Roles of 5HT1A receptor in CNS neurogenesis and ADAM21 in spinal cord injury.

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ROLES OF 5HT1A RECEPTOR IN CNS NEUROGENESIS
AND ADAM21 IN SPINAL CORD INJURY

By
Sheila Ann Arnold
B.A., University of Louisville, 2003
M.S., University of Louisville, 2006

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Doctorate of Philosophy

Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky

August 2011
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July 21, 2011

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DEDICATION

To God, all for your glory.

"The LORD is my rock, my fortress and my deliverer; my GOD is my rock, in whom I take refuge. He is my shield and the horn of my salvation, my stronghold."

(Psalms 18:2)

To Seth,

when life gives you lemons, God helps make them the best lemonade. Always look to God for guidance, as he will never lead you astray.
ACKNOWLEDGEMENTS

I would like to thank my mentor, Theo Hagg, for his guidance not only in science but also in life. I would also like to thank my committee members for their time and assistance in this journey over the last five years. Mostly, I would like to thank my entire family, especially my parents Charles Arnold, Karen Arnold, Marcia Potts, and Tom Potts, for always being there at a moment's notice to lend a helping hand, watch Seth, talk, or just listen. Lastly, I would like to thank my wonderful son who has been patient and understanding while working on this dissertation. I know that was more patience and understanding than most six year olds possess.
ABSTRACT

ROLES OF 5HT1A RECEPTOR IN CNS NEUROGENESIS
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July 21, 2011

These studies set out to identify strategies to rescue and repair the adult nervous system. First, we investigated the role of ciliary neurotrophic factor (CNTF) in 5HT1A receptor-induced neurogenesis in the rodent brain. Systemic treatment with an agonist, 8-OH-DPAT, increased neurogenesis only in rats and not mice, and only in one of the two neurogenic regions. This increase was not mediated by CNTF. These data suggest that translation of 5HT1A-based studies to human cell replacement therapies should be reconsidered. Secondly, the role of the plasticity-associated metalloprotease ADAM21 after spinal cord injury was investigated by comparing ADAM21-deficient mice to their wildtype littermates. No differences in behavioral or histology were found. However, a comprehensive metalloproteinase gene array revealed that ADAM21 regulates a cluster of inflammatory genes following injury. This leaves a potential for discovery of specific pharmaceutical ADAM21 inhibitors to reduce detrimental inflammatory processes following spinal cord injury.
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CHAPTER ONE

INTRODUCTION

General

Plasticity persist in the adult brain, mainly in two distinct regions known as the subventricular zone (SVZ) and the subgranular zone (SGZ). With the discovery of neural stem cells in the adult brain, the concept of neuronal replacement via engraftment of these cells into the damaged region has sparked excitement. However, in light of problems faced with engraftment (tumor growth, misguidance, cell death, cognitive impairment, and lack of sufficient functional recovery), many scientists have refocused their attention to neuroprotection in order to prevent the initial loss of neurons and regeneration using the endogenous cells to alter the neurogenic potential. The focus for regeneration in many neurodegenerative diseases stem from the idea that the newly born neurons in the SVZ and SGZ could replace the lost neuronal population under the correct conditions or in a special environment. Aside from prevention of the initial injury in spinal cord injury, there is a small window of opportunity for limited neuroprotection of the neurons that were spared in the initial injury. Secondary injury, which is caused by inflammation in response to the initial injury, causes further damage that could potentially be prevented thus neuroprotective. However, plasticity also occurs following spinal cord injury that could have the potential to either cause regeneration of the damaged or restore function to lost/damaged axons or it can cause increases in pain perception. This introduction will review topics about CNS neurogenesis, neurodegenerative diseases, the possible link between 5HT1A receptor induced
neurogenesis and CNTF. Additionally, there will be a brief introduction to spinal cord injury, the Metzincin superfamily, matrix metalloproteinases, ADAMs and the possible role in plasticity ADAM21 could have following spinal cord injury.

Neurogenesis Hypothesis

Neurogenesis

The thought that the brain is malleable or able to change is a relatively modern idea. Though proposed in 1890 by William James in The Principles of Psychology, proof of neurogenesis was not shown until Smart in 1961 found new neurons in the subependymal layer of mice. Altman and Das similarly found new neurons in the hippocampus of rats and showed dividing cells of not only glial origin but also proliferation of new neurons which continued throughout life in post-natal mammals refuting the dogma that neurogenesis ceased upon maturation (Smart, 1961, Altman and Das, 1965). Although these two papers are most commonly cited as being the first to discover neurogenesis in the adult brain, Smart references papers written by Opalski in 1934 and Kuhlenbeck in 1944 as referring to “the subependymal cell plate...a potential source of undifferentiated cells which may on occasion give rise to neoplasms.”

However, the idea of adult neurogenesis was still controversial even after other laboratories had replicated this phenomenon (Hinds, 1968b, a, Kaplan and Hinds, 1977). Eventually, it became accepted that new neurons are continually added to the brain circuitry throughout life. Neurogenesis in the adult mammalian brain is now widely
known to occur in two distinct and unique regions of the brain—the lateral wall of the ventricles, known as the subventricular zone (SVZ), and in the subgranular zone (SVZ) of the dente gyrus in the hippocampal formation (Smart, 1961, Altman and Das, 1966, Altman, 1969, Lois and Alvarez-Buylla, 1994, Eriksson et al., 1998, Goldman, 1998, Gage, 2000, Alvarez-Buylla and Lim, 2004, Hoglinger et al., 2004, Lie et al., 2004, Sanai et al., 2004, Ming and Song, 2005, Hagg, 2009, Kriegstein and Alvarez-Buylla, 2009, Suh et al., 2009, Ma et al., 2010, Soumier et al., 2010). In non-human primates, neurogenesis in the SGZ of the hippocampal formation of marmoset monkeys was found to be decreased with stress as was shown in other animals like the tree shrew and rat (Gould et al., 1997, Gould et al., 1998). Although neurogenesis was found in the SVZ and SGZ in some animals, the absence of neurogenesis in the adult human was still central dogma until relatively recent years. Now it is known that neurogenesis does occur in humans as was shown in the postmortem hippocampal formation of the brain in adult human cancer patients that received BrdU as a way to track the proliferation rate of their cancer (Eriksson et al., 1998). However, there are conflicting reports of the presence or lack thereof of a rostral migratory stream in humans (Bedard and Parent, 2004, Sanai et al., 2004, Curtis et al., 2007b, Sanai et al., 2007).

In the SVZ, there has been controversy over what cell type is the neural stem cell. A report published in 1999 indicated that ependymal cells were responsible for the generation of new neurons which migrate to the olfactory bulb (Johansson et al., 1999). However, shortly thereafter, a study was published showing data in support of type B cells (SVZ astrocytes) and not ependymal cells are the cell type of origin of the neural stem cells in the SVZ (Doetsch et al., 1999). These type B cells give rise to both type A (migrating neuroblasts) and type C (precursor cells). The concept of the SVZ astrocytes is the more widely accepted explanation for the origin of the neural stem cells in the SVZ; however, this controversy has yet to be fully resolved since there is a possibility
that these two populations in question are one and the same (Chojnacki et al., 2009). Likewise, in the hippocampal formation there are type 1 and type 2 which are similar to the type B and type C cell, respectively, in the SVZ (Zhao et al., 2008, Suh et al., 2009). Both type B and type 1 cells resemble radial glial cells found in development. The finding that astrocytes are the neural stem cells responsible for the neurogenesis in the SVZ and SGZ means that in cases of neurodegenerative diseases, the astrocytes which are spared can possibly be exploited in order to replace neurons that are being lost.

In rodents, the majority of adult neurogenesis occurs in the SVZ at a rate of approximately 10 times more than in the SGZ (Lois and Alvarez-Buylla, 1994, Cameron and Mckay, 2001, Winner et al., 2002, Rao and Shetty, 2004, Abrous et al., 2005). The newly born neuroblasts migrate from the SVZ along the rostral migratory stream to the olfactory bulb (OB) and from the SGZ into the neighboring granule cell layer, respectively, where they either become integrated as neurons or die (Cameron et al., 1993, Luskin, 1993, Lois and Alvarez-Buylla, 1994, Morshead et al., 1998, Gould et al., 1999, Hastings and Gould, 1999, Dayer et al., 2003). The new neurons from the SVZ migrate to the olfactory bulb become 90% GABAergic and 10% dopaminergic neurons while the new neurons in the SGZ become excitatory glutamatergic granule neurons (Knobloch and Jessberger, 2011).

**Neurodegenerative diseases**

Neurogenesis creates and sustains hope for people who have neurodegenerative diseases such as Huntington’s disease, Parkinson’s disease, ALS, etc. Although these diseases are different in their origination/development, they all share a common characteristic—loss of neurons. The neuronal population that is lost is different in these diseases. If the endogenous neurogenesis can be directed or focus on
replenishment of neurons lost over the progression of the disease then maybe we can attenuate the progression or quite possibly find a treatment/cure for these diseases. However, first the biology of neurogenesis and its endogenous regulation must first be explored before we can pharmacologically manipulate it in various disease states as opposed to engraftment of neural stem cells. Pharmaceutical intervention has less ethical and safety hurdles to overcome before it can be utilized in neurodegenerative diseases. There is a multitude of safety and ethical concerns with grafting neural stem cells some of which are cognitive impairment, tumor development, migration to unintended areas, undesirable differentiation, and genetic and epigenetic changes in the cell population (Mathews et al., 2008). In about 15% of Parkinson's patients, neural transplantation induced dyskinesia and over time cells inside the neural engraftment take on the pathology of Parkinson's disease (Brundin et al., 2010, Lane et al., 2010). Most concerning is the possibility of the donor cells developing into tumors as was the case with a boy who receive intrathecal and intracerebral injection of fetal human neural stem cells and later developed a donor-derived brain tumor (Amariglio et al., 2009). For these reasons, many scientists have switched their focus from engraftment to stimulation of endogenous targets that promote neurogenesis which now appears to be a more practical approach in light of the growing controversies involving engraftment in humans or human-non-human primates engraftments (Greene et al., 2005, Mathews et al., 2008).

**Ciliary Neurotrophic Factor**

Many endogenous molecules have been found to regulate adult CNS neurogenesis (Lie et al., 2004, Hagg, 2005, Ming and Song, 2005, Hagg, 2009, Suh et al., 2009, Bath and Lee, 2010). Endogenous ciliary neurotrophic factor (CNTF) is
responsible for approximately 30% of neurogenesis in the SVZ as shown in CNTF−/− mice and by blocking CNTF antibody injections (Emsley and Hagg, 2003, Yang et al., 2008). We have focused our attention on CNTF because it is produced almost exclusively in the nervous system (Stockli et al., 1989, Ip, 1998) and would therefore be a good target for indirect stimulation of neurogenesis by administration of systemic small molecule drugs. By indirectly stimulating CNTF in the specific environment in which we wish to induce a neurogenic response, we can avoid issues with low CNS bioavailability and undesirable side effects associated with systemically delivered CNTF such as fever, fatigue, and weight loss (Miller et al., 1996, Thoenen and Sendtner, 2002). In culture, CNTF drives precursor cells to differentiate into astrocytes (Bonni et al., 1997).

However, an intraventricular injection of CNTF increased proliferation in the SVZ, but did not seem to affect the normal neuronal cell fate (Emsley and Hagg, 2003). Thus, CNTF may be a good target for indirect stimulation to make more new neurons in the adult CNS.

Previously, we have shown that dopamine from the nigrostriatal projections induce neurogenesis via D2 receptors and that this is entirely mediated by astroglial CNTF (Baker et al., 2004, Yang et al., 2008). This is consistent with the fact that D2 receptors inhibit cAMP (Vallar and Meldolesi, 1989, Kalkman et al., 2003), whereas CNTF is under negative regulation by cAMP in astrocytes (Carroll et al., 1993, Rudge et al., 1994). Astrocytes are our target instead of neurons because the astrocytes are spared in neurodegenerative diseases whereas the neurons are dead, dying or dysfunctional therefore they would not be a good target for neuroregeneration. With further research, the astrocytes can be pharmacologically induced to become a factory for endogenous production of various factors such as CNTF. In order to decrease systemic side effects of the drugs used to stimulate the D2 receptor, a low dose combination with a similar acting drug might be more beneficial.
Serotonin

The serotonin receptors, 5-hydroxytryptamin receptor or 5HT receptors are a group of G coupled protein receptors (excitatory and inhibitory) with the exception of 5HT3 which is a ligand-gated Na+ and K+ cation channel (Maricq et al., 1991). Serotonin receptors include 7 different families 1, 2, 3, 4, 5, 6, and 7 with subtypes signified with a, b, c, etc. (Hoyer et al., 2002, Savitz et al., 2009). 5HT receptors have a wide array of functions ranging from modulation of mood (anxiety and depression), cognition, learning, memory, aggression, appetite, migraines, REM sleep, pain processing, and release of a number of neurotransmitters and hormones. 5HT receptors, in the periphery, facilitate smooth muscle contraction, platelet aggregation, gastrointestinal function, and impact upon pain processing. The 5HT1 receptor family is characterized as a seven transmembrane domain receptor which upon activation, couples to regulatory G proteins that negatively regulates/inhibit adenylyl cyclase (AC) resulting in a decrease of cAMP (Mendez et al., 1999, Azmitia, 2001, Kalkman et al., 2003, Vanhoose et al., 2004). We would expect this decreased cAMP to increase CNTF as we have seen with the D2 receptor leading to an increase in neurogenesis (Yang et al., 2008).

Of this family, we have chosen to pursue serotonin receptor 5HT1A because of its regulatory role in decreasing cAMP and its location in the two neurogenic niches of the brain. When most people talk about the 5HT1A receptor they are referring to the neuronal 5HT1A receptor; however, the 5HT1A receptors were also found to be expressed on subset of GFAP+ cells in the lateral septal nucleus and the polymorphic layer of the DG (Whitaker-Azmitia et al., 1993). In addition to astrocytes with 5HT1A receptors, neuronal 5HT1A is found in the serotonergic projections from the raphe nuclei to the SVZ overlap with dopaminergic fibers in the SVZ where neurogenesis occurs and where a high expression of CNTF is found; likewise, in the hippocampus, neuronal
5HT1A is located in the soma and dendrites of large interneurons (Azmitia et al., 1992). It is important to know the location and type of neuronal 5HT1A receptor since the autoreceptors found in the serotonergic projections from the raphe nuclei to the SVZ have the potential to inhibit or “put the brakes on” serotonin induced activity and their regulation can be affected by 5HT1A agonist; however, the 5HT1A receptors in the hippocampal formation are not affected by agonist treatment as they are heteroreceptors (Riad et al., 2001). Interestingly, 5HT1A receptor is found in highest abundance in astrocytes in the two regions of the brain where neurogenesis occurs throughout life. The 5HT1A receptor is found in ninety percent of the astrocytes that are located in the lateral septa which is adjacent to the subventricular zone; however the greatest abundance of the 5HT1A receptor is in the polymorphic layer or hilus of the DG (Whitaker-Azmitia et al., 1993).

**Pharmacological stimulation of serotonin receptors and neurogenesis**

Depression is thought to cause a decrease in neurogenesis or vice versa, but the exact link between these two is still unclear (Kempermann et al., 2008, Thomas and Peterson, 2008). Furthermore, neurogenesis as an indicator for effective antidepressant activity of serotonin is still under debate. In 129Sv/Ev mice, the antidepressant effects of Fluoxetine was seen to increase neurogenesis; however, within the same lab this finding could not be replicated with BALB/cJ mice (Santarelli et al., 2003, Holick et al., 2008) or BALB/cJ and C57BL/6J mice in another laboratory (Navailles et al., 2008). Serotonin or activation of the serotonin receptor has been shown to increase neurogenesis in both the SVZ and SGZ in adult rats (Brezun and Daszuta, 1999, Brezun and Daszuta, 2000, Banasr et al., 2004, Daszuta et al., 2005, Huang and Herbert, 2005, Zhang et al., 2009, Soumier et al., 2010). Additionally, a landmark study (Santarelli et
al., 2003) showed hippocampal neurogenesis in adult mice, and the effects of antidepressants, are regulated specifically through the 5HT1A receptor, using 5HT1A-/mice and 8-OH-DPAT, which is a selective agonist for the 5HT1A receptor (Middlemiss and Fozard, 1983). In Chapter 2, our studies into the role of serotonin 1a agonist, 8-OH-DPAT, in regulating CNTF and adult neurogenesis in the SVZ and SGZ of mice and rats will be discussed.

**Spinal Cord Injury**

Spinal cord injury begins with the shear mechanical injury which causes tissue damage, i.e., neuronal and glial death, damage to axons, and destruction of blood vessels at the injury site. The initial insult is just the beginning of the pathology that ensues afterwards. Subsequently, a secondary injury occurs due to inflammation in response to the tissue damage. This inflammation has been shown to have both detrimental and beneficial properties (Donnelly and Popovich, 2008, Kigerl et al., 2009). This inflammation can continue chronically after injury (Fleming et al., 2006). Within two weeks a glial scarring begins to form around the epicenter isolating it from the healthier tissue (Berry et al., 1983).

The initial inflammatory response begins within an hour of the primary injury as indicated by a massive rise in TNFα mRNA (Harrington et al., 2005) along with IL-6 and IL-1β mRNA (Donnelly and Popovich, 2008). Furthermore, in mice, astrocytes are thought to be responsible for the initiation of the inflammatory response (Pineau et al., 2010). It is these glial cells that can release cytokines such as TNFα, a key player in the synaptic plasticity, inflammatory pain, and allodynia (Peng et al., 2006, Zhang et al., 2011b). Several ADAMs (a disintegrin and metalloproteinase) such as ADAM10, ADAM17 (aka TACE), and ADAM19 are known to be involved in the ectodomain
sheding of TNFα (Hooper et al., 2005). MMP9 (matrix metalloproteinase 9) plays a detrimental role and a general metalloproteinase inhibitor greatly improve outcomes following spinal cord injury (Noble et al., 2002). Metalloproteinases are central regulators of tissue remodeling, inflammation, plasticity, and signal transduction. In the CNS, metalloproteinases have implications in a vast array of diseases to include cerebral ischemia, spinal cord injury, multiple sclerosis, epilepsy, neuropsychiatric disorders, and neurodegenerative disorders (Rivera et al., 2010). Although some studies have been done on a select few metalloproteinases, they are highly under studied in the CNS, particularly, the ADAMs family. Therefore, they make a great topic for further investigation.

![Diagram of Wildtype and ADAM21 Hypothesis](image)

### The Metzincin Superfamily

The metzincin superfamily is comprised of several families, two of which are the Matrixins and Adamalysin families (Bode et al., 1993, Gomis-Ruth, 2009). The metzincin superfamily consists of multi-domain proteins that are named for the conserved Methionine in the zinc dependent catalytic site of the metalloproteinase which
contains an extended zinc binding motif (HEXXHXXGXXH) followed by the highly conserved "Met-turn" (a 1,4-β-turn found directly below the zinc binding site which contains Methionine in the 3 position) (Bode et al., 1993, Stocker and Bode, 1995, Stocker et al., 1995, Gomis-Ruth, 2009, Tallant et al., 2010). There are four endogenous inhibitors of metalloproteinases, tissue inhibitors of metalloproteinases (TIMP1-4), which can modulate the activity of the metalloproteinases (Brew and Nagase, 2010). The Matrixin family is comprised of several subfamilies of matrix metalloproteinases (MMPs) which include collagenases, gelatinases, membrane-type MMP, stromelysins, matrilysins, enamelysins, metalloelastases and others (Vu and Werb, 2000). The Adamalysin family is comprised four subfamilies: ADAM proteins (a disintegrin and metalloproteinase), ADAMTS proteins (a disintegrin and metalloproteinase with thrombospondin motif), ADAMTSL proteins (with thrombospondin-like motif), and SVMP proteins (snake venom metalloproteinase) (Stocker et al., 1995, Rivera et al., 2010).

**ADAM proteins**

The ADAM proteins contain a prodomain, metalloproteinase domain, disintegrin domain, cysteine rich region, EGF-like repeat, transmembrane domain, and cytoplasmic tail (Wolfsberg et al., 1995, Yang et al., 2006, Edwards et al., 2008, Klein and Bischoff, 2011). The ADAMs are most widely known for their sheddase activity which cleaves off the ectodomain of a membrane bound protein and releasing it into the extracellular space. The ADAMS family includes a group of enzymes known as α-secretases that cleave amyloid precursor protein. It has been suggested that ADAM9, ADAM10, and ADAM17 may form a complex that is associated with decreases of amyloid β peptide (Aβ), thus reducing plaque formation in the normal brain (Asai et al., 2003, Postina et al.,
Ectodomain shedding by ADAMs and other sheddases is used for releasing signaling molecules, cell adhesion molecules, receptors, and cleavage of other extracellular proteins (Hooper et al., 2005). This gives ADAMs the ability to regulate gene transcription, signaling and cellular adhesion. ADAM17 was the first ADAM in which a substrate was found and has since been the main focus among ADAMs due to its sheddase activity on TNFα (Black et al., 1997, Moss et al., 1997). Following spinal cord injury, TNFα is increased within an hour whereas TNFR1 does not increase in the spinal fluid until 3 to 6 hours after initial insult (Harrington et al., 2005). TNFα induces expression of ADAM8 which cleaves TNFα receptor 1 (TNFR1), TNFR1 irreversibly binds free TNFα resulting in feedback inhibition (Bartsch et al., 2010). Our lab has shown ADAM8 expression to increase in endothelial cells and be involved in angiogenesis following spinal cord injury (Mahoney et al., 2009). A substrate of ADAM10 is fractalkine (CX3CL1), a chemokine that induces inflammation and leukocyte migration (Hundhausen et al., 2007); it can be released from neurons to contribute to the pain pathway as shown by intrathecal injection of fractalkine resulting in thermal and mechanical allodynia (Milligan et al., 2004, Milligan et al., 2005). This substrate is common between ADAM10 and ADAM17; however, ADAM10 has constitutive activity towards fractalkine whereas ADAM17’s activity is inducible (Garton et al., 2001). After spinal cord injury the metalloproteinase domain of ADAMs can play a diverse role in the regulation of many processes including inflammation and pain development.

The metalloproteinase domain of ADAM proteins is confined by the pro-domain containing a cysteine switch rendering it inactive until cleavage of the prodomain occurs. ADAM8 and ADAM28 have been shown to have autocatalytic activity, removing the prodomain without the aid of another protein (Howard et al., 2000, Schlomann et al., 2002). In humans, several ADAMs, ADAM2, ADAM11, ADAM18, ADAM22, ADAM23, and ADAM32, are predicted to have an inactive metalloprotease domain due to the
absence of a the Zn-binding motif seen in other ADAMs (Andreini et al., 2005, Hooper et al., 2005) which would suggest that in those ADAMs, the primary function of the protein would most likely involve cell-cell or cell-matrix interaction via integrins binding to the disintegrin domain.

Next to the metalloproteinase domain, ADAMs contain a characteristic disintegrin domain found only in snake venom metalloproteinases (SVMP) and ADAMs. The disintegrin domain was first found in SVMP; the disintegrin domain binds integrins on platelets in order to prevent (thus, dis-integrin) fibrinogen binding resulting in anticoagulant properties (Gould et al., 1990). This type of domain is unique in transmembrane proteins due to the combination with the metalloproteinase and the disintegrin loop. The disintegrin loop found in ADAMs and PIII SVMP have a distinctive alignment of the cysteine residues linked by disulfide bonds (Gould et al., 1990, Calvete et al., 2003, Hooper et al., 2005). With the exception of ADAM15, none of the ADAMs contain a traditional RGD ligand binding site in the disintegrin domain (Kratzschmar et al., 1996), but the disintegrin loop structure (canonical disintegrin loop motif; CRXXCDXXEXC) containing alternative integrin binding sequence (mostly ECD or XCD) are also capable of integrin binding (Bigler et al., 2000, Zhu et al., 2000, Evans, 2001, Hooper et al., 2005, Lu et al., 2007).

As mentioned above, ADAM8 is present in endothelial cells and thought to be involved in angiogenesis following spinal cord injury, but whether the metalloproteinase or the disintegrin domain is involved in the angiogenic response is not yet clear (Mahoney et al., 2009). After an injury to the brain, a beta3-containing integrin found on astrocytes binds to Thy-1 resulting in conversion of astrocytes into reactive astrocytes (Avalos et al., 2004). Additionally, ADAM8 and ADAM33 have been associated with the inflammatory asthma although the mechanism of action is not clear (King et al., 2004, Haitchi et al., 2005, Jie et al., 2009, Koller et al., 2009). If we could elucidate the
mechanism by which these molecules participate in the disease process, we could apply that knowledge to other systems as well. Moreover, the unique combinations of these various domains make ADAMs a particularly good target for therapeutic drug intervention. Currently, selectivity of metalloproteinase inhibitors is a problem with the high degree of homology at the catalytic site (Fisher and Mobashery, 2006, Corbitt et al., 2007, Jacobsen et al., 2010). However, some degree of selectivity was achieved with Gl254023X which inhibits ADAM17 and ADAM10, but is preferential to the constitutive activity of cleavage of fractalkine by ADAM10 (Ludwig et al., 2005). For the disintegrin, small peptides that mimic the disintegrin loop can be used as a competitive antagonist similar to the study with a small peptide resembling the ADAM8 disintegrin binding domain which resulted in enhanced protection of experimentally induced autoimmune diseases (Schluesener, 1998). Therefore, drug development against the metalloproteinase and disintegrin domain holds promise for later clinical use.

Metalloproteinases in Spinal cord injury

Spinal cord injury has not been widely investigated in terms of ADAM protein involvement. Apart from expression data, the literature on ADAM proteins following a spinal cord injury thus far is limited to our lab’s finding that ADAM8 has a potential role in angiogenesis with selective up-regulation localized in the endothelial cells of blood vessels following spinal cord injury (Mahoney et al., 2009). In an unpublished subsequent study, we found no overt phenotype following a contusive spinal cord injury in ADAM8 deficient mice. Unlike ADAMs, a few MMPs have been found to play a significant role in resulting pathology after a spinal cord injury (Zhang et al., 2011). For example, MMP9 has been found to be up regulated after spinal cord injury within 24 hours after spinal cord injury and deletion of MMP9 lead to an increase in functional
recovery and reduced inflammation due to a reduced barrier disruption (de Castro et al., 2000, Noble et al., 2002). Whereas, MMP2 expression was delayed compared to MMP9 expression and did not increase until day 5 and in MMP2 deficient mice there was an compensatory increase in MMP9, decreased white matter sparing, impaired functional recovery and increased reactive astrogliosis (de Castro et al., 2000, Hsu et al., 2006). MMP9 and MMP2 give an example of how a metalloproteinase can be deleterious or beneficial. Due to the similarities in the metalloproteinase domains of MMPs and ADAMs, we can hypothesize that the metalloproteinase activity of any ADAM would have a potential beneficial and/or detrimental role in the pathological events following spinal cord injury. ADAM21 contains a catalytically active metalloproteinase (Hooft van Huijsduijnen, 1998) and is found to be present in highly plastic regions in the developing and adult brain such radial glia lineage cells in the neurogenic niche of the subventricular zone and in new primary olfactory neurons and their growing axons (Yang et al., 2005). After spinal cord injury there is a shift in the balance of excitatory and inhibitory innervation due to the damage or death to the neurons. In addition, as the inflammatory processes begin astrocytes increase and can become reactive releasing excess modulatory factors. This can exacerbate the balance of excitatory and inhibitory signals leading to sprouting of pain fiber into the area of the spinal cord responsible for responses to non-noxious stimuli (Kuner, 2010). Since ADAM21 is present where noxious pain fibers reside in the dorsal horn of the spinal cord, they are likely to be involved in the inflammation or plasticity that results after insult to the spinal cord. The localization of ADAM21 in the adult CNS leads to the possibility that ADAM21 has a role in coordinating the repair/migration processes that follow spinal cord injury. Therefore, inhibition of ADAM21 could be important to preventing chronic pain or inflammation after spinal cord injury.
In the study in Chapter 3, we have used ADAM21-deficient mice to examine the role of ADAM21 following a contusive spinal cord injury in locomotion, balance, thermal nociception, as well as histological outcomes: spared white matter, CGRP and GFAP at the epicenter and the penumbra. In Chapter 4, we investigated the possible compensation of other metalloproteinases and the regulatory role of ADAM21 following spinal cord injury in ADAM21-deficient mice. Chapter 5 discusses the implications of the findings from Chapter 2, 3, and 4 and their potential implications to humans.
CHAPTER TWO

SEROTONIN 1A RECEPTOR INCREASES SPECIES- AND REGION-SELECTIVE ADULT CNS NEUROGENESIS, BUT NOT THROUGH CNTF

INTRODUCTION

Neurogenesis in the adult mammalian brain occurs predominantly in the SVZ of the lateral ventricles and the SGZ of the hippocampal dentate gyrus (Ming and Song, 2005, Hagg, 2009, Kriegstein and Alvarez-Buylla, 2009, Suh et al., 2009, Soumier et al., 2010). SVZ neuroblasts migrate along the rostral migratory stream to the olfactory bulb and SGZ neuroblasts migrate into the neighboring granule cell layer where they become integrated neurons or die (Luskin, 1993, Lois and Alvarez-Buylla, 1994, Winner et al., 2002).

Many endogenous molecules regulate adult CNS neurogenesis (Lie et al., 2004, Hagg, 2005, Ming and Song, 2005, Hagg, 2009, Suh et al., 2009, Bath and Lee, 2010). CNTF is responsible for ~30% of SVZ neurogenesis as shown in CNTF/-/- mice and by antibody injections (Emsley and Hagg, 2003, Yang et al., 2008). We study CNTF because it is produced almost exclusively in the nervous system (Stockli et al., 1989, Ip, 1998) and would therefore be a good target for indirect stimulation of neurogenesis by systemic small molecule drugs. We found that nigrostriatal projections induce neurogenesis via D2 dopamine receptors which is entirely mediated by astroglial CNTF (Baker et al., 2004, Yang et al., 2008). This is consistent with the fact that D2 receptors inhibit cAMP in astrocytes (Vallar and Meldolesi, 1989, Kalkman et al., 2003), whereas CNTF expression is inhibited by cAMP in astrocytes (Carroll et al., 1993, Rudge et al.,
1994). CNTF is predominately produced by astrocytes (Sendtner et al., 1994, Dallner et al., 2002) which are spared in most neurodegenerative diseases. Therefore, they are ideal pharmacological targets to serve as CNTF “factories” to enhance endogenous neurogenesis. This would also circumvent the peripheral side effects and low bioavailability seen with systemic administration of CNTF (Thoenen and Sendtner, 2002).

We wanted to find additional cAMP-inhibiting drugs to increase CNTF, as they could be combined with low doses of D2 agonists to reduce systemic drug doses and, consequently, side effects. 5HT1A is expressed on a subset of astroglia throughout the brain, in addition to neurons in the raphe nuclei (Whitaker-Azmitia et al., 1993). Their activation decreases cAMP levels (Mendez et al., 1999, Azmitia, 2001, Kalkman et al., 2003, Vanhoose et al., 2004). Colocalization of 5HT1A and GFAP is among the highest in the polymorphic layer of the dentate gyrus (Whitaker-Azmitia et al., 1993). Interestingly, serotonergic and dopaminergic projections to the SVZ overlap where neurogenesis and high expression of CNTF are located (Hagg, 2005). Activation of 5HT1A increases neurogenesis in the SVZ and SGZ in rats (Brezun and Daszuta, 1999, Banasr et al., 2004, Huang and Herbert, 2005, Soumier et al., 2010). A landmark study (Santarelli et al., 2003) showed that hippocampal neurogenesis in 129SvEv mice and the effects of antidepressants are regulated through 5HT1A, using 5HT1A-/- mice and 8-OH-DPAT. Acute but not chronic 8-OH-DPAT treatment increases SGZ proliferation in C57BL/6J mice (Klempin et al., 2010). Here, we tested whether CNTF mediates the neurogenic effects of 8-OH-DPAT in the SVZ and SGZ of adult C57BL/6J and 129SvEv mice, as compared to rats.
MATERIAL AND METHODS

Animals

A total of 116 mice were used, i.e., 64 C57BL/6J adult male mice (8-12 wks, 20-30g, stock # 000664, Jackson Laboratory, Bar Harbor, ME, USA), 40 129SvEv adult male mice (model # 129SVE-M [129S6/SvEvTac], 11 wks, 20-30 g, Taconic, Hudson, NY, USA), and 12 wild type background of our CNTF mice (essentially C57BL/6) were used. In addition, 10 adult male Sprague-Dawley rats (280-350g, Harlan, Indianapolis, IN) were used. The average weight of the animals in each experimental group was the same. All invasive procedures in mice were performed under deep anesthesia obtained by an intraperitoneal injection of 0.4 mg/g body weight Avertin (2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline, Sigma-Aldrich, St Louis, MO, USA). Rats were anesthetized with an intramuscular injection of a mixture (62.5 µg/g weight of the animal) containing 5 ml of ketamine (500 mg/ml, Hospira, Lake Forest, IL), 0.5 ml of acepromazine (10 mg/ml, ButlerSchein, Dublin, OH), and 1.2 ml xylazine (20 mg/ml, Akorn, Decantur, IL) diluted in 13.3 ml of 0.9% saline. All animal procedures were performed according to University of Louisville Institutional Animal Care and Use Committee protocols and the National Institutes of Health guidelines.

5HT1A agonist treatment and BrdU injection protocols

The animals were given saline or (R)-(+)8-Hydroxy-DPAT dissolved in saline (1 mg/kg/day, Cat # H-140; Sigma-Aldrich, St. Louis, MO), a full and specific 5HT1A agonist and active enantiomer of (±)-8-hydroxy-DPAT. It was administered either i.p. once daily for 3 days or s.c. via Alzet pump (Cat # 1003D with a flow rate of 1 µl/day for 3 days, or Cat # 1002 with a flow rate of 0.25 µl/day for 14 days or Cat #: 1004 with a flow rate of 0.11 µl/day for 28 days; Durect Corp., Cupertino, CA). The 14 day pumps
were replace on day 14 with a new 14-day pump with fresh reagent for the 28-day infusion in C57BL/6J mice. 5-Bromo-2'-deoxyuridine (BrdU; Cat #: B5002, Sigma-Aldrich, St. Louis, MO) was injected at 50 mg/kg twice daily for 3 days with the final injection 2 hrs before processing for histology.

**mRNA measurements by quantitative real-time reverse transcription-PCR (qPCR)**

To obtain total RNA, each sample was isolated from freshly dissected 0.5 mm wide SVZ strips or the entire hippocampal formation, flash frozen using liquid nitrogen, and isolated from a commercial kit (Cat #: 74104, Qiagen, Valencia, CA). Briefly, 1 μg RNA was DNAsed (Cat #: 18068-015, Invitrogen, Carlsbad, CA) and used as templates for reverse transcription, which included 1 μl of a 0.5 μg/μl random primers (Cat#: C1181, Promega, Madison, WI), 1 μg total RNA, 5 μl of 5x buffer, 1.25 μl of 10 mM dNTP mix, 2.25 μl RNase free water, and 1 μl (200 units) of Moloney Murine Leukemia Virus Reverse Transcription (M-MLV RT, Cat #: M170, Promega, Madison, WI) and heated at 37°C for 1 hr. Water was used as a no-template control instead of total RNA. The qPCR was performed using primer sets specific for CNTF (Mm00446373_m1 and Rn00755092_m1), Nos1 (Mm00435175_m1 and Rn00583793_m1), HTR1A (5HT1A; Mm00434106_s1 and Rn00561409_s1), GapDH (4352339E), and Ywhaz (Rn00755072) (all from Applied Biosystems, Carlsbad, CA).

**Histology**

Mice were transcardially perfused with ice-cold PBS, pH 7.4, until the liver became clear (approx. 10-20 ml) followed by 10 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected out and immediately placed in ice-cold 4% paraformaldehyde to postfix overnight followed by cryoprotection in 30% sucrose in
0.1 M phosphate buffer overnight. Serial coronal brain sections of 30 µm thick were cut on a Leica SM 2000R freezing stage sliding microtome and stored in anatomical order in 24-well plates filled with Millonig's buffer. Double immunofluorescent staining for GFAP (MAB3402, 1:1000, Millipore, Billerica, MA) and 5HT1A (AB5406, 1:300, Millipore, Billerica, MA) was performed using appropriate secondary antibodies conjugated with Alexa488 (1:500 goat anti-mouse) and Alexa546 (1:500; goat anti-guinea pig; Molecular Probes; Eugene, OR). For BrdU staining, starting at a random point along the rostrocaudal axis of the brain sections stained included those approximately between stereotaxic coordinates (caudal to Bregma) 1.18 to 0.14 mm for SVZ and -1.34 to -3.08 mm for hippocampal formation in the mouse (Franklin and Paxinos, 1996). Sections for rats were approximately between 1.6 to 0.2 mm for SVZ and -2.3 to -4.52 mm for hippocampal formation in the rat (Paxinos and Watson, 1986). Every sixth section (180 µm apart) through the SVZ and hippocampal formation was immunostained against BrdU (MAB3510, mouse IgG, clone BU-1, 1:100,000; Millipore, Billerica, MA). Briefly, brain sections were incubated in 50% formamide in 2X SSC at 65°C for 2 h, rinsed in fresh 2X SSC, incubated in 2N HCl at 37°C for 30 min, neutralized in 0.1 M boric acid, pH 8.5, for 10 min. Next, sections were sequentially incubated in 10% normal horse serum for 30 min, primary antibody overnight at 4°C, biotinylated horse anti-mouse IgG (1:800; Vector Laboratories, Burlingame, CA) for 1 hr, and avidin-biotin complex conjugated with peroxidase for 1 hr (1:600; Vector Laboratories). All incubations were carried out at room temperature unless otherwise noted. In between steps, the sections were rinsed 3 x 10 min. The chromogen reaction was performed with 0.04% 3, 3'-diaminobenzidine (D5637, Sigma-Aldrich) solution containing 0.06% nickel ammonium sulfate and 1% hydrogen peroxide in 0.05 M Tris buffer-HCl. Sections were then rinsed in 0.1 M phosphate buffer, mounted on gelatin coated glass slides in anatomical order, and cover slipped in Permount (SP15, Thermo Fisher Scientific, Pittsburg, PA).
Unbiased stereological counts and statistics

The number of BrdU+ nuclei in the SVZ of each brain was estimated using a motorized Leica DMIRE2 microscope and an unbiased optical fractionator stereological method (Stereologer; Stereology Resource Center, Chester, MD) (Baker et al., 2004). For the SVZ, the reference space was defined as an ~50 μm-wide strip of the entire lateral of all the lateral ventricle encompassing dorsoventrally the ventral tip and the dorsolateral triangular regions of the lateral ventricle, rostrocaudally from the genu of the corpus callosum to the caudal end of the decussation of the anterior commissure, and laterally the boundary between the SVZ and striatum. Within the reference space, BrdU-positive nuclei were counted in defined frames (frame size: 2000 μm², frame height: 15 μm, guard height: 2 μm, frame spacing: 100 μm) and the total number of BrdU-positive cells in a brain was calculated by the software as: n = number of nuclei counted × 1/section sampling fraction × 1/area sampling fraction × 1/thickness sampling fraction. A modified unbiased stereology protocol was used to count the BrdU+ cells in the SGZ of the dentate gyrus (West et al., 1991). Briefly, the total number of BrdU+ cells between the granule cell layer and hilus of the dentate gyrus that were within two cells body distance from the granular layer of the dentate gyrus was counted bilateral from the stereological coordinates previous stated. The total number of cells is calculated by the modified formula for rare events: n = number of nuclei counted × 1/section sampling fraction = total number of cells counted × (1/(1/6)). All analysis was done bilaterally except for in rats, in which a unilateral count was performed due to the other half of the brain was used for mRNA analysis. All analyses were performed blindly. Statistical analyses were performed with the Student’s unpaired t-test using Excel software (Microsoft, Redmond, WA). A value of p < 0.05 was considered statistically significant.
RESULTS

5HT1A receptors co-localization in SVZ astrocytes in C57BL/6J mice

Astrocytes in different areas of the brain are heterogeneous populations that can express different amounts and types of receptors (Emsley and Macklis, 2006). Interestingly, 5HT1A is found in highest abundance in astrocytes of rats in the two regions of the brain where neurogenesis occurs throughout life (Whitaker-Azmitia et al., 1993). In mice, 5HT1A staining also co-localized with GFAP in the SVZ (Figure 1A-C). This was confirmed in confocal microscopy z-stack images showing that most of 5HT1A staining is co-localized with GFAP in addition to a few putative axon terminals, presumably presynaptic receptors of serotonergic projections (arrows in Figure 1D-F). However, colocalization with synaptophysin remains to be done. In the dentate gyrus, 5HT1A immunostaining is co-labeled with GFAP, including the SGZ (Figure 1G-I).

5HT1A agonist 8-OH-DPAT does not increase neurogenesis in adult C57BL/6J mice

We intended to test the role of 5HT1A directly in CNTF/- mice and their littermates but wanted to first test 8-OH-DPAT in less expensive C57BL/6J mice. The latter are the most commonly used strain in neuropharmacological research and our normal strain that we currently and previously have used to show changes in SVZ proliferation after 3 day drug treatments (Emsley and Hagg, 2003, Yang et al., 2008). Male C57BL/6J adult mice received 1 mg/kg/daily over 3 days using subcutaneous Alzet osmotic mini-pump containing saline or saline plus the active enantiomer of 8-OH-DPAT. This dose was used by others who have shown 8-OH-DPAT to promote neurogenesis in both rats and mice (Santarelli et al., 2003, Soumier et al., 2010). BrdU was injected
twice daily at 50 mg/kg i.p. per injection. After an unbiased stereological analysis, we found no differences in either BrdU+ cells in the SVZ (Figure 2A) or SGZ (Figure 2B). Separately, we used higher doses and found that 3 or 10 mg/kg per day bolus injections in the wild type background of our CNTF mice (essentially C57BL/6) also did not induce neurogenesis (data not shown; n=4 per group). The osmotic pump was chosen in order to give a constant level of drug over the 3-day drug treatment in light of the 26 min half-life of 8-OH-DPAT in the brain (Perry and Fuller, 1989). Others have shown that the SGZ neurogenesis was increased following 28-day osmotic pump administration of 1 mg/kg/day by osmotic pump (Santarelli et al., 2003). Therefore, C57BL/6J mice received saline or 8-OH-DPAT for 28-days using two 14-day Alzet osmotic pumps. To identify proliferating cells, BrdU was injected twice-daily 50mg/kg i.p. over the last 3 days with the last injection two hours before sacrifice. The 8-OH-DPAT mice did not show an increase in BrdU+ nuclei in the SVZ (Figure 2C) or SGZ (Figure 2D). Lastly, we did not observe the typical serotonergic behavior (forepaw treading, flat body posture, and weaving of the head) that has been described in rats (Tricklebank et al., 1985) in any of these 8-OH-DPAT-treated mice.

**8-OH-DPAT does not increase CNTF expression in C57BL/6J mice**

It was possible that 8-OH-DPAT failed to induce neurogenesis because it does not induce CNTF. We had shown that increases in CNTF cause increased neurogenesis (Emsley and Hagg, 2003, Yang et al., 2008). There were not changes in CNTF mRNA in the SVZ and hippocampal formation either acutely after 3-day infusion (Figure 3A) or chronically after 28-day infusion (Figure 3B) in C57BL/6J mice. In the same tissue, we measured 5HT1A mRNA to confirm there was not a down-regulation of the receptor after the administration of 8-OH-DPAT. After 3 days infusion, 5HT1A was
decreased by ~20% in the SVZ (Figure 3C), but not the hippocampal formation (Figure 3D). Lastly, to find additional evidence for the expected effects of 8-OH-DPAT, we measured neuronal Nitric Oxide Synthase (nNOS) mRNA levels in the same tissue, as these were shown by others to decrease in the hippocampus of B6129SF2 mice 1 day after treatment and to diminish completely by 7 days (Zhang et al., 2010). In our C57BL/6J mice, nNOS mRNA did not decrease after acute (Figure 3E) or chronic (Figure 3F) administration of 8-OH-DPAT.

**8-OH-DPAT does not stimulate neurogenesis or CNTF in adult 129SvEv mice**

It is possible that our negative results after 8-OH-DPAT treatment were related to the C57BL/6J strain. We, therefore, repeated the 8-OH-DPAT experiment of the study by Santarelli et al. (2003), in which they used male 129SvEv mice to show that 8-OH-DPAT increased neurogenesis specifically through 5HT1A. Here, male 129SvEv mice from the same vendor were first treated with a 3-day i.p. injection of saline of 1 mg/kg 8-OH-DPAT. There was no significant change in the BrdU+ nuclei for the SVZ (Figure 4A) or SGZ (Figure 4B). Given the fact that the Santarelli et al. (2003) infused for 28 days using Alzet pumps, we next also infused saline or the same dose, 1 mg/kg/day, of 8-OH-DPAT. There was no significant change seen in BrdU+ nuclei in either the SVZ (Figure 4C) or SGZ (Figure 4D). Since we did not see changes in neurogenesis, we measured CNTF mRNA levels and found no effects in either the SVZ or the hippocampal formation of acute (Figure 5A) or chronic (Figure 5B) 8-OH-DPAT treatment groups. 5HT1A mRNA levels were not affected after acute (Figure 5C) or the chronic (Figure 5D) treatments in the SVZ or SGZ. As in the C57BL/6J mice, the nNOS mRNA levels for both acute (Figure 5E) and chronic (Figure 5F) groups were not significantly affected by
the 8-OH-DPAT treatment. As in the C57BL/6J mice, we did not observe the typical serotonergic behavior in any of the 8-OH-DPAT-treated 129SvEv mice.

8-OH-DPAT increases neurogenesis in adult rats, but only in the SGZ and not through CNTF

Despite bioactivity in mice as shown by the decrease in 5HT1A after acute treatment with 8-OH-DPAT in C57BL/6J mice, we wanted to further verify that 8-OH-DPAT was active. The literature on the use of 8-OH-DPAT in rats is much more extensive, including behavioral effects (Tricklebank et al., 1985) and its ability to increase hippocampal neurogenesis (Banasr et al., 2004, Huang and Herbert, 2005, Soumier et al., 2010). Thus, male Sprague-Dawley rats were injected daily i.p. with saline or 1 mg/kg 8-OH-DPAT over 3 days. Within minutes of 8-OH-DPAT injection the rats exhibited typical serotonergic behavior, verifying that our drug was effective at eliciting a serotonergic response. The number of BrdU+ nuclei in the SGZ was increased by 43% after the 3-day treatment (Figure 6B, D, and F). In contrast, the SVZ showed no change in the number of BrdU+ nuclei (Figure 6A, C, and E). Since we found that 8-OH-DPAT increased neurogenesis in the rat SGZ, we assessed whether this was mediated by an increase in CNTF. However, there were no changes in CNTF mRNA levels in the SVZ or hippocampal formation (Figure 7A). To test whether the difference between the SGZ and SVZ neurogenesis was related to the presence of functional 5HT1A, we measured the effects of 8-OH-DPAT on nNOS mRNA levels. The nNOS mRNA levels were decreased by 50% in the hippocampal formation, but were not affected in the SVZ (Figure 7B). Lastly, 5HT1A mRNA levels were checked to see if there was a down regulation of the receptor. 5HT1A was not significantly changed in the SVZ or SGZ (Figure 7C).
DISCUSSION

Like others, we find that 8-OH-DPAT increases neurogenesis in the adult rat SGZ (Banasr et al., 2004, Huang and Herbert, 2005, Soumier et al., 2010). We wanted to define the role of CNTF in this neurogenic response, given the commonality between 5HT1A and D2 receptors, i.e., both lowering cAMP and found on astrocytes. CNTF seems not involved in the neurogenic response to 8-OH-DPAT, as it did not increase in the hippocampal formation. The lack of changes in CNTF expression in rats and mice, and in the SVZ and hippocampal formation suggests that, unlike the D2 receptor (Yang et al., 2008), 5HT1A does not inhibit cAMP in astrocytes in vivo. In fact, a recent study showed that the R-isoform of 8-OH-DPAT did not decrease forskolin-stimulated increases in cAMP in the membranes isolated from rat dorsal raphe nucleus (Valdizan et al., 2010). If not CNTF, what then might regulate the neurogenic response to 5HT1A agonist in the rat SGZ? The difference between the neurogenic response in the SVZ and SGZ and its correlation with the decrease in nNOS mRNA levels suggests that 5HT1A activation in the SGZ decreases nNOS signaling, which results in an increase in neurogenesis. In fact, NO appears to reduce proliferation and nNOS inhibition in the CNS of adult mammals causes an increase in neurogenesis in the SVZ (Matarredona et al., 2005) and hippocampal formation (Nakagawa et al., 2002a, Nakagawa et al., 2002b, Gass and Riva, 2007, Gur et al., 2007). In cultured hippocampal neurons, 8-OH-DPAT was shown to decrease nNOS, which decreases NO production, increasing phosphorylation of cAMP response element binding protein (CREB) which presumably increases transcription (Zhang et al., 2010). Furthermore, in CREBαα mutant mice there was a 52% increase in BrdU+ nuclei in the SGZ compared to wild-type mice (Gur et al., 2007). Moreover, a decrease in neurogenesis was observed when CREB activity was
down regulated by expressing a dominant-negative CREB (Nakagawa et al., 2002a, Nakagawa et al., 2002b). Pharmacological inhibition of NO by L-NAME was shown to increase neurogenesis in the SVZ of adult mice (Romero-Grimaldi et al., 2008). This suggests that since there were no changes observed in the mRNA levels of nNOS after 8-OH-DPAT treatment in the SVZ of rats and the SVZ and SGZ of mice that 5HT1A does not modulate their nNOS levels. Besides its classical inhibition of adenylyl cyclase (Cornfield et al., 1991), 5HT1A also can couple to other signaling pathways such as phospholipase C (Fargin et al., 1989), mitogen-activated protein kinases (Della Rocca et al., 1999) and AKT (Cowen et al., 2005), which play a role in adult neurogenesis (Ma et al., 2009, Kinsler et al., 2010, Manning et al., 2010). These pathways could signal independent of adenylyl cyclase or converge on different adenylyl cyclase subtypes to increase or decrease cAMP (Raymond et al., 1999). Although the exact intracellular mechanism of action of 8-OH-DPAT on neurogenesis in the rat SGZ remains to be determined, the finding that it does not act through CNTF should enable the development of drug combinations. For example, one could expect an additive effect on the levels of SGZ neurogenesis by combining 5HT1A agonist and D2 agonists, the latter stimulating neurogenesis by increasing CNTF (Yang et al., 2008).

In rats, 8-OH-DPAT was shown to increase BrdU+ nuclei in the SVZ 4 hours after i.p. injection although the data for longer times were not reported (Banasr et al., 2004). Our 3-day treatments did not result in any changes in SVZ BrdU+ nuclei. Other than strain differences, we do not know the reason for this apparent discrepancy. Thus, the reason for the lack of a neurogenic effect of 8-OH-DPAT in the rat SVZ remains to be determined, but the presence of 5HT1A in the SVZ suggests that they are not functional in the SVZ astrocytes under normal conditions. Others have shown that astrocytes from 2-day old Sprague-Dawley rat pups cultured from various other brain regions express non-functional 5HT1A in culture (Hirst et al., 1998). If so, this would suggest that
astrocytes in different CNS regions can function entirely different, as also suggested by their different phenotypes (Emsley and Macklis, 2006). Another possibility is that 5HT1A in the two different regions of the brain have differences in intracellular coupling and/or signaling as it has been shown with different cell types in vitro (Raymond et al., 1999). The activity of 5HT1A was shown to be impacted by alterations in any of the guanine nucleotide-binding protein G(i), alpha subunit (Gia), in particular, alterations in the levels of Gia1 was shown to have a greater impact on 5HT1A activity (Liu et al., 1999, Lin et al., 2002, Valdizan et al., 2010). It remains to be determined whether there are different levels of the Gi/Go protein isoforms accessible to the 5HT1A for coupling in the SVZ versus the SGZ, but it would explain why there is an apparent difference in functional activity. Another possibility is that 8-OH-DPAT acts on other, differently expressed, receptors in the two structures. The R-enantiomer of 8-OH-DPAT is considered to be the prototypical selective agonist for 5HT1A as shown by radio ligand binding (Middlemiss and Fozard, 1983). However, the use of transgenic mice and pharmaceutical inhibitors have shown that 8-OH-DPAT has agonistic activity on 5HT7 (Hedlund et al., 2004) and α2-adrenoceptors (Heusler et al., 2010). Finally, the difference in the neurogenic response between the SVZ and SGZ most likely represents the different physiological roles of the two systems, and differences in the serotonergic neurons that innervate these regions. In turn, it means that hippocampal neurogenesis can be stimulated by 5HT1A agonists without affecting the olfactory neurogenesis, perhaps opening up opportunities for region-selective drug treatments to increase new neuron formation.

Our results also point to remarkable differences between rats and mice with regards to the 5HT1A function and its role in neurogenesis. The lack of any effects of 8-OH-DPAT in our mice, other than a 20% down-regulation of 5HT1A, suggests that the receptors are not fully functional in these strains of mice. Previously, 8-OH-DPAT
administration was shown to induce hypothermia in mice and rats to a different extent, suggesting the 5HT1A heterogeneity is present between mice and rats (Moser, 1991). In their landmark paper, Santarelli et al. (2003) report that the same dose and duration of 8-OH- DPAT causes an increase in BrdU+ nuclei in the SGZ of 129SvEv mice but not in 5HT1A-/- mice. The neurogenic effect of 8-OH- DPAT was replicated in 129SvEv mice within the same laboratory (B. Samuels, personal communication). A few differences might explain the apparent discrepancy between our studies. First, we used the active enantiomer whereas they used the racemic mixture of 8-OH- DPAT. It is unlikely that the S-enantiomer, a partial agonist, would have stimulated neurogenesis through other receptors or mechanisms, as it was ineffective in 5HT1A-/- mice (Santarelli et al., 2003). Another potential difference is the stress caused by their behavioral testing resulted in a depressed baseline of neurogenesis in controls. Stress can have both deleterious and stimulatory properties depending on the type (Sahay and Hen, 2007, Schoenfeld and Gould, 2011). The 129SvEv mice might be unique as the neurogenic effect of fluoxetine (Santarelli et al., 2003) is not seen in BALB/cJ mice within the same laboratory (Holick et al., 2008) or BALB/cJ and C57BL/6J mice in another laboratory (Navailles et al., 2008).

Our results are consistent with the finding that a 7 day treatment of 8-OH- DPAT did not increase neurogenesis whereas a single injection increased proliferation by 55% at 24 hr in the SGZ of C57BL/6 mice (Klempin et al., 2010). Our 3-day data suggest that more than a single injection might be ineffective as a neurogenic treatment. Whatever the differences, we propose that 5HT1A does not play a role in normal neurogenesis in adult mice, although it has a clear role in rats. In fact, 5HT1A-/- mice show no differences in the number of BrdU+ nuclei under baseline conditions (Santarelli et al., 2003).

In light of the differences between rats and mice, what are the potential implications for the role of 5HT1A in neurogenesis in humans? This has important implications for the depression field because of the unresolved debate about whether or
not changes in neurogenesis play a role in depression (Kempermann, 2008, Thomas and Peterson, 2008). 5HT1A protein has an 89% homology between rats and humans, 94% between rats and mice, and 86% between mice and humans (Clustal W Software, European Bioinformatics Institute, Cambridge, UK). However, there could be differences in the ligand binding regions or availability of coupling intracellular proteins. Also, rats and humans have a different neuroanatomical distribution in 3H-8-OH-DPAT binding (Duncan et al., 1998). Rats have high binding in the dentate gyrus molecular layer whereas humans have relatively low binding. Humans and rats have high binding in CA-1 stratum radiatum, but only humans have high binding in the CA-1 pyramidal cell layer. These differences could play a role in augmenting or diminishing the neurogenic effects seen in rats and make translation to humans more uncertain. We propose that based on the major species differences much more research is needed to define the potential role of 5HT1A in human hippocampal neurogenesis and the consequences of such a speculative mechanism for clinical depression.
Figure 1. 5HT1A is present in astrocytes in the SVZ and SGZ of adult C57/B6J mice. 5HT1A (A) and GFAP (B) are co-localized (C) in the SVZ.

Confocal microscopy shows that 5HT1A (D) and GFAP (E) are almost exclusively co-localized (F), as indicated in the z-stack, except for a few putative axon terminals, presumably presynaptic receptors of serotonergic projections (arrows). In the dentate gyrus, 5HT1A (G) and GFAP (H) also shows co-localization (I), including in the SGZ. Scale bar 100 μm (A-C, H-I), 10 μM (D-F). CC = corpus callosum, GCL = granule cell layer, H = hilus, LV = lateral ventricle, STR = striatum.
Figure 2. 5HT1A agonist 8-OH-DPAT does not increase proliferation in C57B/6J mice.

R-8-OH-DPAT was administered s.c. with a dose of 1 mg/kg delivered via Alzet osmotic pump for either 3 days or by two 14-day Alzet pumps for a total of 28 days to C57B/6J male mice. There were no significant changes in BrdU+ nuclei acutely after 3 days, in the SVZ (A) or SGZ (B) or chronically after 28 days in the SVZ (C) or SGZ (D). BrdU was given i.p. twice daily for 3 consecutive days with the last injection 2 hours before euthanasia and processing for histology. n= 5, 6 in each group for 3 day and 28 day, respectively.
Figure 3. 8-OH-DPAT does not alter CNTF or nNOS mRNA, but reduces 5HT1A mRNA levels in C57B/6J mice.

CNTF mRNA levels were not changed acutely (A), after 3 days, or chronically (B), after 28 days of 5HT1A agonist treatment in the SVZ and hippocampal formation. 8-OH-DPAT did cause a significant decrease in 5HT1A mRNA levels (C) (p=0.0499) in the SVZ but not the hippocampal formation after 3 days of 1 mg/kg of 8-OH-DPAT. 5HT1A mRNA levels did not change in the SVZ or HF after 28 days of 8-OH-DPAT infusion (D). nNOS mRNA levels in the SVZ and hippocampal formation did not significantly change acutely (E) or chronically (F). * signifies p<0.05, n= 4, 5, 6 in each group for saline 3 day, 8-OH-DPAT 3 day, and 28 day, respectively.
Figure 4. 8-OHDPAT does not increase proliferation in the SVZ or SGZ in 129SvEv mice.

R-8-OH-DPAT was administered with a dose of 1 mg/kg delivered either by daily i.p. injection for 3 days or s.c. via Alzet osmotic pump for 28 day to 129SvEv male mice. There were no changes in the number of BrdU+ nuclei in the SVZ (A) or SGZ (B) acutely or the SVZ (C) or SGZ (D) chronically. n= 5 in each group.
Figure 5. 8-OH-DPAT does not alter mRNA levels of CNTF, 5HT1A or nNOS in 129SvEv mice.

CNTF mRNA levels were not changed acutely (A), after 3 days, or chronically (B), after 28 days of treatment in the SVZ and hippocampal formation. 8-OH-DPAT did not cause a significant decrease in 5HT1A mRNA levels in the SVZ or hippocampal formation after 3 days (C) or 28 days (D) of 1 mg/kg of R-8-OH-DPAT. nNOS mRNA levels in the SVZ and hippocampal formation did not significantly change acutely (E) or chronically (F). n=5 in each group.
R-8-OH-DPAT was administered with a dose of 1 mg/kg delivered by daily i.p. injection for 3 days in Sprague-Dawley rats. BrdU labeling in the SVZ (A) and SGZ (B) of saline treated rats. BrdU labeling in the SVZ (C) and SGZ (D) of 8-OH-DPAT treated rats. There was no significant difference in the number of BrdU+ nuclei in the SVZ of rats (E). There was a significant 43% increase in the number of BrdU+ nuclei in the SGZ in rats (F). * signifies p<0.05, n= 5 in each group. Scale bar 500 μm (A and C) and 100 μm (B and D).
**Figure 7.** 8-OH-DPAT induces rat SGZ neurogenesis, decreases nNOS mRNA levels, but does not increase CNTF mRNA levels.

A) There was no difference in CNTF mRNA levels in the SVZ or in the hippocampal formation, where neurogenesis was observed in the SGZ after 8-OH-DPAT treatment. B) There was a significant decrease in nNOS mRNA levels only in the hippocampal formation and not in the SVZ. C) There were no changes in 5HT1A mRNA levels in the SVZ or hippocampal formation. * signifies p<0.01, n= 5 in each group.
CHAPTER THREE

ADAM21-DEFICIENT MICE HAVE NO APPARENT PHENOTYPE BEFORE OR AFTER SPINAL CORD INJURY

INTRODUCTION

The ADAMs are multi-domain transmembrane proteins, constituting a subfamily of the metzincin superfamily, and mostly known for their proteolytic sheddase activity and their integrin-binding properties (Wolfsberg et al., 1995, Yang et al., 2006). MMPs are similar in the N-terminus to ADAMs as they contain a prodomain and metalloproteinase domain, but ADAMs also are comprised of several additional domains: disintegrin, cysteine rich, EGF-like repeat, and cytoplasmic tail (Edwards et al., 2008). The ADAMs have been linked to several disease processes such as asthma (King et al., 2004, Haitchi et al., 2005), inflammation (Charrier-Hisamuddin et al., 2008, Koller et al., 2009, Pruessmeyer and Ludwig, 2009), wound healing (Charrier et al., 2005, Hodgkinson et al., 2010), cancer (Lu et al., 2008), and neurological diseases (Gerst et al., 2000, Barrette et al., 2010). The metalloproteinase is of interest because of the potential for drug intervention with a small molecule that could inhibit its catalytic activity.

MMPs have been widely studied in the brain (Agrawal et al., 2008) and found to be differentially regulated after spinal cord injury (Zhang et al., 2011a). Deletion of the metalloproteinase genes MMP9 (Noble et al., 2002) and MMP12 (Wells et al., 2003) resulted in improved functional recovery and reduced/attenuated barrier disruption following spinal cord injury. Conversely, deletion of MMP2 resulted in impairment of
locomotor recovery, reduced white matter sparing, and widespread reactive astrogliosis following spinal cord injury (Hsu et al., 2006).

However, the literature on ADAMs after spinal cord injury, with the exception of expression data, is limited to our finding that ADAM8 is association with angiogenesis after spinal cord injury (Mahoney et al., 2009). ADAM8-/- mice do not have an overt phenotype following spinal cord injury (Arnold and Hagg, unpublished data). ADAM21 is of interest because of the robust immunostaining in highly plastic areas: new adult primary olfactory neurons and their axons growing into the CNS, radial glial cells during postnatal development, and tanycyte-like cells in the adult subventricular zone where neurogenesis occurs (Yang et al., 2005). ADAM21 has a catalytically active metalloproteinase (Hooft van Huijsduijnen, 1998). This evidence leads to the possibility that like its MMP relatives, ADAM21 could be involved in the processes which occur after spinal cord injury and contribute to secondary degeneration, repair, or plasticity.

In the EAE model of demyelination, TIMP-1 deficient mice exhibited a delay in myelin formation which correlated with the decreased number of astrocytes found in the white matter (Crocker et al., 2006, Moore et al., 2011). Thus inhibition of metalloproteinases might result in increased spared white matter which would result in locomotor recovery following spinal cord injury. Additionally, CGRP can be induced by cytokines such as TNFα (Schafers et al., 2003) resulting in hypersensitivity to non-noxious stimuli (alldynia). Since several ADAMs have been linked to activation of TNFα, it is plausible that activation of TNFα or other pro-inflammatory cytokines could be a mechanism in which ADAM21 could have a role in pain development after spinal cord injury (Karkkainen et al., 2000, Doedens et al., 2003, Hooper et al., 2005, Hikita et al., 2009, Pruessmeyer and Ludwig, 2009). Notch is another known target of ADAMs, in particular ADAM10 and possibly ADAM17, which requires shedding of its ectodomain before a second cleavage is required for release of Notch intracellular domain by γ-
secretase (Wen et al., 1997, Qi et al., 1999, Brou et al., 2000, Mumm et al., 2000, Deuss et al., 2008). Modulations by metalloproteinases in Notch signaling following spinal cord injury could affect cell proliferation, angiogenesis, or transcription of proteins (Yamamoto et al., 2001, Fassbender et al., 2011a). Lastly, ADAM21’s presence in radial glial cells, which could potentially act as neural stem cells in the adult spinal cord resulting in neuronal repair and regeneration after spinal cord injury.

Here we investigated the functional outcomes after a T9 spinal cord injury in adult ADAM21-deficient mice by assessing locomotion, balance, and fine motor skills, as well as thermal nociception over a 6-week period. Additionally, we measured histological outcomes such as spared white matter, and the area occupied by CGRP and GFAP immunostaining at and around the injury epicenter.

METHODS

Animals

A total of 55 mice were used, i.e., adult male and female littermates from each genotype were age and gender matched. They were bred in house from heterozygous B6.129P2-ADAM21tm1Dgen/J mice (stock# 006431, Jackson Laboratory, Bar Harbor, ME, USA; crossbred a minimum of 3 times with C57B/6 mice) and crossbred in house 2 more times with C57B/6J mice (stock# 000664, Jackson). The deleted gene was replaced by a Lac-z containing cassette. The genotyping protocol was supplied by Jackson laboratory (refer to http://jaxmice.jax.org/protocolsdb/f?p=116:2:442721623684068::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:1256,006431). All animal procedures were performed according to University of Louisville Institutional Animal Care and Use Committee protocols and the National Institutes of Health guidelines.
Spinal cord injury

Mice were anesthetized by an intraperitoneal injection of 0.4 mg/g body weight Avertin (2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline, Sigma-Aldrich, St Louis, MO). A 50 kdyn T9 contusion injury was made using an Infinite Horizons (IH; Precision Systems and Instrumentation, LLC, Lexington, KY) impactor after a laminectomy was made at T9 spine level. Sham animals received the same treatment as injured animals short of the impact on the spinal cord. All animals received subcutaneous injection of 2 ml saline (1 ml before and after injury) and 0.1 ml of gentamicin (20 μg/ml stock, ButlerSchein, Dublin, OH). Gentamicin was also given on day 2 and 4 post injury. Bacitracin ointment (Qualitest Pharmaceuticals, Huntsville, Al) was applied to the sutured wound area. Once animals were fully awake, twice-daily analgesia was administered subcutaneously for the first 48 hours of a 0.1 ml of a 15 μg/ml stock solution of buprenorphin (Reckitt Benckise, Hull, England). Animals were placed 5 per cage on Alfa Dry bedding and recovered on a water circulating heating pad overnight. Animal bladders were manually voided, as needed, once or twice daily until automatic voiding returned spontaneously, usually within 7-10 days. Metal sutures were removed between 7 and 10 days post injury. As an indicator of overall health and growth of the animals, the weight was checked prior to and after injury. Animals from each group continually gained weight and no significantly different weight gain was observed between genotypes.

BMS analyses

Mice were tested for hindlimb locomotor function before injury to establish a baseline, and weekly after injury beginning at 7 days using the Basso Mouse Scale (BMS) (Basso et al., 2006). Animals were place in a 47-inch metal pool and observed
for 4 minutes by two trained individuals who were blinded to genotype and injury. Only individuals certified by Dr. Michelle Basso at Ohio State University were used in the behavior testing. Animals were given a score ranging from 0 to 9, with a score of zero having no movement of the hindlimbs and 9 signifying a normal animal with proper hindlimb coordination, trunk stability, paw placement, and tail position. Animals with a score of greater than or equal to 5 received a subscore ranging from 0 to 11.

**Beam Walk analyses**

The balance and fine motor skills were tested at baseline and weekly after injury using a graded series of suspended on 47 cm long metal beams of various widths: 4mm, 8mm, 12mm, 16mm, and 20mm, which was modified from the previously described beam walk method (Hill et al., 2009). In short, the narrowest beam a mouse could walk across was used for testing. The animals were scored based on beam size and the number of errors while crossing the beam. The final score was obtained by averaging the scores of two trials obtained on smallest crossable beam for each animal. Details of the scoring sheet are described elsewhere (Hill et al., 2009).

**Hargreaves analyses**

Thermal nociception thresholds in mouse forepaws and hindpaws were evaluated using a Hargreaves device (IITC 390 Plantar Test; IITC Life Sciences, CA). The intensity of the heat source was set at 30%. The mice were placed in a Plexiglas testing chambers with a glass floor that was maintained at 37°C. The mice were allowed to acclimate to the apparatus for twenty minutes. The radiant heat source was placed alternately under the plantar surface of each forepaw and hindpaw until the animal withdrew its paw from the stimulus. The latency time was recorded for each forepaw...
and hindpaw. There was a cutoff time of twenty seconds in order to avoid tissue
damage. At each time point, testing was repeated five times per animal with a minimum
of 5 minutes between each trial. The latency of withdrawal for each paw was averaged.
Mice were tested before injury to establish at baseline and after injury weekly beginning
at 7 days.

**Tail Flick**

Thermal nociception thresholds in the mouse tail was evaluated using Tail Flick
Analgesia Meter (Columbus Instruments, Columbus, OH) with the intensity set at 6
resulting in a baseline of around a 2 second withdrawal time. Mice were restrained in
the Tailveiner (TV-150, Braintree Scientific, Braintree, MA) and the tail was placed in the
groove with a shuddered light. Once the shutter was turned off, the tail-flick response
was measured using automatic detection. The trial was repeated 3 times for each
animal with no less than 5 minutes between trials. Mice were tested at baseline and
weekly after injury.

**Histology**

Mice were anesthetized and transcardially perfused with 20 ml of ice-cold PBS,
pH 7.4, and 10 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer.
Afterwards, a 1 cm segment of the spinal cords was carefully dissected out, post-fixed in
4% paraformaldehyde overnight, and subsequently stored in Millonigs solution. Before
freezing, the spinal cord segments were cryoprotected in 30% sucrose in 0.1 M
phosphate buffer overnight followed by embedding in Tissue Freezing Medium (TFM-C,
Triangle Biomedical Sciences, Durham, NC). Twenty consecutive transverse 20 μm
sections per 1 mm were cut on a cryostat, mounted on charged microscope slides, and
stored at -20°C until staining. Additionally, the spinal cords of a group of naïve animals from each genotype was also cut in 20 μm sections from T7 to T11 and every section was collected. One in every 10 sections from the naïve spinal cords was used for staining.

A modified eriochrome cyanine staining protocol was used to detect white matter tracts. Briefly, one in every 5 sections was thawed for 1 hour at 37°C, immersed in xylene (2 x 30 minutes), followed by a graded alcohol series (3 min 100% EtOH, 3 min 70% EtOH, 3 min 50% EtOH, 2 min dH2O), 10 minutes in eriochrome cyanine reaction RC stain (0.16% eriochrome cyanine RC in 0.4 FeCl₃ and 0.4% H₃SO₄), rinsed twice in tap water, differentiated for 30 seconds in 0.5% NH₄OH, rinsed twice in tap water to stop differentiation, and allowed to dry overnight at room temperature. Slides were then placed in xylene for 10 minutes and cover slipped using Permount (SP15, Thermo Fisher Scientific, Pittsburg, PA). The area of most myelin loss was used to determine the epicenter by calculating total number of pixel stained using manual threshold in ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, http://imagej.nih.gov/ij/, 1997-2011.)

Adjacent sections were stained for GFAP, CGRP, β-galactosidase (from the Lac-z reporter gene), and ADAM21. Sections were thawed for one hour and the dried Tissue Freezing Medium was carefully removed with forceps. In short, the sections on the slides were circled with a hydrophobic barrier using a Super PAP Pen (Cat.# 008899, Invitrogen, Carlsbad, CA), rinsed twice with 0.1 M Tris-Buffered Saline containing 0.3% Triton-X (TBS-Tr), incubated 1 hr in 5% donkey serum in TBS-Tr at room temperature, incubated overnight in 4°C primary antibody containing 5% donkey serum in TBS-Tr and mouse anti-GFAP (1:1000, Cat.# MAB3402, clone GA5, Millipore, Billerica, MA), rabbit anti-GFAP (Cat.# Z0334, Dako, Carpinteria, CA), rabbit anti-CGRP (1:1000, Cat.#, Millipore, Billerica, MA), mouse anti-β-galactosidase (Cat.# 23781, Promega, Madison,
or rabbit anti-ADAM21 (1:1000, Cat.# AB19001, Millipore, Billerica, MA). Next, the slides were rinsed 3 times in 0.1 M Tris-Buffered Saline (TBS), incubated for one hour containing 5% donkey serum in TBS at room temperature with donkey anti-mouse or donkey anti-rabbit secondary Alexa Fluor antibody (1:200, Cat.# A-21202, A-21203, and A-21206, Invitrogen, Carlsbad, CA), rinsed 3 times in TBS, and cover slipped using Fluormount (Cat.# 0100-01, Southern Biotech, Birmingham, Alabama). Images for analysis were taken with a 10x objective for eriochrome cyanine staining and GFAP immunostaining and a 20x objective for CGRP immunostaining on a DM 6000 Leica upright microscope using the automated stitching Surveyor software (Objective Imaging, Cambridge, UK) in conjunction with an Oasis Automation Controller (Objective Imaging, Cambridge, UK) which drives the motorized stage. CGRP images from the second series of naive mice were taken using a z-stack of 15 μm with a 5 μm step.

Histology image analysis

The total number of pixels of spared white matter visualized by eriochrome cyanine staining and GFAP immunostaining was calculated using manual threshold in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.) Fast Fourier Transform (FFT) using Image Pro Plus software (version 6.2, Media Cybernetics, Inc, Bethesda, MD) was used on injured spinal cords that were immunostained with CGRP. In short, pictures were converted to monochrome, followed by FFT with a spectrum gain of 70 and an area of interest (AOI) of an ellipse radius of 5.5 which corresponds to 1638 pixels per period. Frequencies within the radius were cut. Then an inverse FFT was applied to the original image reducing the signal to noise ratio. Then the total number of pixels was calculated using the manual threshold in ImageJ. The AOI, dorsal horn of the spinal
cord was boxed with two boxes (2500px x 2500px) originating at the central canal and extending across each dorsal horn was drawn. The naïve spinal cords were analyzed using the same method as the spinal cord injured with the exception of the Fourier transformation. The two sets of tissue analyzed for CGRP immunostaining, spinal cord injured and naïve, were not stained at the same time or analyzed by the same person. The three-fold difference in total stained area is most likely due to higher signal of CGRP labeling. All sections within a group were analyzed by the same person to decrease variability.

Statistical analysis

A repeated measure ANOVA over time with covariate analysis on gender, age, and weight with a paired Student’s post-hoc t-test was used to determine significance in all behavior data. For histological data, an unpaired Student's t-test was performed between genotypes. A value of $p \leq 0.05$ was considered statistically significant. All values are reported with standard error of the mean.

RESULTS

ADAM21 localization

ADAM21 immunostaining in uninjured wildtype adult mice was found in laminae I and II of the dorsal horns of the spinal cord (Figure 8A and B), the region of nociceptive innervation, and in the ependymal cells around the central canal (Inset of Figure 8B). Similar to the wildtype mice, ADAM21 immunostaining is present in ADAM21-deficient mice in the dorsal horns (Figure 8D and E) and in the ependymal cells surrounding the central canal (Inset of Figure 8E). The ADAM21-deficient mice contain a LacZ reporter gene within the insertion cassette enabling localization of ADAM21 expression by
immunostaining for its β-Galactosidase (β-Gal) protein product. As expected, no immunostaining was present in the spinal cords of wildtype mice (Figure 8C). In the sham or naïve ADAM21-deficient mice, β-Gal immunostaining was present in tube-like structures that appeared to be associated with blood vessels (Figure 8F, G and H; indicated by arrow in G and H). In the injured spinal cord, the β-Gal immunostaining was occasionally seen in cells with a single leading process similar to a migrating cell were seen in the white matter (Figure 8I). In addition, β-Gal immunostaining appeared in cells with morphology similar to microglia at the epicenter, rostral, and caudal to injury site (Figure 8J).

Surprisingly, the β-Gal immunostaining in the ADAM21-deficient spinal cords did not show the same pattern as seen with the ADAM21 antibody in naïve or injured spinal cords (Figure 8A, B, D, and E). Coupled with ADAM21 immunostaining in the ADAM21-deficient mice and lack of co-labeling with the β-Gal reporter gene, leads us to believe the ADAM21 immunostaining in which our original hypothesis was based from does not detect ADAM21 protein.

Behavioral comparison between ADAM21-deficient and wildtype mice.

As expected, 1 week after spinal cord injury there was a significant decrease in the BMS scores in both ADAM21-deficient and wildtype animals that remained significantly different from baseline 6 weeks after injury (Table 1). However, there was not a significant difference between these two groups at any time over the 6 weeks following the spinal cord injury. Of note, BMS scores are higher than normally seen due to mild injury indicated by the low displacement values of the animals in this study. Likewise, Beam walk had a significant decrease in scores 1 week following spinal cord injury in both genotypes and remained significantly different over the 6 weeks following
the injury (Table 1). ADAM21-deficient mice did not gain additional gross or fine locomotor function beyond the spontaneous recovery consistently seen in mice.

At 1 week post injury, there was a significant drop in withdrawal time following thermal stimuli; however, no significant differences was found between ADAM21-deficient and wildtype mice prior or after spinal cord injury in the Hargreaves test on the hindpaws (Table 1). Tail Flick did not show any differences between genotypes or within a genotype from baseline at any time over the 6 weeks following injury (Table 1). The decrease in hindpaw withdrawal time indicts hypersensitivity following spinal cord injury in both ADAM21-deficient and wildtype mice.

**Epicenter histology: comparison between ADAM21-deficient and wildtype mice.**

The area of white matter sparing was assessed by eriochrome cyanine staining. There was no significant difference between the spared white matter in ADAM21-deficient compared to wildtype animals at the injury epicenter, 1 mm rostral, 1 mm caudal or the total penumbra (1 mm rostral and caudal combined) to the injury (Table 2). In both genotypes the amount of spared white matter was approximately 50% of sham which is consistent with the relatively high BMS scores, indicating a mild injury.

CGRP immunostaining was evaluated in order to investigate the possibility of ADAM21 induced sprouting in the dorsal horns. At the epicenter, CGRP was decreased significantly from the penumbra probably due to the loss of neurons at the compression site shortly after injury. The total area of CGRP immunostaining was not found to be significantly different after injury between ADAM21-deficient and wildtype mice at epicenter, 1 mm rostral, 1 mm caudal, or total penumbra (Table 2). Although the degree of CGRP staining extending past Laminae II was not quantified, there were no obvious indications of sprouting in either genotype. The difference of the CGRP immunostaining
in the sham animals could not be statistically analyzed due to the small sample size in the wildtype shams (n=2). The sample size was small due to unexpected deaths in the wildtype shams; in addition, additional wildtype shams were not available due to lack of extra wildtype littermates of ADAM21-deficient mice. The values in the sham-operated ADAM21+/- mice were similar to those of the ADAM21-deficient mice (data not shown).

GFAP increased significantly at the epicenter of the injury, as expected, due to the infiltration of glial cells and formation of the glial scar following spinal cord injury. However, the area of GFAP immunostaining was also not found to be significantly different between genotypes after injury or in the shams in the epicenter, 1 mm rostral, 1 mm caudal, or the total penumbra (Table 2). Since there were only two sham-operated wildtype mice, we repeated the CGRP analysis on a new set of naïve spinal cords using a more precise tissue section sampling method to eliminate variations due to the laddering effect seen in CGRP staining. In the naïve spinal cords, there was no difference between genotypes in total area of CGRP immunostaining from T7 to T10, the area of the injury epicenter and penumbra of the other groups of mice (Table 3). This suggests that the CGRP values seen in the two sham-operated mice were artifactually low.

DISCUSSION

Surprisingly, β-Gal, a reporter for ADAM21 protein in the ADAM21-deficient mice showed expression in the null mouse that had a different cellular distribution than was found with the ADAM21 antibody in wildtype mice. Since the ADAM21-deficient mice contain a LacZ reporter gene, we presume that the β-galactosidase immunostaining shows the areas and cell types that produce ADAM21. From the β-Gal immunostaining ADAM21 expression appears to be localized in blood vessels in the naïve animal and
activated microglia, and migrating neurons in the injured animal, although this remains to be verified by markers to co-labeling for blood vessels and microglia. However, the antibody staining of the dorsal horn and ependymal cells seems to be non-specific for the ADAM21 protein due to its presence in ADAM21-deficient mice and the lack of any colocalization with the reporter, β-Gal. As mentioned in Chapter 4, a truncated ADAM21 transcript is still present in the ADAM21-deficient mice. Since the ADAM21 antibody stained the last 13 amino acids of the protein, this in addition to the truncated ADAM21 transcript could explain why there is immunostaining in the ADAM21-deficient mice. However, the only explanation for why the β-Gal does not colabel with ADAM21 is that the ADAM21 antibody is not detecting the ADAM21 protein. This could explain the absence in a phenotype involving sprouting and pain development because it seems that ADAM21 is not localized in the dorsal horns.

Originally, due to the location in the brain of ADAM21 antibody immunostaining in radial glial lineage cells in the SVZ and growing axons in the olfactory bulb, we believed that ADAM21 could regulate plasticity and subsequent pain development following spinal cord injury by regulation of cytokines such as TNFα or CX3CL which require ectodomain shedding for activation. The initiation of the inflammatory response is thought to originate with astrocytes following injury (Pineau et al., 2010); astrocytes which are damaged or at the site of damage can release cytokines such as TNFα in response to the injury which explains the increase seen in TNFα at 1 hour following spinal cord injury (Harrington et al., 2005, Peng et al., 2006, Zhang et al., 2011b). However, since the ADAM21 antibody is probably not an accurate depiction of cellular expression of ADAM21, this explains why our hypothesis was wrong.

Here we evaluated the potential role of ADAM21 in the intact adult animal and after spinal cord injury using ADAM21-deficient mice. We found no differences between adult ADAM21-deficient mice and their littermates under normal or injury conditions in
locomotion and coordination, fine motor and balance or thermal nociception. The similar extent of white matter sparing between the genotypes is consistent with the similar BMS scores, as these two are known to correlate (Basso et al., 2006, Han et al., 2010). Our data from two different thermal nociception tests and the CGRP immunostaining argue against the possibility of ADAM21 being involved in sprouting and pain development. Since our original hypothesis suggested ADAM21 protein in radial glial cells we analyzed normal distribution of astrocytes and their responses to injury using GFAP immunostaining. However, there were no significant differences in the area of GFAP seen at the injury epicenter and penumbra between the ADAM21-deficient mice and their wildtype littermates. Thus, based on these results, ADAM21 does not seem to play a role in spinal cord development or in responses to injury during adulthood. One caveat to this conclusion is the fact that our injuries turned out mild, lacking clear signs of sensory sprouting. Therefore, it is possible that ADAM21 plays a role in more severe injuries, for example in the allodynia that develops.

Similar to ADAM21, ADAM9 was found to be widely expressed in the brain and spinal cord, but no phenotype was found in development or in naïve adult ADAM9 knockout animals (Weskamp et al., 2002). In addition, ADAM8, a catalytically active metalloproteinase (Fourie et al., 2003), has been implicated in the inflammatory response of asthma (King et al., 2004, Dehmel et al., 2007), but when knocked out there was no apparent phenotype either by defect or pathologically (Kelly et al., 2005). However, when ADAM8 deficient were crossed with wobbler (WR) mice, double mutant had a more severe disease phenotype than ADAM8+/- WR mice and resulted in a dramatic decrease in survival (Bartsch et al., 2010). The role of ADAM9 and ADAM8 was not found to be essential for normal development or in adult mice; however, the role of ADAM8 was elucidated only after the cross with another mutant mouse. This is probably best highlighted in the case of ADAM9, ADAM12, ADAM15, ADAM17, and
ADAM19. ADAM12 and ADAM15 were found not to have overlapping roles in with ADAM9, ADAM17, and/or ADAM19 as combination of ADAM12 and/or ADAM15 did not increase the severity when combined with combinations of ADAM9, ADAM17 and/or ADAM19 lines (Horiuchi et al., 2005). Through different combinations of the preceding 5 ADAMs, it was discovered that ADAM9 and ADAM19 have compensatory or redundant roles (Horiuchi et al., 2005). Similar to the ADAMs mentioned above, ADAM21 could have an overlapping or redundant role with another ADAM. In a separate study, we have not found any metalloprotease gene which solely compensates for the lack of ADAM21 before or after spinal cord injury in this mouse line (Chapter 4). However, this does not preclude the possibility of redundancy across several ADAMs causing the deletion of ADAM21 to show no evident pathological phenotype.

In conclusion, it seems that ADAM21 does not play an essential role in the development of the mouse, as is the case with ADAM10 and ADAM17 or essential in the recovery after spinal cord injury. However, the function of ADAM21 may be revealed through combining the ADAM21-deficient line with that of another ADAM deficient line like was seen with other ADAM deficient mouse lines or by using a different outcome measure that was not covered in this study. Alternatively, ADAM21 might have both beneficial and detrimental roles after spinal cord injury which results in the neutral phenotype of the ADAM21-deficient mice.
Figure 8. ADAM21 and β-Galactosidase immunostaining in the adult spinal cord in sham and spinal cord injured mice.

Transverse sections of the mouse spinal cord were stained with ADAM21 antibody. ADAM21 immunostaining of the intact spinal cord of wildtype (WT) (A and B) and ADAM21-deficient (KO) mouse (D and E): punctated immunostaining is present in the dorsal horn of wildtype mouse and ADAM21-deficient mouse. Labeling in the ependymal cells of the central canal is present in wildtype and ADAM21-deficient mouse, Inset of B and E, respectively. B-Galactosidase immunostaining in the intact spinal cord of wildtype (WT) (C) and ADAM21-deficient (KO) mouse (F). β- Gal immunostaining in the intact cord shows vessel-like structures (G and H) and in the injured spinal cord of ADAM21 deficient mice shows possible microglial cell in the white matter (I and J). WT = wildtype mouse and KO = ADAM21 deficient mouse. Scale bars for A, C, D and F, shown in A, represents 20 μm, for B and E, shown in, represents 20 μm, and for G-J, shown in G, represents 5 μm.
<table>
<thead>
<tr>
<th></th>
<th>BMS</th>
<th>Beam Walk</th>
<th>Hargreaves (sec)</th>
<th>Tail Flick (sec)</th>
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<tr>
<td>Baseline</td>
<td>9.0 ± 0.00</td>
<td>24.8 ± 0.09</td>
<td>3.43 ± 0.29</td>
<td>1.92 ± 0.08</td>
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<td>Week 1</td>
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<td>2.36 ± 0.20</td>
<td>1.86 ± 0.10</td>
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<td>Week 2</td>
<td>6.0 ± 0.50</td>
<td>7.5 ± 1.36</td>
<td>2.73 ± 0.22</td>
<td>1.69 ± 0.07</td>
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<td>Week 3</td>
<td>7.3 ± 0.37</td>
<td>9.3 ± 1.30</td>
<td>2.62 ± 0.17</td>
<td>1.69 ± 0.07</td>
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<tr>
<td>Week 4</td>
<td>6.7 ± 0.34</td>
<td>10.6 ± 1.34</td>
<td>2.89 ± 0.27</td>
<td>1.91 ± 0.11</td>
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<tr>
<td>Week 5</td>
<td>7.1 ± 0.31</td>
<td>11.8 ± 1.77</td>
<td>2.86 ± 0.13</td>
<td>1.78 ± 0.09</td>
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<td>Week 6</td>
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<td>11.2 ± 1.55</td>
<td>2.94 ± 0.23</td>
<td>1.84 ± 0.12</td>
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<td>Baseline</td>
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<td>Week 1</td>
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<td>12.2 ± 1.94</td>
<td>2.85 ± 0.24</td>
<td>2.06 ± 0.10</td>
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</table>

Table 1. Behavioral assessments between ADAM21-deficient mice and wildtype littermates.

There is no statistical significant difference between wildtype and ADAM21-deficient mice in BMS, Beam Walk, Hargreaves, and Tail Flick. Weeks 1-6 in both wildtype and ADAM21-deficient mice are significantly different from baseline for BMS, Beam Walk, and Hargreaves. Scores for BMS and Beam walk in arbitrary units ± SEM and scores for Hargreaves and Tail Flick are in seconds ± SEM. n =13 and 10 for injured wildtype, and injured ADAM21-deficient, respectively. Data for shams is not shown. n = 2 and 3 for wildtype and ADAM21-deficient, respectively.
Table 2. Histological analyses.

There was no difference between wildtype and ADAM21-deficient mice in spared white matter, GFAP, and CGRP. The epicenter of injured within each genotype was significantly different from 1 mm Rostral, 1 mm Caudal, and penumbra (1 mm Rostral + 1 mm Caudal) in all 3 analyses. White matter sparing is in mm² and GFAP and CGRP are in um² ± SEM with the exception of wildtype sham due low number of animals. n = 2, 3, 13 and 10 for sham wildtype, sham ADAM21-deficient, injured wildtype, and injured ADAM21-deficient, respectively.
Naïve levels of CGRP were analyzed to confirm no differences of CGRP at basal levels. There was no significant difference between wildtype and ADAM21-deficient animals at T7 through T10. All values are given in $\mu m^2 \pm SEM$. n=4 in all groups.

<table>
<thead>
<tr>
<th>CGRP $\mu m^2$</th>
<th>$+/+$</th>
<th>$-/-$</th>
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<tbody>
<tr>
<td>T7</td>
<td>13,438 ± 2,980</td>
<td>14,662 ± 963</td>
</tr>
<tr>
<td>T8</td>
<td>14,626 ± 2,708</td>
<td>15,539 ± 1,968</td>
</tr>
<tr>
<td>T9</td>
<td>14,550 ± 2,677</td>
<td>14,622 ± 1,568</td>
</tr>
<tr>
<td>T10</td>
<td>15,532 ± 2,559</td>
<td>13,706 ± 2,074</td>
</tr>
</tbody>
</table>

n = 4 in all groups.

Table 3. CGRP immunostaining in naïve ADAM21-deficient and wildtype littermates.
CHAPTER FOUR

COMPREHENSIVE AND QUANTITATIVE METALLOPROTEINASE GENE ANALYSES REVEAL A REGULATORY ROLE OF ADAM21 IN NORMAL AND INJURED MOUSE SPINAL CORD

INTRODUCTION

The ADAM proteins belong to the adamalysin family of the metzincin or zinc-dependent metalloproteinase superfamily which also includes the MMPs. ADAMs have a disintegrin and metalloproteinase domain, as well as cysteine-rich, EGF-like module, transmembrane and cytoplasmic signaling domains (Wolfsberg et al., 1995, Yang et al., 2006). The adalamysin subfamilies also include the ADAMTS (containing a thrombospondin repeat and lacking the EGF-like, transmembrane, and cytoplasmic domains) and ADAMTSL (lacking the pro-metalloproteinase and disintegrin-like domains) (Tang and Hong, 1999, Apte, 2009). The catalytic activity of all the metalloproteinases is inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Brew and Nagase, 2010).

ADAMs are best known for their role as sheddases in activating cleavage-dependent membrane proteins such as Notch and TNFα (Reiss and Saftig, 2009). MMPs and secreted ADAMTS proteins are best known for extracellular matrix digestion during tissue remodeling, process outgrowth, and cell migration (Pilcher et al., 1999, Curry and Smith, 2006, Amalinei et al., 2007, Ihara and Nishiwaki, 2007). Another unique feature of ADAMs is the disintegrin domain which binds integrins (Bigler et al., 2000, Evans, 2001). Little is known about the mechanism of action or function of all but
a few metalloproteinases in general or specifically in the nervous system (Yang et al., 2006, Yong et al., 2007). Several ADAMs have been associated with diseases or disease pathology such as asthma/inflammation (Van Eerdewegh et al., 2002, Booth et al., 2007, Koller et al., 2009, Mukhopadhyay et al., 2010) and neurological diseases (Gerst et al., 2000, Barrette et al., 2010).

In the injured spinal cord, MMP2 and MMP9 were found to be acutely upregulated in blood vessels, macrophages and astrocytes and to play a predominantly detrimental role (de Castro et al., 2000, Noble et al., 2002, Goussev et al., 2003, Hsu et al., 2006, Zhang et al., 2011a). Increased MMP12 expression by microglia has a detrimental role as shown in injured MMP12-/- mice (Wells et al., 2003). Increased ADAM8 expression is exclusively localized to endothelial cells after spinal cord injury and possibly has a role in angiogenesis (Mahoney et al., 2009). In humans, MMP1, MMP2, MMP9, and MMP12 are expressed in response to spinal cord injury (Buss et al., 2007). Previously, changes in 22 MMPs have been documented up to 5 days following compression injury in adult mice (Wells et al., 2003). Others performed an Affymetrix gene array analysis after a contusive spinal cord injury in adult mice (GEO Profiles accession GDS2159) which did not include all metalloproteinases and was not quantitative or verified.

We have shown robust ADAM21 immunostaining in continuously generated primary olfactory neurons and their growing olfactory bulb axons, as well as in radial glial lineage cells in the neurogenic subventricular zone of adult mice (Yang et al., 2005). Its localization in highly plastic areas of the adult CNS raised the possibility that ADAM21 might play an orchestrating role in repair processes after spinal cord injury. ADAMs could regulate expression of other metalloproteinases for example through activation of the Notch or TNFα pathways (Samuel et al., 2005, Jie et al., 2009, Bartsch et al., 2010, Li et al., 2011).
Here, we used a focused comprehensive quantitative Real-Time RT-PCR (qPCR) gene microarray to document changes in ADAM, ADAMTS, MMP, and TIMP expression following a contusive spinal cord injury in adult mice. We also determined the potential role of ADAM21 in regulating other metalloproteinases under normal and injury conditions using ADAM21 wildtype and homozygous ADAM21-deficient littermates.

MATERIAL AND METHODS

Animals

A total of 40 mice were used, i.e., adult male and female homozygous ADAM21-deficient and wildtype littermates were age and gender matched. They were bred in house from heterozygous B6.129P2-ADAM21tm1Dgen/J mice (stock# 006431, Jackson Laboratory, Bar Harbor, ME, USA; crossbred twice with C57B/6 mice) and crossbred in house 3 more times with C57B/6J mice (stock# 000664, Jackson). The B6.129P2-ADAM21tm1Dgen/J (ADAM21-deficient) mouse line was produced by Deltagen, Inc. which performed a preliminary characterization showing no overt phenotype in multiple organs and physiological measurements (http://www.informatics.jax.org/external/ko/deltagen/142.html). All animal procedures were performed according to University of Louisville Institutional Animal Care and Use Committee protocols and the National Institutes of Health guidelines.

Spinal cord injury

Mice were anesthetized by an intraperitoneal injection of 0.4 mg/g body weight Avertin (2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline, Sigma-Aldrich, St Louis, MO, USA). A laminectomy was made at T9-T10 spine level and a 50
kdyn T9 contusion injury was made using an Infinite Horizons (IH) impactor with steel stabilizers inserted under the transverse processes one vertebra above and below the injury in order to stabilize the spine. Sham animals received a T9-T10 laminectomy only. Animals received 0.1 ml of a 20 μg/ml stock of gentamicin (ButlerSchein, Dublin, OH) and 2 ml saline injected subcutaneously (1 ml before and after injury). After the wound was sutured in layers, Bacitracin ointment (Qualitest Pharmaceuticals, Huntsville, Al) was applied to the wound area and the animals recovered on a water circulating heating pad. Analgesics were given twice daily for first 48 hours of 0.1 ml of a 15 μg/ml stock solution of buprenorphin (Reckitt Benckise, Hull, England). Analgesia was only given after the animal was fully awake due to a lethal interaction with Avertin. Manual bladder expression occurred, as needed, once or twice daily until automatic voiding returned spontaneously, usually within 7-10 days.

**BMS analyses**

The mice were tested for hindlimb locomotor function before injury to establish a baseline, and at 7 days, and 14 days post-injury using the Basso Mouse Scale (BMS) (Basso et al., 2006). In short, two people trained and certified by Michelle Basso at Ohio State University observed mice for 4 minutes in a 47-inch diameter dry pool. The scores range from 0 - 9, where 0 is completely paralyzed and 9 being normal coordination, paw placement, truck stability and tail up. Subscores are given when the animals reach 5 and above on the BMS scale.
Tissue collection and mRNA measurements by quantitative Real-Time reverse transcription-PCR (qPCR)

On the day of tissue collection, animals were anesthetized and perfused with ice-cold PBS. A 4 mm section of spinal cord containing the injury epicenter was freshly dissected out and flash frozen using liquid nitrogen until further processing. Total RNA was isolated using a commercial kit (Cat #: AM1924, Ambion, Austin, TX). In short, 1 µg of DNase was added to RNA (Cat #: 18068-015, Invitrogen, Carlsbad, CA) and used as templates for reverse transcription, which included 1 µl of a 0.5 µg/µl random primers (Cat#: C1181, Promega, Madison, WI), 1 µg total RNA, 5 µl of 5x buffer, 1.25 µl of 10 mM dNTP mix, 2.25 µl RNAse free water, and 1 µl (200 units) of Moloney Murine Leukemia Virus Reverse Transcription (M-MLV RT, Cat #: M170, Promega, Madison, WI) and heated at 37°C for 1 hr. The qPCR was performed using TaqMan® Universal PCR Master Mix (Part Number: 4304437), TaqMan® Array Micro Fluidic Cards, Format 96-a (Part Number: 4342259) and primer sets (see Table 4) from Applied Biosystems, Carlsbad, CA. All cards were run on an Applied Biosystems® 7900HT Fast Real-Time PCR System. Data was analyzed using the SDS 2.1 Relative Quantification software by the ΔΔCT/comparative CT method. All samples were normalized to GAPDH; sham spinal cord CT values of the same genotype were used as the calibrator. Statistical analyses were performed with the Student's unpaired t-test using Excel software for ADAM21-deficient sham versus wildtype sham (Microsoft, Redmond, WA). For all other comparisons, two-way ANOVA with Tukey post-hoc was used to compare genotype and changes over time. A value of p ≤ 0.05 was considered statistically significant.
**Housekeeping genes and negative control**

There were 4 housekeeping genes included on the qPCR array. They included hypoxanthine phosphoribosyl transferase 1 (Hprt1), Beta-glucuronidase (Gusb), Glyceraldehyde 3-phosphate dehydrogenase (GapDH), and 18S ribosomal RNA (18S). Of these housekeeping genes, only two had steady CT values among all samples: GapDH and 18S. However, GapDH was the least variable after spinal cord injury at 7 and 14 days and thus used for normalization. In addition, our negative control for the qPCR array was human CNTF and was undetectable on all arrays.

**RESULTS**

**Temporal mRNA profile in wildtype littermates after spinal cord injury**

Major changes (more than two-fold) in gene expression profile occurred in 19 metalloproteinase genes following spinal cord injury in wildtype mice (Figure 9; Table 5). Seventeen metalloproteinases were increased more than two-fold at 7 and/or 14 days. Of these, ADAM8, ADAM33, and MMP12 were up regulated more than 10-fold compared to the uninjured sham mice at either or both day 7 and 14 post-injury. Only 3 genes were decreased more than two-fold following injury, i.e., ADAM23, ADAMTS17, and MMP17. Surprisingly, ADAM21 was not detectable using the Mm01309314_m1 assay which spans exon1 and exon2. However, in a separate qPCR analysis of the same RNA ADAM21 was detectable using the Mm00480375_s1 assay which measures only exon2. There was no change in the expression level of ADAM21 after injury.

We also measured expression levels of the 4 known endogenous metalloproteinase inhibitors. Only TIMP1 expression changed more than two-fold, increasing by almost 20-fold following spinal cord injury compared to sham. Also included were a few potential substrates of ADAMs to support future investigations into
their potential interactions. Thus, as expected, TNFα was highly up regulated at 7 and 14 days after injury. Alpha-2 macroglobulin (A2m) was also increased at 14 days whereas alpha-synuclein (SNCA) showed a large decrease at 7 and 14 days post-injury compared to sham. Alpha-1 antitrypsin (Serpina1e) was not detectable in either sham or injured spinal cord.

**Sham-operated ADAM21-deficient mice have few changes compared to wild-type littermates**

Under normal conditions, only one gene, ADAMTS16, was significantly higher (1.7 fold) in ADAM21-deficient than in wild-type littermates. However, 4 genes, ADAM19, MMP17, MMP24, and TIMP4 were found to be significantly decreased in the ADAM21-deficient sham-operated mice.

Surprisingly, ADAM21-deficient mice had an ADAM21 transcript detectable by the Mm00480375_s1 assay (beyond the deletion-insertion locus) which was confirmed by endpoint PCR of different regions of the 3' ADAM21 transcript (data not shown). This transcript was increased 3-fold in sham animals. However, we confirmed that the LacZ/Neo cassette was inserted into the ADAM21 gene in the middle of the metalloproteinase domain and prior to the catalytic site (data not shown). A theoretical in-frame ADAM21 start codon would make this a metalloprotease-null aberrant transcript.

**ADAM21-deficient mice have reduced expression levels of metalloproteinases after spinal cord injury**

In ADAM21-deficient mice, mRNA levels of 4 metalloproteinase genes, ADAM8, ADAM33, ADAMTS14, and MMP12 were more than 2 fold lower than their wildtype
littermates following spinal cord injury. Of these, ADAM8, ADAM33, and MMMP12, were decreased by more than 10 fold compared to their injured wild-type littermates. Two metalloproteinase genes (MMP10, MMP27) were detected at all time points in ADAM21-deficient mice, but not detectable at any time point of their wild-type littermates. ADAM28 expression was not seen in wild-type mice, even after spinal cord injury but was seen in some sham-operated ADAM21-deficient mice (4 out of 7) and some injured mice after 14 days (2 out of 6).

TIMP expression levels were not found to be different in injured ADAM21-deficient mice. Of the metalloproteinase substrates measured here, only TNFα had expression levels more than two fold different in injured ADAM21-deficient mice compared to injured wild-type littermates. In fact, TNFα expression levels in the injured ADAM21-deficient mice were 10-fold less than that seen in injured wild-type mice.

Only 8 metalloproteinase genes had no detectable expression in sham or injured mice irrespective of the presence or absence of ADAM21 or spinal cord injury. These included ADAM2, ADAM26b, ADAM34, ADAM36, MMP1a, MMP1b, MMP20, and MMP21. There were 15 metalloproteinases present in only a few samples and therefore could not be statistically analyzed in this array. These included ADAM3, ADAM5, ADAM6A/ADAM6B, ADAM7, ADAM24, ADAM25, ADAM26A, ADAM28, ADAM29, ADAM30, ADAM38, ADAM39, ADAMDEC1, ADAMTS13, and MMP7.

**Locomotor function**

As expected for a moderate injury, the BMS scores in the wildtype mice decreased to 4.1 and 3.7 at 7 and 14 days post injury, respectively. Despite the striking differences in gene expression levels, there were no significant differences between genotypes in the baseline or post-surgery BMS scores of sham-operated mice or after a T9 contusive
spinal cord injury at 7 or 14 days (Figure 10). Additionally, at 14 days post-injury only 3 out of 13 mice had high enough BMS scores to have a subscore, 2 of 7 animals were wildtype, and 1 of 6 was an ADAM21-deficient mouse which was not significantly different.

DISCUSSION

The ADAM21-deficient mouse results suggest that ADAM21 normally up-regulates the expression of a substantial number of metalloproteinase genes following spinal cord injury. This is somewhat surprising as one of the greatest hurdles using knockout mice is the frequent redundancy or overlap of function between proteins due to their high homology within the domain structures and sheer number of metalloproteinases. Thus, we propose that ADAM21 is an important regulator after spinal cord injury. Of particular interest, a cluster of genes known for their role in inflammation (ADAM8, ADAM12, ADAM33, MMP12, TNFα (King et al., 2004, Dehmel et al., 2007, Jie et al., 2009, Koller et al., 2009, Mukhopadhyay et al., 2010, Parameswaran and Patial, 2010)), exhibited a robust increase in injured wild type littermates, which was significantly attenuated in injured ADAM21-deficient mice. In addition, ADAM8, ADAM12, and ADAM33 are within the same phylogenetic group (Hooper et al., 2005). This inflammatory gene cluster seems unique in that ADAM8, ADAM12, ADAM33, MMP12, and TNFα are the only ones so far that have been linked to asthma (Shapiro and Owen, 2002, Van Eerdewegh et al., 2002, Holgate et al., 2004, Haitchi et al., 2005, Berry et al., 2007, Koller et al., 2009, Holgate, 2010, Mukhopadhyay et al., 2010, Paulissen et al., 2011). Under normal conditions (sham-operated), these genes were not changed in ADAM21-deficient mice. This raises the possibility that ADAM21 plays a central role in inflammatory responses under pathological conditions. However, only 6
papers have been published so far on ADAM21 (Hooft van Huijsduijnen, 1998, Poindexter et al., 1999, Liu and Smith, 2000, Seldin et al., 2000, Yang et al., 2005, Yi et al., 2010), mostly describing its presence in the testis, and its functional roles in different organs remains to be investigated.

It remains to be determined how ADAM21 would regulate the expression of the other metalloproteinase genes. The ADAM21-deficient mice contain a Lac-z gene insertion within the metalloproteinase domain with a potential metalloproteinase-dead read-through to the end of the gene. This suggests that the regulation of the other genes is normally through the metalloproteinase domain possibly by its sheddase activity to release growth factors or other cleavage-dependent proteins. Moreover, ADAM21 does not have sequences in its C-terminus that are known to allow binding of intracellular signaling molecules. The absence of ADAM21 after spinal cord injury caused a dramatic decrease in TNFα gene expression levels possibly because of its normal sheddase activity on CD14 and/or Notch. CD14, expressed mainly on macrophages, co-signals through TLR leading to an increase in Notch and NF-κB (Beutler, 2000, Monsalve et al., 2009). In addition to TNFα expression, the Notch signaling pathway has been linked to ADAM12 expression (Li et al., 2011) and indirectly linked to the expression of ADAM8, ADAM33, and MMP12 through increases in downstream targets of Notch such as IL-4 and AP-1 (King et al., 2004, Samuel et al., 2005, Tanaka et al., 2006, Jie et al., 2009). The decrease seen in ADAM8 in the absence of ADAM21 was not decreased to the same degree as that of the other genes in the inflammatory cluster. This could be the result of TNFα induced up regulation in ADAM8 (Bartsch et al., 2010). ADAM8 then cleaves TNFαR1 which can then bind soluble TNFα, thereby desensitizing a cell to the action of TNFα, and causing an apparent feedback loop (Schlomann et al., 2000, Bartsch et al., 2010).
Of note was the finding that ADAM21 deletion did not affect expression of the ADAMTS family members, except for ADAMTS19, following spinal cord injury. The expression levels of some ADAMTSs have been shown to be regulated by induction by IL-1 or down regulation by hypermethylation (Kuno et al., 1997, Wagstaff et al., 2011). Our unpublished data (Chapter 3) suggest that ADAM21 in the injured spinal cord is located in cells resembling microglia at the injury site. Astrocytes and microglia are known to be rapid responders to spinal cord injury and to be intricately involved in initiating inflammation (Brambilla et al., 2005, Kigerl et al., 2009, Loane and Byrnes, 2010, Pineau et al., 2010). Thus, astrocytic and microglial ADAM21 is the most likely candidate for playing a central orchestrating role in the secondary responses to injury. As with other ADAMs which have enzymatic, adhesive and other signaling properties, the physiological targets and mechanism of action of ADAM21 remains to be determined.

We did not observe significant differences in the locomotor function of the ADAM21-deficient mice compared to their wild-type littermates despite the major differences in levels of inflammatory gene expression. A contusive spinal cord injury quickly evolves and progresses into a secondary injury due to inflammation, degradation of both blood vessels and axons and ultimately leading to increased cell death (Hall and Springer, 2004, Hagg and Oudega, 2006, Bramlett and Dietrich, 2007, Fassbender et al., 2011b). The extent of inflammation is thought to predict the functional outcomes and experimental anti-inflammatory treatments are beneficial (Popovich et al., 1999, Weaver et al., 2005, Gonzalez et al., 2007). On the other hand, certain aspects of acute inflammation are thought to be beneficial (Barrette et al., 2007, Kigerl et al., 2009). For example, macrophages remove toxic debris from the injured spinal cord (Popovich and Jones, 2003). The role of the individual ADAMs has been proposed to be both beneficial and detrimental (Yong, 2005, Rivera et al., 2010). For example, although ADAM10 and
ADAM17 promote pro-inflammatory signaling events (including TNFα shedding), they also have protective effects in the injured nervous system (including shedding of amyloid precursor protein, N-cadherin, and Ephrins) (Pruessmeyer and Ludwig, 2009). Further research into the effects ADAM21 has on regulating other proteins involved in inflammation following a spinal cord injury could elucidate the differential processes involved in the inflammatory response.

In the current study no apparent phenotype was seen in the naïve (see also http://www.informatics.jax.org/external/ko/deltagen/142.html) or injured ADAM21-deficient mouse, raising the possibility that one of the other metalloproteinases had compensated for the lack of ADAM21. In sham animals, there were only two genes with significant increases in mRNA levels. ADAM28 was only detected in ADAM21-deficient mice under normal or injury conditions, but only in a subset of mice. The presence or absence of ADAM28 did not predict the expression levels of the genes regulated by the absence of ADAM21. This suggests that ADAM28 had not compensated for the lack of ADAM21. ADAMTS16 showed an increase (70%) in ADAM21-deficient mice under sham conditions. It has been linked to a decrease in cell proliferation and migration when overexpressed in chondrocyte cell lines (Surridge et al., 2009). After spinal cord injury, ADAMTS16 was not changed in wild-type mice but was reduced to 63% (p<0.05, Table 5) in the ADAM21-deficient mice. This suggests that ADAMTS16 did not compensate under injury conditions, which leads us to believe that under normal conditions, it did not compensate in the absence of ADAM21.

In the wild-type mouse, most of the changes following spinal cord injury involved an increase in expression, suggesting that the metalloproteinases play an important role in the responses to injury. Our results are similar to the Affymetrix Mouse Genome 430 2.0 Array data from injured wild-type C57BL6 mice (GEO Profiles accession GDS2159). For example, ADAM8, ADAM12, ADAM33, MMP12, which we showed all have an
increased expression of more than 10 fold, was 11,163, 435, 298, respectively. Our results also confirm reported increases in ADAM8 (Mahoney et al., 2009), MMP2 (Goussev et al., 2003), MMP10, MMP11, MMP12 and MMP13 (Wells et al., 2003), and MMP14 (Mahoney et al., 2009). There are some differences in changes of 6 genes, such as MMP3 and MMP8, at 7 days compared to with the other TaqMan microarray analysis 5 days post-injury (Wells et al., 2003) most likely because of the different type of injury. ADAM10 and ADAM17, both associated with sheddase activity on substrates such as amyloid precursor protein, Notch, and TNFα in the brain (Asai et al., 2003, Pruessmeyer and Ludwig, 2009), increased by around 2-fold after spinal cord injury suggesting that they may play similar roles under that pathological condition.

Of particular note, MMP10 expression was “turned on” at 7 and 14 days post injury. MMP10, also known as Stromelysin-2 or SL-2, has been associated with cell migration (Pilcher et al., 1999, Krampert et al., 2004). In spinal cord injury, MMP10, like ADAM8, seems to play a role in angiogenesis (Heo et al., 2010). Here, MMP27 was another newly expressed gene after spinal cord injury. To date, not much is known about MMP27 other than expression profiles (Bernal et al., 2005). We suggest that MMP10 and MMP27 warrant further study as to their role after spinal cord injury. Despite the many members, most metalloproteinases were expressed at some point before or following spinal cord injury suggesting that this is a very important class of proteins in neural injury.

We also measured the expression of the TIMPs which are natural metalloproteinate inhibitors (Brew and Nagase, 2010). The main response was in TIMP1, showing an almost 20 fold increase following spinal cord injury in wild type mice. This increase would predominantly counteract MMPs (Brew and Nagase, 2010). TIMP1 has a more restricted inhibitory range than the other 3 TIMPs, showing low affinity for MT−MMPs, MMP14, MMP16, MMP19, and MMP24, and is present in plastic regions of
the adult CNS (Rivera et al., 1997, Fager and Jaworski, 2000, Homebeck et al., 2005, Nagase and Murphy, 2008). However, TIMP-1 has stronger affinity for MMP3 and MMP7 than other TIMPs (Hamze et al., 2007). Thus, it could play similar roles in the injured spinal cord. The TIMP1 levels were also increased in the injured ADAM21-deficient mice although at only 60-70% of wild type. How this would affect the activity of the metalloproteinases to affect differential function in the ADAM21-deficient mice remains to be determined. The increase in TIMP1 expression seen in the present study seems to correlate to its proposed involvement in glial scar formation (Ahmed et al., 2005). However, TIMP1 may also act through a mechanism independent of metalloproteinase inhibition (Ogier et al., 2005, Ogier et al., 2006). TIMP2 was increased more in the injured ADAM21-deficient mice than in the wild type mice. In humans, TIMP2 has inhibitory actions on all MMPs and ADAM12 (Jacobsen et al., 2008, Brew and Nagase, 2010). TIMP3 is generally thought of as an ADAM and ADAMTS inhibitor in addition to its inhibition of MMPs (Amour et al., 2000, Hashimoto et al., 2001, Kashiwagi et al., 2001, Wang et al., 2006, Wisniewska et al., 2008).

In conclusion, our data suggest that ADAM21 plays significant role in regulating expression of other metalloproteinases perhaps in a nodal manner, as ADAM21 appears to be an important regulator of a cluster of genes associated with the immune response to injury. Our comprehensive quantitative gene analyses also provide a platform for further study into the role of metalloproteinases after spinal cord injury.
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*Table 4. Genes analyzed.*

Assay and reference sequence numbers for all the genes that were analyzed by in the Applied Biosystems microfluidic gene Taqman array plates. * = assays that were used in an additional analysis which were performed in triplicate with GAPDH as the reference assay. Mm = Mus musculus, Hs = Homo sapiens.
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Figure 9. Gene profile of all genes that evoked a consistent signal among the samples following a T9 contusive spinal cord injury.

Each graph displays the changes in gene expression in wildtype (WT) or ADAM21-deficient (KO) after injury at 7 and 14 days. In addition, the black triangle signifies the ratio in gene expression between the ADAM21-deficient and the wildtype mice in the absence of an injury. All values shown are average fold change ± SEM. n= 8, 8, and 7 for ADAM21-deficient sham, 7 days, and 14 days post injury, respectively and n= 6, 4, and 7 for wildtype sham, 7 days, and 14 days post injury, respectively.
Figure 9 Continued
Figure 9 Continued
Figure 9 Continued
Figure 9 Continued
Table 5. Fold changes and p-values of the data in Figure 9.

Genes are grouped in numerical order by different subfamilies of metalloproteinases, metalloproteinase inhibitors, metalloproteinase substrates, housekeeping/reference genes and the negative control, human CNTF. Increases greater than 2-fold are highlighted in yellow, decreases greater than 2-fold are highlighted in red and statistically significant p-values (less than 0.05) are highlighted in green. Significance was determined by changes with a p < 0.05 calculated by an unpaired Student’s t-test. n= 8, 8, and 7 for ADAM21-deficient sham, 7 days, and 14 days post injury, respectively and n= 6, 4, and 7 for wildtype sham, 7 days, and 14 days post injury, respectively. Asterisks = genes that had expression in only a few samples. ND = Not detectable, NA = Not applicable due to lack of significance in ANOVA, and NS = Not significant.
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Table 5 Continued
Figure 10. Locomotor function in wildtype and ADAM21-deficient mice did not differ before or after spinal cord injury.

The BMS scores were not significantly different between genotypes at baseline or after injury. As expected at 7 and 14 days post-injury the BMS scores are decreased significantly from that of shams. All values are reported as average BMS score ± SEM.
CHAPTER FIVE

DISCUSSION

General

Summary of findings in Chapter 2

In Chapter 2, we examined the possibility that stimulation of the 5HT1A receptor causes a decrease in cAMP resulting in an increase in CNTF leading to an increase in neurogenesis as seen with stimulation of the D2 receptor (Yang et al., 2008). As shown by others, we saw an induction of neurogenesis in the SGZ of rats after stimulation of the 5HT1A receptor by the known agonist, 8-OH-DPAT (Banasr et al., 2004, Huang and Herbert, 2005, Soumier et al., 2010). However, we found that unlike the D2 receptor, neurogenesis in the rat SGZ is not mediated by CNTF. In addition, stimulation of the 5HT1A receptor via intraperitoneal injection of 8-OH-DPAT does not induce neurogenesis in the SVZ and SGZ of mice or the SVZ of mice or rats. Therefore, 5HT1A receptor stimulation results in an increase in neurogenesis in the hippocampal formation of rats at dose of 1 mg/kg/day, which is not mediated by CNTF.

8-OH-DPAT does not increase neurogenesis in the naive mice

The initiation of the study in Chapter 2 was due in part to the Santarelli (2003) paper, which showed increased neurogenesis in the SGZ of 129Sv/Ev
mice after treatment with 8-OH-DPAT, a 5HT1A agonist, which was diminished in the 5HT1A receptor knockout mice (Santarelli et al., 2003). Due to the lack of neurogenic response in the 5HT1A knockout mice, the 5HT1A receptor seemed to be solely responsible for the induction of neurogenesis by 8-OH-DPAT in the SGZ. However, we were not able to replicate the data showing neurogenesis in the SGZ of 129Sv/Ev mice following 5HT1A receptor stimulation by 8-OH-DPAT (Santarelli et al., 2003). In our laboratory, we observed no change in neurogenesis in the SVZ or SGZ after administration of (R)-(+) 8-OH-DPAT in either C57BL/6J or 129Sv/Ev mice either acutely (3 days) or chronically (28 days).

So why would we not see increased neurogenesis after administration of the 5HT1A agonist, 8-OH-DPAT, in mice when the neurogenic response of 8-OH-DPAT was seen in wildtype mice and abolished in the 5HT1A knockouts? There could be a couple of possible reasons for the discrepancy in neurogenic effects of 8-OH-DPAT between our studies. First, the Santarelli (2003) paper had used the racemic mixture while we used (R)-(+) 8-OH-DPAT. In humans, (S)-(--) 8-OH-DPAT binding affinity is similar to that of (R)-(+) 8-OH-DPAT; however, it is approximately 50% less efficacious or potent than (R)-(+) 8-OH-DPAT on the 5HT1A receptor (Cornfield et al., 1991, Yoshitake and Kehr, 2004). This would mean that although the two enantiomers bind equally to the receptor, in the racemic mixture half of the bound receptors produced only 50% activity to the stimuli resulting in 25% less intrinsic activity than the R-enantiomer alone. Furthermore, in rats, the racemic mixture is 2-fold less potent than the R-
enantiomer at reducing dorsal raphe nucleus firing acutely (5 minutes following injection) (Lejeune et al., 1997). Presumably, this would also mean that the racemic mixture could have a 2-fold decrease in the negative feedback produced by the autoreceptors. However, it is unlikely that this is the reason for the discrepancy between our two studies.

A second and more likely explanation for the discrepancy could be due to the behavioral/stress tests performed on in the Santarelli paper. Their goal was to examine the effects of 5HT1A receptor on depression. Therefore, they were not working on naïve mice, but stressed mice. Stress has been shown to affect production and survival of new neurons (Schoenfeld and Gould, 2011). The behavioral analysis used in the Santarelli paper includes unpredictable stressor test, novelty-suppressed feeding test, and cue fear test. Additionally, the unpredictable stressor test involved changing the environment surrounding the mice by altering the sawdust bedding, cage tilting, predator sounds, and modifying to the light cycle. Our studies did not contain any behavior/stress testing. Additionally, personal correspondence with Benjamin Samuels from Rene Hen’s group, suggested that the increased stress from the behavioral tests might have lowered the baseline level of neurogenesis in controls. Therefore, 8-OH-DPAT stimulation of the 5HT1A receptor could be involved in the restoration of neurogenesis to basal levels or prevented the decrease in neurogenesis since behavior testing occurred after administration of 8-OH-DPAT.

Although it is plausible that either the racemic mixture and/or the behavior testing caused the discrepancy in between the studies in Rene Hen’s lab and our
lab, the discrepancy in neurogenic responses is most likely due to the stress-induced decrease in basal level of neurogenesis observed in the Santarelli paper. In order to prove this hypothesis, we could look at changes in neurogenesis triggered by stress from behavioral analysis like what was done in the Santarelli paper. This could be accomplished using four experimental groups. The first two groups would not have any behavior testing and would be administered either saline or 8-OH-DPAT over 4 weeks. The next two groups would be subjected to behavior tests that cause behavioral/stress tests that mimic depression in mice with one group receiving saline and the other receiving 8-OH-DPAT. By analyzing the changes in neurogenesis in each of these groups, we could determine whether 8-OH-DPAT prevents decreases in neurogenesis caused by conditions that are thought to mimic depression. In addition, if 8-OH-DPAT would be given following the induction of depression like behavior in mice, this would determine if 8-OH-DPAT is protective against reduction in neurogenesis or can be an anti-depressant by increasing neurogenesis. If 8-OH-DPAT were found only to prevent the decline in neurogenesis as a result of depression instead of increasing neurogenesis, this would suggest that although depression decreases neurogenesis, the antidepressant activity is not related to neurogenesis. This would not be useful in humans as pretreatment for depression and would only be applicable in people with recurrent depression. Conversely, if 8-OH-DPAT does indeed increase neurogenesis back to basal level, it would suggest that neurogenesis plays a role in recovery from depression. In humans, this would mean that further investigation into drugs that
would increase neurogenesis could be useful in the treatment of depression. Therefore it is important to investigate which mechanism is correct for 5HT1A receptor stimulation so this knowledge can be used to treat depression in human.

Region-selective difference of 8-OH-DPAT effects on neurogenesis in Sprague Dawley rats

Neurogenesis in the SGZ of rats after stimulation of the 5HT1A receptor with the racemic mixture of 8-OH-DPAT has been established previously (Banasr et al., 2004, Huang and Herbert, 2005, Soumier et al., 2010). Therefore, we wanted to test (R)-(+) 8-OH-DPAT in rats as a positive control for bioactivity. Within seconds of drug administration the typical serotonergic behavior of weaving head, flat body posture and forepaw treading (Tricklebank et al., 1985) was observed in the rats which contrasts to the lack of physical response seen in mice after 8-OH-DPAT administration. In addition, neurogenesis in the SGZ was significantly increased after only 3 days of 8-OH-DPAT treatment. This confirmed that our drug was indeed active and induced neurogenesis in response to drug treatment that we were able to quantify using unbiased stereology. However, we did not see any neurogenesis in the SVZ as had been reported previously at 4 hours after 8-OH-DPAT treatment (Banasr et al., 2004). After personal communication with Dr. Annie Daszuta, we found several possible explanations for the discrepancy in our findings. These differences include differences in strain, drug, and quantification of neurogenesis. In the Banasr (2004) paper, they had used the adult Wistar rat and racemic (±)-8-OH-DPAT.
The differences between the racemic mixture and R-enantiomer were discussed earlier in Chapter 5. Surprisingly, the quantification for cell proliferation in all other regions discussed in the Banasr paper was by unbiased stereology with the exception of in the SVZ where total number of BrdU cells was reported per SVZ per section with a total of 5 sections counted per animal. Since we used stereology, a more accurate quantification of neurogenesis, this could be a major area for discrepancy and might suggest that their conclusion with regards to the SVZ was incorrect.

Stereology was designed to estimate, for example, the total number of cells in an anatomically defined area. The balance is in having the appropriate sampling of a region to encompass an accurate sample of biological variation. Under sampling and/or oversampling can result in an inaccurate portrayal of the total number of cells across a region. Therefore, counting every cell present in a section and averaging the total number of cells per section might be a precise method, but it is unlikely that it is accurate due to the absence of estimation in the third dimension. This is due to the inherent biological variation in that cells are not generally evenly distributed in all three axes or even in the same manner across animals; therefore, by sampling in the middle of an anatomical region leads potentially to over estimation and a slight shift toward the edge of the region could cause under estimation. Thus, in addition to systematic sampling across the entire area of tissue, the equation used in the total estimation across the anatomical region takes into account the sampling frequency and tissue
sampling fraction which minimize the biological variation in order to quantitatively and accurately estimate the number of cells in the region of tissue analyzed.

Lastly, at 4 hours there would be an acute response to the 5HT1A agonist to the local 5HT1A receptors, presynaptic receptors located on astroglial cells, in the SVZ resulting in an increase in neurogenesis (Banasr et al., 2004). However, it is possible that by 3 days the 5HT1A autoreceptors, presynaptic receptors on the projections from the raphe nucleus, have been up regulated in response to drug administration and their inhibitory function is predominating in the SVZ (Kinney et al., 2000). Additionally, the regulation of 5HT1A signaling in the hippocampal formation is not affected by the autoreceptor which also explains are response to 8-OH-DPAT in the SGZ but not in the SVZ (Riad et al., 2001, Banasr et al., 2004). Therefore, we conclude that 5HT1A receptor stimulation can induce neurogenesis in the SGZ, but not in the SVZ at 3 days in rats. In order to circumvent the negative feedback in the SVZ, lowering the dose or giving intermittent treatment could potential result in decrease in the negative feedback. However, the most effective way would be to give pindolol, a nonselective beta-blocker and partial beta-adrenergic receptor agonist, to augment the effectiveness of 5HT1A agonist treatment (Kinney et al., 2000). Although the mechanism of action of pindolol’s augmentation of SSRIs and 5HT1A agonists is still in question, it has been shown clinically to bypass the need for desensitization of the autoreceptors resulting in an almost immediate clinical effect as contrasting the delayed effectiveness normally seen with SSRl drug regimens (Kinney et al., 2000).
It seems that in rats at least there is region specificity. Although humans are reported to have neurogenesis in the hippocampal formation, it is difficult to conclude the possible outcome for humans in light of the species differences seen between mice and rats. It goes back to the age old question as to which more closely resembles what occurs in humans. In order to get closer to an answer for whether 5HT1A induced hippocampal neurogenesis is present in humans, the next step would be to look at 5HT1A agonists in higher mammals or move directly to non-human primates which would more accurately predict the likelihood of effects on neurogenesis in humans. If region specificity is also found in non-human primates and used to infer the actions of 5HT1A agonists in human, this would limit treatment to diseases with neuronal loss in the hippocampal formation such as Alzheimer's disease since there is loss of neurons in the hippocampal formation among other regions of the brain. Therefore, treatment of a 5HT1A agonist might improve memory and learning and decrease aggression in Alzheimer's patients if 5HT1A can elicit a neurogenic response in the hippocampus (Lai et al., 2003).

Although we were not able to increase neurogenesis in the SVZ, the most active proliferative region of the brain, it is a very important target and potentially the most effective region to study for neuron replacement. This is due to the endogenous activity already present in the SVZ such as the ability for new neurons to migrate great distances under normal circumstances. In order to develop neuron replacement therapies, continuation of studies which investigate possible pathways that can be exploited in order to manipulate and/or augment
neurogenesis in the SVZ is essential. Under the right circumstances, some diseases states could benefit from increasing neurogenesis in the SVZ such as stroke, Huntington’s, epilepsy, and aging (Curtis et al., 2007a). Furthermore, it is important to investigate and understand the differences of these two regions of the brain such as endogenous regulation by receptors that either stimulate or inhibit neurogenesis and what types of cells are produced in each region. As more information is attained on the regulation of neurogenesis, we can apply it to humans in an effort to control the migration and integration of these new neurons to better rescue the damage or non-functioning portions of the brain in neurodegenerative disease.

5HT1A receptors and depression

The 5HT1A autoreceptors are not present in the hippocampal region while the SVZ is under constant regulation by these autoreceptor unless desensitization occurs. However, the hippocampal formation is under the control of the 5HT1B autoreceptor (Malagie et al., 2001). The 5HT1A receptor function and regulation seem to vary depending on the region of interest (Riad et al., 2001) which is probably due to the density and localization of the 5HT1A receptors. It is hypothesized that the variability in patient responses including delay in onset of therapeutic effect to the treatment with selective serotonin reuptake inhibitors in depression is due to the negative feedback of autoreceptors (Blier et al., 1998). In a recent study, animals with low and high amounts of 5HT1A autoreceptors were found to respond differently to stress.
(Richardson-Jones et al., 2010). The lower the amounts of autoreceptors present gave better serotonergic tone and responded better to stressors and SSRI treatment (Richardson-Jones et al., 2010). Thus, the level of autoreceptors present could be the main cause of the vast degree of variability in the effectiveness with anti-depressants drug regimens. Pindolol, primarily known for being a non-selective beta blocker, is thought to accelerate the therapeutic effect of antidepressant therapies by inhibition of autoreceptors which reduce firing of serotonergic neurons resulting in potentiation of the effects of SSRI treatment by increasing the serotonergic tone of the system (Berney et al., 2008).

Furthermore, return of autoreceptor function could also explain the development of resistance after chronic administration of antidepressants.

In general, the depression field has ignored the presence of 5HT1A receptors on astrocytes and focused on neuronal 5HT1A receptors. Although 5HT1A receptors are in high abundance on glial cells (Whitaker-Azmitia et al., 1993), most effects of serotonin are assumed to be as a result of stimulation of neuronal 5HT1A. In addition, serotonin autoreceptors have attracted attention over the last decade because of the inhibitory effects on the serotonin pathway and resulting delay in the therapeutic effect of antidepressants such as SSRIs. The reason for ignoring the glial component of the serotonergic system is unclear. However, astrocytes provide support for neurons through release of neurotrophic factors, cytokines, and calcium flux. Therefore, astrocytes should be considered part of the neuronal network. In neurodegenerative diseases, the neurons are dead, dying, or not functioning properly and astrocytes are relatively
unaffected. This puts astrocytes in an advantageous position of being available and accessible as a tool to promote neurogenesis.

Caveats and future directions for further investigation in neurogenesis

As mentioned earlier in this chapter, stimulation of 5HT1A receptor might be protective in stress-induced decreases in neurogenesis. In order to investigate this further, we could use 5HT1A agonist, 8-OH-DPAT, and antagonist, WAY100135 or (-)-tertatolol (another beta-blocker with 5HT1A inhibitory activity), to look at the potential for 5HT1A receptor increases in neurogenesis after a perturbation of the system like a stroke. Strokes located in different areas of the brain could provide an opportunity to look at regional differences of 5HT1A. The murine 5HT1A receptor might not play a role in regulating neurogenesis under normal circumstances, but potentially could be inducible following an insult. However, the drug administration would need to follow the insult/injury to be clinically relevant which contrasts with the Santarelli paper where 8-OH-DPAT was given prior to induction of stress. Therefore, it is possible that 8-OH-DPAT is required to be present before the insult. However, it could be possible for 5HT1A stimulation following the insult could still result in protection from subsequent neuronal loss or decreases in neurogenesis.

Additionally, in our hands, a decrease in nNOS seemed to predict neurogenesis. In fact, nNOS might be a better target for increasing neurogenesis by using an inhibitor such as L-NAME which has been shown to
increase neurogenesis in the SVZ of mice (Romero-Grimaldi et al., 2008). L-NAME is a competitive inhibitor of NOS, but unfortunately lacks specificity between iNOS, nNOS, and eNOS. The lack of specificity comes with a multitude of side effects such as impairment of memory retrieval/acquisition and blockade of non-noradrenergic, non-cholinergic nerves leading to erectile dysfunction and impaired bowel motility (Moore and Handy, 1997). While these side effects do not preclude the need for further study, they would decrease patient compliance and potentially prevent FDA approval with a non-specific NOS inhibitor. Therefore, further study into other receptors that could inhibit nNOS or more selective nNOS inhibitors would be useful to study in an effort to find additional drugs that could be used in conjunction with a D2 agonist to promote increases in neurogenesis and prevent systemic side effects.

Due to the degree of complexity of the 5HT1A pathway, glial 5HT1A responses are difficult to tease out of the responses seen with 5HT1A agonist. It seems likely that the lack of neurogenic effects in the SVZ after 8-OH-DPAT systemic administration could be attributable to inhibition by 5HT1A autoreceptors. This could be confirmed by knocking out the autoreceptors by denervation of the raphe nuclei using the serotonergic neurotoxin, 5,7-dihydroxytryptamine which alone has been shown to increase neurogenesis. In addition by varying the degree of denervation, one could potentially see a dose dependent increase in neurogenesis. In our studies, we saw a region and species specificity to the activity in response to the 5HT1A agonist, 8-OH-DPAT. There are also marked differences in the responses between mice and rats
which were described in Chapter 2. Therefore, further research is needed in order to tease out the specific mechanism of action of the 5HT1A receptor in specific regions of the brain. However, one must question the validity of studying the 5HT1A receptor in rodents when there is such a wide range in responses to stimulation. Although the 5HT1A receptor has 89% homology between rats and humans and 86% homology between mice and humans, there seems to be drastic differences in the function in different species. The lack of serotonergic syndrome in mice given up to 10 times the normal dose of 8-OH-DPAT given to rats leads me to believe that the murine 5HT1A receptor does not have the same biological function as in humans and rats. Since both rats and humans can exhibit serotonergic syndrome in response to serotonin, rats seem to be the better choice for further study into the function of the 5HT1A receptor before studying this paradigm in non-human primates or humans.

What would I have done differently in Chapter 2?

One of the biggest problems with Chapter 2 is the lack of a biological effect in the mouse. In order to understand the activity of 8-OH-DPAT activity in the mouse, it would have been nice to have added a couple of experiments that looked at the bioactivity of 8-OH-DPAT on mice. First, our hypothesis was that the decrease in cAMP following 5HT1A stimulation would cause an increase in CNTF. However, we never measured cAMP. If we had measured cAMP levels we might have seen, for instance, that there was no change in cAMP. Then we could have either adjusted the dosage or found another drug to test for changes
in cAMP before doing so much work looking at mRNA and neurogenesis analysis. In addition, mild hypothermia was reported in mice following treatment with 8-OH-DPAT. It would have been relatively simple to test the temperature of the animals periodically throughout the study for changes in the basal body temperature. A change would indicate bioactivity. Lastly, I could have added another experimental group that combined 8-OH-DPAT with a 5HT1A antagonist to determine if the autoreceptors were causing the lack of effect in mice.

**Summary of findings from Chapter 3 and 4**

In Chapter 3 we found that the absence of ADAM21 protein did not result in a function phenotype measured by two locomotors tests, BMS and beam walk, and two thermal nociception tests, Hargreaves and tail flick. Histologically, we found no significant difference between genotypes in spared white matter, GFAP immunostaining, or CGRP immunostaining. The neutral phenotype was thought to be a result of compensation by another zinc metalloproteinase; however, we found no evidence of compensation on the mRNA level for the absence of ADAM21. Interestingly, we found a massive decrease in several genes which have been associated with inflammation, i.e., ADAM8, ADAM33, MMP12, and TNFα. These changes lead us to believe that ADAM21 has a regulatory role in the inflammatory gene response following spinal cord injury. Thus far, these changes in mRNA have not given a functional phenotype, but become the basis for further research into the role of ADAM21.
Hypothesis for potential role of ADAM21 in pain development following spinal cord injury

As mentioned in Chapter 3 and 4, robust ADAM21 immunostaining was found in highly plastic areas of the brains (Yang et al., 2005). In addition, it is predicted to have a catalytically active zinc metalloproteinase site which pharmacologically makes it a great target for inhibition by a small molecule. Our original hypothesis was based off the ADAM21 immunostaining in growing axons and presence in radial glial lineage cells. We wanted to investigate the possibility that ADAM21 was involved in sprouting after spinal cord injury. Upon immunostaining for ADAM21 in the spinal cord it was found to be localized in laminae I and II of the dorsal horn and in the ependymal cells surrounding the central canal. The staining in the dorsal horn was punctated and occasionally resembled fibers. Since nociceptive fibers (C and Aδ fibers) correspond to the location of ADAM21, we chose to investigate the role of ADAM21 in the pain response after spinal cord injury using ADAM21-deficient mice. Conversely, we now believe that the ADAM21 immunostaining is an artifact and does not resemble the true localization of ADAM21 in the spinal cord due to the lack of co-labeling of ADAM21 and β-galactosidase immunostaining. As the basis for our original hypothesis turned out to be false, it makes sense that we did not see changes in sprouting of CGRP in the dorsal horn or changes in GFAP since this is not the region or cell type the ADAM21 protein is located.
ADAM21 deletion and spinal cord injury

A cluster of genes known to be linked with inflammation was greatly increased in wildtype, but considerably reduced in their response to injury in the ADAM21-deficient mice (i.e., ADAM8, ADAM12, ADAM33, MMP12, and TNFa). Increases in inflammation would presumably cause increases in pain and decrease in locomotor function. Likewise, decreases in inflammation should produce decreases in pain and improved locomotor function, but this was not the case with ADAM21 deficient mice. ADAM21 lacked a functionally significant difference in pain development after spinal cord injury as we found no changes in thermal nociception measured by Hargreaves and tail flick tests following spinal cord injury. In addition, ADAM21-deficient mice responded similarly to the spinal cord injury as the wildtype littermates in all parameters tested.

A possible explanation for the absence of a function phenotype is that the changes in mRNA as a result of the lack of ADAM21 protein did not translate into a reduction in inflammation. Until we are able to look at the immunological response in the ADAM21-deficient mice, this conclusion is purely speculative. As discussed in Chapter 4, ADAMs and other metalloproteinases have been shown to overlapping physiological functions [Horiuchi, 2005 #2187, but there was no evidence of compensation by another zinc metalloproteinase. Yet other proteins could compensate or regulate the changes resulting from a lack of ADAM21 such as TIMPs, a group of endogenous metalloproteinase regulators. Although there were changes in 3 of the 4 TIMPs following spinal cord injury, there was not a significant difference in the changes in TIMPs between genotypes. The
consistent inhibitory tone of the system could account for the lack of a phenotype if the changes in metalloprotease were inhibited to the same extent in both wildtype and ADAM21-deficient mice. For instance, TIMP1 could mitigate the different changes in metalloproteinases resulting in the neutral outcome that was observed. In order to confirm this is the case, a double knockout of TIMP1 (or other TIMPs) and ADAM21 should be compared. However, combinations of small changes in TIMPs and metalloproteinases could account for no lack of compensatory mechanism in ADAM21 deficient mice.

Another possibility is the fact that we are looking at mRNA levels at 7 and 14 days post injury. It is possible that an increase/decrease in mRNA does not translate into an increase/decrease in protein levels/activity. Therefore, the changes in mRNA could be transient and any affect as a result of these acute changes could be diminished over the 6 weeks. In addition, we only looked at these two early time points which could not be giving us a full picture as to what is going on in the system.

Spinal cord injury resulted in numerous changes in metalloproteinase activity, but none of these changes resulted in a functional phenotype. Due to the complexity of the regulatory mechanisms of metalloproteinases, it is plausible that other proteins not included our array could compensate the lack of a phenotype such as feedback or feed forward loops like seen with ADAM8, TNFα, and TNFR1. In addition, regulation of both beneficial and destructive metalloproteinases and/or TIMPs could explain the neutral phenotype. An example of this would be the feedback loop of ADAM8 and TNFα that was
discussed in Chapter 4 (Bartsch et al., 2010). Although there is no evidence of compensation of ADAM21, the complexity of the regulation of the metalloproteinases by not only each other, but also TIMPs and other proteins make finding a definitive compensatory mechanism challenging.

**Caveats pertaining to ADAM21-deficient mouse**

During the course of the array portion of the study, it was discovered that exon 1 of ADAM21 was not present in the spinal cord. This was not unexpected since it was reported that exon 1 of ADAM21 in the testis was only present in germ cells (Yi et al., 2010). After obtaining an assay for exon 2, we found expression of ADAM21 in both wildtype and ADAM21-deficient mice. However, under further investigation of the mRNA from ADAM21-deficient mice, we found that the insertion site of the LacZ cassette was in the middle of the metalloproteinase domain effectively eliminating the potential for metalloproteinase activity. We also found that starting at the beginning of the disintegrin domain, mRNA transcription resumed to include the rest of the C-terminal portion of the ADAM21 transcript. Upon further investigate a theoretical promoter region followed by a putative start codon was found downstream of the metalloproteinase catalytic site. However, this is highly speculative until we are able to determine if this truncated transcript is translated into protein. Unfortunately, additional ADAM21-deficient mice were not available to confirm presence of a truncated ADAM21 protein. Additionally, our antibody for ADAM21 shows similar immunoreactivity in wildtype and ADAM21-deficient mice. This
means either that our antibody is not specific or it is detecting a truncated ADAM21 protein. Lastly, β-galactosidase immunostaining revealed a different expression pattern than was seen with the ADAM21 antibody. This probably means that the ADAM21 antibody is not specific and would explain why our original hypothesis failed—it was based off false information. Therefore, the exact type of genetically modified ADAM21 we have obtained is still in question. We have two options available: a metalloproteinase-null ADAM21 and the function of the other domains remain intact or we could have an ADAM21-deficient mouse that has truncated ADAM21 mRNA that lacks the ability to be translated into protein. We plan to investigate further these two possibilities by using another antibody for ADAM21 which recognizes the metalloproteinase domain which will be validated by β-galactosidase immunostaining. If this antibody has the same immunostaining as the β-galactosidase antibody then the ADAM21 antibody that targets the C-terminus, PSGPKETKASSPG, is not specific to ADAM21. Of note, BLAST analysis of this 13 amino acid sequence produced only one exact match, ADAM21, but included partial matches to 140 other mouse proteins. If, however, the pattern of staining with the new ADAM21 antibody matches our C-terminus ADAM21 antibody, then there could be some alterations in the expression pattern of β-gal such as different regulation, transport, or processing. Lastly, if the truncated protein is present, this would suggest that the changes in inflammatory gene were a result of the lack of metalloproteinase activity of ADAM21. Whereas, a knockout of the entire protein
does not allow one to discern which domain is responsible for the gene changes that occurred after spinal cord injury.

Future directions

The data from the ADAM21 deficient mice suggests that ADAM21 does not play a role in pain development or influence the recovery of locomotor or fine motor control following spinal cord injury. In apparent contradiction, ADAM21 could potentially be involved in the inflammatory response following spinal cord injury. Massive changes in metalloproteinases and TNFα point to ADAM21 having a regulatory role following spinal cord injury. Although there were other changes in mRNA levels of other metalloproteinase, the magnitude of change seen in this cluster of inflammatory genes points to a more direct relationship between them and ADAM21. Further validation is still needed. Validation of these results could be accomplished by the following techniques: western blot, ELISA, or immunostaining for the genes of interest. In addition, following up with the use of CD45 and CD68 immunostaining (microglia and monocytes/macrophages, respectively) in the spinal cord tissue of ADAM21-deficient mice as an indicator of the degree of inflammation would give us a better idea as to the degree of inflammation in these mice compared with wildtype littermates. If ADAM21 is indeed a master regulator of inflammation following spinal cord injury, this could open up a new avenue for manipulation of inflammatory processes by blocking ADAM21 activity with a small molecule could potentially provide an avenue for reduction of inflammation. First, the pro-
inflammatory activity of ADAM21 would need to be narrowed down to the domain responsible. Although this would need further investigation to confirm, more than likely the metalloproteinase domain is the primary domain responsible for changes seen in the inflammatory genes upon deletion of ADAM21. Confirmation could be done by point mutations of the catalytic site of ADAM21 resulting in a metalloproteinase dead protein.

Although there are metalloproteinase inhibitors, there is not an ADAM21 metalloproteinase specific inhibitor to date. The use of broad-spectrum metalloproteinase inhibitors would inhibit large groups of metalloproteinases and is not desirable. However, continued effort has been put into design of inhibitors of specific metalloproteinases although selectivity of such an inhibitor is a great obstacle due to the high degree of homology of the catalytic site of zinc metalloproteinases (Fisher and Mobashery, 2006, Corbitt et al., 2007, Jacobsen et al., 2010). Non-specific metalloproteinase inhibition results in detrimental side effects such as MMP-induced musculoskeletal syndrome which can cause muscle stiffness, loss of range of motion in large joints, joint swelling, and soft tissue pain as seen in rats (Renkiewicz et al., 2003) and humans (Coussens et al., 2002, Krzeski et al., 2007). Therefore, specific inhibitors would reduce side effects like those mentioned above. The most successful method for increased specificity in the development of metalloproteinases has been accomplished using bioinformatics to model the catalytic site. The catalytic site contain 6 recognition "pockets" around the catalytic zinc and the distance between these pockets vary among the different metalloproteinases (Dorman et al., 2010).
utilizing this difference and the computing power of computers, potential selective small molecules can be found and tested for selectivity and bioavailability. Alternatively, small peptide based drugs could be designed to bind to more specific regions around the catalytic site to block access.

The question remains whether we could reduce the chronic inflammation after spinal cord injury which has been shown to be detrimental consequences in the long-term for spinal cord injury patients. A specific inhibitor of ADAM21 metalloproteinase would have many hurdles to overcome before it could be brought to the clinic. Once a suitable candidate is identified, the timing of when to begin treatment of the inhibitor and the duration of treatment would need to be determined. Some inflammatory processes are beneficial and the exact time when beneficial inflammation turns detrimental is still under investigation (Popovich and Jones, 2003, Crutcher et al., 2006, Sandhir et al., 2011). Is it required for the rest of the patient’s life to keep the chronic inflammation at bay? These question still need to be answered and I believe it is important to work to find inhibitors specific to different metalloproteinases than could be used not only in spinal cord injury but also asthma, cancer, periodontal disease and many more diseases as the metalloproteinases are involved in a vast range of diseases.

What would I have done differently in the Chapter 3 and 4?

If I could change the order in which I looked at the role of ADAM21 we might have more answers than questions at this point. Initially upon receiving the
ADAM21-deficient mice, I should have confirmed by qPCR that ADAM21 mRNA was not present. This way I would have been able to figure out whether or not the truncated transcript of ADAM21 is translated into a truncated protein or degraded. This would have allowed me the time to explore what type of genetically modified mouse we had before spending countless hours doing a spinal cord injury study which left many more questions than it answered due to the current uncertainty of the mouse strain. In the process of investigating this mouse strain, I would have run a western to confirm protein sizes and the absence or presence of a truncated protein and done β-galactosidase immunostaining from the start to look which cells produced ADAM21. This would have led to the inconsistency in the β-galactosidase and ADAM21 staining. All of which could have been confirmed before years of work went in to a mouse that may or may not be a true knockout. Of course looking back, it would have been nice to have done the comprehensive array following spinal cord injury prior to the 6 week behavior study on pain and plasticity in order to formulate a better experiment that could have better answered what role ADAM21 has after a spinal cord injury.
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