid Percussion Injury

A total of 32 rats received LFPI except for sham. After the craniotomy and the Luer-Lock hub was sealed and cemented to the skull, a Luer-lock fitting filled with 0.9% saline was attached to the hub to test the integrity of the seal. A lateral fluid percussion injury device (Figure 2.1) was used for administrating fluid percussion injury (FPI) on all injured animals in the study. The fluid pulse is first generated by the impact of the falling pendulum on the stationary fluid filled acrylic cylinder, then traveled across the acrylic cylinder onto the target which is the animal receiving the injury, the impulse is amplified by a pressure transducer amplifier and measured by an oscilloscope (Tektronix). The device was calibrated and prepared by adjusting the angle of pendulum to the device and sending and monitoring a few test pulses. The adjustment process was repeated until the optimum targeted fluid pulse with moderate severity (2.2 ± 0.02 atm) was achieved. Once the device was prepared and the isoflurane was turned off, the animal showed paw reflex and then was connected to the device by the Luer-lock fitting to the hub attachment on the animal and the injury was delivered. Immediately after injury, the animal was detached from the device and transferred to the surgical table and the righting time was recorded.
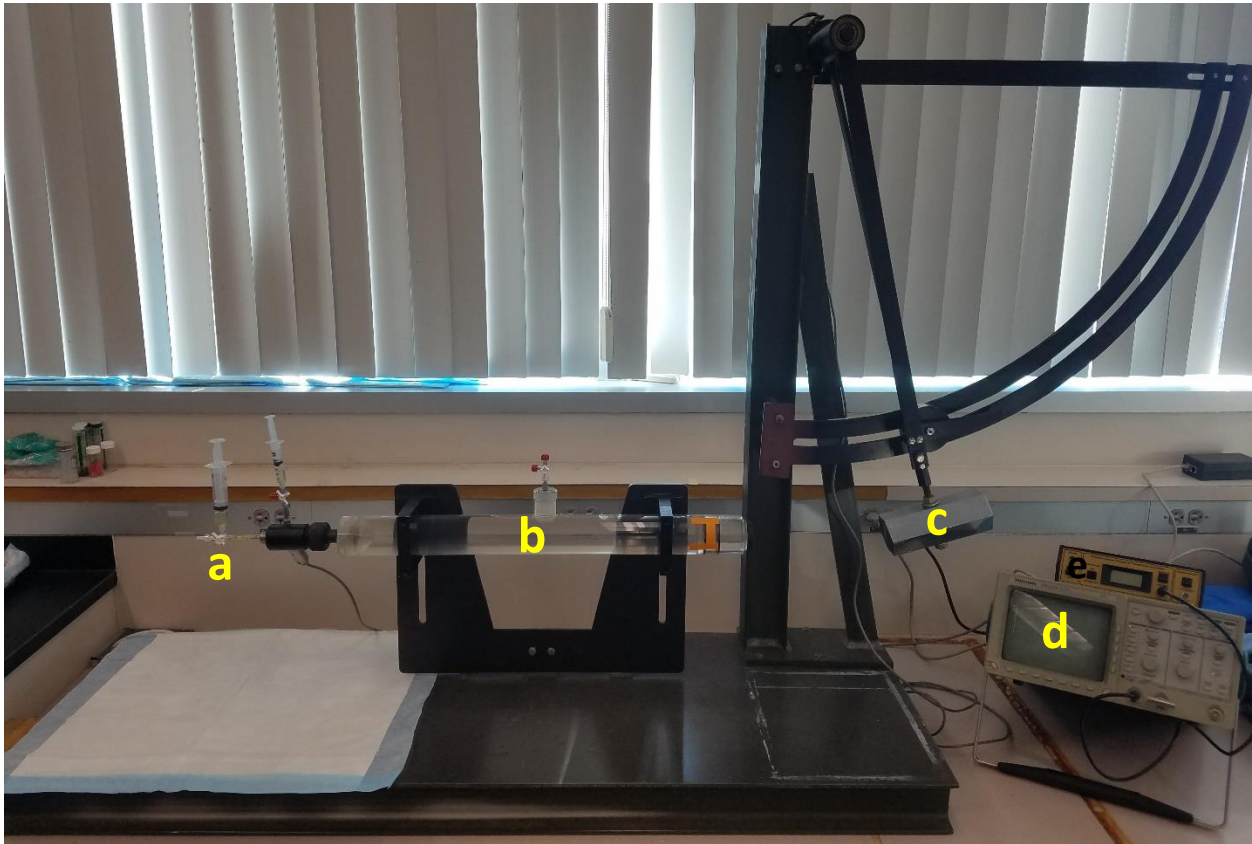
Figure 2.1. Lateral fluid percussion injury device. Consists of (a) a Luer-lock fitting for animal attachment, (b) a fluid filled acrylic cylinder, (c) a pendulum, (d) an oscilloscope, (e) an amplifier. The device is custom built for a laboratory use in Virginia Commonwealth University by a company currently known as Custom design & Fabrication.

Intraventricular Osmotic Mini-pump Infusion

A total of 28 injured rats received infusion, 12 rats received Notch1 agonist (Notch1 activation antibody), 12 rats received Notch1 antagonist (recombinant Jagged 1 fusion protein), and the remaining 4 animals received vehicle. Notch1 infusion were prepared by reconstituting the drug with the vehicle-a sterile artificial CSF (148mM NaCl, 3mM KCL, 14mM CaCl2, 0.8mM, MgCl2, 1.5mM Na2HPO4, and 0.2 mM NaH2PO4 [pH 7.4]). The concentration for Notch1 activation antibody (Notch extracellular clone 8G10, cat# MAB 5414, Millipore) infusion dose was: 0.5µg/ml, 2µg/ml or 4µg/ml. Recombinant human Jagged1/Fc chimera (cat# 1277-JG, R&D System) was first incubated with anti-human Fc antibody (cat# I8885, Sigma-Aldrich) for 1hr on ice at a ratio of 2:1, and the final desired concentration of Jagged-1 Fc was prepared by reconstituting with a sterile artificial CSF. The final concentration for Jagged1 Fc infusion was: 25µg/ml, 50µg/ml or 100µg/ml. Before infusion, all mini-osmotic pumps used for Notch1, Jagged1 or vehicle infusion were first primed in a warm water bath (37°) for 2 hours. The infusion was administered for 7 consecutive days at a flow rate of 0.5µL/h.

BrdU Injections

BrdU (5-bromo-2-deoxyuridine, Sigma-Aldrich Co.) which incorporates into the DNA of mitotically dividing cells and labels them permanently is used as a cell proliferation marker for the study. All rats received a single daily intraperitoneal (IP) BrdU injections at the dose of 50 mg/kg for 7 consecutive days, the last injection was given 2h before the animal was sacrificed.

Sacrifice and Tissue Processing

Rats were deeply anesthetized in a plexiglass chamber with isoflurane. After reaching

deep unconsciousness, a surgical gaze soaked in 100% isoflurane was wrapped around the nose

of the animal to keep from re-gaining consciousness. The animal was first perfused

transcardially with 400 mL of phosphate-buffered saline (PBS) then with 400mL of 4%

paraformaldehyde in PBS. The brain was harvested and transferred into a plastic container pre-

filled with 4% paraformaldehyde in PBS and stored at 4°C for 48 h before sliced. Vibratome

(Leica) was used for slicing brain into 60µm coronal sections which were collected and placed

into five 24 well plates pre-filled with 0.01% sodium azide in PBS solution. The sections were

stored at 4°C and used as needed for immunostaining procedures.

BrdU Immunostaining

Six sequential sections spaced 480um between containing hippocampi were selected for

BrdU immunostaining from animals of all groups. A five-day procedure of BrdU immunostaining

were followed. The brain sections were washed with PBS for 5 min twice, then denatured in

50% formamide (Sigma) for 1 h at 65°C. They were rinsed with 2X SSC on a shaker for 2 min in

room temperature twice. The sections were then incubated for further denaturation in 2N HCl

for 30 min at 37°C. They were rinsed with PBS for 5 min on a shaker at room temperature twice.

Then the sections were quenched in 3% hydrogen peroxide (Sigma) for 1 h on a shaker at room

temperature. Finally, they were rinsed with PBS + 0.3% Triton100 solution for 10 min on a

shaker at room temperature thrice. The sections were then blocked with blocking buffer (PBS +

0.3% Triton100, 5% horse serum) over night at 4°C. Following that, the sections were incubated

with BrdU primary anti-body solution for 48 h on a shaker at 4°C. The primary antibody solution

is prepared with monoclonal mouse anti-BrdU antibody (Invitrogen) in blocking buffer at 1:2000

dilution. After that, the sections were first brought back to room temperature, then washed

with PBS + 0.3% Triton100 on a shaker for 10 min thrice. Sections were blocked with blocking

buffer for 3 h on a shaker at room temperature. Afterward they were placed in secondary

antibody solution on a shaker over night at 4°C. The secondary antibody solution is prepared

with Biotin-conjugated anti-mouse IgG antibody (Vector) in blocking buffer at 1:200 dilution. On

day five, the section were brought back to room temperature, then washed with PBS for 5 min

on a shaker thrice. The sections were then incubated with ABC solution for 2 h on a shaker at

room temperature. ABC solution was prepared with Avidin-biotin complex regent kit (Vector)

both A and B reagent were mixed in PBS at 1:200 dilution 30 min prior to use. After 2 h of

incubation in ABC solution, the sections were washed with PBS for 10 min, repeated thrice

before placed in DAB solution (Sigma). DAB reaction was controlled by simultaneously

observing color reaction in sections under dissecting microscope. Upon reaching the adequate

reaction time, sections were then washed with PBS for 10 min on a shaker at room

temperature, repeated thrice. Sections were mounted under dissecting microscope using paint

brushes onto super-frosted microscope slides. Once mounted, they were left out for air dry

overnight. The mounted slides were counterstained with 0.1% cresyl violet before cover slipped

using Permount (Fisher).

DCX, Ki67 Immunostaining

Six sequential hippocampal sections 480um in between from animals from all groups

were selected for immature neuronal marker doublecortin (DCX) or cell prolfeiration marker

Ki67 immunostaining. Similar five-day procedure was followed as BrdU immunostaining with

exclusion of formamide and HCl denaturing steps. For DCX, the primary antibody solution is

prepared with polyclonal goat anti-DCX antibody (Santa Cruz) in blocking buffer at 1:1000

dilution, the secondary antibody solution is prepared with Biotin-conjugated anti-goat IgG

antibody (Vector) in blocking buffer at 1:200 dilution. For Ki67, the primary antibody solution is

prepared with rabbit anti-Ki67 antibody (Abcam) in blocking buffer at 1:500 dilution, the

secondary antibody solution is prepared with Biotin-conjugated anti-rabbit IgG antibody

(Vector) in blocking buffer at 1:200 dilution.

Stereological cell quantification

All stained sections were observed under an inverted light microscope (1X71, Olympus).

Visio pharm program (Denmark) stereology software was used for cell quantification for

counting individual cells in the DG granular zone (GZ) and hilus region in both ipsilateral (injured

side) and contralateral sides. First, 4x objective was used to identify and locate targeted

hippocampus, the region of interest was outlined via drawing tool. The GZ which includes both

SGZ and granular cell layer (GCL) was outlined and counted. For all three markers, the hilus

region were also outlined and counted. Cell counting was done with 40x objective. Each

individual BrdU+, Ki67+ or DCX+ cells were counted in the counting frame with the dissector

height set at 15 μm and any cells outside of dissector counting frame was omitted in the process. The brain section thickness was measured from five random locations of the tissue. Each five focal point were measured and averaged for the section thickness. The average thickness (t) of the brain was obtained by averaging thickness of all five sections. Estimation of total number of cells (n) per brain were obtained by $n= \Sigma Q^{-} \cdot (t/h)(1/asf)(1/ssf)$. ΣQ^{-} represents the total number of counted cells. *asf* represents average sampling fraction, which is set to one since the entire region was counted in this study. *ssf* represents sampling section fraction which is also set to 0.125, since only five sections were used per brain each representing 1/8 of total hippocampus.

Statistical Analysis

The cell proliferation data was analyzed using SPSS software to determine a pairwise significances between all nine groups including 3 doses of Notch agonist, 3 doses of Notch antagonists, TBI only, TBI+Vehicle, and sham. A one-way analysis of variance (ANOVA) test was performed with *post-hoc* Tukey's honestly significant difference (HSD) test. A *p* value less or equal to 0.05 was considered statistically significant.

Chapter 3 – Results

The aim of this study is to determine whether Notch signaling is responsible for injury-induced neural stem cell proliferation in the neurogenic niche. To test this, Notch1 agonist (Notch1 antibody) or antagonist (recombinant Jagged1 fusion protein) at three different doses was infused into the lateral ventricle following TBI for 7-days. To confirm whether similar level of injury was received among TBI animals, post-injury righting time was analyzed between the drug infusion groups and the vehicle infusion groups. The righting time, which is the time that the rat spontaneously turns from a supine position to a natural position, believed to be correlated with animal's neural deficits, is regarded as an indicator for severity of injury (Hamm, 2001). ANOVA test revealed that right time had no significant differences between groups (p=0.125), suggesting that all TBI animals received similar severity of injury.

To assess the effect of Notch agonist and antagonist infusion on neural stem cell proliferation and generation of new neurons in the dentate gyrus (DG) of the hippocampus, sections were immunolabeled with three different markers including BrdU, Ki67 and DCX.

Effect of Notch inhibition or activation on cell proliferation at 7-day post-injury –Ki67 study

To examine the cell proliferation at the time of sacrifice (7 days post-injury), a proliferation marker Ki67 was used. Ki67 protein which is strictly associated with cell proliferation and exclusively detected within the nucleus during interphase, however most of the protein is relocated to the surface of the chromosomes during mitosis (Scholzen, 2000), making an ideal marker for examining cell proliferation at the time of sacrifice. Proliferating

cells were labeled at the granular zone (including the subgranular zone and the granular cell layer) and the hilus region of hippocampal dentate gyrus in both hemispheres. The immunostaining patterns demonstrated Ki67-positive cells are predominately localized in subgranular zone (SGZ) and some scattered in hilus region (Fig. 3.1). Among all groups (sham, TBI-only, TBI-vehicle, TBI-Notch1-0.5μg/ml, TBI-Notch1-2μg/ml, TBI-Notch1-4μg/ml, TBI-Jagged1-25μg/ml, TBI-Jagged1-50μg/ml, TBI-Jagged1-100μg/ml), more Ki67+ cells were observed in injured-only group and in TBI-Notch1-2μg/ml group. (Figure 3.1.a-i).

Stereological quantitative analysis of Ki67-positive cells in the DG revealed that in the ipsilateral granular zone, higher number of Ki67+ cells were only observed in TBI-only animals compared to sham and other groups. TBI-vehicle animals had less Ki67+ cells compared to TBI-only group. Compared to TBI vehicle group, injured animal with Notch1 activator at 3 doses, the 2μg/ml group had higher number of Ki67+ cells, suggesting Notch1 antibody only at the 2μg/ml is sufficient to enhance cell proliferation, lower dose at 0.5μg/ml had no effect whereas a higher dose at 4μg/ml had detrimental effect. For Notch1 antagonist Jagged-1 Fc infusion, lower dose at 25μg/ml had slightly less Ki67+ cells compared to TBI-vehicle group, whereas 50μg/ml or 100μg/ml group showed no change. Similar pattern of Ki67+ cell counting was found in the contralateral granular zone with higher number of Ki67+ cells in the TBI-only and TBI-Notch1-2μg/ml groups compared to all other groups. (Fig. 3-2 a-b) in comparison to both sham and injured animal that received 7-day infusion of vehicle, the increased numbers of Ki67-positive cells in injured only animals weren't statistically significant, the same was also true for increased in numbers of cells observed in injured animals that received 7-day infusion of Notch1-2μg/ml. However, a significant decrease in numbers of Ki67-positive cells was observed

in injured animals that received 7-day infusion of Notch1-4µg/ml ($p < 0.01$) compared to injured

only animals (Figure 3.2.a-b). Further comparison revealed a significant decrease in numbers of

Ki67-positive cells in injured animals infused with Jagged1-25µg/ml ($p < 0.01$), Jagged1 50 µg/ml

($p < 0.05$) in ipsilateral and Jagged1-25µg/ml ($p < 0.05$) in contralateral side to the injured

hemisphere. Different cell proliferation responses were observed between two Notch agonist

doses, while injured Notch1-2µg/ml infused animals displayed a significant increase in Ki67-

positive cells compared to injured only animals, injured Notch1-4µg/ml infused animals

displayed a significant decrease in numbers of cells, the difference between two Notch doses

were considered significant ($p < 0.05$). However, no differences were observed between Notch

antagonist doses. Quantitative analysis of cell proliferation in the hilus region (Figure 3.2c-d)

revealed no significant differences among groups).

In the hilus regions, in both ipsi- and contralateral hemisphere, slightly higher number of

Ki67+ cells were found in the Notch1-2ug/ml group, however, no statistical significance was
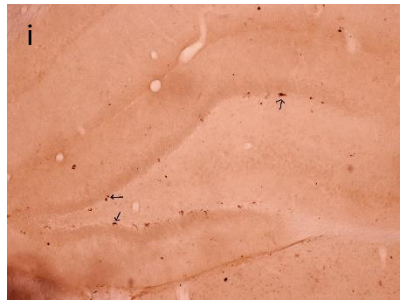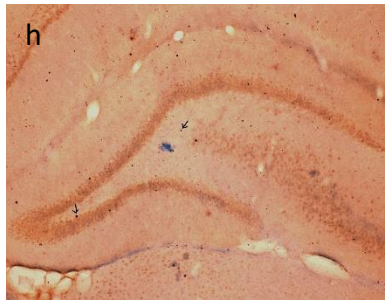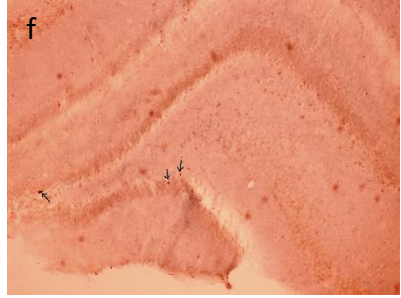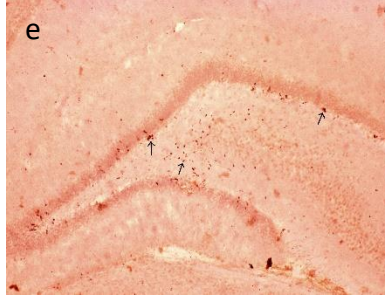
found among all group comparisons (Fig3-2-c&d).
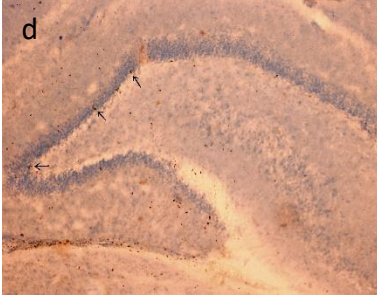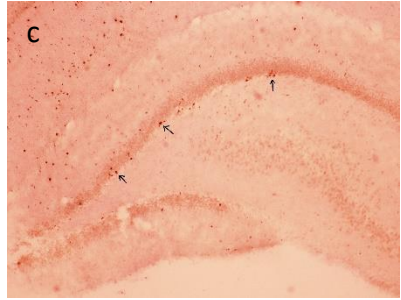
Figure 3.1. Cell proliferation at 7th day post-injury. Images of coronal sections of ipsilateral dentate gyrus showing Ki67 staining patterns including **(a)** sham animal, **(b)** an injured only animal, **(c)** an injured animal who received 7-day vehicle infusion, and injured animals who received 7-day infusion of either Notch1 receptor agonists: **(d)** Notch1-0.5μg/ml, **(e)** Notch1-2μg/ml, **(f)** Notch1-4μg/ml, or Notch1 receptor antagonists: **(g)** Jagged1-25μg/ml, **(h)** Jagged1-50μg/ml, **(i)** Jagged1-100μg/ml. Immunostaining patterns demonstrate increased nu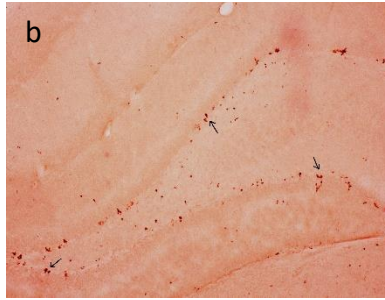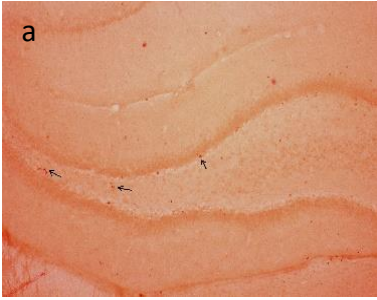mbers of Ki67-positive cells, mainly localized in SGZ and some scattered in hilus region, were observed in injured only animal compared to sham or other injured animal that received infusion of either vehicle or Notch ligands. Increased numbers of Ki67-positive cells were also observed in injured Notch1-2μg/ml infused animals, showing more scattered patterns in the hilus region compared to injured only animal.

a

Cell Proliferation in IP granular zone 7 days post injury

Legend:
- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

Y-axis: Ki67+ Cells

X-axis: Ipsilateral GZ

b

Cell Proliferation in CT granular zone 7 days post injury

Legend:
- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

Y-axis: Ki67+ Cells

X-axis: Contralateral GZ

c

Cell Proliferation in IP hilus 7 days post injury

Ki67+ Cells

Ipsilateral hilus

- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

d

Cell Proliferation in CT hilus 7 days post injury

Ki67+ Cells

Contralateral hilus

- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
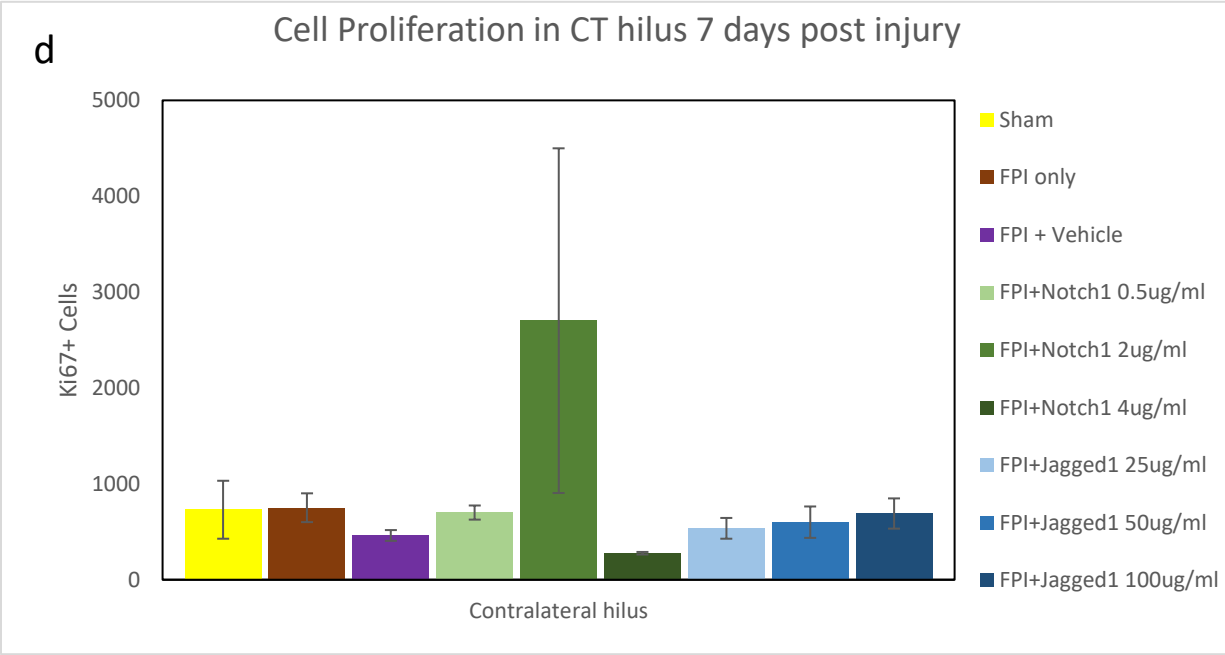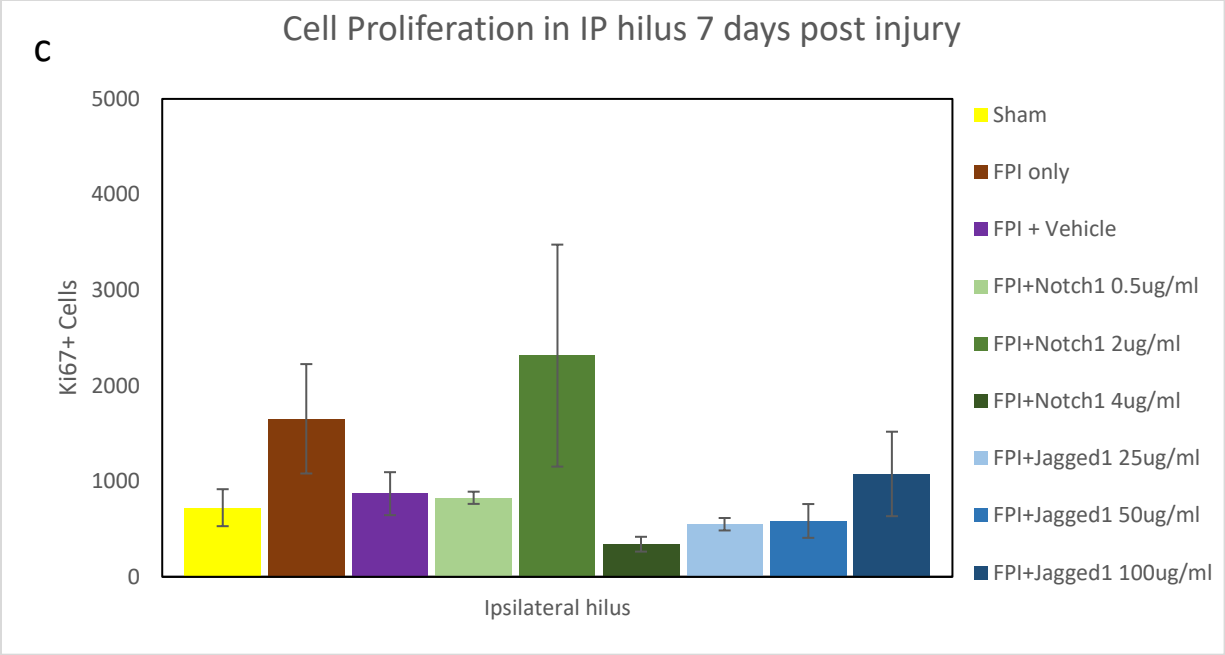- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

Figure 3.2. Quantification of Ki67 positive cells in dentate gyrus at 7th day post-injury.

Quantitative analysis of cell proliferation in granular zone displayed no significant differences between the sham animals and all injured animals, although increase in numbers of Ki67-positive cells were observed in TBI-only and Notch1-4μg/ml animals compared to sham, no significant differences were observed due the variation of the Ki67-positive cells within each group. No differences were observed between the injured vehicle infused animals and the injured Notch agonist or antagonist infused animals. **(a)** In the ipsilateral granular zone, the injured animals that received 7-day infusion of Notch1 agonist or antagonist displayed a significant decrease in numbers of Ki67-positive cells compared to injured only animals: Notch1-4μg/ml ($p < 0.01$), Jagged1-25μg/ml ($p < 0.01$), Jagged1 50μg/ml ($p < 0.05$). A significant decrease in numbers of Ki67-positive cell is observed between two injured Notch agonists infused animals, Notch1-2μg/ml and Notch1-4μg/ml ($p < 0.05$). **(b)** In the contralateral granular zone, the injured animals who received 7-day infusion of Notch1 agonist or antagonist infusion displayed significant decrease in numbers of Ki67-positive cells compared to injured only animals: Notch1-4μg/ml ($p < 0.05$), Jagged1-25μg/ml ($p < 0.05$). A significant decrease in numbers of Ki67-positive cell is observed between two Notch agonists infused animals, Notch1-2μg/ml and Notch1-4μg/ml ($p < 0.05$). Quantitative analysis of cell proliferation in **(c)** ipsilateral hilus zone and **(d)** contralateral hilus zone both reveled no changes in numbers of Ki67 positive among the groups.

Effect of Notch inhibition or activation on accumulated cell proliferation and survival in the DG

7 days following -TBI – BrdU study

To examine the accumulated cell proliferative response following Notch1 manipulation, a cell proliferation marker BrdU was given I.P, daily for 7 days during Notch1 agonist or antagonist infusion period. When injected, 5-bromo-2-deoxyuridine (BrdU), a thymine analog incorporates itself into newly synthesized DNA during S phase substituting for thymine. The degree of BrdU incorporation was detected by BrdU antibody staining. Proliferating cells were labeled at the granular zone and the hilus region of hippocampal dentate gyrus in both hemispheres. The immunostaining patterns demonstrated BrdU-positive cells are mainly localized in SGZ and some scattered in hilus region. Compared to sham animal, higher numbers of BrdU-positive cells were observed in, injured only, injured animals that received 7-day infusion of vehicle, and injured animals that received 7-day infusion of Notch1-2μg/ml (Fig. 3.3, a-f). Less BrdU-positive cells were observed in injured animals that received 7-day infusion of Jagged1-50μg/ml compared to all other groups (Fig 3.3.a-f).

From preliminary Ki67+ cell quantification data, Notch1 antibody at 2μg/ml or Jagged-1 Fc at 50μg/ml were selected as the best dose for Notch1 and Jagged-1, respectively, for further data analysis. Quantification analysis of BrdU-positive cells has shown: 1). In ipsilateral granular zone compared to sham group, significantly increased numbers of BrdU+ cells were found in injured only ($p<0.01$), injured vehicle ($p<0.05$), and injured Notch1-2μg/ml infusion ($p<0.05$). , whereas compared to other injured groups, a significant decrease in numbers of BrdU-positive cells were observed in injured animals that received 7-day infusion of Jagged1-50μg/ml (Fig. 3.4-a). In the contralateral granular zone, similar pattern of BrdU+ cell counting was found.

Compared to sham animals, a significant increase in numbers of BrdU-positive cells was observed in injured only animals ($p < 0.01$), injured animals that received 7-day Notch1-2μg/ml or vehicle infusion ($p < 0.05$). In comparison to injured only animals, a significant decrease in numbers of BrdU-positive cells were observed in injured animals that received 7-day infusion of Jagged1-50μg/ml. No significant differences were observed between injured vehicle infused animals and the injured Notch agonist or antagonist infused animals (Figure 3.4.b). 2). In the hilus region, quantitative analysis of BrdU-positive cells (Figure 3.4c-d) revealed that compared to sham animals, injured only animals, injured-Notch1-2ug/ml group and injured-vehicle group had significant increase in the numbers of BrdU-positive cells ($p < 0.01$). In comparison to injured only animals, a significant decrease in numbers of BrdU-positive cells were observed in injured animals that received 7-day infusion of Jagged1-50μg/ml ($p < 0.01$). However, no significant differences were observed between injured vehicle infused animals and the injured Notch agonist or antagonist infused animals.
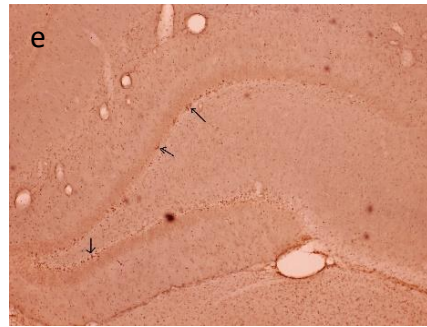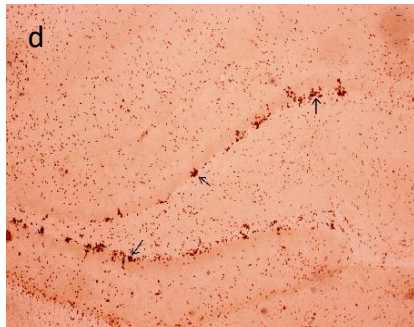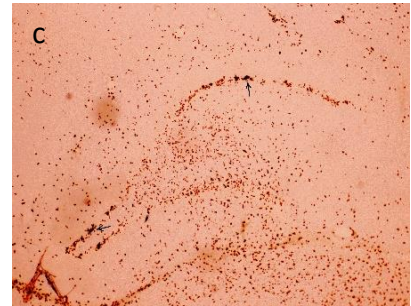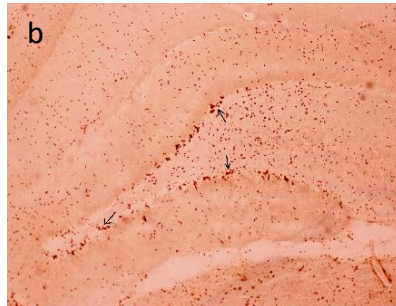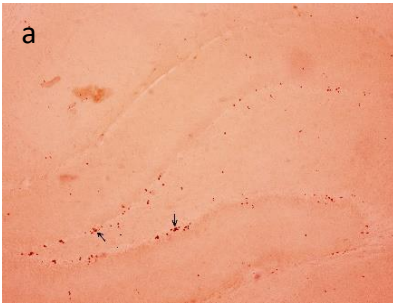
Figure 3.3. Cell proliferation for 7 days post-injury. Images of coronal sections of ipsilateral dentate gyrus showing BrdU staining patterns including (**a**) sham animal, (**b**) an injured only animal, (**c**) an injured animal who received 7-day vehicle infusion, **(d)** an injured animal that received 7-day infusion of of Notch agonist, Notch1-2μg/ml, **(e)** an injured animal that received 7-day infusion of Notch antagonist, Jagged1-50μg/ml. Immunostaining patterns demonstrated BrdU-positive cells are mainly localized in the SGZ and scattered in the hilus regions of injured only and injured animal that received 7-day vehicle or Notch1-2ug/ml infusion, compared to sham. . Injured Jagged1-50μg/ml infused animals displayed decrease in numbers of BrdU-positive cells in comparison to sham, injured only, and injured animals that received infusion of either vehicle or Notch1-2μg/ml (BrdU-positive cells indicated by arrows).

Cell Proliferation in IP granular zone 7 days post injury

Cell Proliferation in granular zone 7 days post injury

c

Cell Proliferation in IP hilus 7days post injury

Ipsilateral hilus

- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 2ug/ml
- FPI+Jagged1 50ug/ml

d

Cell Proliferation in CT hilus 7 days post injury

Contralateral hilus

- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 2ug/ml
- FPI+Jagged1 50ug/ml
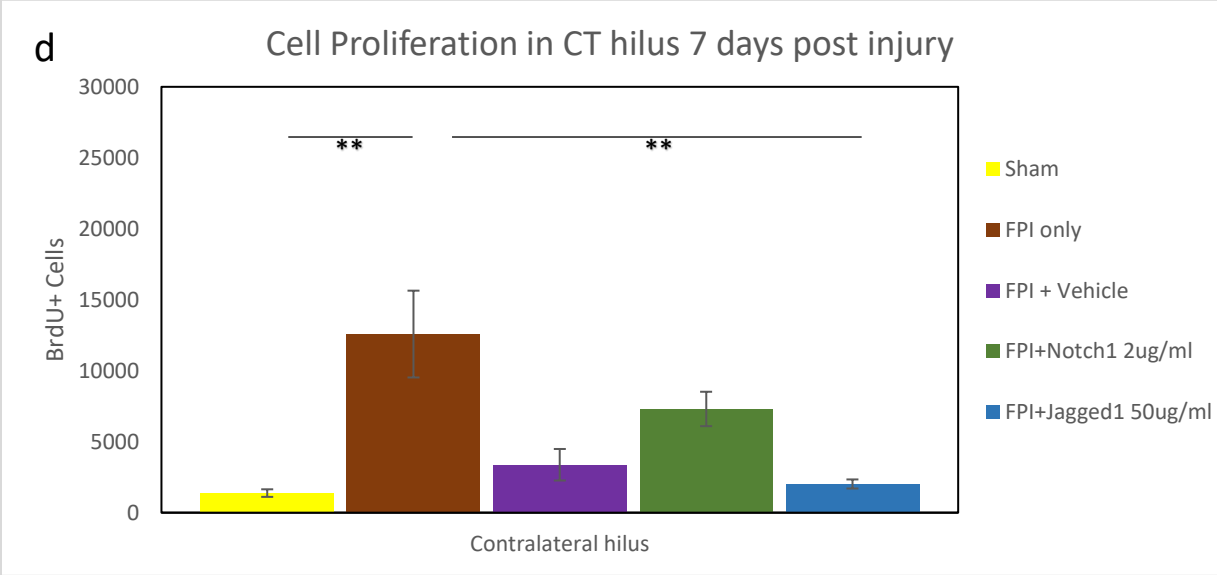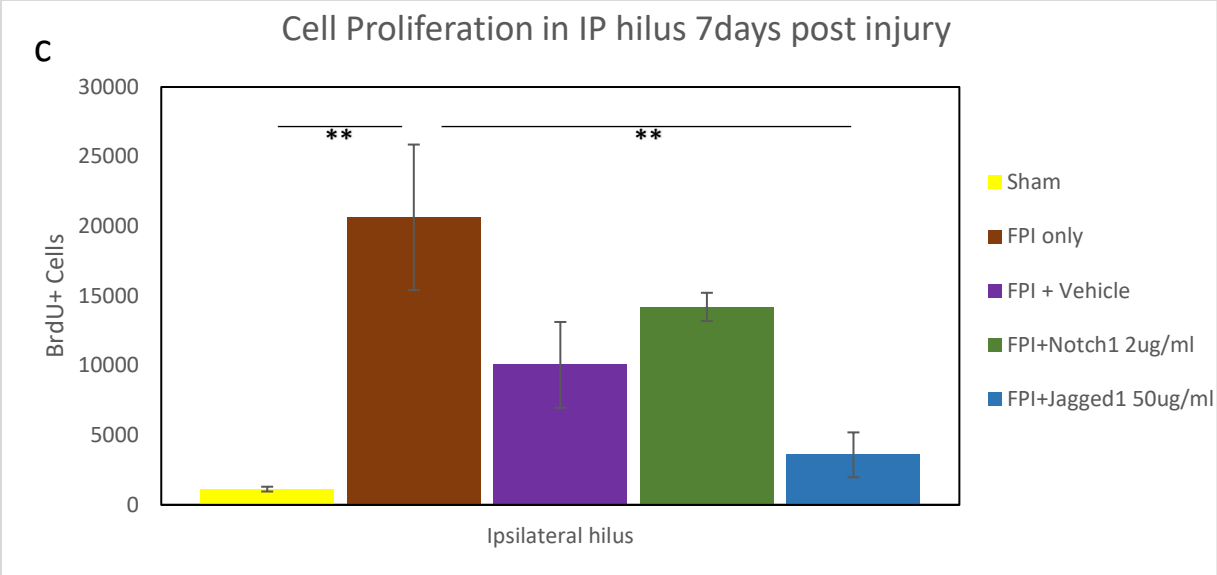
Figure 3.4. Quantification of BrdU positive cells in dentate gyrus for 7 days post-injury. (a) Quantitative analysis of cell proliferation in ipsilateral granular zone reveled that compared to sham animals, a significant increase in numbers of BrdU-positive cells were observed in injured only animals ($p < 0.01$), and the injured animals who received 7-day infusion of either vehicle ($p < 0.05$), or Notch1-2μg/ml ($p < 0.05$). In comparison to injured only animals, a significant decrease in numbers of BrdU-positive cells was observed in the injured animals with 7-day infusion of Jagged1-50μg/ml ($p < 0.01$). No significant differences were observed among injured vehicle infused animals and injured Notch agonist or antagonist infused animals. **(b)** Quantitative analysis of cell proliferation in contralateral granular zone reveled that compared to sham animals, a significant increase in numbers of BrdU-positive cells was observed in injured only animals ($p < 0.01$), and the injured animals who received 7-day Notch1-2μg/ml ($p < 0.05$). In comparison to injured only animals, a significant decrease in numbers of BrdU-positive cells was observed in the injured animals with 7-day infusion of Jagged1-50μg/ml ($p < 0.01$). No significant differences were observed among injured vehicle infused animals and injured Notch agonist or antagonist infused animals. **(c-d)** Quantitative analysis of cell proliferation in the hilus region reveled that compared to the sham animals, a significant increase in the numbers of BrdU-positive cells was observed in injured only animals ($p < 0.01$). Increase in the numbers of BrdU-positive cells was also observed in injured vehicle infused animals and injury induced Notch1-2μg/ml infused animals compared to sham, however, there was no statistical significance due to the big standard deviation within group. In comparison to injured only animals, a significant decrease in numbers of BrdU-positive cells was observed in the injured

Jagged1-50μg/ml infused animals ($p < 0.01$) in both the ipsilateral and contralateral hilus regions.

Effect of Notch inhibition or activation on generation of new neurons-DCX study

The proliferated cells in neurogenic regions often differentiate into neuronal cell lineage. The neuronal precursor cells and neuroblasts transiently express, a migration associated, microtubule binding protein called doublecortin (DCX) in adult mammalian brain (Brown et., al 2003). DCX is often used as a marker for neurogenesis for this exclusiveness expression of the protein in immature neurons (Tzeng et., al 2016). To examine the effect of Notch ligand infusion on hippocampal neurogenesis, DCX antibody was used to label newly generated neurons in dentate gyrus of sham, FPI only animals, and FPI animals that received either vehicle or Notch ligands infusion. The staining patterns and quantification of DCX-positive neurons were compared among the groups. Furthermore, DCX-positive neurons with each dendritic orientation (horizontal, vertical, and mixed) were also quantified and compared.

New neurons labeled with DCX were located at the granular zone and the hilus region of hippocampal dentate gyrus in both hemispheres. The immunostaining patterns demonstrated DCX-positive neurons were predominately localized in granule cell layers displaying dendritic extensions of various orientation. Increased numbers of DCX-positive neurons were observed in almost all injured animals compared to sham animals, with greater numbers of newly generated neurons expressed in injured only, injured vehicle infused, and injured Notch1-2μg/ml infused and 4μg/ml infused animals. However, decreased numbers of DCX-positive

neurons were observed in injured animal that received 7-day infusion of Jagged1-50µg/ml compared to sham (Figure.3.5. a-i)

Quantification analysis of DCX-positive neurons has shown: 1). In ipsilateral granular zone, the increase in the numbers of new neurons was significantly higher in injured vehicle infused animals ($p < 0.01$), injured Notch1-4µg/ml ($p < 0.01$), or Jagged1-25µg/ml ($p < 0.05$) infused animals in comparison to sham animals (Figure 3.6.a). In the contralateral granular zone (Figure 3.6.b), compared to sham, a significant increase in numbers of DCX-positive neurons were observed in injured animals that received 7-day infusion of either vehicle ($p < 0.05$) or Notch1-4µg/ml ($p < 0.05$) (Figure 3.7.b). However, no significant difference was observed between injured only animals, injured vehicle infused animals, or injured Notch ligand infused animals.

2). In the ipsilateral hilus region (Figure 3.6.c), in comparison to sham, a significant increase in the numbers of DCX-positive neurons was observed in injured animals that received 7-day infusion of either vehicle ($p < 0.01$), or Notch1-4µg/ml ($p < 0.05$). In comparison to injured vehicle infusion animals, injured animals that received 7-day infusion of Notch1-2µg/ml displayed a significant decrease in numbers of DCX-positive neurons ($p < 0.05$) (Figure 3.7.c). In the contralateral hilus region (Figure 3.6.d), in comparison to sham animals, a significant increase in numbers of DCX positive neurons were observed in injured animals that received 7-day infusion of either vehicle ($p < 0.01$) or Notch1-4µg/ml ($p < 0.01$). In comparison to Injured only animals, a significant increase was also observed in injured vehicle infused animals ($p < 0.01$) and injured Notch1-4µg/ml infused animals ($p < 0.01$) (Figure 3.7.d). No significant

difference was observed between injured vehicle infused animals and injured Notch ligand
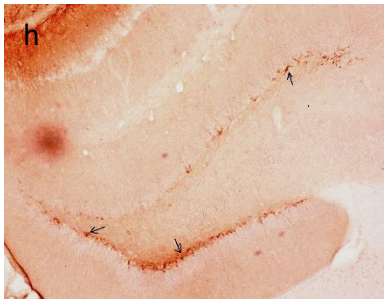
infused animals.

.
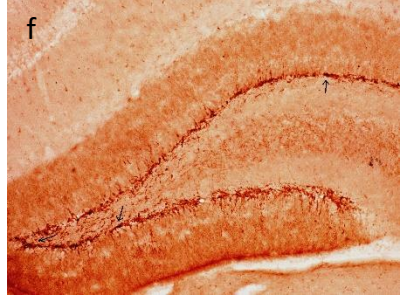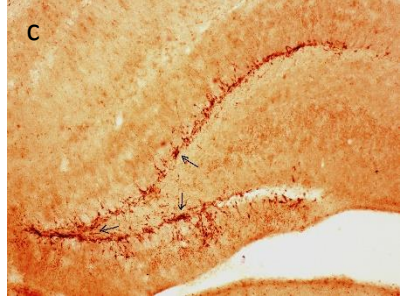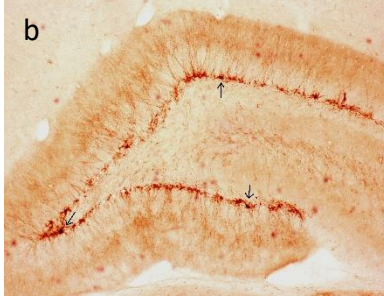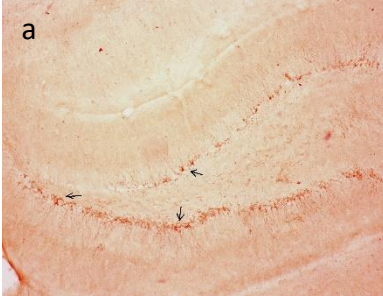
Figure 3.5. Generation of new neurons at 7 days post-injury. Images of coronal sections of ipsilateral dentate gyrus showing DCX positive neurons (indicated by arrows) including **(a)** sham animal, **(b)** an injured only animal, **(c)** an injured animal that received 7-day vehicle infusion, injured animals that received 7-day infusion of Notch1 agonist: **(d)** Notch1-0.5µg/ml, **(e)** Notch1-2µg/ml, **(f)** Notch1-4µg/ml, or Notch1 antagonist: **(g)** Jagged1-25µg/ml, **(h)** Jagged1-50µg/ml, **(i)** Jagged1-100µg/ml. Immunostaining patterns demonstrate newly generated neurons were predominately localized in granular zone displaying dendric extensions. Increased numbers of DCX-positive neurons were observed in the granular zone of almost all injury received animals compared to sham, except Jagged1 50 µg/ml infused animals, which displayed decrease in numbers of DCX-positive neurons. Greater numbers of DCX-positive neurons were observed in injured animals which received infusion of either vehicle or Notch 4 µg/ml (DCX-positive neurons indicated by arrow).

Generation of New Neurons in IP GZ 7 Days Post Injury

Generation of New Neurons in CT GZ 7 Days Post Injury

c Generation of New Neurons in IP hilus 7 Days Post Injury

Ipsilateral hilus

Legend:
- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

d Generation of New Neurons in CT hilus 7 Days Post Injury

Contralateral hilus

Legend:
- Sham
- FPI only
- FPI + Vehicle
- FPI+Notch1 0.5ug/ml
- FPI+Notch1 2ug/ml
- FPI+Notch1 4ug/ml
- FPI+Jagged1 25ug/ml
- FPI+Jagged1 50ug/ml
- FPI+Jagged1 100ug/ml

Figure 3.6. Quantification of newly generated neurons in dentate gyrus at 7 days post-injury.

(a) In the ipsilateral granular zone, compared to sham, a significant increase in the number of DCX-positive neurons was observed in injured animals that received 7-day infusion of either vehicle ($p < 0.01$), Notch1-4μg/ml ($p < 0.01$) or Jagged1-25μg/ml ($p < 0.05$). No significant difference was obse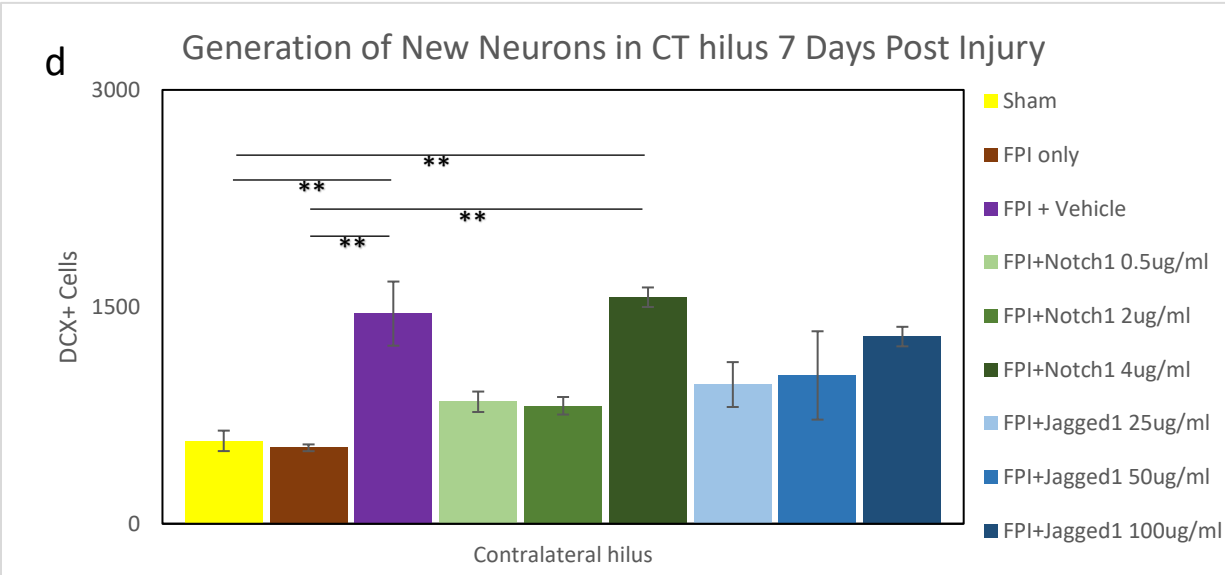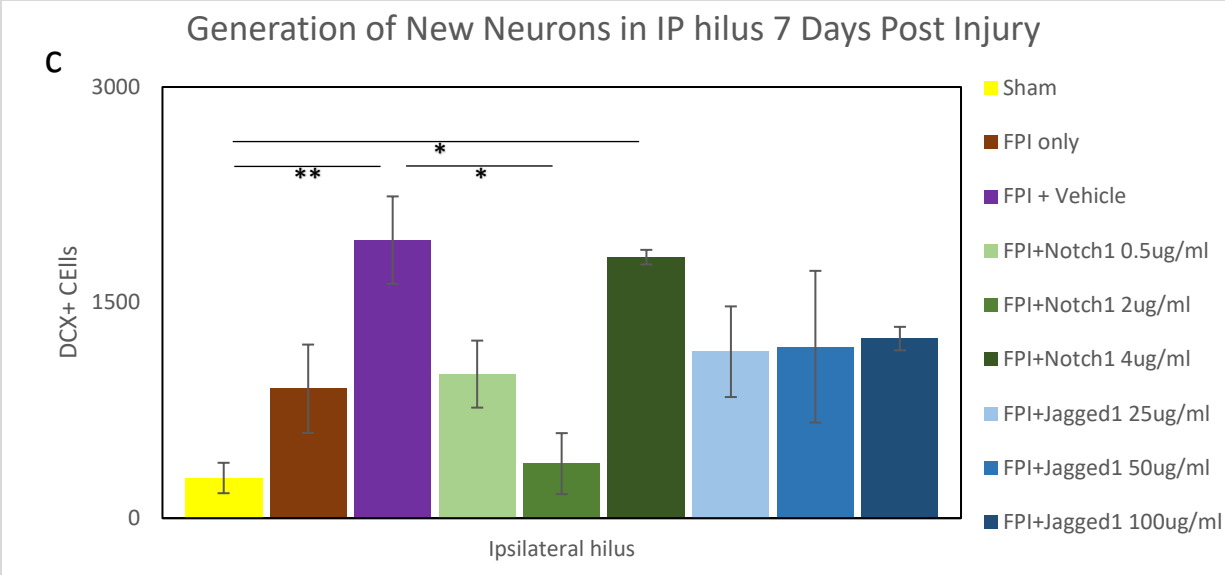rved between injured only animals, injured vehicle infused animals, or injured Notch ligand infused animals. **(b)** In the contralateral granular zone, compared to sham, a significant increase in the number of DCX-positive neurons was observed in injured animals that received 7-day infusion of either vehicle ($p < 0.05$) or Notch1-4μg/ml ($p < 0.05$). No significant difference was observed between injured only animals, injured vehicle infused animals, or injured Notch ligand infused animals. **(c)** In the ipsilateral hilus region, in comparison to sham, a significant increase in the numbers of DCX-positive neurons was observed in injured animals that received 7-day infusion of either vehicle ($p < 0.01$) or Notch1-4μg/ml ($p < 0.05$). In comparison to injured vehicle infusion animals, injured animals that received 7-day infusion of Notch1-2μg/ml displayed a significant decrease in numbers of DCX-positive neurons ($p < 0.05$). No significant difference was observed between injured only animals and injured animals that received infusion of either vehicle or Notch ligands. **(d)** In the contralateral hilus region, in comparison to sham animals, a significant increase in numbers of DCX positive neurons was observed in injured animals that received 7-day infusion of either vehicle ($p < 0.01$) or Notch1-4μg/ml ($p < 0.01$). In comparison to Injured only animals, a significant increase was also observed in injured vehicle infused animals ($p < 0.01$) and injured Notch1-4μg/ml infused animals ($p < 0.01$). No significant difference was observed between injured vehicle infused animals and injured Notch ligand infused animals.

Chapter 4- Discussion

Published studies have found that TBI enhances neural stem cell proliferation in the neurogenic regions, and this endogenous neurogenic response contribute to cognitive recovery following TBI (Sun et al., 2007). Unpublished study from our group has further found that TBI induces upregulation of Notch signaling pathway protein expression, and this is correspondent to neural stem cell proliferation observed following TBI. As Notch signaling is critical for neurogenesis in both developing and adult brain, we speculate that Notch pathway activation plays an important role in TBI-induced neurogenic response. In this study we utilized a Notch agonist and an antagonist to assess the role of Notch signaling in post-TBI neurogenesis in the hippocampus. We found that manipulation of Notch pathway activation can affect TBI-induced cell proliferation. Specifically, intraventricular infusion of Notch agonist (Notch1 antibody) for 7-day post-injury did not further augment injury-induced accumulated cell proliferation, however, 7-day infusion of Notch antagonist post-injury greatly reduced injury-induced cell proliferation. We also found that ambiguous dose related responses wherein Notch agonist dose with higher and lower doses induce opposite effect on cell proliferation indicating more complicated mechanism involved.

Studies have confirmed that cell proliferation persists throughout life in neurogenic niches such as SVZ and DG of mammalian and human brain (Boldrini et al., 2018, Gage 2000, Sun et al., 2016), and induction of proliferation and neurogenesis were observed in these neurogenic niche post TBI (Sun et al., 2005). Studies show the cell proliferation and differentiation in adult neurogenic niche is maintained by neural stem cell (NSC) in development and adulthood (Gage, 2000, Kreigstein, and Alvarez-Buylla, 2009). From the

embryonic development, it has been long known that, a highly conserved Notch signaling

pathway functions as a core signaling system for maintenance and cell fate decision of NSCs

(Artavanis-Tsakonas et al., 1999). Emerging evidences also support the involvement of Notch

signaling pathway in regulating adult NSCs, the Notch pathway proteins are expressed in

germinal zone of both embryonic and adult brains (Stump, 2002), and recent transgenic mice

study has confirmed the involvement of Notch signaling in regulating maintenance of postnatal

NSCs (Imayoshi et al., 2010). Moreover, Notch signaling pathway is also responsible for

regulating neurogenesis in neurogenic niches during development and adulthood (Zhang et al.,

2015).

The highly conserved cell to cell mediated canonical Notch signaling pathway is known

to be responsible for regulating NSCs in neurogenic niche due to its downstream effectors such

as *Hes* family, which is also known to regulate NSCs to take on either neuronal fate or maintain

proliferative state (Kageyama et al., 2007). Generally, Notch ligand-receptor binding activates

Notch signaling pathway and maintains NSCs into proliferative state, by upregulating *Hes1* that

is known to repress its own activity, it is known to induce neuronal cell differentiation by

default. While unbound Notch receptors are thought to be constantly internalized and recycled,

resulting the unrepressed Hes1 activity to induces cell differentiation (Kageyama et al., 2007,

Zhang et al, 2014). Emerging evidences support the role of Notch signaling pathway in

regulating NSC in neurogenic niches in development and adulthood, however the involvement

of Notch signaling pathway in injury induced NSC proliferation in the neurogenic niche is not

clear.

In the current study, we examined the effect of Notch pathway activation or inhibition on hippocampal neurogenesis and particularly assessed the optimal dose of the Notch agonist and antagonist on cell proliferation. We selected the doses based on published studies and used the cell proliferation marker Ki67 as the read out following 7-days infusion of the agonist or the antagonist. We found that cell proliferation in the DG of hippocampus at day 7 post-injury, was augmented by 7-day infusion of Notch agonists at the dose reported by other (Notch 1 -2ug/ml) and a further lower dose (Notch1-0.5μg/ml). This is consistent with ligand-activated Notch signaling downstream effects. However, a higher dose of Notch agonist (Notch 1-4μg/ml) infusion caused detrimental effect on injury induced cell proliferation in neurogenic niche, this suggests that the Notch1 signaling in regulating post-injury neurogenesis is more complicated than previously reported with over activation-inducing inhibition. As only a few published studies which explored Notch activation on neurogenesis using the same Notch1 antibody in other injury models all used Notch1 antibody at 2ug/ml (Sun et al., 2013,), and no literatures have reported the inhibitory effect of high dose Notch activation, our finding needs to be further confirmed. For Notch inhibitor, the dose of 50ug/ml was used by others (Sun et al., 2013, Wang et al., 2009), however, our Ki67 data found that 7-day infusion of Notch antagonist at lower dose of 25ug/ml significantly reduced injury-induced cell proliferation, whereas higher dose at 50ug/ml or 100ug/ml of Notch antagonist showed no effect on injury induced cell proliferation on 7th day, further suggesting a narrow window for pharmacological manipulation of Notch pathway activation. It is noticed that the Ki67 data only represented the snapshot of cell proliferation at the time of perfusion, meaning 7 days after injury. Our previous study using pausing BrdU-labeling has shown that LFPI-induced cell proliferation in rats lasts at

least for 7 days (Sun et al., 2005), our Ki67 data of TBI only group is in agreement with this observation. However, what is intrigue is that all other injury groups with intraventricular infusion except Notch agonist at 2ug/ml had lower number of Ki67+ cell counts compared to the injury only group. This data suggests that extra surgical manipulation such as implantation of cannula and infusion of materials into the lateral ventricle affects cell proliferative response in the DG. As our study only observed NSC proliferation in the DG, the other neurogenic region-the SVZ, which is directly in contact with the lateral ventricle, was not assessed, this speculation is not conclusive.

In pharmacological aspects, presence of ligand in high concentration generally means the better chance for ligands to bind to a receptor and able to induce its downstream effect. Although this is not always the case, the opposite effect that was produced between two Notch agonist doses (Notch1-2μg/ml) and (Notch1-4μg/ml) seems inexplicable. However, it is known that other Notch receptors are also expressed in adult hippocampus, Notch1 and Notch3 are expressed in dentate gyrus, and Notch2 is expressed in granular cell layer (Irvin et al., 2001). It is also reported that these receptors share Notch ligands such as Delta1, Jagged1, and Jagged2 which known agonist for Notch1 and Notch3 receptors (Shimizu, 2000). Luciferase reporter studies also revealed that the transcriptional activities are markedly different from each other and dependent on different promoters, also Notch1 and Notch3 activities were reduced by expression of Notch2, suggesting each Notch receptor has a diverse role in the downstream gene expression, and *Hes1* and *Hes5* gene expression maybe complexly dependent on various factors such as combination of Notch receptors (Shimizu, 2002). Perhaps 7-day infusion of higher concentration exogenous Notch1 agonist (Notch1-4μg/ml), Notch1 activation antibody

that exclusively detects & binds to Notch1 receptor only, may have led to overly occupied

Notch1 receptors, forcing other endogenous Notch ligands to bind to other Notch2, or Notch3

receptors, involuntarily activating them. Adding to the fact that Notch activity is already

elevated due to TBI (Tatsumi, 2010) and upregulation of Notch ligands from other intersecting

signaling pathways possibly upon onset of TBI (D'Souza et al., 2010, Hurlbut, 2007, Table 1.1)

these factors collectively could have created a mayhem of Notch activities between Notch1,

Notch2, Notch3 downstream expressions leading to the detrimental effect on injury induced

cell proliferation, since co-expression of Notch2 reduced activity of Notch1 and Notch3

(Shimizu, 2002).

For Notch1 antagonist dose response study, it is not clear why higher dose has no effect

on cell proliferation inhibition. The exogenous Notch antagonist, Jagged1 fusion protein binds

to all Notch receptors, possibly to Notch1 and Notch3 with higher affinity since Jagged1 is one

of known ligands for those receptors (Shimizu, 2000). Affinity between Jagged1 fc, Deltalike1,

Jagged1 and Jagged2 are unknown, but exogenous inhibitors such as Jagged1 fusion protein are

often engineered to possess higher affinity to receptors than other endogenous counterparts

inhibiting Notch1 and possibly Notch3. The interaction of downstream signaling pathways

between reduced activity of Notch1 and Notch3 and possible increased activity of Notch2

receptors are unknown.

To quantify accumulated cell proliferation and surviving cells in the DG of hippocampus,

we used BrdU as the marker. Due to the labor intensity and time limitation, we only quantified

the number of BrdU+ cells at one dose for both Notch agonist and antagonist. We found that

BrdU cell counts were increased compared to sham in all injury group except the Notch

antagonist treated group. This confirmed that there is an injury-induced cell proliferation in the DG during the 7-day period post-injury. However, infusion of Notch agonist does not further augment this injury effect. As TBI enhances Notch expression in the neurogenic regions, the Notch effect on NSC proliferation is probably already saturated, thus exogenous Notch activator would not have further effect.   The BrdU data has showed that 7-day infusion of Notch antagonist at 50μg/ml greatly reduced injury induced cell proliferation suggesting that Notch signaling is indeed involved in NSC proliferation following injury.

Notch signaling activation or inhibition on generation of new neurons in the DG of hippocampus was assessed by quantifying the number of DCX+ cells. We found that in correlation to cell proliferation, that animals groups with lower number of Ki67 cell counts had higher number of DCX+ cells (TBI-veh, TBI-Notch1-4ug/ml), whereas animals groups with higher Ki67+cell counts (TBI-only, TBI-Notch1-2ug/ml) had lower number of DCX+ cells in both the granular zone and hilus regions. This suggests that when NSC proliferation is increased, less cells are differentiated (Notch activation), whereas when NCS proliferation is inhibited, more cells are becoming differentiated (Notch inhibition). This confirms the important role of Notch in maintaining the proliferation pool of NSCs, as studies have reported enhanced neurogenesis (NSCs going on differentiation into neurons) in the neurogenic niche in the absence of Notch activity (Borghese et al., 2010, Chen et., al 2014, Falk et al., 2012, Wang et al., 2004).

Further work to complete the quantification of the number of BrdU+ cells at other two doses of Notch agonist and antagonist will provide better information about the dose response effect of Notch activation/inhibition on neurogenesis. Further studies involving various Notch agonists targeted for Notch1, Notch2, and Notch3, and antagonists for DSL ligands including

Delta-like1, Jagged1, and Jagged2 would allow closer examination of Notch signaling pathways involved with injury induced cell proliferation in neurogenic niche. Moreover, proliferated cells could be double labeled with cell proliferation marker and neuronal /glial markers to further investigate injury induced neuronal or glial cell fate induced by downstream effectors of each signaling pathways by Notch1, Notch2, or Notch3 receptors. Further, in combination of receptors to discover possible downstream interactions among Notch receptors that are expressed in the adult DG of hippocampus. This clarification could potentially allow precise pharmaceutical manipulation of brain's innate ability to repair and regenerate post-TBI.

Summary

Notch signaling pathway maybe involved in injury induced NSC cell proliferation in the neurogenic niche. Our data showed varying results confirming the involvement of Notch singling pathway in injury induced NSC cell proliferative response. However, some intriguing data indicated a more complicated interaction of other Notch receptors in neurogenic niches, which shares same ligands and possibly can produce different downstream effects depending on their own signaling mechanisms and effectors involved. Further studies and understanding of each Notch receptors could potentially allow precise pharmaceutical manipulation of brain's innate ability to repair and regenerate post-TBI.

Vita

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