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**Osteopontin Expression During the Acute Immune Response Mediates Reactive Synaptogenesis and Adaptive Outcome**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

Julie Lynn Chan

B.S. Neurobiology, University of California, Irvine, 2005

Director: Linda L. Phillips, Ph.D.

Professor, Department of Anatomy and Neurobiology

Virginia Commonwealth University  
Richmond, Virginia  
August 2013

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## List of Abbreviations

A $\beta$	..... amyloid-beta
ACH	..... acetylcholine
ACHR	..... acetylcholine receptor
AD	..... Alzheimer disease
ADAM	..... a disintegrin and metalloproteinase
AIDS	..... acquired immunodeficiency syndrome
AMPA	..... $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	..... analysis of variance
APP	..... amyloid precursor protein
BBB	..... blood brain barrier
BDNF	..... brain-derived neurotrophic factor
CA	..... cornu ammonis
CAM	..... cell adhesion molecule
CCI	..... controlled cortical impact
CHI	..... closed head injury
CNS	..... central nervous system
CSF	..... cerebrospinal fluid
CTE	..... chronic traumatic encephalopathy
CSPG	..... chondroitin sulfate proteoglycan

DA ..... dopamine

DAI ..... diffuse axonal injury

DG ..... dentate gyrus

DIG ..... digoxigenin

EAA ..... excitatory amino acid

EAE ..... experimental autoimmune encephalomyelitis

EC ..... entorhinal cortex

ECM ..... extracellular matrix

ETA-1 ..... early t-lymphocyte activation 1

GAG ..... glycosaminoglycan

GCL ..... granule cell layer

GCS ..... Glasgow Coma Scale

GF ..... growth factor

GFAP ..... glial fibrillary acidic protein

GLM ..... general linear model

HI ..... hypoxic-ischemic injury

HIV ..... human immunodeficiency virus

HOAC ..... glacial acetic acid

HSPG ..... heparan sulfate proteoglycan

IBA1 ..... ionized calcium binding adaptor molecule 1

IA ..... impact acceleration

IFN ..... interferon

IGF-1 ..... insulin-like growth factor 1

IHC .....	immunohistochemistry
ICP .....	intracranial pressure
IL .....	interleukin
IL-1RA .....	interleukin 1 receptor antagonist
IML .....	inner molecular layer
IP .....	intrapertoneal
ISH .....	<i>in situ</i> hybridization
KD .....	kilodalton
KO .....	knockout
LCN2 .....	lipocalin 2
LOC .....	loss of consciousness
LPS .....	lipopolysaccharide
LTP .....	long-term potentiation
MANOVA .....	multivariate analysis of variance
MAP .....	microtubule associated protein
MBP .....	myelin basic protein
MCAO .....	middle cerebral artery occlusion
MCAO/R .....	middle cerebral artery occlusion with reperfusion
MEOH .....	methanol
MI .....	myocardial infarction
ML .....	molecular layer
MMP .....	matrix metalloproteinase
MPTP .....	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MT-MMP .....	membrane-type matrix metalloproteinase
MS .....	multiple sclerosis
MTBI .....	mild traumatic brain injury
MWM .....	Morris water maze
NCC .....	neural crest cell
NGAL .....	neutrophil gelatinase-associated lipocalin
NMDA .....	N-methyl-D-aspartate
NMDAR .....	N-methyl-D-aspartate receptor
NMJ .....	neuromuscular junction
NOR .....	novel object recognition
NOS .....	nitric oxide synthase
NSC .....	neural stem cell
OB .....	olfactory bulb
OEC .....	olfactory ensheathing cell
OML .....	outer molecular layer
OPN .....	osteopontin
PAGE .....	polyacrylamide gel electrophoresis
PB .....	phosphate buffer
PBS .....	phosphate buffered saline
PCR .....	polymerase chain reaction
PD .....	Parkinson disease
PNN .....	perineuronal net
PNS .....	peripheral nervous system

PTSD ..... post-traumatic stress disorder

QRT-PCR ..... quantitative reverse transcriptase polymerase chain reaction

RGD ..... arginine-glycine-aspartate

ROD ..... relative optical density

ROS ..... reactive oxygen species

R-OPN ..... recombinant osteopontin

SAH ..... subarachnoid hemorrhage

SCI ..... spinal cord injury

SEM ..... standard error of the mean

SGZ ..... subgranular zone

SIBLING ..... small integrin-binding ligand n-linked glycoproteins

SN ..... substantia nigra

SPP1 ..... secreted phosphoprotein 1

SVZ ..... subventricular zone

TAI ..... traumatic axonal injury

TBI ..... traumatic brain injury

TBI+BEC ..... central fluid percussion injury + bilateral entorhinal cortex lesions

TGF- $\beta$  ..... transforming growth factor-beta

TNF ..... tumor necrosis factor

UEC ..... unilateral entorhinal cortex lesion

WB ..... western blot

WT ..... wild type

6-OHDA ..... 6-hydroxydopamine



Abstract

**OSTEOPONTIN EXPRESSION DURING THE ACUTE IMMUNE RESPONSE  
MEDIATES REACTIVE SYNAPTOGENESIS AND ADAPTIVE OUTCOME**

By Julie Lynn Chan, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Major Director: Linda L. Phillips, Ph.D.  
Professor, Department of Anatomy and Neurobiology

Traumatic brain injury (TBI) is a worldwide epidemic as the number of victims living with the resulting cognitive and physical impairments continues to rise, principally due to limited treatment options which fail to address its multifaceted sequelae. By approaching TBI therapy from a molecular standpoint, we have the opportunity to develop a better understanding of the mechanisms which prevent effective recovery. With this information, we can move toward the identification of novel therapeutic treatments which target specific molecules to improve patient outcome following TBI. Here, we focused on the therapeutic potential of osteopontin (OPN), an extracellular matrix (ECM) protein which is a substrate of several matrix metalloproteinases (MMPs), and capable of acting as both a cytokine and modulator of axonal outgrowth during synaptic recovery. The ECM and its components are of particular interest with respect to selecting novel TBI therapeutics since this network has been implicated in neuronal plasticity

during both development and following central nervous system (CNS) insult. In this dissertation study, the temporal and spatial profile of OPN expression, its protein and transcript localization within reactive glia (IBA1 positive microglia or GFAP positive astroglia), and its interaction with the cytoarchitectural protein microtubule associated protein 1B (MAP1B) after injury were each compared under conditions of deafferentation induced synaptogenesis in the rat. Two TBI models were employed: one exhibiting adaptive synaptic plasticity (unilateral entorhinal cortex lesion, UEC), and the other generating maladaptive synaptic plasticity (central fluid percussion injury followed by bilateral entorhinal cortex lesions, TBI+BEC), in each case targeting 1, 2, and 7d postinjury intervals. In addition, we examined the potential for converting the adaptive response to one of maladaptive plasticity by attenuating immune reactivity through acute administration of the tricyclic antibiotic minocycline, utilizing a dosing paradigm previously demonstrated to reduce inflammation. To more clearly confirm that OPN has a role in successful synaptic regeneration, we developed a colony of OPN knockout (KO) mice which were used to profile synaptic structure and functional outcome under conditions of UEC-induced synaptogenesis.

In Chapter 2, we report that full length OPN responds robustly in the acute (1-2d postinjury) degenerative period following UEC and TBI+BEC. After UEC, time-dependent differences were observed for two alternative MMP-processed OPN forms, including early increase in a RGD-containing 45 kD, integrin binding fragment (1d), and delayed increase in a C-terminal 32 kD OPN peptide (7d). OPN transcript was also elevated acutely after UEC, a finding which was pronounced in enriched dentate molecular layer (ML) fractions. Parallel immunohistochemistry (IHC) and *in situ* hybridization localized OPN protein and transcript to reactive glia following UEC. This localization was concentrated within microglia which

delineated the border between the intact and deafferented ML, a pattern which was less pronounced in maladaptive TBI+BEC animals. The timing of this glial movement suggests that OPN regulates microglial migration and, potentially, could act as an astrokine to recruit activated astrocytes to influence subsequent synaptic regeneration. MAP1B staining confirmed dendritic loss during axonal degeneration and dendritic atrophy, with a reemergence during collateral axonal sprouting. However, OPN colocalization with MAP1B was minimal, suggesting a minor role for OPN in reorganization of dendritic/axonal cytoarchitecture in this model of deafferentation. Minocycline reduced acute OPN protein response 2d after UEC, and caused a more random OPN positive glial distribution, similar to that of the maladaptive TBI+BEC. The role of OPN in the inflammation-directed degeneration of terminals is supported by reduced MMP-9 activity which is temporally correlated with the reduction of MMP-generated OPN lytic fragments (45 kD). Interestingly, this reduction of integrin-binding OPN peptide also matched the impaired removal of presynaptic terminals, evidenced by diminished synapsin 1 clearance in animals which received postinjury minocycline.

In Chapter 3, we sought to more precisely evaluate the role of OPN following deafferentation utilizing wild type (WT) C57BL/6 and OPN knockout (KO) mice subjected to UEC, comparing the spatio-temporal injury response between WT and KO. To do this we profiled several outcome measures which assessed OPN role in different aspects of recovery: 1) expression of select proteins important in various stages of synaptic recovery, 2) glial response, 3) cognitive recovery, and 4) MMP enzymatic activity. Compared to WT mice, OPN KO mice did not show significant differences in the acute injury-induced alteration of proteins important to cytoarchitectural reorganization (MAP1B) or stabilization of the synaptic junction (N-cadherin). However, both Western blot and IHC analyses showed OPN KO mice had impaired

presynaptic terminal clearance, supported by attenuated synapsin 1 breakdown, a result quite similar to that of the minocycline-treated rats with OPN reduction in Chapter 2. This impaired degeneration in OPN KO mice at 2d postinjury correlated with IHC evidence for altered microglial morphology, and hippocampal function assessed by the novel object recognition (NOR) task. Our NOR results confirmed cognitive dysfunction in OPN KO mice during the 4-21d period of synapse reorganization after UEC. In addition, OPN KO decreased MMP-9 activity, an effect associated with reduced MMP-9-bound lipocalin 2 (LCN2), a persistently activated form of that MMP. These latter findings further support the hypothesis that MMP processing of OPN contributes to effective regenerative response after injury.

Collectively, the studies presented in the two chapters of this dissertation provide evidence that OPN is a critical element in the acute immune response following injury-induced CNS deafferentation. They suggest that the cytokine can be produced by reactive microglia, may mediate cell migration and acute degenerative clearance, potentially serves as an astrocyte to recruit those glia to sites of synaptic repair, and disrupts these processes when it is either reduced or ablated. Interestingly, this OPN role in synaptogenesis appears to involve ECM interaction with MMP-9, possibly regulated by LCN2. Most importantly, OPN involvement seems to affect the time-dependent progression of synaptic repair, an effect which can be measured by efficacy of functional outcome

## CHAPTER 1

### INTRODUCTION

## **TRAUMATIC BRAIN INJURY**

### Impact, Epidemiology, and Classification

Traumatic brain injury (TBI) is a serious global health issue with an estimated 10 million new incidents a year (Hyder et al., 2007), 1.7 million of which occur in the United States alone (Faul et al., 2010). TBI affects people of all backgrounds and geographic locations, culminating in a vast number of incidents which exceed that of spinal cord injury (SCI), HIV/AIDS, breast cancer and multiple sclerosis (MS) combined (Brain Injury Awareness Association). Further compounding the impact of new injuries are the lasting effects of TBI, including long term cognitive and motor deficits. These impairments often require victims to undergo extensive psychological and physical therapy (Hyder et al., 2007; Asikainen et al., 1998; Faul et al., 2010; Weightman et al., 2010), nearly tripling (5.3 million) the number of Americans who currently live with the effects of TBI. Together, the subsequent cost of rehabilitation and loss of productivity has resulted in a large financial burden in excess of \$60 billion annually (Langlois et al., 2006; Finkelstein et al., 2006; Rutland-Brown et al., 2006). While these estimates are limited to the direct effects of TBI, the economic and social impact may be even greater, up to \$221 billion, when factoring in lost quality of life (Coronado et al., 2012).

TBI is defined as any process that damages the brain following trauma such as a blow to the head, penetrating injury, or merely rotational forces which involve rapid acceleration and deceleration (Silver et al., 2005). While everyone is susceptible to TBI, the most vulnerable cohorts are men, young individuals, and the elderly. Those 0-4 years, adolescents, and seniors over 75 years comprise the largest portion of TBI-related injuries each year (Langlois et al., 2006). The leading cause of TBIs is falls, while motor vehicle accidents and blows to the head comprise the majority of all other annual incidents (Langlois et al., 2006). Due to the varied

nature in which TBI is acquired, it presents an assorted constellation of symptoms and signs (Saatman et al., 2008) which manifest in an estimated 52,000 deaths, 275,000 hospitalizations, and 1.3 million emergency room visits each year (Rutland-Brown et al., 2006). Further, TBI is a silent epidemic due to the high incidence of cases which are undetected and/or untreated, a number which remains unknown (Goldstein, 1990; Coburn, 1992). In addition to civilian injury, recent attention has focused on the impact of TBI on military personnel returning from combat. In 2009 alone, 28,000 soldiers were diagnosed with a TBI, many whom suffer long term co-morbidities alongside other psychological and psychiatric symptoms of war (Department of Defense 2013). Given the expanding societal impact of TBI, this epidemic requires improved diagnostics for more specific treatments that lead to better functional recovery and outcomes. TBI classification is considered one of the greatest barriers to effective TBI therapeutics due to the inherent heterogeneity of this disease (Saatman et al., 2008). Clearer criteria to improve the classifications of TBI would greatly assist in the development of individual TBI therapies for improved outcome.

Currently, TBI is classified into broad categories of severe, moderate, and mild. These groups are based on clinical scores utilizing the Glasgow Coma Scale (GCS) which assesses brain function and alertness through eye opening, motor response to pain, and verbal responses. Patients with a GCS score of 8 or less are considered to have a severe injury, while those with a score of 9-12 have a moderate injury, and those with a GCS 13 or greater are considered to have a mild injury. Unfortunately, the GCS does not account for the pathophysiological or pathoanatomical features of head injury sequelae, placing importance on molecular assessments which may further define additional biological markers key to future therapeutic management (Andriessen et al., 2010; Saatman et al., 2008). This approach is particularly important in the

treatment of mild traumatic brain injury (mTBI) which may occur with minimal loss of consciousness (LOC) or lack of LOC altogether, often permitting this type of injury to go unnoticed. Acute medical consequences from TBI can range from motor impairment to cognitive dysfunction, and may include disruption of sensory perception, psychiatric disturbances (Hurley and Taber, 2002; Bryant et al., 2010), sleep disorders, post-traumatic stress disorder (PTSD), and seizures (Annegers et al., 1998; Benedictus et al., 2010; Greenwald et al., 2012) however, there is limited knowledge regarding the symptoms and long term outcome of mTBI (Senathi-Raja et al., 2010; Shenton et al., 2012; Vanderploeg et al., 2005). Clinical evidence indicates that the effects of TBI are not only acute, but victims are at increased risk for a myriad of long term neurological and psychiatric diseases (Rogers and Read, 2007; Halbauer et al., 2009; Bryant et al., 2010) including depression (Rapoport, 2012), epilepsy (Annegers et al., 1998; Ferguson et al., 2010; Frey, 2003), and Alzheimer disease (Mortimer et al., 1985; Fleminger et al., 2003; Sivanandam and Thakur, 2012). Given the potential for a number of long term complications related to TBI, and recognition that mTBI may often go undiagnosed and untreated, a number of studies have investigated the role of repeated concussive or subconcussive injury in athletes or military personnel. Interestingly, these patients did not have profound acute or intermediate symptoms, but suffered from a progressive dementia, chronic traumatic encephalopathy (CTE) (Goldstein et al., 2012; Saulle and Greenwald, 2012; McKee et al., 2009). Given recent evidence that neurodegenerative disease can manifest years after the initial TBI insult, and the importance of using pathoanatomical features in the classification of TBI, it is clear that understanding the molecular mechanisms which underlie TBI pathobiology and recovery must be investigated to improve the detection and treatment of TBI which improve functional recovery (Saulle and Greenwald, 2012; Goldstein et al., 2012; Saatman et al., 2008).



## Pathophysiology

As previously stated, TBI is a heterogeneous process which includes a wide range of events including skull fracture, cerebrovascular injury, and synaptic damage. TBI pathology may be categorized as focal or diffuse, while the molecular processes which determine patient outcome are classified as primary or secondary. Primary injury includes mechanical damage that occurs at the moment of impact, while secondary insults are characterized by downstream processes (Werner and Engelhard, 2007). During a blow to the head or rapid acceleration and deceleration, the brain contacts the hard skull, creating focal brain injury injuries on opposite sides of the brain, also known as coup and contrecoup (Silver et al., 2005; Andriessen et al., 2010). These focal injuries often include laceration, contusion, and hemorrhage, the latter of which induce brain damage best studied in models of ischemia and stroke (Lin et al., 1993). While gross damage is a factor in TBI outcome, current therapies are focused on the molecular pathobiology which underlies the lasting impairment seen in TBI patients (Povlishock and Katz, 2005; Silver et al., 2005; Smith et al., 2013). Specific to TBI, brain movement within the rigid skull induces mechanical injury which causes a number of events such as cell membrane damage and subsequent excitatory amino acid (EAA) release. Also, there is often breakdown of the blood brain barrier (BBB), further exacerbating the brain's vulnerability to neuroexcitotoxicity (Schmidt and Grady, 1993; Farkas et al., 2006; Habgood et al., 2007; Povlishock et al., 1978) and cytokine/chemokine distribution, inducing yet another level of chemical neurotoxicity. In contrast to focal insult, these chemical imbalances and stretching of axons or the shearing of fibers against the cranial vault can also result in a diffuse axonal injury (DAI) (Strich, 1961; Gennarelli et al., 1982; Adams et al., 1991), pathology characterized by hallmark axonal bulb swellings (Povlishock et al., 1983; Povlishock and Kontos, 1985). While DAI is a classic injury

used to confirm TBI in postmortem brains of trauma patients, this pathology also leads to downstream events of synaptic deafferentation and neuronal cell death as axonal transport and targeted synapses become interrupted and damaged (Povlishock et al., 1992; Povlishock and Katz, 2005).

### Neuroexcitation Following Traumatic Brain Injury

While many deleterious processes occur during TBI, one of the first to occur is the release of multiple neurotransmitters, EAAs, and cytokines. Neurotransmitters have been well-documented in synaptic transmission and intracellular cell function (Campbell, 1983; Young, 1992; Tymianski, 1996; Tymianski and Tator, 1996), and have been hypothesized in the regulation of neuronal dysfunction underlying human TBI (Hayes et al., 1988; Lyeth et al., 1988; McIntosh et al., 1988; Faden et al., 1989; Miller et al., 1990). This excitotoxic hypothesis was first proposed in 1983 by Rothman and Olney based on the neurotoxic potential of glutamate binding to NMDA receptors (NMDAR) (Rothman and Olney, 1986; Rothman and Olney, 1987; Bullock et al., 1998). Following TBI, ionic dysregulation not only occurs within the axon, but within astrocytes, and throughout the brain, and is characterized by an early release of multiple EAAs, and a rapid increase in extracellular aspartate and glutamate (Faden et al., 1989; Hayes et al., 1992). Excessive neurotransmitter release as a consequence of dysfunctional glutamate receptor binding has been described in conflicting reports which cite location-specific reduction of EAA binding to NMDAR within the hippocampus (Miller et al., 1990), as well as prolonged activation of glutamate receptors in injury-induced receptor function (Hayes et al., 1988). The classic hypothesis of increased EAA receptor activation is comprised of two components where the first phase involves an alteration in  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations inducing immediate cell

swelling and cell death, while the second phase is  $\text{Ca}^{2+}$ -dependent cell degeneration (Arundine and Tymianski, 2004). Specifically, increased  $\text{Ca}^{2+}$  induces downstream neurotoxic cascades including mitochondrial dysfunction and stimulation of calpains, nitric oxide synthase (NOS), and other kinases known to play a role in structural damage (Arundine and Tymianski, 2004). Isolated studies have shown that mechanical cell deformation alone is responsible for altered glutamate receptor function (Zhang et al., 1996) and  $\text{Na}^+/\text{Ca}^{2+}$  exchange in cortical astrocytes (Floyd et al., 2005), and neuronal stretch enhances AMPA receptor-mediated currents (Goforth et al., 1999). Further, evidence in experimental models of mTBI have demonstrated the excessive release of acetylcholine and other EAAs in the hippocampus (Gorman et al., 1989; Katayama et al., 1989), and also show that treatment blocking these neurotransmitter receptor subtypes can decrease long term cognitive deficits (Hayes et al., 1986; Lyeth et al., 1988). This detrimental release of EAAs is exacerbated by breakdown of the BBB (Tanno et al., 1992; Shapira et al., 1993), an event which further induces the release of cytokines (Das et al., 2012) and other plasma proteins (Hoshino et al., 1996). Together this cytotoxic cascade compounds TBI insult, manifesting in vasogenic edema presenting as elevated intracranial pressure (ICP) in the clinic which leads to further secondary injury when left untreated (Klatzo, 1987; Bullock et al., 1998; Schilling and Wahl, 1999).

### Traumatic Brain Injury Inflammatory Response

Mechanisms underlying BBB breakdown are currently unknown, however reduced BBB integrity after TBI is likely due to altered NOS-mediated release of nitric oxide (NO), a molecule key in the regulation and maintenance of cerebral blood flow and ionic membrane flow (Janigro et al., 1994; Kobari et al., 1994; Cobbs et al., 1997; Lu et al., 2001). BBB disruption is

detrimental as this structure normally provides a stable neurochemical environment, essential for optimal central nervous system (CNS) function (Saunders et al., 1999; Habgood et al., 2007). When intact, the BBB harbors numerous intercellular tight junctions which prevent the transmission of large molecules (Ek et al., 2003; Ek et al., 2006), but following injury, permits potentially toxic molecules to enter the CNS, and impair normal function (Habgood et al., 2007). Certain molecules which pose such a threat are cytokines and chemokines released during post-traumatic CNS inflammation involving glial cell activation and the recruitment of leukocytes (Morganti-Kossmann et al., 2001; Ziebell and Morganti-Kossmann, 2010). In models of closed head injury (CHI), studies indicated an increase in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a cytokine important in the inflammatory immune response during injury and repair, (Hofman et al., 1989; Shohami et al., 1994), IL-6, a cytokine secreted by CNS cells, reportedly elevated following human TBI (Breder et al., 1988; Spangelo et al., 1990; Young et al., 1992; Frugier et al., 2010), and IL-1, a prototypic inflammatory cytokine which acts in the brain to regulate the acute immune response (Rothwell and Luheshi, 2000). In addition, even diffuse TBI models show a neuroinflammatory response as early as 6 hours postinjury, characterized by microglial activation localized to injured axons (Kelley et al., 2007).

While the inflammatory response is likely initiated in attempt to promote neurological repair mechanisms (Correale and Villa, 2004; Lucas et al., 2006), it remains to be determined whether TBI-induced inflammation plays a protective or deleterious role (Shohami et al., 1999; Morganti-Kossmann et al., 2002) as cytokines have the ability to promote both neurotoxicity and neuroprotection (Morganti-Kossmann et al., 2007). The initiation of early inflammatory activation is thought to be induced by several factors including reactive oxygen species (ROS) and NO, and is supported by both resident microglia (Juliet et al., 2008) and astroglia which have

the ability to secrete cytokines after monitoring the injured environment (Singhal et al., 2002; Cederberg and Siesjo, 2010). Microglial response is instrumental in the removal of damaged neurons and cellular debris, necessary for the progression of effective tissue remodeling. During this process, however, microglia are also responsible for the release of ROS, NOS, and glutamate, which, as previously mentioned, may be detrimental to neuronal recovery (Kreutzberg, 1996; Ziebell and Morganti-Kossmann, 2010). Similar to EAA release, cytokines can be responsible for trauma-induced edema in the brain, subsequent increased ICP (Yamasaki 1992), and potentially contribute to delayed neuronal damage (Lucas et al., 2006; Cederberg and Siesjo, 2010). Initial studies evaluating inflammatory response after TBI indicated a deleterious role as IL-1 was found to exacerbate neuronal injury (Rothwell, 1999). Additional animal studies support the damaging effects of inflammatory cytokines by showing an improved post-TBI neurological outcome when the acute inflammatory response is attenuated by over-expressing interleukin-1 receptor antagonist (IL-1ra) (Relton and Rothwell, 1992; Loddick and Rothwell, 1996; Tehranian et al., 2002; Lucas et al., 2006) or pharmacologically inhibiting TNF- $\alpha$  (Shohami et al., 1997). More recent studies utilizing S-nitrosoglutathione, a metabolite of NO/glutathione reaction which nitrosylates proteins as a protective strategy show increased neurological repair after TBI, protecting the CNS against excitotoxicity, inflammation, and ROS (Khan et al., 2011). Further, anti-inflammatory treatments aimed at developing therapy for human TBI have demonstrated transient neuroprotection after injury.

Minocycline, a tetracycline derivative capable of inhibiting the immune response and inactivating microglia, has been used to test inflammatory role after TBI. There is published evidence that it is neuroprotective in instances of SCI (Stirling et al., 2004; Teng et al., 2004), excitotoxicity (Tikka and Koistinaho, 2001; Tikka et al., 2001) and ischemic injury (Arvin et al.,

2002; Wang et al., 2003; Xu et al., 2004; Morimoto et al., 2005), components of heterogeneous TBI insult. Until recently, the positive effects of attenuating the early immune response with minocycline were largely understudied. Original studies by Sanchez Mejia indicated a decrease in IL-1 $\beta$  was linked to improved neurological function and decreased lesion volume following CCI (Sanchez Mejia et al., 2001). More recently the neuroprotective effects of acute minocycline administration has been replicated in studies of CHI (Bye et al., 2007), including evaluation of reduced cognitive and anxiety states (Siopi et al., 2012) and long term locomotor recovery (Homsy et al., 2009; Homsy et al., 2010). Together, these studies suggest the positive effects of immune regulation in fostering functional recovery following TBI.

Contrary to evidence that the inflammatory response produces deleterious effects, additional reports indicate cytokines may also be neuroprotective during the acute immune response. Anti-inflammatory cytokines such as IL-10 provide neuroprotection following TBI (Knobloch and Faden, 1998; Csuka et al., 1999; Kremlev and Palmer, 2005) whereas deficiency in pro-inflammatory IL-6 is detrimental to recovery (Penkowa et al., 2000). Studies utilizing TNF or TNF receptor KO mice indicated transgenic animals had exacerbated injury characterized by extensive BBB damage and impaired neurological function compared to wild type (WT) mice (Scherbel et al., 1999; Sullivan et al., 1999). Finally, studies in cuprizone models of demyelination found that TNF- $\alpha$  promoted proliferation of oligodendrocytes to conduct remyelination within the corpus callosum (Arnett 2001), supporting the hypothesis that there are alternative cytokine receptors which facilitate opposing deleterious and protective roles (Correale and Villa, 2004). The complexity of this immune response is seen with reported studies in chronic inflammatory neurodegenerative disease, which suggest that chronic inflammation (Wirths et al., 2010) and activation of microglia (von Bernhardi, 2007; Block et al.,

2007) may underlie the long term detrimental effects of the immune response (Holmin and Mathiesen, 1999; Bartfai et al., 2007; Ramlackhansingh et al., 2011). During TBI recovery, the combination of EAA toxicity (Miller et al., 1990; Arundine and Tymianski, 2004), BBB breakdown (Habgood et al., 2007), and excessive cytokine and chemokine release (Shohami et al., 1994; Morganti-Kossmann et al., 2002) may also lead to swelling and neuronal cell death (Dietrich et al., 1994; Raghupathi, 2004; Katayama et al., 1990), a process which also occurs as a result of apoptotic processes (Belousov et al., 2012; Dietrich et al., 1994; Kermer et al., 1999; Raghupathi, 2004). Together, this suggests further studies must be completed to understand the mechanisms by which cytokines and their receptors interact in specific environments to induce neurodegenerative or neuroprotective processes after TBI.

#### Traumatic Brain Injury Induces Neuronal Cell Death

Following TBI in both humans and experimental animal models, a degree of cell death is exhibited among neurons, oligodendrocytes, and astrocytes. Certain areas within the brain are particularly vulnerable to cell death, namely the hippocampus, cortex, cerebellum, and thalamus (Adams et al., 1985; Kotapka et al., 1992; Ross et al., 1993). In experimental animal models of TBI, injured neurons begin to swell, and can exhibit neurodegeneration as early as minutes to hours after initial insult (Sutton et al., 1993; Dietrich et al., 1994; Hicks et al., 1996). It has been postulated that injured cells undergo a spectrum of cell death mechanisms ranging from apoptotic to necrotic pathways (Raghupathi et al., 2002; Portera-Cailliau et al., 1997; Nicotera et al., 1999). Apoptotic mechanisms in the injured brains of rodents have been observed following mild lateral fluid percussion injury, particularly in subcortical white matter and cortical neurons (Raghupathi et al., 2002). Interestingly mechanisms such as caspase- and calpain-mediated cell

death have been shown to play a role in both necrosis and apoptotic pathways (Squier et al., 1994; Wang, 2000). While both mechanisms appear to affect cell death after TBI, it appears that apoptosis is prominent in ischemic mechanisms of injury (Du et al., 1996), while necrosis potentially plays a large role in cell death after moderate to severe TBI (Conti et al., 1998; Newcomb et al., 1999). Independent of the mechanism behind cell death, it is a large component of the consequences of TBI and may even manifest in delayed or chronic neuronal degeneration over time (Hicks et al., 1996; Conti et al., 1998).

### Traumatic Brain Injury-Induced Axonal Injury

In addition to cell death, injury to the axonal compartment also occurs. Current literature has demonstrated that even mTBI induces diffuse axonal injury (DAI) without loss of consciousness (Browne et al., 2011), and may be responsible for long term cognitive damage (Molgaard et al., 1990; Hillier et al., 1997; Asikainen et al., 1998) or predisposition for other neurodegenerative disorders (Graves et al., 1990; Fleminger et al., 2003). DAI is a major component underlying the morbidity and mortality linked to TBI, and as a result has been the focus of multiple imaging, biomarker, and high throughput studies in attempt to generate novel therapeutic TBI treatments (Smith et al., 2013). Rapid acceleration and deceleration of the head subjects the brain to mechanical forces, deforming the axons which become particularly vulnerable in such situations (Gennarelli et al., 1982; Meaney et al., 1995; Smith et al., 1997; Smith and Meaney, 2000). DAI is a condition where damaged axons manifest local axonal swellings that appear like a beaded string, injury most commonly found in white matter tracts such as the corpus callosum, parasagittal white matter, internal capsule, and thalamus (Graham et al., 1988; Adams et al., 1989; Adams et al., 1991; Graham et al., 1995; Gultekin and Smith,



1994). The diffuse nature of DAI is characterized by the integration of both damaged and intact axonal fibers, a term utilized only in humans and large gyrencephalic animal models. This is opposed to rodent TBI models which have less widespread axonal damage, but rather focal fiber damage termed traumatic axonal injury (TAI) (Smith et al., 2013).

Original postmortem histological identification of human DAI utilized hematoxylin and eosin, silver staining, the Palmgren technique, and/or myelin staining to locate axonal and preterminal swellings, as well as the histological confirmation of Wallerian degeneration (Strich, 1961; Adams et al., 1989; Rand and Courville, 1946). These techniques, however, have been replaced by amyloid precursor protein (APP) staining in both humans and experimental animal models (Gultekin and Smith, 1994; Sherriff et al., 1994; Gentleman et al., 1993; Gentleman et al., 1995; Grady et al., 1993; Graham et al., 2004; Ng et al., 1994; Christman et al., 1994). APP staining is now the gold standard in identifying DAI/TAI due to the easily identifiable APP pooling at local swelling following injury-induced disruption of anterograde axonal transport (Povlishock, 1992; Povlishock and Christman, 1995). APP antibody detection of axonal injury has been a cornerstone development, paving the way for subsequent studies which have extended the knowledge regarding persistent traumatic injury and its role in the late development of neurodegenerative disorders (Chen et al., 1999; Chen et al., 2004; Johnson et al., 2012a; Johnson et al., 2012b). APP staining can be identified in two types of TBI pathology: as retraction bulbs in disconnected axons, and axonal varicosities in intact axons (Adams et al., 1989; Smith and Meaney, 2000; Povlishock et al., 1983; Povlishock and Kontos, 1985; Povlishock and Becker, 1985; Povlishock and Katz, 2005). Reactive axonal swelling in injured axons often progress to axonal disconnection, supporting development of partial transport interruption at multiple locations (Tang-Schomer et al., 2012). Pathologically, these reactive axonal swellings appear as

early as a few hours postinjury, and began to enlarge within the first 2d after injury. Further, more severe injury induces  $\text{Ca}^{2+}$  dysregulation, contributing to the disruption of the spectrin and ankyrin network which occurs concurrently with mitochondrial and cytoskeletal damage, sealing off the distal end of the swelling resulting in the hallmark axonal bulb (Povlishock et al., 1983; Povlishock and Kontos, 1985; Povlishock et al., 1992; Povlishock and Katz, 2005). Importantly, this chemical imbalance has been found to be the key in triggering the cytoskeleton breakdown and progressive swelling which leads to cognitive and neuronal impairment (Povlishock, 2000; Stone et al., 2004; Marmarou et al., 2005; Buki and Povlishock, 2006). Together, neuroexcitation and the inflammatory process which incite neuronal swelling, as well as subsequent neurodegeneration and axonal swelling, ultimately induce complete axotomy similar to that of Wallerian degeneration (Povlishock et al., 1992). This process leads to downstream degeneration of axon terminals and synaptic deafferentation, pathology which occurs in both human injury and experimental models of TBI (Grady et al., 1989; Kotapka et al., 1992; Steward, 1989; Erb and Povlishock, 1991).

## **NEUROPLASTICITY AND RECOVERY FROM TRAUMATIC BRAIN INJURY**

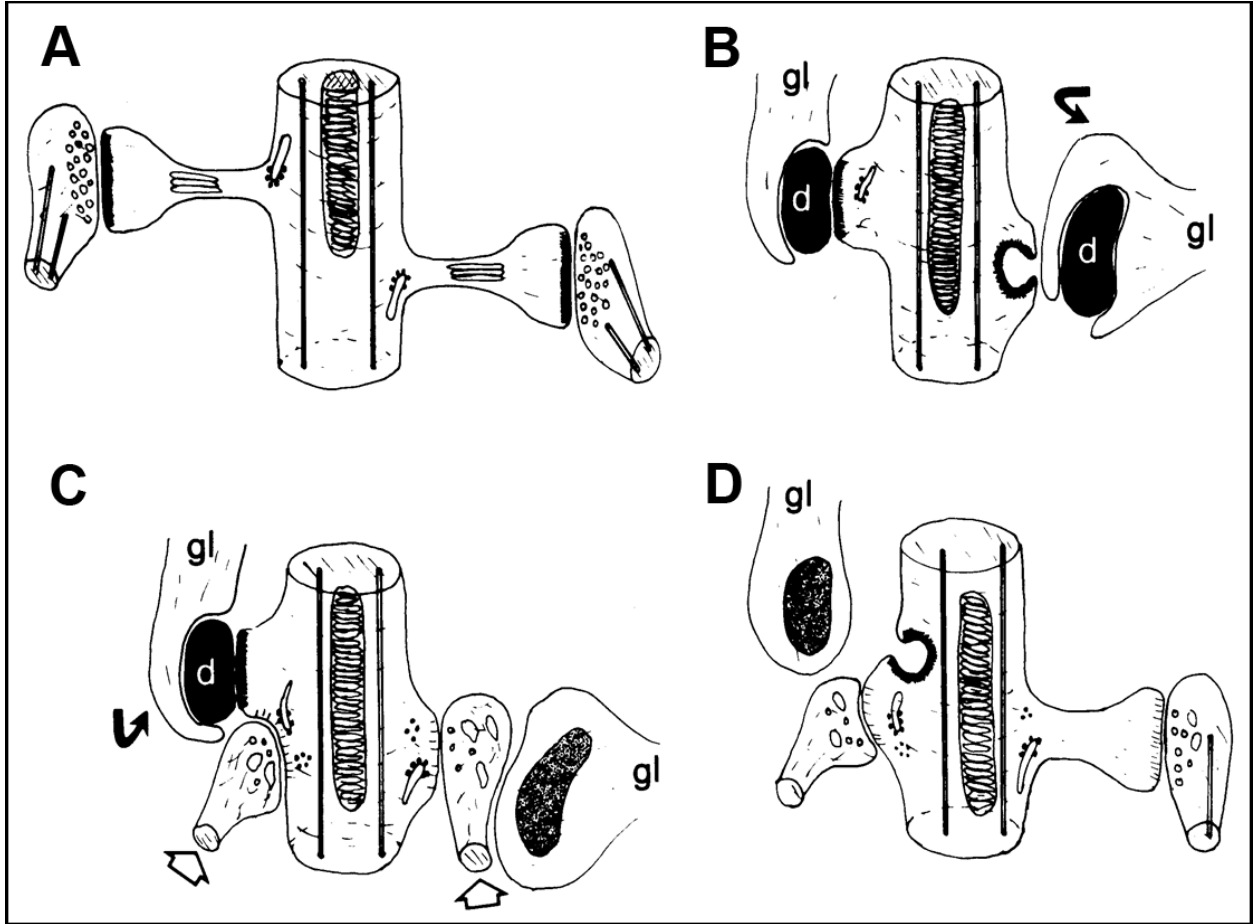
### Deafferentation-Induced Synaptogenesis

Following traumatic insult, the CNS undergoes inherent cell death and a loss of synaptic connections as a result of axonal damage and deafferentation (Erb and Povlishock, 1991; Povlishock and Katz, 2005). Restoring neuronal function requires the synaptic networks to transition from injured synapses to initiating the synaptic recovery process. Despite initial theories describing the adult CNS as a hard-wired structure, neuronal plasticity in mature systems has become an accepted theory since the first description of experience-dependent

plasticity describing the modification of synapses following activation (Hebb, 1949; Keyvani and Schallert, 2002). After traumatic injury, early studies within the spinal cord (Liu and Chambers, 1958), brain stem (Goodman and Horel, 1966), septal nuclei (Raisman, 1969), superior colliculus (Lund and Lund, 1971), and the hippocampal dentate gyrus (Steward, 1976; Lynch et al., 1976; Scheff et al., 1977; Christman et al., 1997; Hulsebosch et al., 1998; Harris et al., 2010a) have demonstrated that the CNS is a plastic structure with the capacity to rewire lost connections, and does so in time-dependent stages. As previously described, traumatic insult to the CNS induces a number of pathophysiological cascades, DAI, and subsequent deafferentation, processes which then initiate reactive synaptogenesis. This recovery following injury occurs in well-defined phases within the local extracellular matrix (ECM). Recovery is mediated through several cell types, and depends upon a variety of molecules, such as cytoskeletal proteins, cell adhesion molecules (CAMs), growth factors, matrix proteins, and matrix enzymes like the matrix metalloproteinases (MMPs) to effect synaptic reorganization of dendritic and somatic synaptic contacts. To further understand this process of injury-induced reactive synaptogenesis, both human TBI victims and experimental TBI models which express the deafferentation underlying clinical deficits have been used to evaluate the specific cellular and synaptic players following traumatic injury (Cotman and Nadler, 1978; Grady et al., 1989; Steward, 1989; Phillips and Reeves, 2001).

As noted above, several areas of the brain are particularly vulnerable to TBI-induced damage including the cortex, cerebellum, thalamus, and hippocampus (Adams et al., 1985; Kotapka et al., 1992; Ross et al., 1993). Given the importance of these structures in key brain functions, these locations serve as useful models to study the process of synaptic and neuronal recovery after injury. Specifically, studies utilizing rodent models of synaptic deafferentation

**Figure 1.1 Stages of Reactive Synaptogenesis.** Following deafferentation normal synapses (A) first undergo a degenerative phase (1-5d postinjury; B) characterized by reactive glia (gl) which remove degenerating terminals (d). Next, synapses enter the regenerative phase (6-15d postinjury; C) where sprouting of new axons occurs (arrows) before maturing during stabilization (15d+ postinjury; D) (Steward et al., 1988a).



have documented time-dependent stages of synaptic recovery after injury in the dentate gyrus (DG) (Loesche and Steward, 1977) (**Fig. 1.1**). The DG receives the main afferent pathway into the hippocampus, and serves as a structure involved in memory and cognition, often affected in TBI patients. During deafferentation, there is a loss of synaptic input, inducing the death of affected axonal projections and terminals (Cotman et al., 1981). Early after injury (1-5d postinjury) affected synapses enter a degenerative phase where lesioned axon terminals and debris are removed from the site of injury. The next stage is an intermediate stage (6-15d postinjury) when new synapses form by collateral axonal sprouting and terminal generation innervates reorganized postsynaptic sites (Lynch et al., 1976; Steward, 1976; Deller et al., 2007; Frotscher et al., 1997) . Finally, in the third stage, the newly repaired synapses undergo stabilization (15d+ postinjury) where synapses mature and are strengthened both structurally and functionally (Steward and Vinsant, 1983). These specific stages of synaptic reorganization are integral to understanding how synaptic networks recover from injury, and can be further studied to ascertain which molecular players permit effective synaptic reorganization within the local ECM.

During the acute postinjury degenerative period, neurons become deafferented, and their synaptic structure undergoes altered morphology. One of the key features contributing to lasting CNS impairment seen in TBI patients is the early loss of synaptic input characterized by initial changes in dendritic shape and spine density, as well as axonal degeneration (Matthews et al., 1976a; Adams et al., 1982; Kim et al., 2006). Underlying these changes in dendritic number and morphology are proteins which likely interact with structural components such as microtubules and intermediate filaments. Recent evidence indicates alterations in microtubule associated proteins (MAPs) which decrease after ischemia (Tanay et al., 2006), but conversely increase

after axotomy of the trochlear nerve (Book et al., 1996) as well as following optic nerve crush (Dieterich et al., 2002). Although these results show opposite effects, they provide evidence for the potential use of MAPs in identifying injured synaptic structures. Further, MAPs may play a role in synaptic recovery as they are important in axonal outgrowth during development and cortical wiring (Gordon-Weeks and Fischer, 2000; Yamanouchi, 2005) as well as after injury (Ma et al., 2000; Ramon-Cueto and Avila, 1999). Dendritic reorganization also involves microtubule plasticity (Tortosa et al., 2011), and is an equally important component in the process of trauma-induced synaptic reorganization demonstrated in several models of CNS deafferentation (Cotman et al., 1981).

#### Inflammation, Neuroglia, and Synaptic Plasticity

During this early phase of dendritic morphological changes and synaptic terminal loss, the inflammatory response is one of the first cellular pathways to respond (Martino et al., 2011; Marchetti and Pluchino, 2013). Acute response after injury and subsequent synaptic recovery is dependent on these early mechanisms which permit the effective conditioning of the ECM. After trauma, the afore mentioned damage to synapses and neurons is significant, requiring the interaction of multiple cell types and molecules such as glia, neurons, and cytokines to repair the injured terminals (Aloisi et al., 1992; Aloisi, 2001). One of the first inflammatory cell types to arrive at the site of injured synaptic terminals in the inflammatory cascade are microglia.

Microglia are well known for their function in inflammatory immune response through their secretion of cytokine and chemokine signals (Aloisi, 2001). In addition to secreting these molecules, microglia are also often the target of these signals through both autocrine and additional paracrine signaling with neurons, astrocytes, and immune cells (Polazzi et al., 2001;

Hanisch, 2002). Microglia can also act as sensors of their environment (Nimmerjahn et al., 2005) and are often the very first responders to trauma regardless of the organ system involved (Kreutzberg, 1996). Although inflammation is a mechanism for repair, it can also serve as a damaging insult. In lipopolysaccharide-induced inflammation, the site is surrounded by activated microglia which can induce neurodegeneration compared to controls (Ekdahl et al., 2003). Prevention of this neurodegeneration was effected through blockade of microglial response, implicating microglial activation in inflammatory neurodegeneration. These glia may also regulate cell death through apoptosis (Piani et al., 1991). Microglia-induced apoptosis, however, can be extremely variable regarding the mechanisms involved. For example, various studies have reported microglial apoptosis through different pathways, including cathepsin B in hippocampal and cerebellar neurons (Kingham and Pocock, 2001), as well as ROS (Colton and Gilbert, 1987), NO, and interleukin 1 $\beta$  (IL-1 $\beta$ ) (Chao et al., 1992; Tikka and Koistinaho, 2001).

In addition to modulating neuronal cell death, microglia can affect neuronal survival through the release of anti-inflammatory factors, and secretion of trophic factors (Polazzi et al., 2001). Initial descriptions of microglial role in synaptogenesis came with cortical development in the early weeks of postnatal life, when maximum microglial numbers correspond with high levels of synaptogenesis in nervous tissue (Steward and Falk, 1986). Temporal correlation of this microglial surge and synaptogenesis in development implies a potential role for microglia in synaptic reorganization after injury. Microglial mechanisms of reorganization are supported by evidence that these cells are capable of secreting many proteins involved in synaptogenesis and neural plasticity, such as brain derived neurotrophic factor (BDNF) and thrombospondin (Chamak et al., 1995; Binder and Scharfman, 2004; Christopherson et al., 2005; Bessis et al., 2007; Lipsky and Marini, 2007). BDNF in particular can be expressed by microglia after TBI



(Nagamoto-Combs et al., 2007) and focal ischemia (Madinier et al., 2009). Studies indicate that interruption of the inflammatory response after focal brain ischemia decreases microglial activation. This decrease in microglial activation results in a decrease in BDNF and synaptophysin expression, known markers of synaptogenesis and neuroplasticity (Madinier et al., 2009; Valtorta et al., 2004). Decreased levels of neurotrophic factors and synaptic markers in the absence of microglial activation indicate that microglia are essential for synaptogenesis. In addition to microglial role in synaptogenesis after ischemia, Moller and colleagues describe microglial secretion of thrombospondins after axotomy of the facial nucleus (Moller et al., 1996). Although it may seem contradictory that these cells appear to have the ability to induce both neurodegeneration and neurogenesis, evidence that not all microglia express the same proteins (Ellison et al., 1998) suggests there are multiple microglial subpopulations exhibiting different roles in neuronal survival or neuronal cell death (Byrnes et al., 2006; Ekdahl et al., 2009). Furthermore, Ekdahl and colleagues (2009) contend that microglial role in neurodegeneration and neurogenesis is dependent on the surrounding pro- and anti-inflammatory molecules. This largely implies that synaptogenesis is sensitive to its environment, and no single molecule inherently plays a defined role in synaptic survival or loss. Overall, this data points to complex repair mechanisms involving microglial surveillance of the injured environment.

As resident macrophages of the brain, microglia play a direct role in the degenerative phase of synaptic reorganization by engulfing debris, degenerating terminals, and damaged synaptic structures (Colton and Gilbert, 1987; Chao et al., 1992; Ramlackhansingh et al., 2011; Bechade et al., 2013; Bessis et al., 2007). Despite the fact that a majority of the microglial literature focuses on their ability to take up and process cell debris, microglia actually have a diverse set of functions as they are able to secrete and interact with a number of molecules. For

example, microglia are also capable of inducing superoxide anions and NO in apoptotic pathways (Colton and Gilbert, 1987; Chao et al., 1992), potentially contributing to the reshaping of injured synapses during trauma-induced reorganization (Ramlackhansingh et al., 2011; Bechade et al., 2013; Bessis et al., 2007). As an immune response cell, many microglia are also capable of secreting and interacting with a number of cytokines and chemokines (Hanisch, 2002). As early mediators of inflammation after trauma, microglia are instrumental in establishing regenerative boundaries of synaptic plasticity by interacting with a number of ECM molecules (Takeuchi et al., 1998). Other types of molecules which are secreted by microglia and function in the first phase of reactive synaptogenesis are the ECM proteoglycans and MMPs. The MMPs play a major role in the degenerative phase as they have the ability to target multiple substrates to break down structural components of the local ECM around synapses (Ethell and Ethell, 2007; Ding et al., 2009; Dziembowska and Wlodarczyk, 2012; Harris et al., 2009). While microglia are key to debris clearance during the acute recovery phase, they are also instrumental in the next phase of reactive synaptogenesis, regeneration (Graeber et al., 1998). The modulation of neurogenesis and synaptogenesis during this regenerative phase is not restricted to microglia, but involves reactive astroglia, which have the ability to modify functional synapses both directly and indirectly (Bechmann and Nitsch, 2000; Christopherson et al., 2005; Ekdahl et al., 2009; Bechade et al., 2013).

Like microglia, astroglia can secrete cytokines (Semple et al., 2010), however their role in recovery after trauma has been traditionally described in terms of glial scar formation, as exemplified with SCI (McKeon et al., 1991). Historically, astrocytes have been considered physiologically important in the maintenance of CNS homeostasis, supporting neurons through nutrition, recycling neurotransmitters, and maintaining the integrity of the BBB (Abbott, 2002;

Pellerin and Magistretti, 2004; Wilhelmsson et al., 2006). Recent studies have further described the importance of astrocytes in neuronal repair, showing their ability to direct synaptic plasticity (Ullian et al., 2001; Christopherson et al., 2005) . Following injury, astrocytes undergo reactive astrogliosis as they respond with a number of morphological and physiological changes, principally increased GFAP supporting hypertrophy and/or thickening and elongation of cellular processes (Amaducci et al., 1981; Baldwin et al., 1996; Wilhelmsson et al., 2006). As a result of their reactive morphology, astrocytes become involved in the neuronal repair and recovery process by first forming a glial scar (Bignami and Dahl, 1976) . While reactive gliosis in the glial scar has often been described as an inhibitory to axonal regeneration, serving as barrier to axonal sprouting (Matthews et al., 1976a; Liuzzi and Lasek, 1987; Silver and Miller, 2004; Goldshmit and Bourne, 2010), it is now thought to also serve a positive role in preventing the spread of injury to neighboring intact tissue (Matthews et al., 1979; Sofroniew, 2005; White and Jakeman, 2008). While such barrier formation may be part of the astrocytic role after TBI, reactive astrocytes also serve an important role in repair through their interaction with a number of ECM proteins and growth factors to facilitate effective neuronal recovery (Laird et al., 2008).

## **EXTRACELLULAR MATRIX AND NEUROPLASTICITY**

The ECM is the lattice network of multiple proteins and molecules which help organize the structure of the brain, as well guide the development, maturation and growth of synapses (Ruoslahti, 1996a; Venstrom and Reichardt, 1993; Dityatev and Schachner, 2003). The ECM is the major structural component providing stability among cells and tissues, however this complex network of neurons and glia appears to play a dominant role in plasticity within the CNS. While it is often described as less organized in the CNS compared to other organ systems,

this may be due to the extensive amount of proteolysis it undergoes during the continuous remodeling necessary for development and the maintenance of ongoing plasticity (Pittman and Buettner, 1989; Romanic and Madri, 1994). Moreover, the ECM in the brain contributes to neuronal homeostasis, and its diffuse distribution of proteins mediate interactions among CNS cellular components (Jones, 1996; Yong et al., 1998). For example, integrins are glycoproteins expressed on the cell surface of CNS neurons, glia, meningeal, and endothelial cells which signal cell-cell and cell-ECM interactions (Hynes, 1992; Milner and Campbell, 2002). Several integrin binding proteins, including cytokines and chemokines, are released into the ECM to mediate such cellular signaling. Structurally, integrins consist of mixed dimers from  $\alpha$ - and  $\beta$  subunits, which introduces another level of variability for ECM interaction, and the ability to affect a number of physiological processes such as cellular organization and synaptogenesis during neuronal development (Miyagoe-Suzuki et al., 2000; Benson et al., 2000; Graus-Porta et al., 2001).

As ECM proteins play a large role in the synaptogenesis during development, they also influence the success of synaptic reorganization following traumatic insult. Throughout reactive synaptogenesis, there are a number of cell types and molecules which act in concert to promote the regeneration of functional synapses. Cells such as microglia (Giulian et al., 1989; Chao et al., 1992; Bessis et al., 2007; Ekdahl et al., 2009), astroglia (Christopherson et al., 2005), and olfactory ensheathing cells (OECs) (Barnett, 2004) are critical players in this process as they have the ability to interact with a number of ECM molecules to direct the different phases of synaptic reorganization (Steward, 1989; Ullian et al., 2001). Specifically, microglial response to injury is characterized by the secretion of a number of molecules including cytokines (Hanisch, 2002), MMPs (Gottschall et al., 1995), and proteoglycans (Yokoyama et al., 2006). In addition,

microglia also interact with these cytokines and matrix molecules to facilitate reshaping of ECM protein structure during the acute degenerative phase (John et al., 2003; Ethell and Ethell, 2007). Following deafferentation-induced degeneration, there is often an increase in reactive astrocyte interaction with synapses, suggesting that the acute immune response may also serve to recruit astrocytes and induce their secretion of growth factors (Goss et al., 1998; Madathil et al., 2013) and MMPs (Wells et al., 1996; Muir et al., 2002) to direct sprouting of new terminals during synapse regeneration (Falo et al., 2008; Warren et al., 2012). With regard to synaptic recovery, these matrix molecules interact with glial cells to establish the integrity and structure of synapses, as well as promote their stabilization and function (Dityatev and Schachner, 2003; Dityatev and Schachner, 2006; Busch and Silver, 2007; Frischknecht and Seidenbecher, 2008). For example, growth factors secreted by astrocytes are well-documented to influence axonal sprouting and neurogenesis after CNS injury (Nieto-Sampedro and Bovolenta, 1990; Deller et al., 2006; Thau-Zuchman et al., 2010). Secreted MMPs can convert pro forms of these molecules into active growth factors, as well as mediate their interaction with other glia-secreted ECM molecules such as proteoglycans and CAMs important for synapse regeneration. Both microglia and astroglia are sources of various proteoglycans and CAMs (Bandtlow and Zimmermann, 2000; Redies, 2000), and *in vitro* studies show that astrocyte cultures can be easily induced to increase their secretion of certain proteoglycans (Snyder et al., 1996; Canoll et al., 1996; Dobbertin et al., 2003).

#### Proteoglycans and Cell Adhesion Molecules Influence Synapse Formation

Proteoglycans are a diverse group of glycoproteins important in forming the structural integrity of the ECM around synapses, and are found in both secreted and transmembrane forms

(Harris et al., 2009). This family of proteins can be subdivided into different groups based on their glycosaminoglycan (GAG) sidechains: chondroitin/dermatan sulfate (CSPG/DSPG), heparan sulfate (HSPG), and keratan sulfate. CSPGs and HSPGs have been studied the most in the context of synaptic plasticity due to their role in synaptic development and proximity to the synapse (Bandtlow and Zimmermann, 2000). One HSPG, agrin, can affect synaptic plasticity through its binding to growth factors and CAMs, inducing growth of shorter, branched axons important for postsynaptic targeting, as well as increased vesicles at pre-and post-synaptic densities (Mantych and Ferreira, 2001). Agrin was first discovered in the ECM of the Torpedo electric organ at the neuromuscular junction (Godfrey et al., 1984), and postulated to be a neuronally secreted protein to direct formation of the postsynaptic membrane at the neuromuscular junction (NMJ). Subsequent cloning of the agrin gene in various systems including the rat (Rupp et al., 1991) and chicken (Tsim et al., 1992) led to the development of agrin KO mice. These agrin-deficient mice failed to form the NMJ, supporting this proteoglycan as a key molecule in synaptic organization (Gautam et al., 1996). Other studies have focused on the acetylcholine receptor (AChR) aggregating properties of agrin, demonstrating its function during the window of development when synaptogenesis occurs (Ma et al., 1994; Stone and Nikolics, 1995; Li et al., 1997). Further, agrin is present in high amounts in areas of adult synaptic plasticity such as in the cortex, hippocampus (O'Connor et al., 1994), and within interneuron synapses (Mann and Kroger, 1996; Gingras and Ferns, 2001; Hoover et al., 2003; Ksiazek et al., 2007). In addition to playing a role in postsynaptic development, CSPGs can promote axonal outgrowth. The CSPG family consists of versican, neurocan, phosphacan, brevican, and NG2, each of which contain a protein core with a GA side chain, but differ in the level of sulfation (Morgenstern 2002). Although CSPGs were initially viewed as inhibitory to

axonal outgrowth during plasticity (Snow et al., 1990; Oohira et al., 1991; Grumet et al., 1993), recent evidence suggests certain types of CSPGs are integral during axonal sprouting following TBI (Harris et al., 2010b), often interacting with CAMs and growth factors during trauma induced-plasticity (Faissner et al., 1994; Bicknese et al., 1994; Sakurai et al., 1997; Schafer et al., 2008). Specifically, versican mRNA was increased within the glial scar 4-14 days following CCI, while decreased protein expression of all CSPGs was evident between 1-14d postinjury. CSPG reduction was concentrated within the pericontusional area, highlighting the importance of spatial and temporal parameters in the evolution of plasticity following TBI (Harris et al., 2009). Further studies support a strict window of spontaneous cortical sprouting, a process which was maximal at 6-14d in pericontusional regions and absent at 28d postinjury (Harris et al., 2010a).

In addition to guiding axonal outgrowth, CSPGs functional domains interact with other ECM molecules near the synaptic junction to permit effective synaptic plasticity (Miyata et al., 2004; Harris et al., 2011), including CAMs which are known to affect brain connectivity during development and throughout life (Doherty and Walsh, 1992; Benson et al., 2000). One CAM family important in synaptic plasticity during both development and after trauma are the cadherins, a subset of  $Ca^{2+}$ -activated cell to CAMs present at the synaptic junction (Beesley et al., 1995; Shapiro and Colman, 1999; Redies, 2000). Specifically, cadherins are active in the hippocampus where they are present within extended filopodia and mature synapses (Takeichi and Abe, 2005), likely regulating postsynaptic dendritic morphology (Togashi et al., 2002). While there are 80 different types of cadherins, roughly 20 of these are so-called 'classic' cadherins, distinguishable from the remaining protocadherins, and have been extensively studied. The classic cadherin nomenclature is derived from the tissue of their original discovery location: E-cadherin (epithelial), N-cadherin (neural), P-cadherin (placental), and R-cadherin (retinal)

(Huntley et al., 2002). Of importance to synaptic plasticity is N-cadherin, a CAM family member which has been localized to optic axons during arborization (Yamagata et al., 1995), as well as in both pre-and post-synaptic sites, and the synaptic cleft of hippocampal synapses (Fannon and Colman, 1996; Bozdagi et al., 2000). While there is evidence that N-cadherins play a key role in the sprouting and synapse reorganization after trauma (Warren et al., 2012), it is not known whether the acute immune signals generated by TBI alter their response during periods of synaptic recovery.

### Matrix Metalloproteinases and Synaptogenesis

Another group of molecules secreted into the ECM by glia are MMP family members, enzymes which can modify the local matrix around the synapse, and interact with growth factors, proteoglycans, and CAMs to facilitate reactive synaptogenesis. The MMPs belong to a family of over 20 secreted  $Zn^{2+}/Ca^{2+}$ -activated proteolytic enzymes which have the ability to modify and/or degrade the ECM in both physiologic and pathogenic conditions (Romanic and Madri, 1994; Asahi et al., 2001a). These proteases are capable of activating and inactivating receptors, adhesion molecules, and growth factors during embryonic development, wound healing, and angiogenesis (Van Hove et al., 2012; Verslegers et al., 2013). Relevant to CNS plasticity, MMPs may act to condition the ECM during the early stages of reactive synaptogenesis, facilitating synaptic recovery after trauma (Wee Yong, 2010). While all MMPs have a similar structural organization, including a signaling peptide and catalytic domain, they may be further subdivided by their substrate specificity into the collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs (MT-MMPs) (Nagase et al., 2006). MMPs are initially produced as inactive zymogens (pro-MMPs) and become active following cleavage of the



propeptide domain, which releases the regulatory  $Zn^{2+}$ -cysteine bond at the catalytic site, permitting functional activity (Van Wart and Birkedal-Hansen, 1990; Becker et al., 1995). MMP proteolytic activity can also be indirectly regulated by a number of mechanisms, including cell compartmentalization, post-translation modification, and endogenous tissue inhibitor of matrix metalloproteinases (TIMPs) which non-covalently bind to MMPs (Baker et al., 2002; Chakraborti et al., 2003).

Considerable attention has been focused on these proteinases and their molecular substrates as effectors of reorganization during synaptic plasticity (Yong et al., 2001; Lo et al., 2002; Dityatev and Schachner, 2003; Yong, 2005; Wlodarczyk et al., 2011). In the CNS, these enzymes are also capable influencing axonal growth, mediate synaptic modification (Szklaarczyk et al., 2002; Vaillant et al., 1999; Fredrich and Illing, 2010), and critical to synaptic recovery following injury (Yong et al., 2001; Dityatev and Schachner, 2003). As previously mentioned, these modulators of synaptic plasticity may be secreted by glia, and stimulate process outgrowth or cell signaling pathways by activating surface receptors (Oh et al., 1999; Giraudon et al., 1997; Sternlicht and Werb, 2001). Prior studies in our laboratory have linked aberrant MMP activity to disrupted synaptic reorganization following hippocampal deafferentation. Membrane type 5-matrix metalloproteinase (MT5-MMP) and a disintegrin and metalloproteinase-10 (ADAM-10), both cleave N-cadherin, a CAM instrumental in synapse stabilization. Contrasting models of adaptive and maladaptive plasticity indicate persistent ADAM-10 expression during maladaptive plasticity is correlated with attenuated N-cadherin production and reduced functional recovery (Warren et al., 2012). Further, time-dependent MMP inhibition can reduce ADAM-10 and shift N-cadherin expression toward that seen during adaptive synaptic recovery, highlighting the significant impact that MMPs can have on synaptic reorganization. Our laboratory and other

groups have identified the stromelysins (MMPs-3,-10,-11) and gelatinases (MMP-2,-9) to be of critical importance in the pathophysiology of CNS injury and disease. While MMP-3 and the gelatinases were initially thought to be detrimental proteases, disrupting the BBB and promoting further injury (Rosenberg et al., 1998; Van Hove et al., 2012), more recent studies support beneficial effects of these MMPs in CNS injury and disease. MMP-3, for example, has been linked to successful axonal outgrowth following sciatic nerve crush (Demestre et al., 2004; Shubayev and Myers, 2004), and MMP-9 mediates dendritic remodeling during long term potentiation (LTP), a cellular model for learning and memory (Bozdagi et al., 2007; Dziembowska and Wlodarczyk, 2012). Together, these studies implicate MMPs, particularly MMP-3 and the gelatinases as players in synaptic reorganization following TBI.

#### Stromelysin-1 (Matrix Metalloproteinase-3) and Synaptogenesis

MMP-3, also known as stromelysin-1, has simple structure consisting of a hemopexin domain attached to the catalytic domain of the base MMP structure (Handsley et al., 2005). While typically found in neurons and glial cells, MMP-3 is capable of acting both intracellularly in dopaminergic neurons (Choi et al., 2008; Shin et al., 2012), as well as extracellularly, cleaving a number of substrates including ECM proteins, growth factors, CAMs, chemokines, and cytokines (Van Hove et al., 2012). Although MMP-3 has been localized to various cell types, it is largely confined to neurons (Wetzel et al., 2003) within the developing healthy CNS, as well as expressed in high amounts during embryonic axonal outgrowth (Pauly et al., 2008). Additional studies detected MMP-3 in most areas of the adult brain, particularly within peripheral cell bodies and axons (Nordstrom et al., 1995), and functional activity confirmed via *in situ* zymography in the neocortex (Gonthier et al., 2007). Further, MMP-3 KO mice exhibit

balance and hind limb motor deficits as adults, implicating this proteinase as key molecule in proper synaptogenic development (Van Hove et al., 2012). MMP-3 has been demonstrated a number of studies to play an important role in CNS development, specifically during neurite extension (Machida et al., 1989) and axonal growth cone guidance and extension (Gonthier et al., 2007). Its role in synapse formation between granule cells and Purkinje cells in the mouse cerebellar cortex (Van Hove et al., 2012) further supports a role for MMP in proper CNS development, a process which may be achieved through the lytic activation of laminin (Okada et al., 1986), tenascin (Imai et al., 1994), or BDNF (Lee et al., 2001), molecules important in synaptic function. In addition, MMP-3 may regulate myelination during development, given the elevated expression in oligodendrocytes and Schwann cells, and ability to promote insulin growth factor (IGF) release (Del Bigio and Jacque, 1995; Muir et al., 2002).

MMP-3 functional role in synaptogenesis and reorganization however is not limited to the developing CNS, but has been shown to be important in plasticity within the adult brain. This MMP is of particular interest due to its increased postinjury expression in neurons and reactive astrocytes, and its targeting of ECM proteins important in axonal and synaptic growth (Imai et al., 1995; VanSaun and Werle, 2000; Muir et al., 2002). Important to synaptic stabilization in the adult mammalian brain are the perineuronal nets (PNNs) comprised of a number of CSPGs, and their binding partners which act in concert to support dendrites and map synaptic contact organization within the ECM. Essential to plasticity, is cleavage of the PNNs, a function accomplished by MMP-3 as evidenced by numerous studies showing that it cleaves all CSPGs (Muir et al., 2002; Durigova et al., 2011). Further, MMP-3 is localized to PNNs during remodeling suggesting MMP-3 may direct synaptic plasticity in the adult brain (Baig et al., 2008). MMP-3 has also been localized to the NMJ in the PNS, and its cleavage of agrin and

intracellular adhesion molecule 5 (ICAM5) suggests an association with synaptic sprouting and dendritic spine maturation respectively (Van Hove et al., 2012). Finally, transient MMP-3 elevation is associated with learning during hippocampal-dependent tasks and inhibition of MMP-3 elicited learning deficits during passive avoidance training together implicate MMP-3 in effective hippocampal learning (Olson et al., 2008). Given that MMP-3 expression and functional activity plays a role in proper synapse development through a number of cellular and molecular pathways during development, it may play an important role in immune regulation of synaptic regeneration following disease or trauma.

While MMP activity appears to play a beneficial role during development, evidence of a role regarding CNS disease and trauma has mixed results. MMPs are elevated in all forms of neurological disorders, and MMP-3 has been identified as a molecule elevated during the inflammatory response related to CNS disease and trauma (Agrawal et al., 2008; Candelario-Jalil et al., 2009). Cell culture studies examining LPS injection in neuronal astrocytes and microglia demonstrate a large increase in MMP-3 (Kim et al., 2007; Kim et al., 2008), supporting the reciprocal activation between glial cells and cytokines or matrix molecules. Specifically, microglia-secreted TNF- $\alpha$  and IL-1 $\beta$  may act on microglia themselves to induce MMP secretion and activation within the ECM (Cross and Woodroffe, 1999; Gottschall and Yu, 1995; Crocker et al., 2006). Further, activated MMP-3 is also capable of triggering microglial production of inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  production (Kim et al., 2005; Kim et al., 2007; Kim and Hwang, 2011), highlighting the complex nature of the molecular interaction between glial cells and MMPs during the acute immune response following CNS injury. Subsequent downstream effects of this response may be detrimental as demonstrated in multiple experimental models of Parkinson disease (PD) which show an increase of MMP-3 contributing

to neuronal damage in the substantia nigra (Sung et al., 2005; Kim et al., 2007; Choi et al., 2008; McClain et al., 2009). Similar effects were seen in human pathology and experimental animal models of AD (Gottschall and Deb, 1996; Yoshiyama et al., 2000). In addition to chronic inflammatory disease, MMP-3 is significantly elevated following traumatic injury in models of ischemia (Rosenberg et al., 2001; Sole et al., 2004), brain contusion (Li et al., 2009), and synaptic deafferentation (Kim et al., 2005). While these various models all exhibit different methods of trauma, and report increased MMP-3 following injury, they do not address whether the MMP role is deleterious or beneficial. Previous studies in our lab indicate the importance of temporal expression of MMPs as MMP-3 mRNA and protein were elevated acutely after adaptive UEC, supporting its role in synaptic recovery after TBI (Falo et al., 2006). Increased MMP-3 expression during degenerative phase of synaptogenesis was present in both adaptive and maladaptive models however MMP-3 elevation persists over time with maladaptive synaptic plasticity, consistent with diminished removal of degenerating pre-synaptic terminals, damaged synapses, and the failure of both structural and functional synapse reformation (Falo et al., 2006). Interestingly, acute postinjury MMP inhibition (MMPi) also attenuates MMP-3 production, and reduces synaptic recovery (Reeves et al., 2003). This result indicates that MMP-3 can serve a beneficial role during reactive synaptogenesis, depending upon its time of activation. Together, these studies point to important MMP-3 interaction with ECM proteins during the acute inflammatory response after injury, and further experiments may elicit the mechanisms behind how this MMP activation mediates synaptic reorganization.

## Gelatinases (Matrix Metalloproteinase-2, -9) and Synaptogenesis

The gelatinases consist of MMP-2 (73 kD) and MMP-9 (92 kD), a subfamily of type IV collagenases studied extensively in multiple physiological processes including bone growth, blastocyst implantation, inflammatory disease, cancer, and cardiovascular disorders (Van den Steen et al., 2002; Stamenkovic, 2003; Klein and Bischoff, 2011). Similar to the stromelysins, their structure is also fairly simple, consisting of a hemopexin domain and fibronectin repeats upstream of the catalytic site (Handsley et al., 2005). Given that gelatinase activity is elevated during the development of pathological disorders, MMP-2 and MMP-9 are good candidates for modulation of the early phases of synaptic reorganization following TBI. High levels of gelatinase mRNA have been documented in the CNS during neuronal development, particularly within the first postnatal week in various cell types such as pericytes and endothelial cells (Ayoub et al., 2005; Planas et al., 2001; Szklarczyk et al., 2002), however, the major sites showing upregulation are astrocytes for MMP-2 and myelinated tracts for MMP-9 (Planas et al., 2001; Szklarczyk et al., 2002). Evidence in the developing CNS indicates that both MMP-2 and MMP-9 direct the migration of neural crest cells (NCCs), neural stem cells (NSCs), neurons, and astrocytes. Interestingly, MMP inhibitor studies showed impaired NCC and NSC migration in avian embryos and embryonic rat cortex respectively, implicating the importance of gelatinase function in proper direction of neural precursor cells (Duong and Erickson, 2004; Ingraham et al., 2011). Similarly, MMP-9 deficient mice and *in vitro* culture assays showed altered granule cell migration (Vaillant et al., 2003; Ayoub et al., 2005), while evidence correlating MMP-2 positive pericytes with vessel sprouting suggests a role in angiogenesis (Girolamo et al., 2004; Virgintino et al., 2007). Finally, MMP-2 and -9 are highly expressed in dendrites, and a number of their substrates are structural cytoskeletal components, growth factors (nerve growth factor,

NGF), CAMs (ICAM-5), or axonal guidance receptors (EphB2), implicating a functional role for these collagenases in dendritic (Tian et al., 2007; Gonthier et al., 2009; Saygili et al., 2011) and axonal outgrowth (Lin et al., 2008). Taken together, these studies, along with evidence of MMP-2 Schwann cell localization and MMP-9 expression in ECM near oligodendrocytes precursors (Uhm et al., 1998; Oh et al., 1999; Ulrich et al., 2005), highlight gelatinase activity in normal CNS development, specifically directing vascularization, cellular migration, myelination, and axonal outgrowth within the ECM.

In addition to influencing CNS development, gelatinase modulation of neurogenesis and synaptic outgrowth has been described during physiological processes and following pathological events. MMP-9, but not MMP-2, appears to play a role in adult plasticity during LTP by regulating extracellular proteolysis and neurotransmitter receptor trafficking and activation (Bozdagi et al., 2007; Michaluk et al., 2009). Further, MMP-9 mRNA is transported and activated following LTP (Sbai et al., 2008; Dziembowska and Wlodarczyk, 2012), and behavioral analysis of MMP-9 KO mice display altered hippocampal-dependent behavioral and cognitive function (Nagy et al., 2006; Meighan et al., 2006). Interestingly, the changes in dendritic spine morphology characteristic of LTP are mediated by integrin-directed modulation of actin filaments (Nagy et al., 2006; Kramar et al., 2006; Kim et al., 2009) which is regulated by MMP-9 proteolytic exposure of integrin ligands such as the Arg-Gly-Asp (RGD) sequence found in numerous ECM proteins (Ethell and Ethell, 2007; Ruoslahti, 1996a). As mediators of LTP synaptic plasticity in the adult brain, the gelatinases also have potential to modulate synaptic regeneration in the injured CNS (Shubayev and Myers, 2000; Szklarczyk et al., 2002; Hughes et al., 2002; Platt et al., 2003).

Several studies have documented increased MMP expression following CNS injury in humans (Clark et al., 1997) and experimental animal models of stroke and ischemia (Rosenberg et al., 1998; Gasche et al., 1999; Heo et al., 1999). Typically, MMPs have been thought of as a deleterious molecule when upregulated after injury due to their promotion of widespread proteolysis and breakdown of the local ECM and tight junctions between cells (Rosenberg, 1995; Rosenberg et al., 1998; Wang et al., 2000; Gursoy-Ozdemir et al., 2004; Michaluk and Kaczmarek, 2007). Also supporting this theory of MMP-9 function are animal studies utilizing gelatinase KO mice, where loss of MMP-2 did not reduced injury effects (Asahi et al., 2001b), but MMP-9 KO mice were protected from the pathology of ischemia (Asahi et al., 2000) and TBI (Wang et al., 2000). Protection from CNS injury has been linked to BBB integrity, where MMP-9 KO mice exhibit decreased white matter infarct volume and reduced myelin basic protein (MBP) fragment proteolysis after injury (Asahi et al., 2001b). This effect may be related to the fact that gelatinases contain a fibronectin-like domain which binds collagens and elastins, making these basement membrane proteins easy targets for MMP-9 modulation of BBB integrity (Conant and Gottschall, 2005; Gidday et al., 2005). While the gelatinases can clearly alter BBB integrity after trauma, their role in synaptic plasticity is not well understood. Nevertheless, a few studies have linked gelatinases to modulation of growth factors (Rosell and Lo, 2008) and inflammatory cytokines through microglial production after trauma (Gottschall and Yu, 1995; Giraudon et al., 1997).

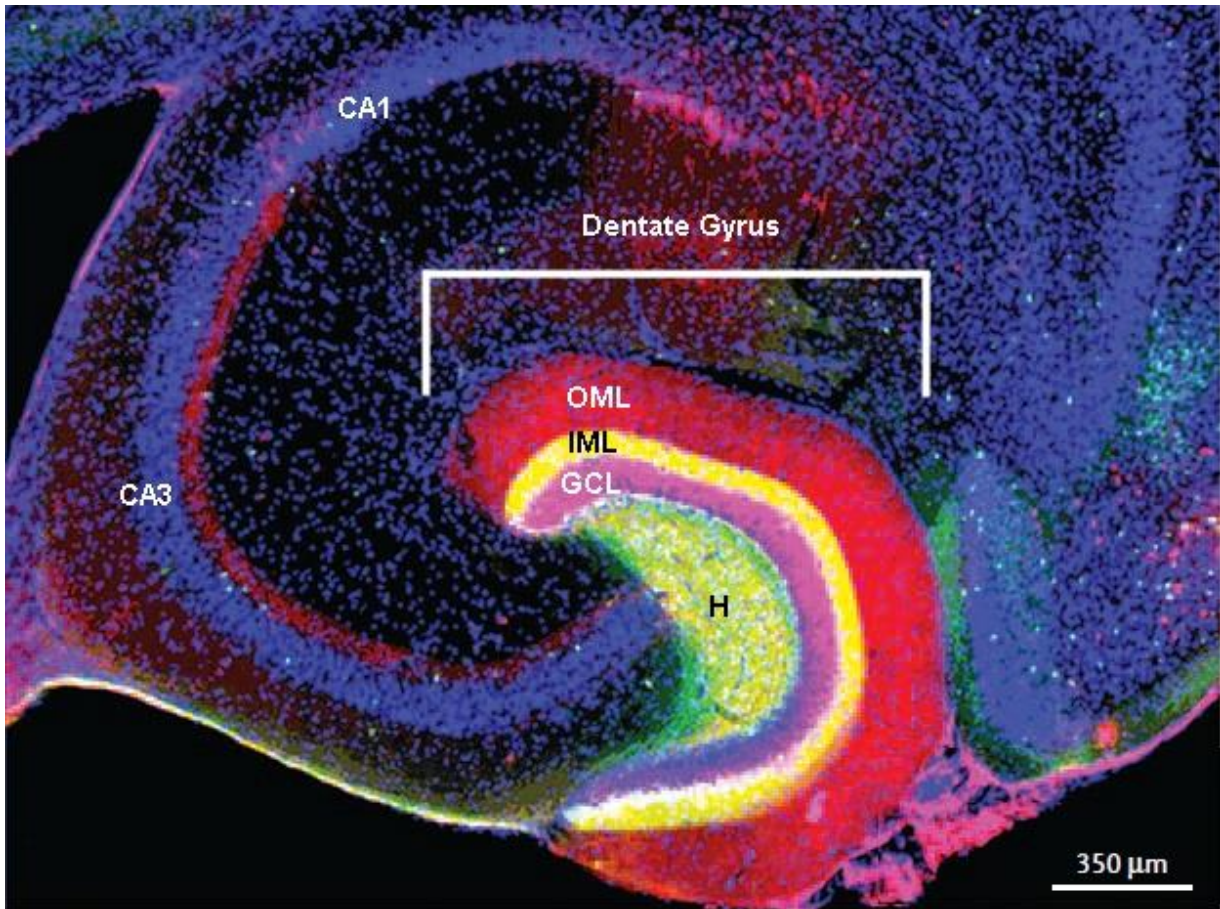


## **THE HIPPOCAMPUS: STRUCTURE AND VALUE AS A MODEL OF SYNAPTIC PLASTICITY AFTER TRAUMATIC BRAIN INJURY**

### Structure and Function of the Hippocampus

The hippocampus is a seahorse shaped structure located in the temporal lobe of humans, a brain region critical to memory formation. The hippocampus and its associated cortical regions together comprise the hippocampal formation, which can be further divided into six anatomically distinct areas: the subiculum, the hippocampus proper, containing four cornu ammonis sectors (CA1 -4), the DG, along with presubiculum, parasubiculum, and entorhinal cortex (EC). The CA fields are comprised of pyramidal neurons whose cell bodies lie within the pyramidal cell layer, while the basal dendrites reside in the stratum oriens and the apical dendrites extend through the stratum radiatum and stratum lacunosum. The DG can be divided into three layers: the hilus, granule cell layer (GCL) which contains the cell bodies of granule neurons, and the molecular layer (ML), containing the dendrites of the granule cells (Amaral and Witter, 1995) (**Fig. 1.2**). The hippocampal functional circuitry consists of synaptic connections involving both internal and external pathways. Externally, information is shared reciprocally between a number of structures including the EC, other components of the limbic system, the association cortices, and septum, all regions which facilitate the processing of memory and emotion. Internally, the principal hippocampal circuitry is processed along a trisynaptic pathway, and transmitted unidirectionally through a series of synaptic connections which receive their primary input from the external EC (Amaral et al., 2007). The secondary axonal input from the medial septal nucleus contains acetylcholinesterase, identifying these as cholinergic projections, which travel dorsally along the fimbria, fornix, supracallosal stria, and ventrally within and around the amygdala, projecting to the inner 1/3 of the molecular layer (IML) of the DG. The major

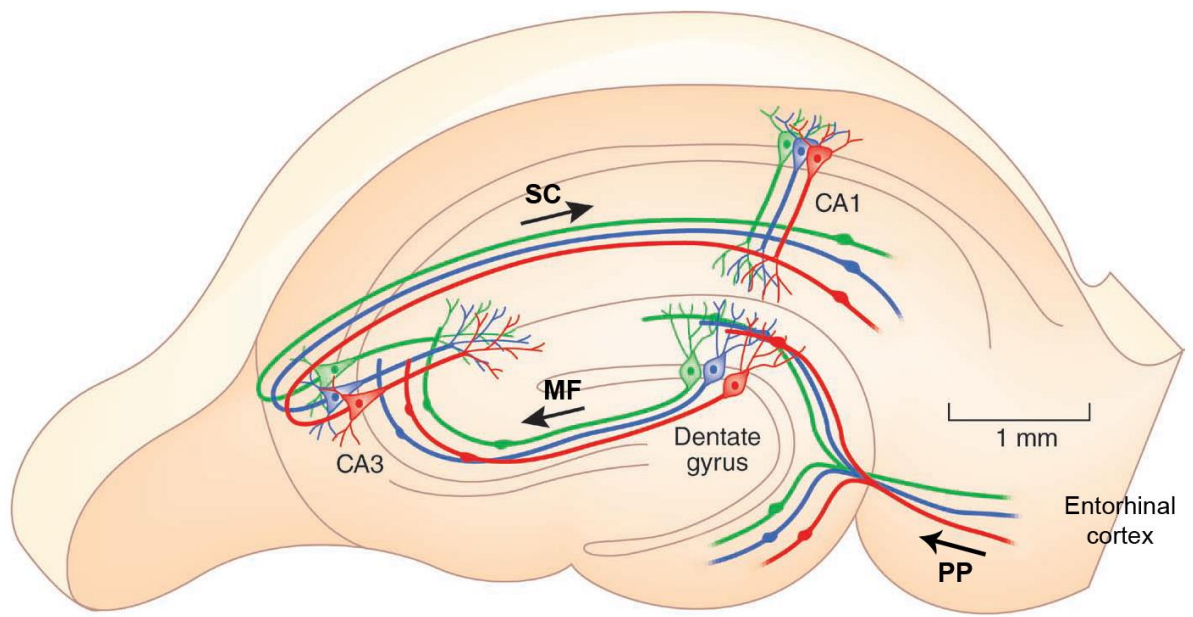
**Figure 1.2 Laminar Structures of the Hippocampus.** Various labeling techniques visualize the arrangement of different laminae in the mouse hippocampus. Within the dentate gyrus (DG), the granule cell layer (GCL) is labeled in pink, with neuronal cell bodies labeled in DAPI (blue). The dendrites of these neurons extend to the inner molecular layer (IML, yellow) and outer molecular layer (OML, red), where they synapse with afferent projections from the entorhinal cortex (EC). The pyramidal layers of CA1 and CA3 are shown in blue. (Forster et al., 2006)



afferent pathway into the hippocampus is known as the perforant path (PP), originating from layers II and III of the EC, innervating the outer 2/3 of the ML (OML) of the DG. More specifically, axons from the lateral EC innervate the outer most third of the ML and the medial axons project to the middle third, collectively deemed the first fiber tract in the hippocampal trisynaptic loop (Hjorth-Simonsen, 1972; Steward et al., 1973).

The tri-synaptic loop (**Fig. 1.3**) is comprised of three pathways which are the major axonal projections and synaptic connections conducting information flow within the hippocampus. As described above, the perforant path is the primary input into the hippocampus and also constitutes the first synapse within the trisynaptic loop. The second portion of the hippocampal circuit conducts information from the DG to the pyramidal cells of CA3 through mossy fibers, which make the second synapse of the loop. Finally, CA3 pyramidal neurons complete the tri-synaptic circuit when their axons converge as Schaffer collaterals onto synaptic junctions onto the CA1 pyramidal dendrites. Ultimately, information is then free to flow out of the hippocampus from CA1, making connections with structures such as those in the limbic circuit. This information circuit within the hippocampus is important due to its role in memory formation and contribution to the limbic system, a circuit which encompasses multiple structures related to memory and emotion. Historically, the hippocampus has been instrumental in documenting the capacity to elicit LTP in cortical neurons, a property which continues to serve as a cellular model for learning and memory (McClelland et al., 1995; Battaglia et al., 2011). LTP has been applied experimentally within the hippocampus to demonstrate that repetitive stimulation of excitatory input leads to an increase in synaptic activity within the DG, suggesting that these neurons can be sensitized or ‘remember’ the previous input and become more responsive (Hall, 1998). The earliest studies of the hippocampus date back to the 1950’s

**Figure 1.3 Schematic of the Trisynaptic Circuit within the Hippocampus.** The major input into the hippocampus arises from the EC via the perforant pathway (PP) into the DG where it makes its primary set of synapses. Information is then transmitted from the DG through the mossy fibers (MF) to CA3 where a second set of synapses occur. Finally, activity is transferred from CA3 through the Schaffer collaterals (SC), synapsing on CA1 dendrites, after which information is free to flow out of the hippocampus (Moser, 2011).



when the structure was first identified as critical to the development in seizures and epilepsy. Original observations of H.M., a patient whom underwent bilateral hippocampal resections to treat intractable seizures, include descriptions of profound anterograde amnesia, pointing to the hippocampus as a key structure in explicit memory formation (Scoville and Milner, 1957).

Similarly, TBI that disrupts hippocampal connectivity produces altered cognitive function in both humans (Nicholson, 2000; Dikmen et al., 2009; Benedictus et al., 2010; Creed et al., 2011; McKee et al., 2009) and experimental animal models (Griesbach et al., 2009; Phillips et al., 1994). It is interesting that the hippocampal circuits involved in memory formation are the same pathways implicated in seizure pathology, as there is a considerable amount of synaptic reorganization during seizure activity (de Lanerolle et al., 2003; O'Dell et al., 2012). Patients who suffer from seizures exhibit aberrant electrical activity, often within the temporal lobe, followed by hippocampal neuronal loss as well as aberrant sprouting, astrogliosis, and microgliosis which have been replicated in kainic acid animal models (Zhang and Zhu, 2011). These synaptic and immune-related changes within the hippocampus following seizure activity are of particular importance to studies of neuroplasticity after brain injury as TBI also affects the hippocampus and can induce epilepsy in humans (Ferguson et al., 2010) and experimental animal models (Kharatishvili et al., 2006).

### Hippocampal Plasticity: Neurogenesis and Synaptogenesis

The hippocampus is a site with extensive neuroplastic potential. The two principal mechanisms which generate this plasticity are neurogenesis and synaptogenesis, each of which are activated after CNS insult, and significantly contribute to the extent of achievable recovery. The hippocampus has a special role in neuronal regeneration as it is one of the limited sites

where neurogenesis occurs in the adult brain (Gould, 2007). The subgranular zone (SGZ) of the DG is capable of generating neuronal precursors, a process which is enhanced by CNS insults such as ischemia (Liu et al., 1998; Jin et al., 2001), neurotoxic lesions (Weinstein et al., 1996; Gould and Tanapat, 1997), as well as both focal (Kernie et al., 2001; Dash et al., 2001), and diffuse TBI (Bye et al., 2011). These proliferating cells are capable of differentiating into either neurons or glia, as well as migrating to injured locations (Magavi et al., 2000), and contributing to cognitive recovery (Sun et al., 2005; Sun et al., 2007). The beneficial role of TBI-induced neurogenesis within the hippocampus is further supported by studies that administered vascular endothelial growth factor (VEGF) after TBI, which produced an increase in neuronal differentiation, decreased lesion volume, and improved neurological function (Thau-Zuchman et al., 2010). Downstream synaptic reorganization is also supported by this neurogenesis, as new neurons generated within the SGZ migrate to the GCL, where they extend their processes to integrate into the circuits of the DG molecular layer and CA regions of the hippocampus proper (Dash et al., 2001; Emery et al., 2005).

In addition to regenerative connections from newly derived neurons, intact, surviving axonal projections also make a significant contribution to the rewiring of hippocampal circuitry after CNS injury. These events occur in the deafferented hippocampus within the same time-dependent phases of reactive synaptogenesis described in detail above. Injury-induced synaptogenesis is a well-documented feature of the hippocampus, as evidenced by previous studies which demonstrate synaptic sprouting and synapse replacement in the DG and CA following TBI (Steward et al., 1988b; Scheff et al., 2005). Also as described in detail above, this reactive synaptogenesis is a process which our laboratory has shown to be mediated, in part, by MMP interaction with ECM proteoglycans and CAMs (Falo et al., 2006; Falo et al., 2008; Harris



et al., 2011; Warren et al., 2012). Moreover, other researchers have found that the acute postinjury phase of this process is heavily reliant on the inflammatory response to induce early phagocytic clearance, a necessary prerequisite for structural and functional recovery after TBI (Morganti-Kossmann et al., 2002). Recent evidence also indicates that the acute TBI-induced inflammation generated with reactive synaptogenesis and neurogenesis may be linked, given that robust microglial cell response was correlated with neurogenesis in the hippocampus following fluid percussion injury (Sun et al., 2005) or impact acceleration (IA) insult (Bye et al., 2011). The further evaluation of specific molecules which direct these time-dependent stages of repair in the hippocampus may lead to potential therapeutic treatments for reducing cognitive deficits seen after TBI. One promising molecule is osteopontin (OPN), a protein secreted into the ECM, capable of functioning as both a cytokine and a modulator of axonal outgrowth following CNS injury (Hashimoto et al., 2003; Plantman, 2012). Given OPN's multiple functions, and its direct link to the inflammatory response, we believe that it is an excellent candidate to investigate as a model of the relationship between the acute immune response and effective hippocampal synaptic reorganization following TBI.

## **OSTEOPONTIN**

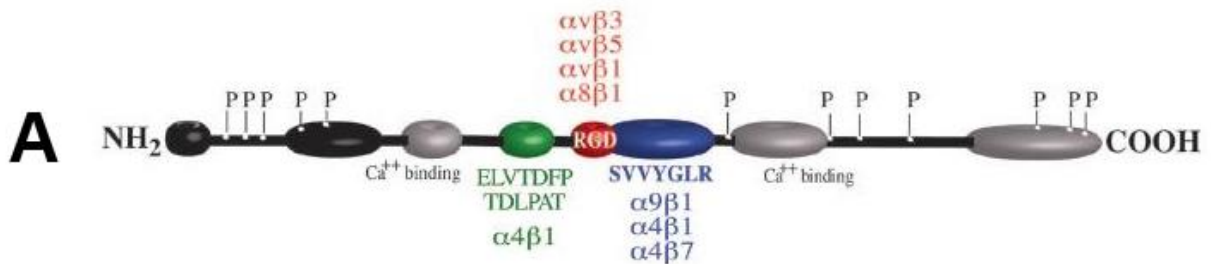
### Discovery, Structure, and Role in Inflammation

OPN is a pleiotropic molecule found in all body fluids which has the ability to act as both a cytokine and tissue repair molecule when released into the ECM (Denhardt and Noda, 1998; Mazzali et al., 2002). Although independently discovered in a number of systems and functional roles, OPN was originally described in bone matrix as secreted phosphoprotein-1 (SPP1), produced by both osteoblasts and osteoclasts during bone remodeling, mediating resorption and

mineralization (Senger et al., 1979; Tezuka et al., 1992). It is also known as bone sialoprotein-1 (BSP-1), as a member of Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLING) family (Fisher et al., 1983), and as early t-lymphocyte activation-1 (Eta-1), a name derived from its function as a cytokine-inducer of macrophage infiltration (Patarca et al., 1989). Structurally, the molecular mass of OPN amino acid sequence is estimated to be around 32 kD, however, more precise matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) studies have predicted a molecular weight ranging from 34.5-37.6 kD, depending on the tissue-specific OPN analyzed (Keykhosravani et al., 2005; Christensen et al., 2007). Despite an estimated molecular weight of about 30 kD, the high level of negatively charged amino acids and the heavy posttranslational glycosylation and phosphorylation of OPN introduce structural variability such that OPN migrates between 45- 75 kD in SDS-PAGE (Kazanecki et al., 2007b). Further, posttranslational modifications are hypothesized to modulate OPN activity, supporting its wide range of function, from cytokine to tissue repair molecule in multiple organ systems (Denhardt and Guo, 1993; Kazanecki et al., 2007b; Wang and Denhardt, 2008). Also critical to OPN's versatile functions are the highly conserved arginine-glycine-aspartate domain (RGD), SVVYGLR, and ELVTDFPTLDLPAT sequences (**Fig. 1.4 A**).

The RGD (or **GRGDS**) sequence is commonly found in ECM proteins, and acts as a ligand recognized by multiple integrin receptors (Ruoslahti, 1996b; Denhardt et al., 2001), allowing OPN to attach to various cell types (Ruoslahti and Pierschbacher, 1987; Denhardt et al., 1995). Specifically, OPN has been shown to bind directly to several integrins including  $\alpha_v(\beta_1, \beta_3, \text{ or } \beta_5)$  and  $(\alpha_4, \alpha_5, \alpha_8, \text{ or } \alpha_9)\beta_1$  (Yokosaki et al., 2005; Liaw et al., 1995a; Bayless et al., 1998; Bayless and Davis, 2001; Scatena et al., 2007; O'Regan and Berman, 2000; Schack et al., 2009; Barry et al., 2000a; Barry et al., 2000b; Hu et al., 1995), facilitating cellular signaling of

**Figure 1.4 Structural Features of Osteopontin.** Colored sequences indicate cell adhesive domains (RGD: red; SVVYGLR: blue) and corresponding integrins which bind these ligands (A). Human OPN sequence below indicates select MMP and thrombin proteolytic sites (B). (Scatena et al., 2007)



**B** MRIAVICFLLGITCAIPVKQADSGSSEEKQNAVSSEETNDFKQETLPSKSNES  
 HDHMDDMDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDSHHSDESDE  
 LVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR SKSKKFRRPDIQ  
 YPDATDEDITSHMESEELNGA YKAIPVAQD LNAPSDWDSRGKDSYETSQ  
 LDDQSAETHSHKQSRLYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSH  
 EDMLVVDPKSKEEDKHLKFRIS HELDSASSEVN

↓ MMPs

▲ Thrombin

osteoclasts, epithelial cells, and smooth muscle cells (Denhardt et al., 1995). To permit these cellular interactions, OPN tertiary structure often requires proteolytic processing to more directly expose the available binding sequences. Two principal mechanisms have been identified in the generation of these functional fragments: thrombin and MMP proteolysis. Thrombin cleavage of OPN is documented in the vascular system. Lysis immediately following the SVVYGLR sequence unfolds the OPN protein backbone, exposing the SVVYGLR integrin binding site (**Fig. 1.4 B**) (Senger et al., 1994; Smith and Giachelli, 1998; Bayless et al., 1998; Scatena et al., 2007; Yokosaki et al., 1999; Barry et al., 2000a; Barry et al., 2000b; Green et al., 2001) to permit the modulation of outgrowth and regeneration in the ECM (Mukherjee et al., 1995; Lefcort et al., 1992; Hynes, 1992; Venstrom and Reichardt, 1993; Martin et al., 2004; Midwood et al., 2004). In addition to thrombin cleavage, OPN is subject to MMP processing where MMPs -2, -3, -7, -9, -12, (Agnihotri et al., 2001; Hou et al., 2004; Dean and Overall, 2007; Goncalves DaSilva et al., 2010) generate functional fragments and permit exposure of integrin binding sites important in cell adhesion (Agnihotri et al., 2001; Goncalves DaSilva et al., 2010; Scatena et al., 2007; Takafuji et al., 2007; Dean and Overall, 2007). Of the known integrin receptors, OPN more selectively interacts with,  $\alpha_v\beta_3$ , a binding motif known to bind molecules such as vitronectin, directing cell migration, a critical aspect of the inflammatory immune response during tissue repair (Preissner and Jenne, 1991; Ria et al., 2002; Yue et al., 1994; Liaw et al., 1995b; Serini and Gabbiana, 1996).

The ability of OPN to function in the early inflammatory response has been well-described in repair after cardiac injury, nephritic injury, and general wound healing (Liaw et al., 1998). After traumatic events such as myocardial infarction or nephritis, elevated OPN transcript and protein was localized to smooth muscle cells and macrophages (Giachelli et al.,

1993; Murry et al., 1994). This acute OPN expression was linked to cell migration to the injury site, as well as formation of the neointima, supporting an important role in the recovery process after trauma (Giachelli et al., 1994; Giachelli et al., 1995). As indicated above, to effect recovery and repair during the immune response, the RGD and SVVYGLR N-terminal sequences within OPN interact with  $\alpha\beta$  integrins to promote cell adhesion and migration of inflammatory and angiogenic molecules to the site of injury (Liaw et al., 1995b; Hu et al., 1995; Smith et al., 1996; Smith and Giachelli, 1998; Denhardt et al., 2001). Given the importance of inflammation in all healing mechanisms (Leibovich and Ross, 1975; Portera et al., 1997), and evidence of OPN as a matrix protein involved in wound healing, this versatile molecule requires further investigation as to its inflammatory role following traumatic CNS injury.

Inflammation is one of the first cellular mechanisms to respond to after CNS injury, and is a response which has also been implicated in the development of chronic neurodegenerative disease. Given the importance of the acute immune response in recovery, OPN signaling cascades are likely to be involved in the synaptic regeneration process following TBI. The first descriptions of OPN immunological activity documented Eta-1 gene upregulation within lymphocytes in response to a rickettsia tsusugamushi, a bacterial infection which elicits a T-cell immune response (Patarca et al., 1989). Subsequent studies identified this Eta-1 gene as OPN, and linked its acute elevation to T-lymphocyte activation and macrophage recruitment, confirming the importance of OPN in the acute T-cell mediated immune response (Singh et al., 1990). OPN's ability stimulate phagocytosis in response to intracellular staph invasion (Schack et al., 2009), and its high expression in Th1 cells during T-cell differentiation further support its role in type-1 immune eradication of intracellular pathogens and delayed hypersensitivity reactions (Nagai et al., 2001). Additional studies in type-1 immune responses to conditions such

as granulomas indicate that OPN exacerbates inflammation by increasing pro-inflammatory cytokine IL-12 and decreasing anti-inflammatory cytokines such as IL-10 (Ashkar et al., 2000). Interestingly, these effects are thought to be mediated by integrin signaling via CD44 receptor binding, a glycoprotein important in the immune response.

### Inflammatory Role in the Central Nervous System

The discovery of OPN as an early activator of T-lymphocytes and evidence of OPN participation in Th1-mediated pathology has led to the exploration of its role in the inflammatory response underlying CNS Th1-mediated diseases such as MS, a progressive neurodegenerative disease characterized by myelin degeneration (Chabas et al., 2001; Vogt et al., 2003). MS is an autoimmune inflammatory disease of the CNS which affects both the brain and the spinal cord. As a progressive disorder, chronic inflammation exacerbates demyelination, and impedes the restorative remyelination process. This progressive inflammation and subsequent demyelination manifests in white matter plaques and axonal damage (Trapp et al., 1998; Pitt et al., 2000), ultimately neurologically disabling the individual. Interestingly, while MS is a chronic disorder, there is variation in the time course of its progression, and many patients will present with intermittent bouts of neurological dysfunction, characterized as a relapsing-remitting form of the disease (Compston and Coles, 2008). Given the importance of the inflammatory response in myelination and axonal injury, OPN role has been studied in both human MS patients and experimental animal models of MS. Clinical investigation indicated detectable OPN levels in both the serum and cerebral spinal fluid (CSF) of MS patients. Interestingly, the highest OPN levels were found in the CSF of patients with active MS lesions (Vogt et al., 2003), and these elevated levels of OPN correlated with the severity of neurological impairment (Chowdhury et

al., 2008). Together, clinical evidence of OPN presence during periods of active lesions and motor dysfunction suggests that this cytokine may play a role in the inflammatory response associated with MS demyelination. The precise OPN role, however, is unclear at this time since demyelination and remyelination occur within close temporal proximity. This uncertainty highlights the need for additional studies to determine whether OPN prolongs inflammation underlying myelin degradation and axonal damage, or if it is functioning as a repair molecule during remyelination to promote axonal recovery.

To better understand OPN role in MS, investigation of the molecular mechanisms surrounding this process have been studied in the experimental autoimmune encephalomyelitis (EAE) model of MS. Although there are several mechanisms which can be used to induce EAE, the most common model utilizes subcutaneous injection of a myelin-based protein to trigger a CNS inflammatory response which mimics the MS pathology seen in patients (Van Epps, 2005). EAE animals expressed OPN in microglia during both relapsing and remitting phases of this pathology, and expression was concentrated within close proximity to MS-like lesions. Interestingly, increased OPN expression is also correlated with worsening neurological symptoms in MS patients (Chabas et al., 2001). Together, these studies suggest OPN participations in the chronic inflammation underlying clinical MS. Similarly, EAE models also mimic the interleukin pathology seen in granulomatous formations (Ashkar et al., 2000). Specifically, OPN acts as pro-inflammatory cytokine, increasing the secretion of other pro-inflammatory cytokines such as interferon gamma (IFN- $\gamma$ ) and IL-12, and down-regulating secretion of anti-inflammatory cytokines such as IL-10 (Chabas et al., 2001; Weber et al., 2002). Based on investigations in MS patients and EAE models, OPN's role in CNS type-1 immune



response appears to involve with the acute mediation of chronic inflammatory signaling which leads to axonal and neuronal damage.

### Role in Neurodegenerative Disease

In addition to chronic autoimmune disease, OPN appears to play a role in the pathogenesis of chronic neurodegenerative diseases such as Parkinson disease (PD) and Alzheimer disease (AD). Characterized by neurological dysfunction, and generally affecting those over 60 years old, PD and AD are the leading neurodegenerative diseases in the world. While AD typically manifests as a neurocognitive disease characterized by dementia and deficits in memory, language, and behavior, the debilitating effects of PD present as motor dysfunction such as resting tremor, bradykinesia, and rigidity (Torrao et al., 2012). One common theory places chronic inflammation as the likely mechanism producing the neurodegeneration which underlies cognitive and motor deficits seen in victims of each disease (Block and Hong, 2005). For example, the characteristic loss of dopaminergic neurons in the substantia nigra (SN) and alpha-synuclein aggregation in Lewy bodies (Torrao et al., 2012) is potentially caused by chronic neuroinflammation which translates into impaired motor activity (Jankovic, 2008).

Evaluation of normal animal and human brains shows that OPN is present within many structures, most prominently expressed in the brain stem, sensory neurons, and deep nuclei of the cerebellum (Shin et al., 1999; Ichikawa et al., 2000). Complimentary studies by Iczkiewicz and colleagues detected OPN protein expression within the intact rat hippocampus and cortex, with the highest levels observed in the basal ganglia and SN, locations vulnerable to the pathology underlying the development of Huntington's disease and PD, respectively (Iczkiewicz et al., 2004). Finally, evaluation of normal primate and human brain indicate a similar presence of

OPN within nigral neurons, but not glial cells, in the SN (Iczkiewicz et al., 2006). This latter finding is of particular interest since OPN has been colocalized to glial cells during active synaptogenic development of the rat brain (Choi et al., 2004), and in experimental models of CNS disease (Van Epps, 2005). These findings further support the hypothesis that OPN function is linked to both immune response and synaptic plasticity within the CNS.

Similar to clinical MS studies, OPN in PD patients was increased in hallmark lesions (Lewy bodies), serum, and CSF compared to control subjects, where OPN serum protein levels correlated with an increase in symptomatic dementia (Maetzler et al., 2007). This clinical evidence coincides with elevated OPN mRNA and protein expression in inflammatory models of PD utilizing direct LPS injection into the SN of rats (Iczkiewicz et al., 2005). LPS injection produces an inflammatory response resulting in the death of dopaminergic neurons, a prototype of PD pathology. Interestingly, both OPN mRNA and protein responded acutely, peaking at 48 and 72 hours respectively. Further, this rise in OPN production was correlated with an increase in OPN positive reactive microglia. Astroglial reactivity also increased, however, this response was much later (120 hours postinjury), and staining for GFAP was not colocalized with LPS-induced OPN. Given this acute selective rise in microglial OPN coinciding with LPS-induced dopaminergic cell death, it is reasonable to predict that OPN may function as a modulator of the SN immune response, likely contributing to the loss of tyrosine-hydroxylase positive neurons in PD (Iczkiewicz et al., 2005). Contrary to these results are studies utilizing subcutaneous injections of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) neurotoxin to induce dopaminergic death in a non-human primate model of PD, where reduced OPN expression was observed within the SN (Iczkiewicz et al., 2007). In this same study the evaluation of postmortem human brain tissue showed decreased OPN levels in remaining dopaminergic

neurons. The fact that OPN is reduced within populations of surviving neurons suggests that it, in some way, influences the extent of neuroprotection, however, OPN may also be involved in recruiting reactive glial cells to the site of injury and the recurrent cycle of cell degeneration. For example, additional studies by Iczkiewicz and colleagues addressed the inflammatory response during neuronal injury through the comparison of 6-hydroxydopamine (6-OHDA) toxin and mechanical lesions of the SN (Iczkiewicz et al., 2007). In both models, OPN was colocalized with activated microglial cells but not dopaminergic, or GFAP positive cells, and dopaminergic cell death was correlated with increased OPN expression and ED1 positive macrophages. Together, these data suggest that OPN plays a role in both pro-inflammatory and anti-apoptotic mechanisms, highlighting the complex nature of OPN's influence on both neuronal recovery and repair. Further, OPN contribution to the recovery process may depend on the injury mechanism which generates the immune response, as seen in experimental animal models of neurodegeneration. Nevertheless, it is undetermined whether OPN is beneficial or detrimental in the context of synaptic regeneration in these models, a question which must rely on further studies investigating the molecular neuropathology underlying the CNS disease.

While both chronic inflammatory diseases and inflammatory diseases of the extrapyramidal system in PD each indicate a role for OPN in the evolution of neuropathology, OPN has not been extensively studied in the development of AD. AD is the leading cause of dementia in the world, with an increased incidence in those over 65 years old. Histopathological hallmarks include the accumulation of extracellular amyloid-beta ( $A\beta$ ) plaques and neurofibrillary tau, which are thought to induce progressive neuronal loss underlying the hippocampal dysfunction and cognitive deficits seen in AD patients (Torrao et al., 2012). Early stages of AD are marked by inflammation and microglial activation, and most recently studies

have linked cognitive performance of AD patients with increased OPN CSF levels (von Bernhardt, 2007; Comi et al., 2010). Anatomical studies have also documented an increase in OPN expression within the pyramidal neurons of the hippocampus, cells which make up a major pathway for memory formation (Wung et al., 2007). Interestingly, this staining was found to correlate with A $\beta$  deposition and age, suggesting that OPN may play an important role in AD disease development or remodeling during neurodegeneration. Given recent evidence linking mild head injury with delayed cognitive impairment, these studies also suggest OPN response may be critical to the development of chronic degenerative pathology sometimes accompanying TBI, such as CTE. CTE is characterized by cortical atrophy and enlarged ventricles caused by repeated blows to the head, such as those suffered by athletes or the military, and like that of AD, patients suffer cognitive deficits which are associated with hyperphosphorylated tau, chronic microglial reactivity, and inflammatory conditions (Blaylock and Maroon, 2011; Saulle and Greenwald, 2012). When these observations are considered together, it is clear that elevated OPN correlates with inflammatory-associated neuropathogenesis in MS, PD, and AD, and likely contributes to the CNS cellular response underlying these debilitating neurological diseases.

#### Role Following Central Nervous System Injury

In addition to the chronic inflammatory response of neurodegeneration, OPN function has been evaluated during the acute immune response in focal models of stroke or ischemia. Initial studies utilizing permanent middle cerebral artery occlusion (MCAO) in spontaneously hypertensive rats reported OPN mRNA elevation within the periinfarct region within 2 hours postinjury. Further, OPN protein was expressed in periinfarct microglia 24 hours after injury, cells which migrated to the center of the infarct by 48 hours. These results demonstrate an acute

trauma-induced OPN response, and migration of OPN containing glia toward the site of injury, similar to the chronic inflammatory OPN response in neurodegenerative disease (Ellison et al., 1998). Global ischemia using a transient 4-vessel occlusion model in rats revealed similar results, specifically documenting increased OPN mRNA in the hippocampal pyramidal layers and striatum within 24 hours following occlusion. As seen with the cellular OPN response in focal stroke, global ischemia also induced OPN positive reactive microglia restricted to the sites of neuronal damage within hippocampal CA1 and CA3 at 3 -7d postinjury (Lee et al., 1999). These studies suggest microglia as principal secretors of OPN during the acute inflammatory response, but interestingly did not detect OPN mRNA within reactive astrocytes present around 15d postinjury. To further understand the potential link between OPN and cognitive damage following stroke or ischemia, mechanisms such as BBB impairment identified in the pathogenesis of vascular dementia were also examined. Interestingly, BBB disruption alone induced elevated levels of OPN mRNA, verified by qRT-PCR (Iwanaga et al., 2008), suggesting cognitive deficits following stroke may be linked to OPN and vessel disruption. Together, evidence that OPN is elevated after stroke or ischemia within microglia supports the idea that OPN, active in the immune response, promotes vascular and neuronal recovery.

Given the similar cellular OPN response to stroke and ischemic, putative mechanisms behind its interaction with reactive glial cells was investigated. While previous studies indicated OPN does not appear to be produced by reactive astroglia, their interaction with OPN is supported by colocalization of the cytokine and  $\alpha_v\beta_3$  on the surface of glial fibrillary acidic protein (GFAP) positive astrocytes at 15d following MCAO (Ellison et al., 1998). The upregulation and colocalization of  $\alpha_v\beta_3$  within reactive astrocytes is also supported by independent studies in non-human primates subject to focal cerebral ischemia (Okada et al.,

1996), and transient forebrain ischemia in the rodent four vessel occlusion and reperfusion model (Kang et al., 2008). Further, OPN and  $\alpha_v\beta_3$  upregulation was observed within the periinfarct region and newly synthesized glial scar, suggesting OPN may modulate axonal or vascular repair at that site (Ellison et al., 1998; Ellison et al., 1999). More specifically, prior studies linking  $\alpha_v\beta_3$  upregulation with angiogenesis suggest OPN/  $\alpha_v\beta_3$  interaction via the SVVYGLR sequence modulates the synthesis or repair of blood vessels following stroke and ischemia (Brooks et al., 1994; Okada et al., 1996; Reynolds et al., 2002; Serini et al., 2006). In addition to modulating angiogenesis, OPN/ $\alpha_v\beta_3$  interaction likely permits OPN to function as an astrocyte and induce chemotaxis of reactive astrocytes to the site of stroke injury (Ellison et al., 1998; Ellison et al., 1999), an effect similar to that of smooth muscle cell mobility after cardiovascular insult (Liaw et al., 1995a; Liaw et al., 1995b). Such OPN/integrin interaction in response to TBI remains to be investigated, but it appears quite likely to occur since OPN binds to many different integrin receptor subtypes in the injury region. For example, recent studies have linked OPN to the promotion of phagocytosis via the  $\alpha_x\beta_2$  integrin receptor during bacterial infection (Schack et al., 2009), as well as the alteration of cell migration (Lund et al., 2013). The fact that microglia express  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptors, and can influence angiogenesis during traumatic inflammation (Milner, 2009), points to their potential for OPN interaction to mediate inflammatory cell signaling such as leukocyte extravasation during the acute immune response following TBI (Jones, 1996; Archelos et al., 1999).

In addition to integrin receptor binding, OPN is also capable of interacting with cell surface CD44 receptors. CD44 was first investigated as a receptor expressed on activated microglia and reactive astrocytes as a modulator of inflammation (Vogel et al., 1992; Foster et al., 1998; Pure and Cuff, 2001). The hyaluronic receptor CD44 is a cell adhesion receptor

linked to macrophage recruitment and facilitation of the acute immune reaction (Weber et al., 1996). Cellular studies indicate that OPN directs chemotaxis and macrophage migration utilizing the CD44 receptor via  $\beta 1$  integrin (Katagiri et al., 1999; Weber et al., 2002; Zhu et al., 2004; Marcondes et al., 2008). Specifically, OPN is known to bind CD44 splice variants, such as CD44v3-6, a form highly expressed on the surface of microglia (Smith et al., 1999; Kang et al., 2008). Although OPN binding to these splice variants has been demonstrated, the exact sequence which forms a ligand for either CD44 or its splice variants has yet to be discovered (Weber et al., 1996; Weber et al., 2002; Takafuji et al., 2007). Interestingly, studies document trauma-induced increase in CD44 and OPN in models of ischemia (Kang et al., 2008). Following ischemic insult, CD44 is colocalized with OPN in microglia at 1 day post-reperfusion, peaking in expression at 3 days, and then remission by the 7<sup>th</sup> day. This early 3d postinjury peak in OPN occurred in the CA1 pyramidal layer, as well as the dentate hilar region of the hippocampus (Kang et al., 2008). Most recently, studies in LPS models of PD document both an increase in OPN and upregulation of CD44 within neurons and glia, which is interpreted as providing a neuroprotective role (Ailane et al., 2013).

While CD44 and integrin receptors link OPN to microglial activation and the acute inflammation associated with tissue degeneration after CNS injury, this same OPN/receptor interaction could play a more positive role in promoting repair during the later stages of synaptic recovery. For example, repair of CNS vascular insult is a complex process, involving a two phases of OPN response in the injured area. As previously mentioned, stroke induces an initial OPN mRNA response linked to the activation and migration of microglia within 24 hours of injury. By contrast, the second phase of OPN expression occurs in reactive astrocytes at the penumbra 5d postinjury, cells which ultimately migrate to the injury core by 15d (Ellison et al.,

1998). These observations suggest that the same microglia which interact with OPN to control acute inflammation can also secrete OPN as an astrokine, promoting chemotactic recruitment of reactive astrocytes to the site of injury (Choi et al., 2007). This idea is supported by the fact that OPN is acutely colocalized with CD44 and microglia, followed by a second colocalization with  $\alpha_v\beta_3$  and astrocytes (Ellison et. al., 1998; Wang et al., 2001; Kang et al., 2008). Similar biphasic response after MCAO followed by reperfusion (MCAO/R) (Okada et al., 1996), or transient 4-vessel forebrain ischemia, shows  $\alpha_v\beta_3$  expression within GFAP positive cells 3-15 postinjury (Kang et al., 2008). This time frame overlaps with the window of synaptic recovery after TBI, further suggesting that OPN could direct alternative cellular responses in order to modulate different phases of synaptogenesis following traumatic insult.

#### Interaction with Extracellular Matrix Proteins and Role in Synaptic Plasticity

During synaptic reorganization, a number of molecules interact to condition the ECM for effective synaptic recovery. While OPN has been linked to neuroplasticity, its expression and potential function models of synaptic deafferentation have yet to be examined. OPN is promising as a mediator of effective synaptic reorganization because: 1) the cells principally involved in molding ECM during synaptic recovery can express OPN, 2) it binds to cell surface receptors utilized by these cells, and 3) it is processed into active functional forms by MMPs in the ECM. Interestingly, recent studies have documented OPN/MMP interaction in models of MS or subarachnoid hemorrhage. For example, in EAE model of MS, MMP-12 cleavage of OPN attenuated inflammatory damage (Goncalves DaSilva et al., 2010), while recombinant OPN (r-OPN) reduced BBB compromise through inhibition of MMP-9 activation (Suzuki et al., 2010b). In addition, OPN interacts with secreted growth factors like fibroblast growth factor to



increase neural progenitor proliferation in cell culture assays (Kalluri and Dempsey, 2012). These observations support OPN interaction with ECM molecules critical to the success of reactive synaptogenesis following TBI.

While the role of OPN in the inflammatory process and its mediation of degeneration are well-documented, there are few studies examining its role during synaptic reorganization. Rodent studies do describe an increase in OPN expression during Wallerian degeneration of the sciatic nerve (Jander et al., 2002), but surprisingly, the elevated OPN levels were expressed in Schwann cells, not infiltrating macrophages. This study is in contrast to another which found macrophage OPN expression following optic nerve crush, suggesting that OPN role may be dependent on CNS or PNS-specific environmental cues such as MMP activity (Kury et al., 2005). OPN expression in Schwann cells has been further investigated in the cuprizone model of demyelination. Cuprizone is a copper chelator toxic to oligodendrocytes, which causes demyelination within the corpus callosum when administered orally (Blakemore, 1972; Ludwin, 1978; Matsushima and Morell, 2001). Due to the spontaneous remyelination upon removal, this model provides a way to study both demyelination and remyelination properties (Arnett et al., 2001; Mason et al., 2001; McMahon et al., 2001). Interestingly, cuprizone treatment induced OPN in microglia and astrocytes surrounding axons undergoing demyelination-remyelination cycles (Selvaraju et al., 2004). In the same study, administration of r-OPN to primary cultures of oligodendrocyte precursors resulted in proliferation and MBP synthesis, suggesting that OPN promotes myelination and axonal repair. Similar OPN response was seen with damaged axons in models of SCI, where clip compression of the spinal cord increases acute OPN mRNA expression within white matter microglia as early as 24 hours postinjury (Hashimoto et al., 2003; Moon et al., 2004). Moreover, OPN levels are increased in the degenerating neurons and axons

of the hippocampus during status epilepticus (Borges et al., 2008), as well as within a subset of microglia following kainic acid administration (Kim et al., 2002). In aggregate, these models show that at least three primary components of TBI pathophysiology, microglial activation, direct axotomy, and excitotoxic insult, all induce OPN expression during the postinjury period of synaptic reorganization.

Other studies examining OPN after the CNS injury also point to its role in both degeneration and regeneration. During the course of trauma-induced remodeling in the CNS, OPN may stimulate neurogenesis as part of effective recovery. In models of forebrain ischemia, striatal OPN upregulation between 3-30 days after reperfusion induces migration of regenerative neuroblasts from the subventricular zone (SVZ) (Yan et al., 2009). Interestingly, blockade of the  $\beta$ 1 integrin receptor halted this progenitor migration, suggesting that OPN/integrin binding plays a role in this early stage of neurogenic recovery (Meller et al., 2005). OPN's neuroprotective effect is also supported by studies administering r-OPN, where administration following subarachnoid hemorrhage (SAH) protects the integrity of the BBB and microvasculature, minimizing extravasation (Suzuki et al., 2010b). Similar to neuroregeneration studies in forebrain ischemia, blockade of the RGD sequence abolished these protective effects, again indicating that OPN/integrin receptor interaction is critical to neuroprotection. Thus, multiple models of CNS disease and trauma point to a wide range of postinjury functions for OPN, most notably microglial recruitment to the site of injury, and the activation of astrocytes to promote regeneration.

## Response after Traumatic Brain Injury

While OPN role during tissue repair has been studied in the context of inflammation and CNS disease, few studies have examined OPN role after TBI. Due to the heterogeneous nature of TBI, OPN has been investigated in the broad context of varied neuronal, axonal, and vascular pathologies. Recent studies include examination of OPN in focal cortical injury models (von Gertten et al., 2005; Shin et al., 2005; Israelsson et al., 2006; Plantman, 2012), as well as related to specific components of TBI pathology such as hippocampal damage (Morita et al., 2008), ischemia (Ellison et al., 1998; Wang et al., 1998; Meller et al., 2005), and axonal injury (Jander et al., 2002; Kury et al., 2005; Hashimoto et al., 2003; Moon et al., 2004; Hashimoto et al., 2005). To date, no studies have examined the details of OPN and its related inflammatory response during synaptic reorganization after TBI. Only three animal models of TBI have been used to investigate OPN response: controlled cortical impact (CCI) which induces cortical contusion, cavitation, and axonal damage (Lighthall et al., 1989), cold probe cryolesion which generates a severe focal cortical lesion with BBB disruption (Sun et al., 2000), and brain stab wound creating physical tissue ablation and significant glial scar formation (Mathewson and Berry, 1985). OPN transcript was reported to increase within the rat cerebral cortex following CCI (von Gertten et al., 2005), and is supported by evidence of transcript increase after severe TBI (Israelsson et al., 2006) and mild blast injury (Cernak et al., 2011). Similar to previously discussed studies in stroke and ischemia, this OPN elevation was detected during the acute degenerative and onset of subsequent regenerative phases (1-7d postinjury), and was concomitant with CD44 elevation for both cold probe cryolesion, CCI, and brain stab wound models (von Gertten et al., 2005; Shin et al., 2005; Plantman, 2012). Further, evidence that this OPN response may be reversed by FK506 immunosuppression (Morita et al., 2008) suggests that

the acute trauma-induced OPN expression is related to inflammation. Interestingly, cold probe cryolesion induction of OPN was similar to that after stroke, where the cytokine was colocalized within microglia early after injury (4-7d), and later at 7-14d within reactive astrocytes (Wang et al., 1998; Shin et al., 2005). More recent studies have focused on OPN role in recovery, showing elevated neuronal, but not astrocytic, OPN expression after brain stab wound, which suggests a role for OPN in the process of neurite sprouting after injury (Plantman, 2012).

In summary, evidence of OPN response in inflammatory CNS disease, models of vascular injury, and recent studies showing OPN elevation after TBI all would support the hypothesis that OPN plays an important role during reactive synaptogenesis. While these studies map the inflammatory role of OPN in response to independent CNS insults, none address the capacity for the OPN to influence synaptic recovery following brain injury. The studies of this dissertation will test the hypothesis that OPN mediates the efficacy of synaptic reorganization induced by TBI, and its involvement can serve to predict adaptive or maladaptive outcome.

## **EXPLORATION OF OSTEOPONTIN ROLE IN SYNAPTOGENESIS AFTER TRAUMATIC BRAIN INJURY**

### Traumatic Brain Injury Models of Adaptive and Maladaptive Plasticity

To investigate the cellular and molecular mechanisms underlying the long term cognitive deficits produced by human TBI, a number of different animal models have been developed, each permitting a specific aspect of the complex TBI pathophysiology to be studied in a controlled fashion. As described above, the CCI and the cold probe cryolesion models induce a focal lesion, concentrated at the cortical surface of the brain. Others, such as blast, impact acceleration, and fluid percussion versions produce a more diffuse injury, permitting the direct

study of concussive insult often present in human TBI. While each of these models is useful in studying the molecular pathways mediating human head injury, they generate diffuse deafferentation and do not permit clear evaluation of targeted synaptic insult and subsequent reinnervation processes. A more precise injury paradigm used to study this targeted synaptic regeneration is the unilateral entorhinal cortex lesion (UEC), a well-established model of reactive synaptogenesis within the hippocampus (Steward et al., 1988b). As described above, the EC comprises the major input into the hippocampus and, when lesioned, 80-90% of the afferent fibers which synapse in the ML of the rodent DG are ablated, inducing synaptic deafferentation. Following UEC, successful adaptive synaptic reorganization occurs in three well-defined stages (Steward and Vinsant, 1983). For the first 4 days after lesion, there is an acute removal of degenerating terminals as well as dendritic retraction and spine remodeling. At 6-7d postinjury, removal of these afferent terminals permits reemergence of new postsynaptic spines along dendrite, reextending into the denervated zone, accompanied by collateral sprouting of new presynaptic terminals (Cotman et al., 1977; Steward, 1989). By 15d, the rate of this ML synaptic reorganization plateaus, and the phase of synapse stabilization and maturation begins. During this adaptive process, glial cells, ECM proteins, and MMPs all interact to generate functional reconnection as demonstrated by immunohistochemistry (IHC) staining (Lynch et al., 1972), ultrastructural analysis (Matthews et al., 1976a; Matthews et al., 1976b), electrophysiological recordings (Reeves and Steward, 1986), and hippocampal-dependent behavioral analysis (Steward, 1976; Loesche and Steward, 1977; Reeves and Smith, 1987). This time-dependent remodeling of the deafferented DG provides an appropriate template to study how adaptive reactive synaptogenesis responds when challenged by the additional neuroexcitation insult of TBI. Our laboratory has developed a model which directly challenges the adaptive remodeling

of entorhinal deafferentation with the excessive neuroexcitation of fluid percussion TBI, the TBI+BEC paradigm (Phillips et al., 1994). This combination generates a reproducible model of maladaptive synaptic plasticity with many of the persistent deficits seen with human TBI. Importantly, it can be directly contrasted with the UEC adaptive plasticity model to identify differences in molecular and cellular response underlying persistent impairment seen after human TBI. In this dissertation, the role of OPN during reactive synaptogenesis will be examined in both the rat UEC and TBI+BEC models. With this comparative approach, we can determine if OPN response differs under conditions of adaptive versus maladaptive plasticity, and if so, identify an additional target for turning maladaptive reorganization into adaptive recovery after TBI.

#### Osteopontin Knockout Mice

To further assess the OPN function in the context of CNS disease and injury, many groups have utilized OPN KO mice. The OPN KO mouse was first generated in 1998 as a germ line SPP1 mutation where 4 of the 7 SPP1 exons were replaced by a neomycin cassette, rendering a null protein in mixed 129S6 Black Swiss mice (Liaw et al., 1998). Currently, these mice are available from Jackson Laboratory as homozygotes on a C57BL/6 background, and are considered viable and fertile with no gross or behavioral abnormalities (The Jackson Laboratory). Due to their normal development and growth, they have been used in numerous studies to assess OPN function in general wound healing (Liaw et al., 1998), kidney repair (Giachelli et al., 1994), and after myocardial infarction (Murry et al., 1994). A number of studies have utilized OPN KO mice to assess OPN response in CNS disease and injury, indicating that OPN may have both beneficial and detrimental roles. These findings again support its diverse

functions, which are considered to depend upon environmental conditions and the form of OPN present. In models of inflammatory CNS disease, OPN appeared to be a detrimental, as OPN KO mice exhibited decreased EAE severity, and immunity to disease progression which would normally lead to death (Chabas et al., 2001). Further, pharmacological treatment of these OPN KO EAE models with exogenous OPN exacerbated disease presentation, supporting the negative effects of OPN within chronic CNS inflammation (Hur et al., 2007). In exploring the hypothesis that OPN KO mice exhibit decreased inflammatory function in models of PD, Maetzler and colleagues described reduced microglial activation, and altered astrocytic response compared to WT mice (Maetzler et al., 2007). Following MPTP administration, longer astrocytic processes and increased neuronal survival in the absence of OPN further suggests this cytokine plays a detrimental role in neurodegenerative disease, much like that of the inflammatory conditions of MS (Maetzler et al., 2007). Contrary to these inflammatory neurodegenerative disease models, stroke, hypoxic-ischemic injury (HI), and SCI models generated in the same OPN KO mice showed reduced injury, suggesting a neuroprotective role for OPN. Specifically, OPN deficient mice exhibited increased thalamic neurodegeneration and NO release compared to WT controls (Schroeter et al., 2006), and *in vivo* studies utilizing clip compression show OPN KO mice had increased locomotor deficits over WT due to greater neuronal loss and less white matter sparing (Hashimoto et al., 2007). Similarly, neonatal OPN KO mice subjected to HI developed increased gray and white matter sensorimotor dysfunction, and decreased cell proliferation and oligodendrocyte generation (van Velthoven et al., 2011).

As in the EAE studies described above, replacing OPN reversed many of the OPN KO effects. For example, OPN KO cultures from mice subject to SCI had decreased levels of MBP, and administration of r-OPN stimulated MBP synthesis (Hashimoto et al., 2005). Other

recombinant studies utilizing MCAO or SAH models also support OPN functioning as a beneficial molecule after injury. They show that postinjury administration of r-OPN reduced weight loss, neurological impairment, infarct size, brain edema, and BBB disruption (Meller et al., 2005; Suzuki et al., 2010a; Suzuki et al., 2010b). Finally, studies in the cuprizone demyelination model suggest OPN is supportive of remyelination and MBP synthesis by showing that administration of r-OPN increased MBP expression, while OPN KO mice had decreased myelin-forming capacity and fewer mature oligodendrocytes (Selvaraju et al., 2004). Together, these results suggest that the OPN KO model can be used to dissect details of OPN's neuroprotective role, such as examining its potential for altering the immune response to reduce neurodegeneration, or for promoting white matter regeneration, myelination, and angiogenesis.

## **SUMMARY AND EXPERIMENTAL HYPOTHESES**

This Introduction has outlined the complexity of TBI and its pathophysiology, components which contribute to the growing economic and societal burden of managing human brain injury. As this epidemic continues, many individuals are at risk and require novel therapies to treat the consequences of TBI. TBI is a heterogeneous insult, involving perturbations in a number of cellular and molecular pathways. Given the myriad of long term effects resulting from these perturbations, this present set of studies will explore how one of the first postinjury changes, acute immune response, determines the quality of synaptic recovery underlying long term outcome. These studies will also test an acute immune mediator for its potential to serve as a therapeutic alternative in patients suffering from TBI.

The inflammatory response is one of the first cell processes to be engaged after CNS injury, and the significance of its contribution following head trauma has been the subject of



considerable debate. In addition to the severe edema, the immune response directs cell migration and clearance injured neurons and axons. While initially supportive, chronic inflammation may be detrimental, leading to prolonged and extensive neurodegeneration and synaptic disruption. Understanding the molecular players which permit effective synaptic reorganization is critical for future therapies. Within the ECM there are several immune regulators of this reorganization, including reactive glia and their secreted cytokines and MMPs. The studies of this dissertation will address the role of OPN, a cytokine already linked to tissue repair in multiple organ systems. A novel function for OPN will be examined, exploring its role as a cytokine and modulator of synaptic reorganization caused by axonal injury. It is hypothesized that OPN is elevated during the acute postinjury immune response, mediating cellular reactions which direct deafferentation-induced degeneration and promote the onset of successful synaptic reformation. A corollary to this hypothesis posits that contrasting models of adaptive and maladaptive plasticity will reveal differences in OPN-directed response to synaptic deafferentation, identifying novel inflammatory pathways which discriminate effective synaptic reorganization. To test this hypothesis, we will first map the spatial and temporal profile of OPN in the rat following UEC and TBI+BEC using Western blot (WB) analysis, IHC, and qRT-PCR. Further analysis of the acute immune response will examine OPN lytic fragments to explore potential cell signaling mechanisms with OPN receptors and determine the extent of MMP/OPN proteolytic interaction. This OPN response will be manipulated by immune suppression with the antibiotic minocycline, after which we will examine how immune activation affects OPN response, and probe for effects on a marker of presynaptic structure during acute postinjury intervals.

In a second set of studies, we will utilize more specific OPN manipulation with OPN KO mice to more accurately establish OPN role during synaptogenesis and address its interaction

with MMPs. We hypothesize that absence of OPN will alter the acute immune response leading to impaired cellular interaction during the acute postinjury period, which will result in poor synaptic reorganization and cognitive dysfunction. To test this hypothesis, we will again use WB and IHC analysis to characterize the temporal and spatial profile of OPN during the acute degenerative phase in OPN KO subjected to UEC, contrasting their response with that of paired WT C57BL/6 mice. Molecules important in synaptic remodeling, including those contributing to cytoskeletal structure, synaptic junction stabilization, and presynaptic terminal formation will be compared between strains. In addition, the effect of OPN KO on postinjury behavioral function will be evaluated using a cognitive test of hippocampal-dependent learning. Finally, gelatin zymography and WB analysis will assess the potential for MMP and its regulator lipocalin-2 to mediate upstream OPN response. Together, these studies will provide insight into how acute OPN expression affects plasticity during the degenerative and regenerative phases of synaptic reorganization. They will determine if OPN can influence effective neurological recovery following deafferentation, highlighting the importance of the acute immune response in effective synaptic reorganization following traumatic CNS injury.

## CHAPTER 2

### OSTEOPONTIN RESPONSE FOLLOWING TRAUMATIC BRAIN INJURY

## ABSTRACT

Osteopontin (OPN) is a pleiotropic molecule which can act as both a cytokine and axonal growth modulator during the acute immune response. OPN has been documented to increase after CNS injury, however, its response during trauma-induced synaptic reorganization is not known. This study sought to determine whether OPN can serve as a functional link between the acute immune response and adaptive synaptic recovery following traumatic brain injury (TBI). We contrasted OPN in two TBI models, adaptive synaptogenesis induced by unilateral entorhinal cortex lesion (UEC), and maladaptive plasticity produced by combined central fluid percussion injury and bilateral entorhinal cortex lesions 24 hours later (TBI+BEC). Our results showed an acute (1-2d postinjury) elevation in both OPN protein and transcript within the hippocampus and its deafferented molecular layer (ML), the site of synaptic reorganization in these models. This acute OPN protein elevation was seen under conditions of both adaptive and maladaptive plasticity. Interestingly, proteolytic fragments of OPN with exposed integrin binding domains were also generated in the ML over the first week postinjury. In the deafferented zone, OPN was primarily localized to glial cell types, including reactive astroglia and active microglia containing OPN transcript. Notably, these OPN positive cells were directed to the border between the injured and intact ML in the adaptive UEC model. This was in contrast to the maladaptive TBI+BEC insult, where OPN label was reduced in reactive microglia, and active glial cells failed to migrate to the boundary zone of ongoing synaptic reorganization. As a marker of deafferentation-induced dendritic morphing, ML microtubule associated protein 1B (MAP1B) was reduced during acute degeneration, and reemerged with axonal sprouting. However, we observed few instances of the potential binding between MAP1B and OPN, suggesting a limited role for OPN in cytoskeletal reorganization after injury. Finally, blunting the immune response

with minocycline reduced OPN response to UEC, attenuating presynaptic terminal removal and producing altered glial cell distribution closely resembling the pattern of the maladaptive injury. In addition, this attenuated immune response reduced injury-induced matrix metalloproteinase (MMP) activity and MMP-generated OPN integrin binding fragments. These results suggest a positive role for OPN in the early immune response, one which facilitates the conditioning of the extracellular matrix to permit effective synaptic reorganization following TBI.

## **INTRODUCTION**

Traumatic brain injury (TBI) is a serious health concern nearly 2 million individuals suffer each year (Faul et al., 2010). This is further compounded by the TBI-induced long term motor and cognitive deficits more than 5.3 million Americans endure, imposing a substantial economic burden estimated at \$60 billion annually due to rehabilitation costs and loss of productivity (Langlois et al., 2006; Rutland-Brown et al., 2006; Finkelstein et al., 2006). Of these deficits, impaired cognition is of particular importance as this has profound effects on the patient's ability to return as a functional member of society. One structure particularly vulnerable to TBI insult is the hippocampus, a brain region important in memory formation and essential to proper cognitive function. Following the initial traumatic insult, the hippocampus becomes damaged through a number of pathophysiological mechanisms, resulting in diffuse axonal damage, neuronal death, and deafferentation. During the recovery and repair of injured synapses, one of the first processes to respond to traumatic central nervous system (CNS) injury is the inflammatory immune response. While the immune response has previously been regarded as a deleterious mechanism following TBI, more recent evidence indicates the acute immune

response is essential to effective synaptic reorganization, directing cytokines and reactive glia to the injured area (Ziv et al., 2006; Derecki et al., 2010).

In an attempt to develop new therapeutics in the treatment of TBI, many injury paradigms have been designed to mimic the pathophysiologic events leading to CNS dysfunction in human TBI. Following traumatic insult, the CNS undergoes a series of well-defined stages of reactive synaptogenesis: degeneration of injured synaptic terminals (1-5d), regeneration of postsynaptic spines and collateral axonal sprouting to form new synapses (6-15d postinjury), and the maturation of stabilized synapses (15d+ postinjury) (Steward et al., 1988b). One model that demonstrates these time-dependent stages of synaptic reorganization is the unilateral entorhinal cortex lesion (UEC), which results in predictable remodeling of injured synapses. Following electrolytic lesion of the EC, 80-90% of the fibers that synapse in the outer molecular (OML) of the dentate gyrus (DG) are ablated. While this model is appropriate to study effective reactive synaptogenesis, it fails to recapitulate the impaired recovery seen in TBI patients. The TBI+BEC model however, pairs a mild-moderate midline fluid percussion injury with bilateral entorhinal cortex lesions (BEC) 24 hours later, inducing diffuse neuroexcitation and deafferentation that leads to persistent structural, functional, and behavioral deficits which mimic the CNS impairment in human TBI (Phillips et al., 1994; Phillips and Reeves, 2001). By contrasting these models of adaptive and maladaptive plasticity, we can determine differences in the expression profile of proteins, potentially identifying candidates which may underlie unsuccessful injury-induced synaptogenesis.

As previously mentioned, the first development in response CNS injury is immune-mediated, a process which occurs during the degenerative phase, and likely sets the stage for synaptic reorganization. Previous studies have identified matrix metalloproteinases (MMPs) and

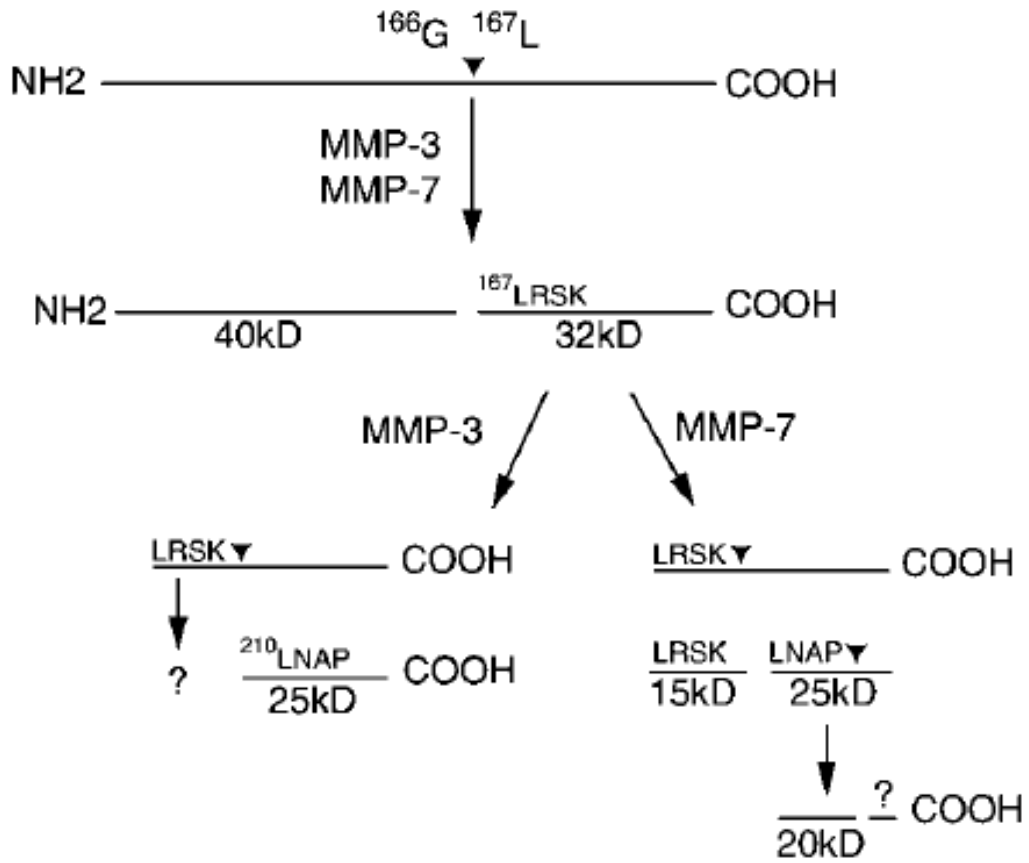
their substrates as important molecules in effective synaptic reorganization after trauma (Falo et al., 2008; Harris et al., 2011; Warren et al., 2012). MMPs are a diverse set of molecules known to function in the immune response through reciprocal interaction with active microglia and proinflammatory cytokines (Kim et al., 2005; Kim et al., 2007; Kim and Hwang, 2011). One cytokine which is a substrate of MMPs (Agnihotri et al., 2001), and capable of acting as a modulator of synaptic outgrowth (Kury et al., 2005; Plantman, 2012), is osteopontin (OPN). This highly negative and posttranslationally modified glycoprotein contains several binding sites which function in integrin receptor signaling when secreted in the extracellular matrix (ECM) (Kazanecki et al., 2007b; Hu et al., 1995; Ellison et al., 1998) (see **Fig. 1.4**). Previous studies have demonstrated acute OPN response in the tissue repair in a number of organ systems including bone (Denhardt and Guo, 1993), heart (Giachelli et al., 1993), kidney (Lopez et al., 1993), and CNS (Wang et al., 1998). Most recently, OPN has been implicated in inflammatory neurodegeneration (Iczkiewicz et al., 2006; Wung et al., 2007; Maetzler et al., 2007), and identified as a molecule significantly upregulated following stroke/forebrain ischemia (Wang et al., 1998; Lee et al., 1999), SCI (Hashimoto et al., 2003; Moon et al., 2004), and TBI (von Gertten et al., 2005; Shin et al., 2005). Additional studies utilizing OPN knockout (KO) mice suggest OPN may be a neuroprotective molecule, functioning during the acute immune response which overlaps with the window of postinjury degeneration, implicating OPN as a potential modulator of effective trauma-induced synaptic reorganization.

In this study, we examined reactive synaptogenesis in rats during the subacute postinjury intervals following TBI to determine if immune-stimulated OPN plays a role in establishing the early phases of synaptic recovery. In preliminary microarray screening experiments, we found significant increase in OPN transcript within the hippocampus at 7d after both UEC (14-fold)

and combined TBI+BEC insult (41-fold) (see **Appendix A**), prompting mapping of its protein expression between during the first week postinjury. We initially profiled the expression of OPN using Western blot (WB) to examine hippocampal extracts from 1, 2, and 7d survivors, contrasting protein levels generated by adaptive UEC and maladaptive TBI+BEC plasticity. Additional blots were probed with an antibody which specifically recognizes MMP-cleaved OPN fragments (**Fig. 2.1**). We used parallel immunohistochemistry (IHC) to determine cellular OPN colocalization in reactive glia and its association with potential cytoskeletal binding partner microtubule associated protein (MAP1B) within the injured dentate gyrus. In addition quantitative real time-polymerase chain reaction (qRT-PCR) was employed to assess changes in OPN transcript at postinjury intervals when the cytokine protein level was highest. Finally, we applied minocycline in the UEC model to test whether blunting immune-driven OPN expression would alter patterns of synaptic recovery, employing synapsin 1 and IBA1 IHC to map differences in synapse and microglia redistribution within the deafferented region. We hypothesized OPN would increase significantly after TBI, likely acting as a cytokine to effect debris clearance during the degenerative phase, and anticipated specific OPN MMP-cleaved fragments would exhibit unique temporal profiles, consistent with the multifunctional capacity of OPN. Further, we posited OPN colocalization within reactive glial cells, where it facilitates chemotaxis and the acute immune response, driving the degeneration of damaged presynaptic terminals after injury, responses we found to be impaired by minocycline immunosuppression. Our results suggest that OPN is an early response molecule, elevated in expression during the acute degenerative phase of reactive synaptogenesis, a protein which localized within activated glia of the deafferented zone, showing distinct differences in expression and tissue distribution when plasticity is maladaptive. Moreover, the increase of integrin-binding OPN fragments in the



**Figure 2.1 MMP/OPN Proteolysis Schematic.** OPN proteolysis occurs through multiple MMPs. This example of chondrocyte OPN proteolysis by MMP-3 and MMP-7 shows the generation of 40 kD and 32 kD fragments after a single cleavage. The 40 kD fragment contains the RGD and SVVYGLR integrin receptor-binding regions while the 32 kD fragment is subsequent to further proteolytic processing.



deafferented zone provides a cell signaling mechanism for this glial migration. These studies are the first to document OPN expression in the acute inflammatory phase of reactive synaptogenesis induced by TBI. Further, we provide evidence that immune-mediated attenuation of OPN expression alters the subsequent pattern of adaptive synaptic reorganization.

## **METHODS**

### **Experimental Animals**

The procedures in this study met national guidelines for the care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. Male Sprague Dawley rats (300-350 g; Harlan Laboratories) were used in these experiments, housed in pairs under a temperature (22°C) and humidity controlled environment with food and water *ad libitum*, and subjected to a 12 hour dark-light cycle. Rats were randomly assigned to one of three groups: UEC (n=78), TBI+BEC (n=32), or Sham-injured (n=32) and evaluated at 1, 2, 4, or 7d postinjury by either WB, IHC, qRT-PCR or gelatin zymography. For the minocycline study, rats were randomly assigned to UEC saline-treated or UEC minocycline-treated groups, and evaluated at 1 or 2d postinjury by either WB or IHC (n=8 per group, total n=32). For all analyses, contralateral hemisphere served as a control for UEC lesioned animals while Sham-injured served as controls for TBI+BEC animals.

### **Rat Unilateral Entorhinal Cortex Lesion**

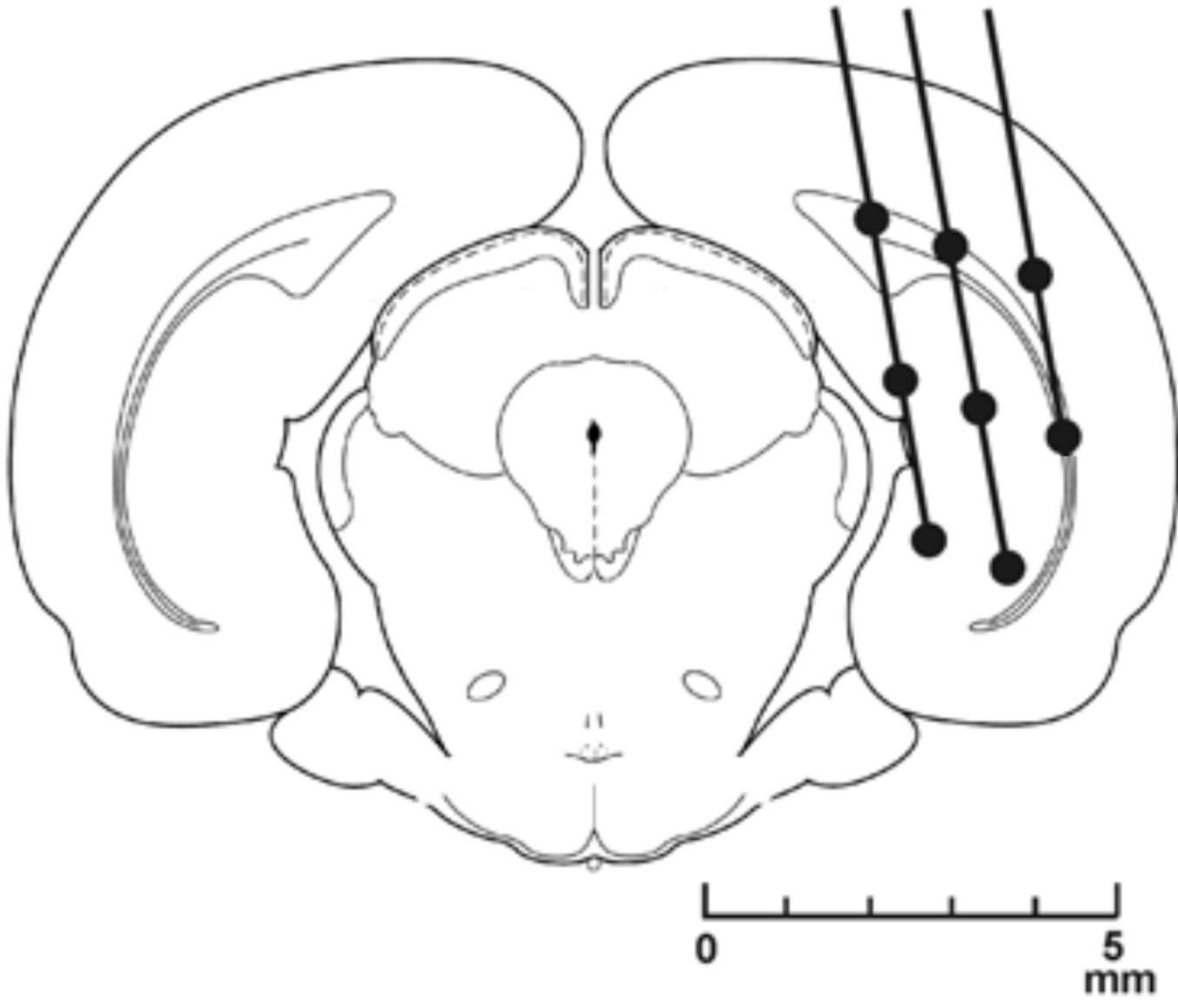
The rat UEC protocol is a modification of that described by Loesche and Steward (1977). Rats were anesthetized with 4% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> for 4 minutes, heads shaved, then maintained on 2% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> delivered

via nose cone for the duration of the surgery. Body temperature was maintained at 37°C via heating pad (Harvard Apparatus, Holliston, MA), and animal heart rate (beats per minute, bpm), arterial oxygen saturation (%), breath rate (breaths per minute, brpm), pulse distention ( $\mu\text{m}$ ), and breath distention ( $\mu\text{m}$ ) were monitored via pulse oximeter (MouseOx; Starr Life Sciences, Oakmont, PA). While under anesthesia, rats were secured in a stereotaxic device, and a midline incision exposed the cranium to permit a 2 mm wide craniectomy to visualize the intact dura mater above the right EC. Electrolytic lesions were delivered via Teflon®-coated wire transmitting 1.5 mA for 40 seconds to eight stereotaxic coordinates: 10° lateral to perpendicular; 1.5 mm rostral to the transverse sinus; 3 and 4 mm lateral to the midline at 6, 4, and 2 mm ventral to the brain surface; and 5 mm lateral at 4 and 2 mm ventral to the brain surface (**Fig. 2.2**). After lesion completion, the electrode was removed, and the incision sutured closed and treated with topical anesthetic and antibiotic. Animals were then housed singly in a heated holding cage during the acute recovery phase and monitored for discomfort or distress before being returned to their home cage.

### Surgical Preparation for Rat Central Fluid Percussion Injury

In preparation for central fluid percussion injury, animals underwent surgical preparation 24 hours prior to injury. Rats were anaesthetized, secured in a stereotaxic apparatus, and vitals monitored as described above for UEC. Under anesthesia, rats received a circular 4.8 mm craniectomy along the sagittal suture midway between bregma and lambda, exposing the intact dura mater. Two 3/16 inch long steel screws were implanted into the skull at a bias 1 cm rostral and caudal to the midline, and the craniectomy fitted with a modified Luer Lock hub (2.6 mm

**Figure 2.2 Rat Unilateral Entorhinal Cortex Lesion Stereotaxic Locations.** 1.5 mA electrolytic lesions of 40 seconds each were delivered to eight stereotaxic sites as shown.



internal diameter) fixed with cyanoacrylate adhesive. To provide additional hub stability during fluid percussion injury, dental acrylic (Coltene/Whaledent, Inc., Cuyahoga Falls, OH) was applied to solidify the Luer Lock hub and screws into a single complex. The incision wound was then sutured closed over the complex and animals were then monitored for post-surgical stress as described above before returning to their home cage.

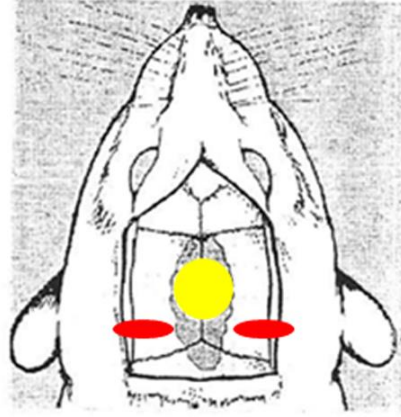
### Central Fluid Percussion Injury

The fluid percussion injury device consists of a weighted pendulum, 0.9% saline-filled Plexiglas cylinder, and is fitted with a metal extra-cranial pressure transducer (Entram Devices, Inc., model EPN-0300-100A). Fluid percussion injury is induced by releasing the metal pendulum from a pre-determined height that coincides with the desired injury severity, striking the rubber piston on one side of the acrylic cylinder to produce a pressure wave and injecting a small volume of saline onto the dura of the animal attached to the metal transducer via the Luer Lock fitting (Dixon et al., 1987) (**Fig. 2.3 B**). Twenty-four hours after craniectomy and hub implantation, animals were anaesthetized 4% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> for 4 minutes, and a scalp incision was made to expose the hub complex. Animals were then attached to the fluid percussion device by filling the hub with sterile 0.9% saline to ensure a seal free of air bubbles. Animals then received a moderate midline fluid percussion injury (2.0±0.1 atmospheres) via saline injection into the closed cranial cavity. The pressure pulse measured by the transducer was displayed on an oscilloscope (Tektronix 1000 S, Beaverton, OR) and the peak of the pressure curve was recorded. Prior to waking, the hub was removed and incision sutured closed and treated with topical triple antibiotic and lidocaine. To confirm injury severity, reflexes (ear, tail, corneal blink) and righting times were recorded prior to acute recovery. Sham

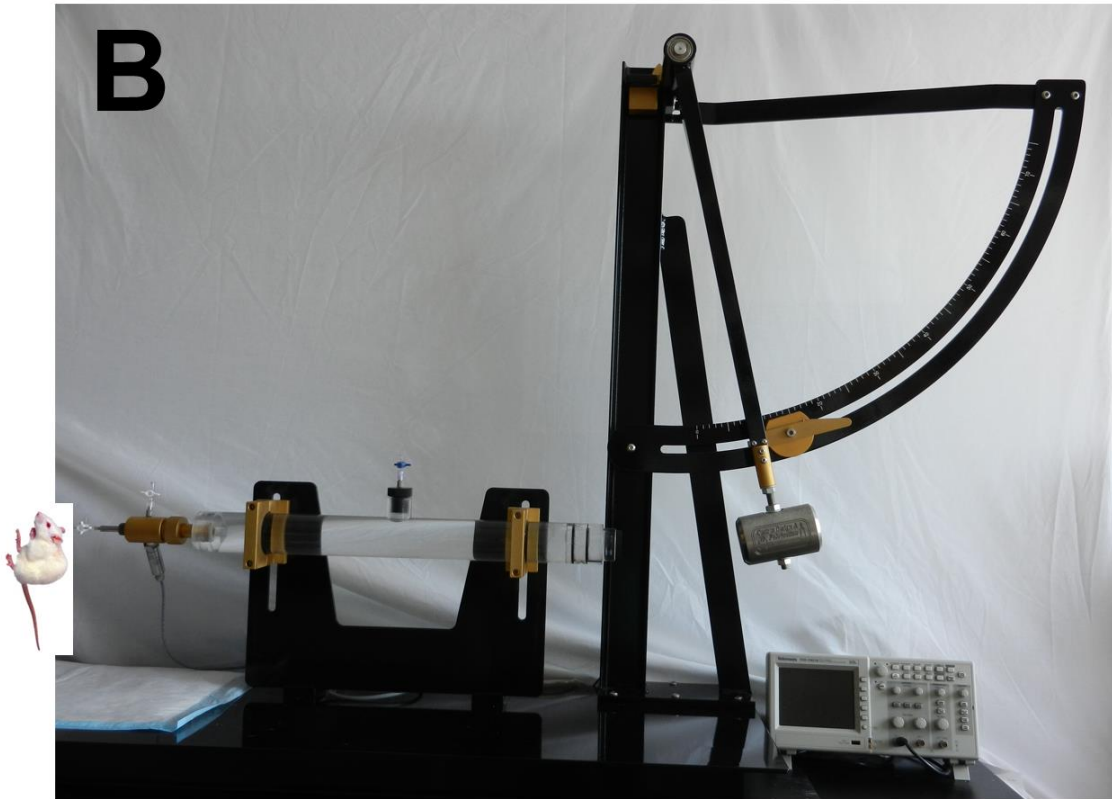
**Figure 2.3 TBI+BEC Injury Locations and Fluid Percussion Injury Device.** Schematic of craniectomy locations (A) for central fluid percussion (yellow circle) and bilateral entorhinal cortex lesions (red ovals) and fluid percussion injury device (B).



**A**



**B**



injured animals underwent the same surgical preparation and hub removal procedures with the exception of the actual central fluid percussion injury.

### Combined Central Fluid Percussion Injury and Bilateral Entorhinal Cortex Lesions

For the TBI+BEC paradigm, rats were first subject to central fluid percussion injury surgical preparation and moderate injury as described above, then at 24 hours post-TBI, subjected to bilateral entorhinal cortex lesions (BEC) utilizing the method described for UEC above with the addition of a second, left entorhinal lesion (Phillips et al., 1994) (**Figure 2.3 A**). Animals were then monitored for distress and discomfort during recovery as previously described.

### Administration of Minocycline

The general immune response was blunted using the tricyclic antibiotic minocycline. Selected rats received two acute intraperitoneal minocycline doses (Sigma-Aldrich, Co., St. Louis, MO) of 45 mg/kg at 30 minutes and 6 hours postinjury, a paradigm previously shown to induce immunosuppression (Homsy et al., 2009). Control animals underwent the same procedure but received a saline vehicle instead of minocycline.

## **Morphological and Molecular Assessments**

### Protein Extraction and Analysis

Selected rats were anaesthetized with 4% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> for 4 minutes, then sacrificed at 1, 2, 4, or 7d postinjury via decapitation (n=4 each whole hippocampus and enriched molecular layer (ML) dissection for UEC, TBI+BEC, Sham; total n=

96). For minocycline studies, rats were sacrificed at 1 or 2d postinjury and hippocampi dissected (n=4 each saline and minocycline treated; total n=16). Whole hippocampi or ML samples were dissected, homogenized on ice in 125  $\mu$ l of T-PER (Thermo Scientific, Rockford, IL), and centrifuged at 8,000 x g for 5 min at 4°C. Supernatant was aliquotted and stored at -80°C for protein quantification, WB, or gelatin zymography analysis. Protein concentration was determined using Thermo Scientific Pierce Protein Assay Reagent (Rockford, IL) and FLUOstar Optima plate reader (BMG Labtech, Inc., Durham, NC).

### Western Blot Analysis

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Thirty  $\mu$ g of protein were prepared for WB with XT sample buffer and XT reducing buffer then denatured at 95°C for 5 minutes. Samples were electrophoresed on 4-12% Bis-Tris Criterion XT gels (200 V x 45 min) then protein transferred to polyvinylidene fluoride (PVDF) membranes (100 V x 60 min). Following transfer, post-blot gels were stained with 0.1% coomassie brilliant blue in destain #1 (40% methanol (MeOH), 10% glacial acetic acid (HOAc) x 30 minutes), then destained at room temperature (#1 x 30 minutes; #2: 7% MeOH, 10% HOAc x 30 minutes) to confirm even transfer of protein. To prevent non-specific protein binding, membranes were first washed in nanopure H<sub>2</sub>O (2 x 5 minutes) and TBS (tris-buffered saline; 1 x 5 minutes), then blocked in 5% milk Tris-Buffered Saline with 0.05% Tween 20 (mTBST). Blots were then separated at the 25 kDa marker and the appropriate pieces incubated overnight at 4°C in mTBST with either anti-OPN (0.25  $\mu$ g/ml, R&D Systems, Minneapolis, MN; 1:300, Rockland Immunochemicals Inc., Gilbertsville, PA) or anti-cyclophilin A (1:2,500, Millipore, Billerica, MA) as a loading control. Blots were washed with mTBST (5 x 5 minutes) then incubated in

appropriate secondary in mTBST (1:10,000 goat anti-mouse; 1:20,000 bovine anti-rabbit, Santa Cruz Biotechnology Inc., Dallas, TX) at room temperature for 1 hour. Finally, blots were washed in TBST (5 x 5 minutes) and incubated with Super Signal Dura West chemiluminescent substrate (Thermo Scientific, Rockford, IL) for signal detection. WB images were captured with Syngene G:Box and positive band signal subjected to densitometric analysis (relative optical density, ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data are expressed as either fold change over controls or percent change relative to paired control cases.

#### Gelatin Zymography Analysis

Twenty  $\mu\text{g}$  of protein from 2d whole hippocampal extracts as described above were prepared with 2x Tris-glycine SDS sample buffer then separated by gelatin electrophoresis at 4°C on Novex® 10% zymogram gels (Life Technologies, Grand Island, NY). Gels were then renatured in Novex® Zymogram Renaturing Buffer (LC2670, Life Technologies, Grand Island, NY) at room temperature before development in Novex® Zymogram Developing Buffer (LC2671) over 6 days at 37°C. Gelatin lysis was visualized with coomassie brilliant blue and purified enzyme run as positive control. Zymogram signal was captured as an inverted image with Syngene G:BOX, and densitometry analyzed as ROD with Gene Tools software (Syngene, Frederick, MD). Enzyme activity data are expressed as percent change relative to paired control cases.

#### Immunohistochemical Analysis

At 1, 2, 4, or 7d (n=4 each UEC, TBI+BEC, Sham; total n= 48) animals were selected for fluorescent IHC analysis. Rats were anaesthetized and sacrificed with a lethal dose of sodium

pentobarbital (90 mg/kg, i.p.). Rat brains were then preserved using transcardiac perfusion with 0.9% saline (500 ml) followed by aldehyde fixative (500 ml 4% paraformaldehyde (PFA) in 0.1 M NaHPO<sub>4</sub>, pH=7.4). Perfused brains were then extracted and placed in fixative for an additional 24 hours before transfer to 0.03% NaN<sub>3</sub> in 1.0 M phosphate buffered saline (PBS). For IHC staining, brains were blocked at the dentate gyrus and 40 µm coronal sections were sliced using the VT1000S microtome (Leica, Buffalo Grove, IL). Slices were then pre-treated in 0.5% peroxidase (30 minutes) and washed in PBS (3 x 10 minutes). To prevent non-specific binding, sections were blocked in Blotto (5% Cold Water Fish Skin Gelatin, Aurion, Netherlands, 5% Triton in 1.0 M PBS) for 30 minutes prior to overnight primary antibody incubation in Blotto (anti-OPN MPIIB10, 1:300, Iowa Hybridoma, Iowa City, IA; anti-IBA1, 1:300, Wako Chemicals, Richmond, VA; anti-gial fibrillary acidic protein (GFAP), 1:300, Dako, Carpinteria, CA; anti-synapsin 1 a/b (N-19, sc-7379), 1:250, Santa Cruz Biotechnology, Inc., Dallas, TX; anti-MAP-1B (H-130, sc-25729), 1:200 Santa Cruz Biotechnology, Inc., Dallas, TX). The next day, tissue sections were washed with PBS, blocked in Blotto at room temperature (30 minutes), then incubated in Blotto with the desired secondary fluorescent antibody (Alexa Fluor® 488 or 594, 1:1,000, Life Technologies, Grand Island, NY) for 1 hour at room temperature. Free floating sections were then washed in PBS (3 x 5 minutes), equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield (Vector Laboratories, Burlingame, CA). IHC signal was visualized using the TCS-SP2 AOBS (Leica, Buffalo Grove, IL) or LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope.

## Quantitative Reverse Transcriptase-Polymerase Chain Reaction

At 1 or 2d after UEC, selected rats were anesthetized, decapitated, and whole hippocampi (n=10) or ML enriched fractions dissected (n=10). Total RNA was extracted from either whole hippocampal or ML enriched dissections with TRIzol® (Life Technologies, Grand Island, NY) were processed for OPN gene assessment utilizing Qc analysis and qRT-PCR with TaqMan® Assay Reagents (Life Technologies, Grand Island, NY). Probes and primer sets for detection of OPN (Rn00582114m1) were obtained from Inventoried Assays (Life Technologies, Grand Island, NY). Probes were labeled at the 5' end with 6-carboxyfluorescein and with a dark quencher at the 3' end. Efficiency was determined with 10-fold serial dilutions of template and cyclophilin A was used as an endogenous control (Pre-developed TaqMan® Assay Reagents; Life Technologies, Grand Island, NY). Experiments were performed in the ABI Prism 7500 Sequence Detection System using the TaqMan® One-Step PCR Master Mix Reagents Kit (Life Technologies, Grand Island, NY). All samples were tested in triplicate under cycling conditions (48°C/30 minutes, 95°C/10 minutes; 40 cycles 95°C/15 seconds, and 60°C/1 minute). Fold change in OPN mRNA expression was calculated by the  $2^{-\Delta\Delta Ct}$  method (Dumur et al., 2009).

## In Situ Hybridization

Sense and antisense probes were generated against full length OPN (SPP1, MRN1768-202780755) and synaptobrevin (Vamp1, MRN1768-202782932) which was used as a positive control (Thermo Scientific, Rockford, IL). Riboprobes were synthesized using digoxigenin (DIG)-tagged dNTPs (Roche, Mannheim, Germany) and the MAXIscript® in Vitro Transcription Kit (Life Technologies, Grand Island, NY) and hydrolyzed to ~500nt as previously described (Su et al., 2010). Selected 40 µm coronal sections from 1, 2, and 7d animals in the

previous IHC studies were mounted free floating in PB on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) as described above, then prepared and hybridized at 65°C as previously described (Yamagata et al., 2002). Bound riboprobes were detected by horseradish peroxidase (POD)-conjugated anti-DIG and fluorescent Cy3 staining with Tyramide Signal Amplification (TSA) systems (PerkinElmer, Shelton, CT). For fluorescent ISH colabeling with IHC, standard IHC was performed following the TSA step above. ISH/IHC signal was visualized using the TCS-SP2 AOBS (Leica, Buffalo Grove, IL) confocal microscope.

### Statistical Analyses

Changes in protein or RNA levels, due to injury or minocycline treatment, were evaluated using the General Linear Model (GLM) programs in SPSS v.11 (International Business Machines Corp., Armonk, NY). The time course of changes in OPN protein and transcript after UEC injury were assessed using a mixed model analysis of variance (ANOVA) with hemisphere (contralateral vs. ipsilateral to injury) as a within-subjects variable, and day postinjury as a between-subjects measure. The significance of changes at specific postinjury days was evaluated as a priori planned comparisons using simple-main effects based on marginal means, and implemented using multivariate ANOVA (MANOVA) routines in SPSS. In experiments which compared animals receiving a TBI+BEC injury to sham-injured animals, protein and RNA changes were analyzed using a completely randomized ANOVA design, with Duncan post-hoc tests used for pairwise comparisons. Results are reported as mean +/- SEM. An alpha level of 0.05 was used in all analyses.

## RESULTS

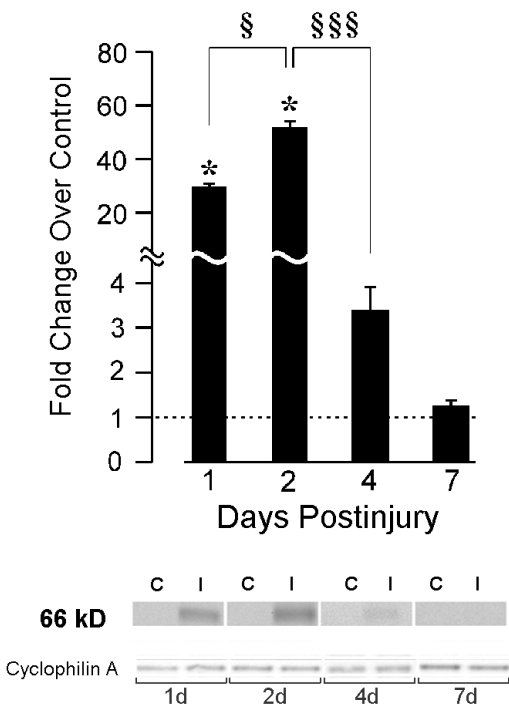
### Osteopontin Expression Is Time Dependent During UEC Reactive Synaptogenesis

We first examined the postinjury time course of OPN protein expression during UEC adaptive synaptogenesis, focusing on acute survival intervals which sample the period of initial immune response (1d) and onset of presynaptic terminal degeneration (2d). These time points were contrasted with a later pre-regenerative interval (4d) and a time point matching the onset of presynaptic terminal sprouting (7d). Using whole hippocampal extracts, we first performed routine WB analysis with polyclonal goat OPN antibody (R&D Systems) which identified full length OPN (66 kD) as the principal signal. In the control samples, OPN signal was near background, supporting low constitutive OPN expression as reported in prior studies of brain tissue (Choi et al., 2004; Iczkiewicz et al., 2004). Results showed a time-dependent increase in full length 66 kD OPN protein (**Fig. 2.4 A**), initiated by 1d ( $29.42 \pm 1.38$  fold,  $p < 0.05$ ), peaking at 2d ( $51.58 \pm 2.57$  fold,  $p < 0.05$ ), and reduced by 4d ( $3.38 \pm 0.53$  fold). At the 7d onset of sprouting, OPN protein was no longer significantly elevated over paired controls ( $2.94 \pm 0.15$  fold). In a parallel experiment, we utilized a polyclonal rabbit OPN antibody (Rockland), which, in our samples, recognized MMP-cleaved OPN fragments of 45 and 32 kD. Here we probed enriched extracts of the deafferented ML, revealing a significant acute elevation in a 45 kD fragment at 1d ( $166.21 \pm 24.05\%$ ,  $p < 0.05$ ) and an increase at 2 and 7d postinjury that did not reach significance ( $109.96 \pm 9.69$ ;  $126.29 \pm 15.27\%$ ). In addition, 32 kD OPN was elevated at 1d postinjury ( $128.75 \pm 12.37\%$ ), significantly reduced at 2d ( $86.90 \pm 3.79\%$ ,  $p < 0.05$ ), then significantly elevated at 7d ( $117.30 \pm 5.63\%$ ,  $p < 0.05$ ) (**Fig. 2.4 B**). As a structural correlate, hippocampal tissue sections were subjected to OPN fluorescent IHC at 1, 2, and 7d postinjury (**Fig. 2.5**). In the control hemisphere, we found OPN signal within granule cell bodies of the

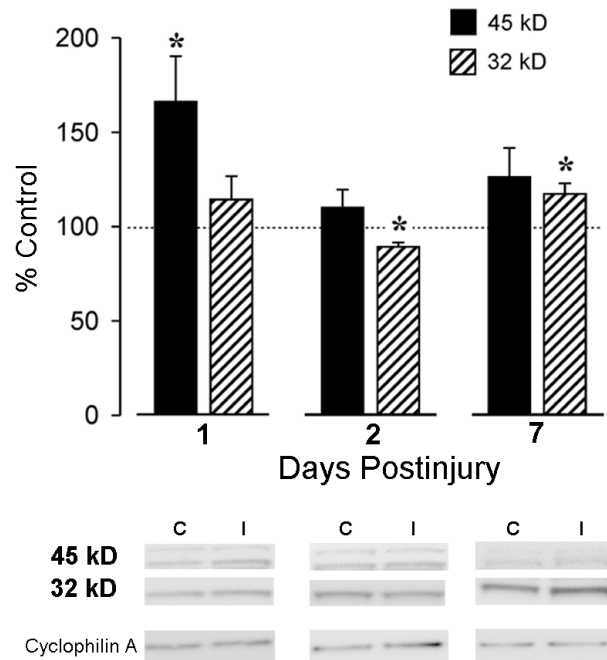


**Figure 2.4 Hippocampal or Dentate Molecular Layer OPN Protein Expression Following Adaptive UEC.** WB analysis of full length 66 kD OPN protein in whole hippocampal extracts (A). UEC induced an acute robust increase in OPN protein, peaking at 2d postinjury compared to contralateral controls. OPN response then showed a significant reduction at 4d and a return to baseline by 7d. Using antibody raised against peptide sequences exposed by MMP proteolysis (B), two principal OPN fragments (45, 32 kD) were identified in extracts of enriched dentate ML. The 45 kD fragment, containing integrin receptor binding RGD sequence, was significantly increased compared to controls 1d postinjury, but not at 2 or 7d. By contrast, the 32 kD C terminal fragment did not change in level at 1d, but was modestly reduced at 2d, with a shift to elevation over controls at 7d following UEC. Results are displayed as fold change over control (A) or percent of control (B), with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$ , relative to paired control cases; § $p < 0.05$ ; §§§ $p < 0.001$ .

**A** Full Length OPN  
UEC Hippocampus

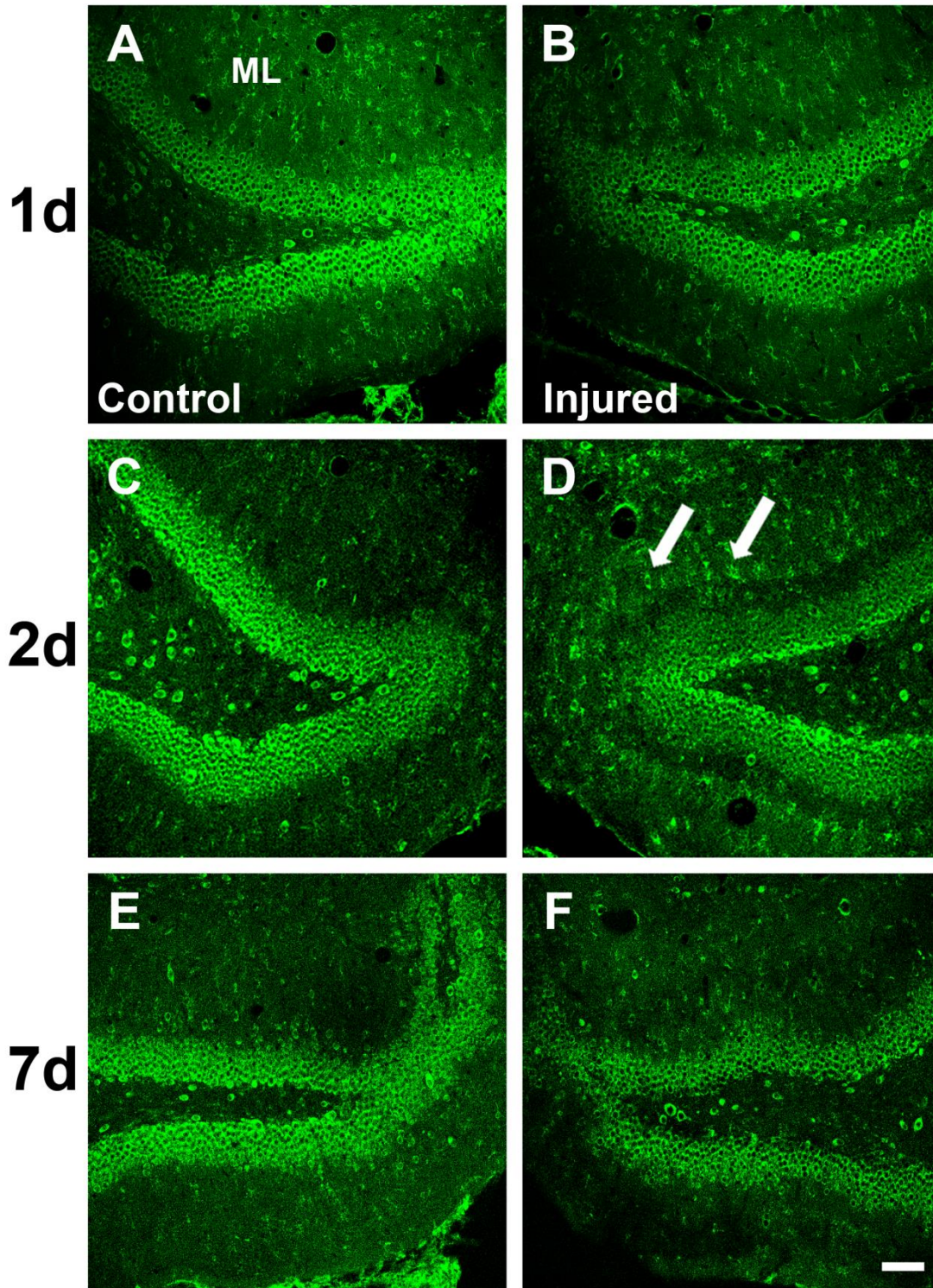


**B** OPN Lytic Fragments  
UEC Molecular Layer



**Figure 2.5 IHC of OPN in the Dentate Gyrus.** Confocal OPN staining in the granule cell layer was observed in all cases, and remained qualitatively unchanged at 1, 2, and 7d postinjury (A-F). Following UEC, OPN increased in the ML between 1 and 2d postinjury returning to baseline by 7d. The 2d OPN signal was pronounced in cell bodies within the deafferented zone (arrows). Scale bar = 75  $\mu$ m.

# UEC



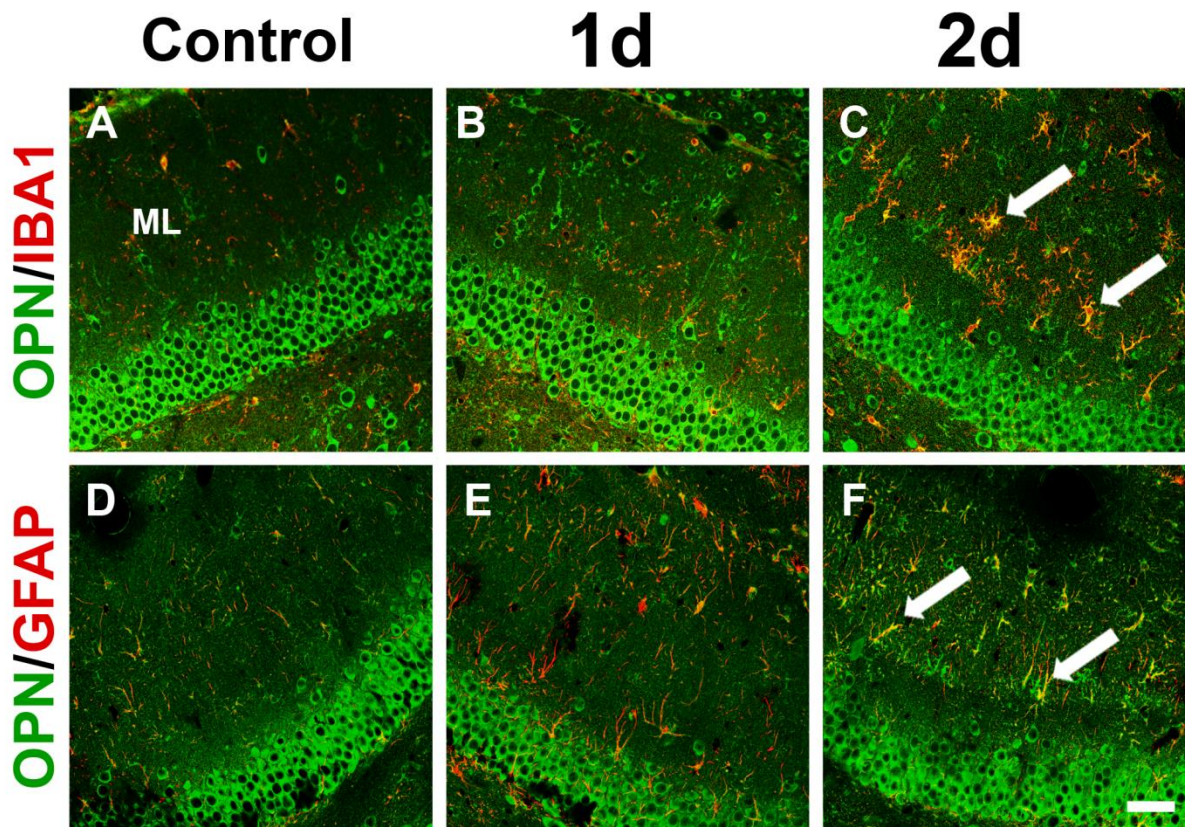
dentate gyrus, with a diffuse, low level label over the dentate ML. At 1d following UEC, we observed no visible differences in OPN staining between injured and control hippocampi (**Fig. 2.5 A-B**), however at 2d there was a prominent increase over the deafferented ML, including both fine punctate label and cellular localization consistent with reactive glia (**Fig. 2.5 C-D**). By 7d postinjury, no qualitative differences were observed between lesioned and control hippocampi (**Fig 2.5 E-F**). OPN signal within granule cell body layers did not change at any postinjury interval. This acute increase in full length OPN 1-2d after UEC is in agreement with reported elevation for other models of traumatic CNS insult (Ellison et al., 1998; Hashimoto et al., 2003; Shin et al., 2005), each reporting onset of OPN elevation within 24 hours after injury. The present results suggest that OPN is part of the acute immune response after deafferentation, and support a potential role for the protein in reactive synaptogenesis. Focal elevation of OPN fragments over regions of synaptic plasticity is consistent with the fact that MMP-3, a matrix enzyme which cleaves OPN to expose integrin binding epitopes, shows similar spatial and temporal increase after UEC (Falo et al., 2006).

#### Osteopontin Is Expressed in Reactive Glial Populations During Synaptogenesis

To determine the identity of OPN labeled cells within the deafferented molecular layer, we performed double label colocalization OPN immunofluorescence experiments using antibodies specific for microglia (IBA1) and astrocytes (GFAP). We found that at 1-2d after UEC, increased OPN staining in the deafferented zone was associated with both reactive microglia (**Fig. 2.6 B, C**) and astroglia (**Fig. 2.6 E, F**), compared to low level OPN signal in paired controls (**Fig 2.6 A, D**). ML OPN increased between 1 and 2d postinjury, where microglia exhibited greater labeling. A subset of reactive microglia migrated to positions

**Figure 2.6 IHC of OPN and Glial Cells in the Dentate Gyrus Following UEC.** Confocal OPN (green) staining increased over the ML after UEC (B, C, E, F) compared to contralateral control hemispheres (A, D). Injury-induced staining was colocalized with activated microglia (red; B, C) and reactive astrocytes (red; E, F), each of which showed cellular alignment at the inner molecular layer (IML) border with the deafferented zone (arrows). Scale bar = 50  $\mu$ m.

# UEC



along the boundary between the outer deafferented dendritic region and the inner non-deafferented ML, marking the interface between intact and damaged synapses. Such alignment was not striking for OPN positive astrocytes. These results suggest that acute OPN increase during deafferentation-induced synaptogenesis is principally glial in nature, and that the potential immune-initiated role of OPN may be mediated through reactive microglia. Similarly, OPN localization within reactive glia was reported for models of CNS cortical injury (Shin et al., 2005; von Gertten et al., 2005; Plantman, 2012), and following both stroke and ischemia (Ellison et al., 1998; Choi et al., 2007). Prior UEC studies show that activated microglia position themselves along dendritic boundaries, potentially interacting with matrix proteins like agrin to direct synaptic reconstruction (Falo et al., 2008), and that reactive astroglia express MMP-3, providing a glial pathway for generating OPN integrin signals (Falo et al., 2006).

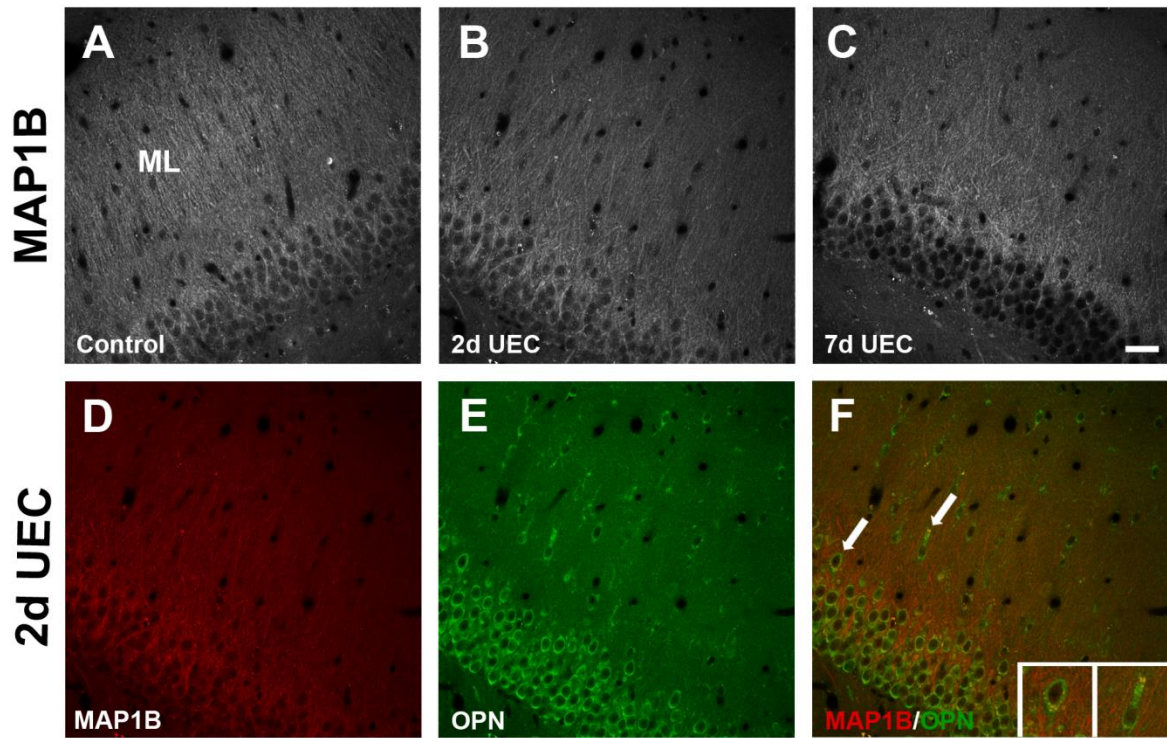
#### Osteopontin Does Not Colocalize with Cytoskeletal Proteins

In a second experiment, we explored the relationship between OPN and the cytoskeletal protein MAP1B during UEC reactive synaptogenesis. Recent reports suggest that intracellular OPN may stabilize cell cytoskeleton through a direct binding with MAP1B (Long et al., 2012). We reasoned that the structural reshaping of dentate postsynaptic dendrites induced by synaptogenesis may involve changes in neuronal OPN/MAP1B distribution. Again, using double label immunofluorescence, we probed the dentate ML for OPN and MAP1B at 2 and 7d after UEC. As expected, we observed a high concentration of MAP1B in primary granule cell dendrites, and along branched dendritic processes of control ML (**Fig. 2.7 A**), consistent with microtubule cytoarchitecture. A progressive aggregation of signal along proximal dendrites and within granule cell bodies was seen at 2d, then a reemergence of signal into the neuropil at 7d



**Figure 2.7 IHC of MAP1B in the Dentate Gyrus.** Confocal MAP1B staining in the injured ML decreased at 2d after lesion (B) relative to control (A), correlated with the retraction of injured dendrites during the degenerative phase. A reemergence of MAP1B staining was observed with the onset of synaptic regeneration at 7d postinjury (C). Confocal overlay of MAP1B (red, D) and OPN (green, E) revealed minimal colocalization (arrows) during both phases of synaptic reorganization (illustrated for 2d in F, with cells enlarged in insets). Scale bar = 30  $\mu$ m; inset 80X.

# UEC



after UEC (**Fig. 2.7 B, C**), consistent with synapse degeneration and dendritic retraction, followed by extension of dendrites at the onset of synapse regeneration, a process well-defined in the UEC model (Steward, 1976; Steward et al., 1988b). Surprisingly, there was little evidence of OPN/MAP1B colocalization in granule cell dendrites, however, isolated cells within the deafferented ML did show cytoplasmic colocalization of the two proteins (arrows, **Fig. 2.7 F**). These results suggest that dendritic interaction between OPN and MAP1B is not a major component of the granule cell reorganization with UEC deafferentation. Cellular OPN/MAP1B colocalization in the ML is consistent with cytoskeletal associated OPN within reactive glia in the deafferented zone.

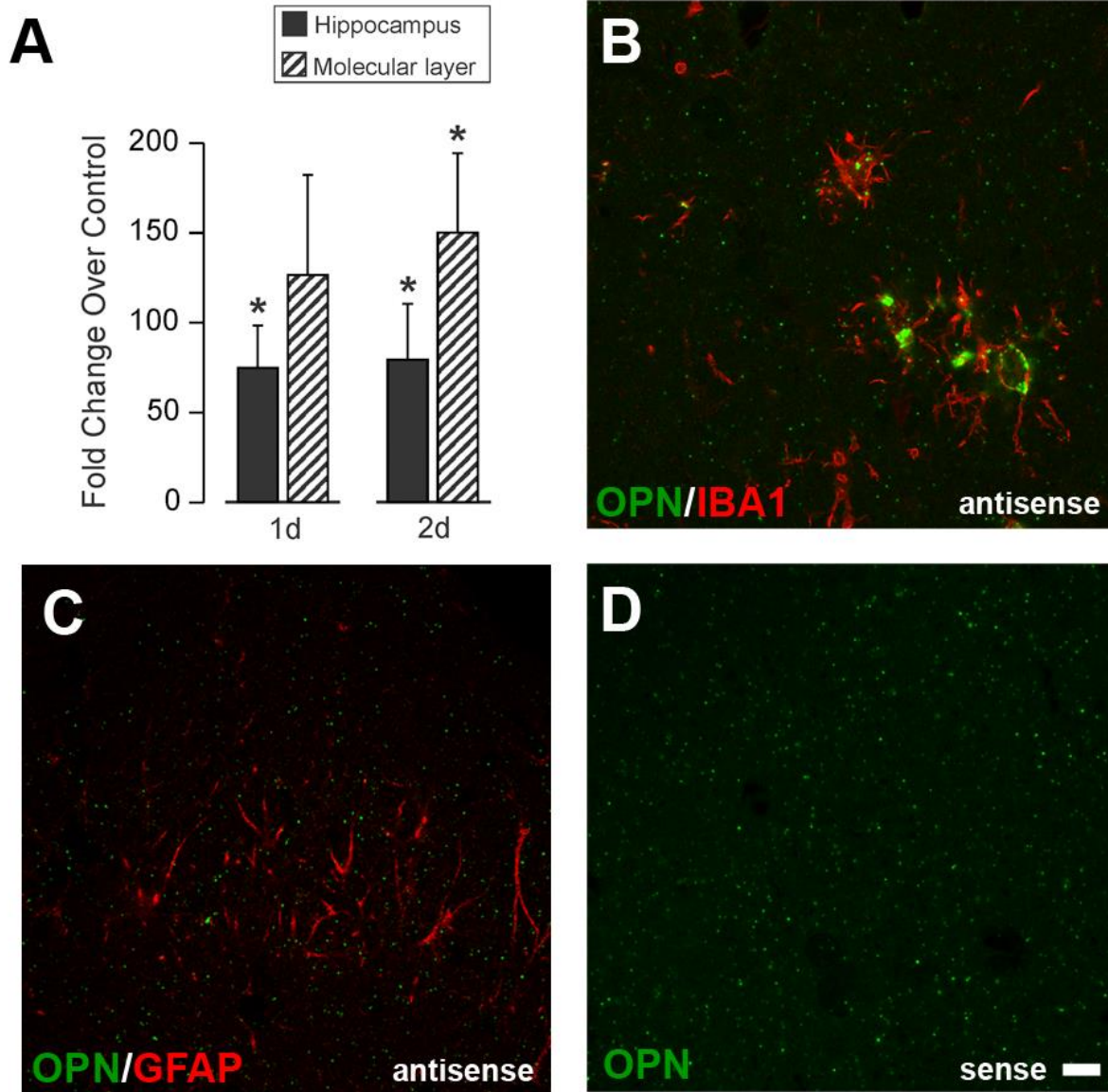
#### Elevated Osteopontin Transcript in Sites of Synaptogenesis Is Localized to Microglia

Given that OPN protein showed significant elevation during the acute postinjury phase of reactive synaptogenesis, and that this elevation was largely cellular in the ML, we applied qRT-PCR and *in situ* hybridization (ISH) methods to assay whether OPN transcription was involved. As noted above, preliminary microarray screening showed an increase in hippocampal OPN mRNA by 14-fold with UEC, and 41-fold after TBI+BEC, while the deafferented ML showed 21-fold and 6-fold elevations, respectively (see **Appendix A**). To examine OPN transcript expression, we performed qRT-PCR on RNA extracts from either whole hippocampi or enriched in ML dissections at 1 and 2d after UEC lesion. Results showed multifold elevation of OPN mRNA in both whole hippocampus and enriched dentate ML relative to paired control extracts (**Fig. 2.8 A**). Significant injury-induced elevation of hippocampal OPN transcript was seen at each time point ( $74.96 \pm 23.33$  fold for 1d;  $79.25 \pm 31.08$  fold for 2d;  $p < 0.05$ ). ML extracts showed higher mean fold changes in OPN transcript than controls ( $126.55 \pm 55.72$  fold for 1d;

**Figure 2.8 OPN Transcript Expression and Microglial Localization Following UEC.**

OPN mRNA was measured by qRT-PCR using RNA extracts prepared from whole hippocampus and enriched ML samples (A). Significant fold elevation in hippocampal OPN transcript relative to paired contralateral controls was observed at both 1 and 2d following UEC. ML samples showed greater variance in OPN transcript, but did trend toward higher levels than in the whole hippocampus. Interestingly, 2d enriched ML had the highest fold increase and was significantly elevated over paired controls. Using DIG-tagged riboprobes for OPN, antisense probe binding and confocal fluorescent detection (green) showed transcript localization within reactive cells of the deafferented dentate ML, identified as activated microglia (red) after post hybridization labeling with IBA1 antibody (B). The same protocol conducted with GFAP antibody staining showed no colocalization of OPN transcript in reactive astrocytes (C). Hybridization signal was validated by the absence of fluorescent signal when control sense strand was applied (D). Results are displayed as fold change over control. \* $p < 0.05$ , relative to paired control cases. Scale bar = 10  $\mu\text{m}$ .

# OPN Transcript



150.10 ± 44.16 fold for 2d, p<0.05), however, only the 2d differences reached statistical significance. When 2d postinjury UEC cases were subjected to ISH with digoxigenin (DIG)-tagged riboprobes for OPN transcript (Su et al., 2010), antisense strand binding was localized within reactive microglia of the deafferented zone, identified by post-hybridization staining for IBA1 (**Fig. 2.8 B**). Parallel hybridized sections were probed with anti-GFAP antibody and no mRNA signal was observed in reactive astrocytes of the ML (**Fig. 2.8 C**). Sense control binding was negative, confirming the specificity of the signal (**Fig. 2.8 D**). These results suggest that reactive microglia are a principal source of OPN during the acute phases of reactive synaptogenesis.

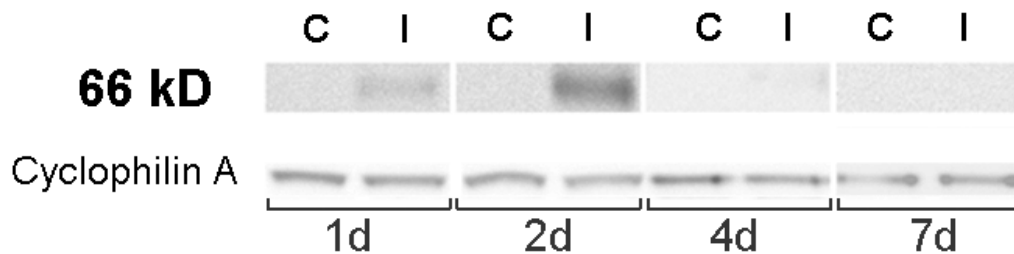
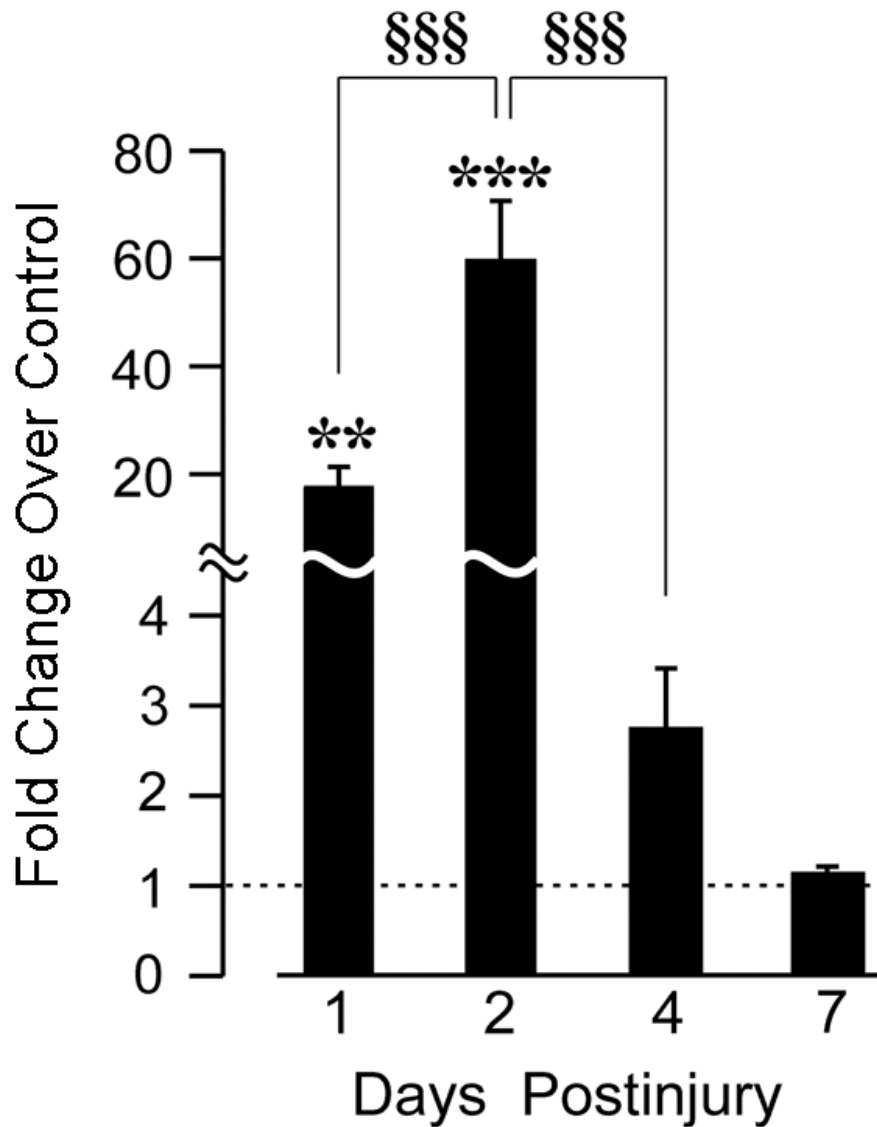
#### Maladaptive Plasticity Alters Distribution, but Not Elevation of Osteopontin

In prior studies we have contrasted UEC adaptive synaptogenesis with the maladaptive synaptic plasticity of combined TBI+BEC insult to explore how the response of molecules controlling synaptic repair differ under maladaptive conditions (Phillips et al., 1994; Falo et al., 2006; Falo et al., 2008; Warren et al., 2012). As for UEC, we profiled the expression of full length 66 kD OPN protein in TBI+BEC hippocampal extracts. Interestingly, we found that the time-dependent OPN elevation was similar to the UEC (**Fig. 2.9**). OPN was elevated by 1d (17.25 ± 3.96 fold, p<0.01), peaking at 2d (59.40 ± 11.12 fold, p<0.001), and significantly attenuated (p<0.001) by 4d (27.32 ± 6.72 fold). As with UEC, 7d OPN protein was no longer different from paired controls (15.30 ± 3.85 fold). These findings suggest that acute OPN expression during reactive synaptogenesis is associated with a broad, 1-4d immune response, which is similar in both models. This pattern is in contrast to the clear differences in expression

**Figure 2.9 Hippocampal OPN Protein Expression Following Maladaptive TBI+BEC.** WB analysis of full length 66 kD OPN protein in whole hippocampal extracts. TBI+BEC induced an acute robust increase in OPN protein, peaking at 2d postinjury compared to sham-injured controls. OPN response then showed a significant reduction at 4d and a return to baseline by 7d similar to that of UEC animals. Results are displayed as fold change over control with representative blot images and cyclophilin A loading controls shown below. \*\*p<0.01, \*\*\*p<0.001, relative to paired control cases; §§§p<0.001.

# Full Length OPN

TBI+BEC Hippocampus





between adaptive and maladaptive plasticity observed for MMPs (Falo et al., 2006; Warren et al., 2012), and their matrix substrates (Falo et al., 2008; Warren et al., 2012). As for UEC, we applied double label OPN immunofluorescence staining in maladaptive TBI+BEC, using IBA1 and GFAP antibodies to identify OPN labeled microglia and astrocytes within the deafferented zone. Consistent with UEC, we observed an increase in reactive microglia and astrocytes over the deafferented zone at 1 and 2d after injury relative to sham controls (**Fig. 2.10**). Interestingly, there was less OPN localized to microglia, and microglia were more randomly distributed within the 2d ML, showing poor alignment at the inner/outer dendritic layer boundary (**Fig. 2.10 C**). This reduced microglial alignment is consistent with the loss of agrin matrix boundary in the TBI+BEC model (Falo et al., 2008). OPN positive astrocytes, however, were more evident at 2d after the maladaptive insult, extending their processes throughout the breadth of molecular layer dendrites (**Fig. 2.10 F**). In contrast to UEC, astroglia were highly reactive and a principal OPN containing cell of the deafferented zone. Together, these findings support a successful immune-driven OPN response in the hippocampus under maladaptive conditions, but one which is marked by an altered cellular expression and tissue distribution.

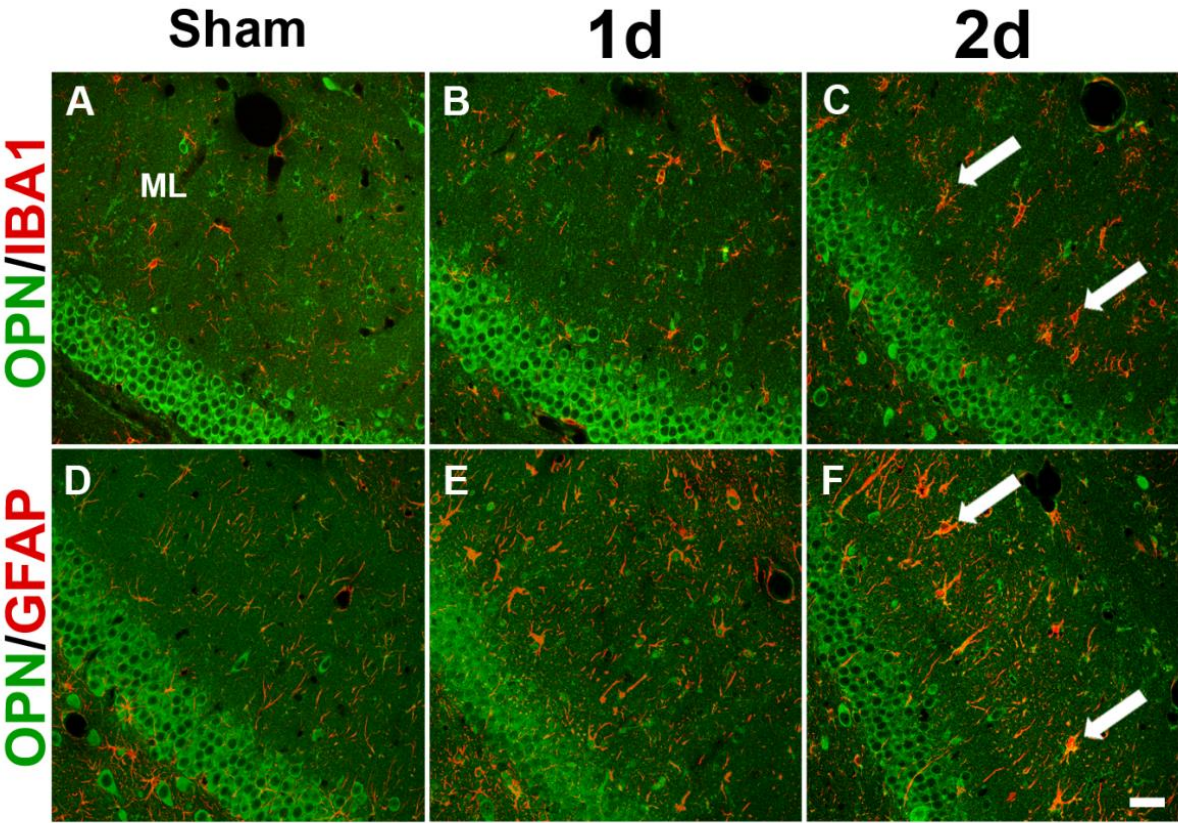
#### Immunosuppression Alters OPN Response and Reactive Synaptogenesis

Collectively, the present data support OPN role in the acute inflammatory response following deafferentation, likely through microglial activation to orchestrate the early stages of synaptic reorganization and reinnervation. Since OPN is highly expressed under conditions of inflammation (Patarca et al., 1989; Hwang et al., 1994; Weber and Cantor, 1996; Chabas et al., 2001), facilitating the migration of macrophages and microglia to sites of injury (Ellison et al., 1998; Shin et al., 2011; Hashimoto et al., 2003), the manipulation of inflammation should affect

**Figure 2.10 IHC of OPN and Glial Cells in the Dentate Gyrus Following TBI+BEC.**

Confocal OPN (green) staining increased over the ML after TBI+BEC (B, C, E, F) compared to sham controls (A, D). As for UEC, OPN was colocalized within activated microglia (arrows; B, C) and reactive astrocytes (arrows; E, F) of the deafferented zone. Scale bar = 50  $\mu\text{m}$ .

# TBI+BEC



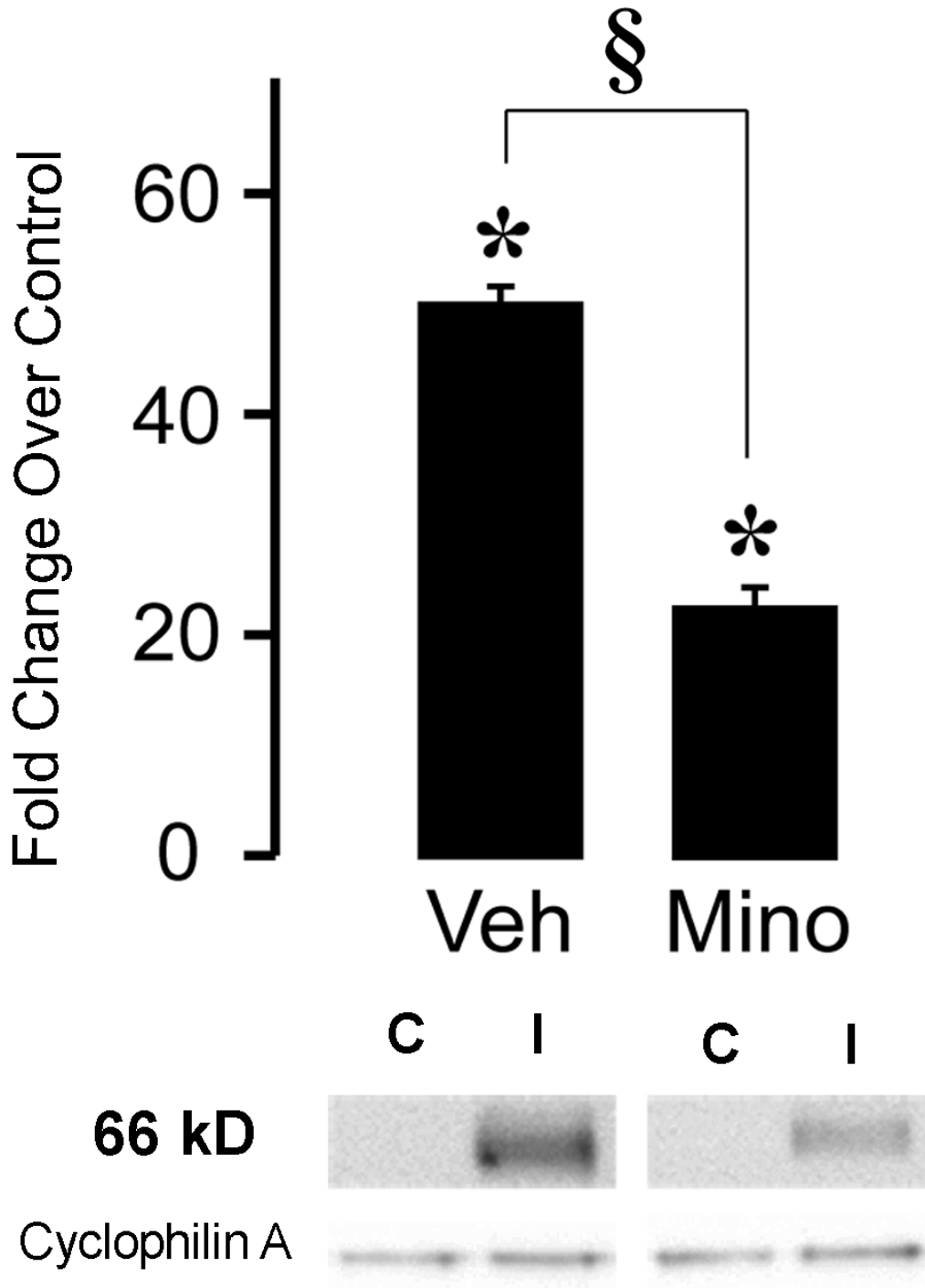
synaptogenesis, which recruits these processes during its initial phases. To test this possibility, we used the tricyclic antibiotic minocycline (delivered acutely at 30 minutes and 6 hours post-lesion) to attenuate inflammatory response, as well as reduce microglial activation (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999). We then examined the evolution of the early phases of UEC-induced synaptogenesis. OPN protein expression was measured in hippocampal extracts by WB, which showed that injury-induced elevation of OPN ( $48.64 \pm 2.24$  fold,  $p < 0.05$ ) was significantly reduced by 53% ( $p < 0.05$ ) following minocycline administration ( $22.64 \pm 1.92$  fold,  $p < 0.05$ ) when compared with vehicle treated cases (**Fig. 2.11**). When double OPN immunofluorescence was performed with IBA1 antibody (**Fig. 2.12 A-D**), we found that OPN positive reactive microglia were more randomly distributed after minocycline (**Fig. 2.12 B, D**). Notably, minocycline-induced alteration in OPN and glial response was correlated with altered presynaptic terminal loss during the early phases of reactive synaptogenesis (boxed area, **Fig. 2.12 F, H**). By 2d after UEC, vehicle treated animals show predicted loss of presynaptic marker synapsin 1 over the deafferented dentate ML (**Fig. 2.12 F**) (Lund and Lund, 1971; Greif and Trenchard, 1988), however, animals with minocycline reduction of OPN showed altered distribution of synapsin 1 positive presynaptic terminals over the deafferented ML (**Fig. 2.12 H**). Further, reduced OPN was also associated with altered synapsin 1 distribution at the inner/outer ML boundary (**Fig. 2.12 F, H**). After minocycline, the presynaptic terminal marker is no longer distinct at this boundary, but rather shows a diffuse, irregular pattern, extending into the proximal deafferented neuropil. This suggests that, even with partial immune inhibition of OPN, pre-synaptic terminal and microglial response to deafferentation is altered, attenuating critical changes during the initial degenerative phases of reactive synaptogenesis.

**Figure 2.11 Minocycline Attenuates Hippocampal UEC-Induced OPN Response.**

Minocycline was administered at 30 minutes and 6 hours postinjury (45 mg/kg, i.p.), a dose previously demonstrated to attenuate inflammatory response, as well as reduce MMP-9 activity. At 2d postinjury, full length 66 kD OPN response was significantly reduced by over 50% with acute administration of minocycline compared to saline-treated controls. Results are displayed as fold change over control with representative blot images and cyclophilin A loading controls shown below. \*p<0.05 relative to control cases; §p<0.05.

# Full Length OPN

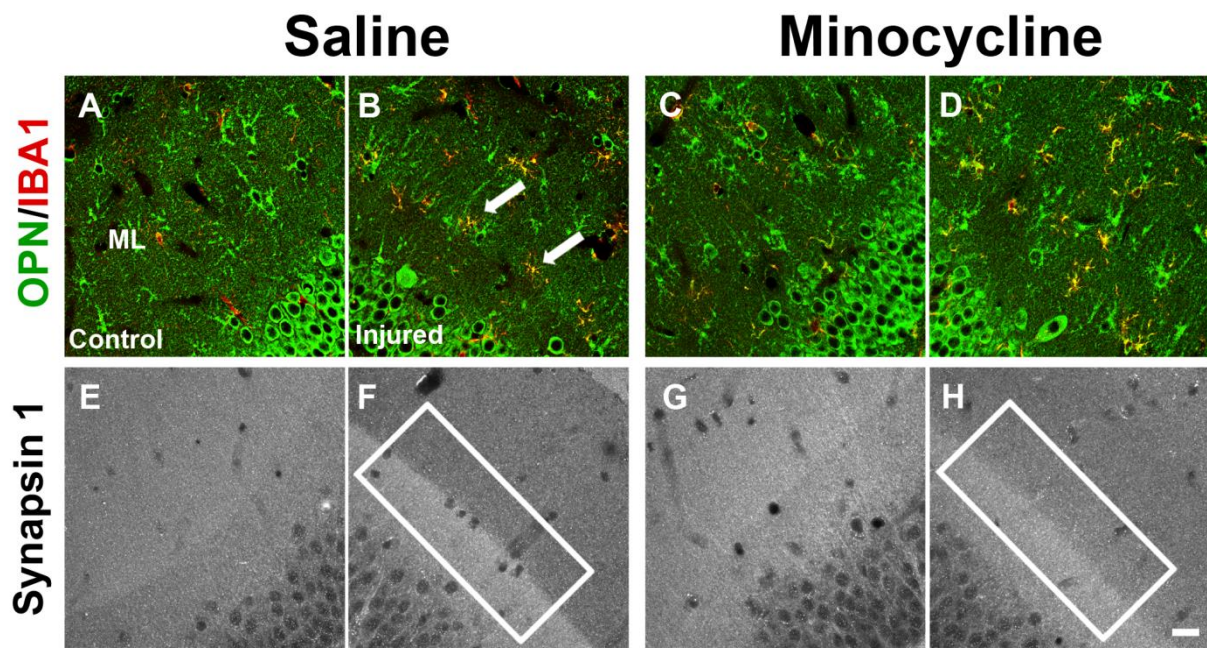
2d UEC Hippocampus



**Figure 2.12 Minocycline Alters Migration of OPN Positive Microglia and Pattern of Presynaptic Terminal Removal in Deafferented Molecular Layer Following UEC.**

Confocal imaging of minocycline-treated animals 2d after UEC lesion (A) showed OPN (green) colocalized within microglia (red), however, these glia appeared randomly distributed in the ML and did not position themselves along the distinct border between the IML and outer deafferented zone, as was observed for injured saline control cases (arrows, B). During the 2d degenerative phase, removal of presynaptic terminals is marked by loss of synapsin 1 staining (F), with a crisp boundary between the intact synapses of the IML and the deafferented zone was disrupted and diffuse, indicating a reduced presynaptic terminal clearance compared to injured saline treated controls (boxed area; F, H). Together, these observations suggest that manipulation of OPN through minocycline immunosuppression disrupts microglial migration to establish tissue boundaries for removal of degenerating terminals in the early phases of reactive synaptogenesis. Scale bar = 20  $\mu$ m.

# 2d UEC





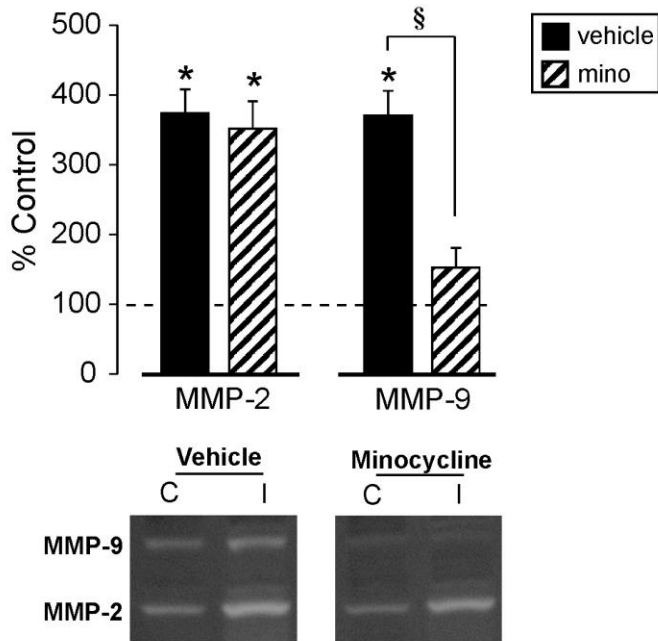
To assess the potential for generation of OPN functional fragments during the acute recovery period of degeneration, we also utilized gelatin zymography and WB analysis to evaluate MMP activity and the presence of OPN fragments. Consistent with previous studies documenting increased MMP activity during the degenerative phase of debris clearance (Kim et al., 2005; Falo et al., 2006), both MMP-2 and MMP-9 activity was significantly elevated within the hippocampus of saline-treated animals at 2d following UEC ( $374.14 \pm 34.05\%$ ;  $370.43 \pm 35.45\%$ ;  $p < 0.05$ ) (**Fig. 2.13 A**). Acute administration of minocycline did not alter MMP-2 activity ( $351.82 \pm 39.46\%$ ), but significantly reduced MMP-9 injury-induced activity by 41.18% ( $p < 0.05$ ) compared saline-treated controls at 2d ( $152.54 \pm 28.11$ ), a finding consistent with previous literature documenting minocycline inhibition of MMPs in other models of CNS trauma and stroke (Machado et al., 2006; Cayabyab et al., 2013). Additional studies evaluating changes in MMP-generated OPN fragments revealed an injury-induced increase in the 45 kD fragment at 2d after UEC in saline-treated animals ( $152.88 \pm 28.27\%$ ,  $p < 0.05$ ), and reduction in the 32 kD fragment ( $90.29 \pm 1.19\%$ ,  $p < 0.05$ ). Similar to minocycline-reduction in MMP-9 activity, the injury-induced expression of the 45 kD form of OPN was significantly reduced by 62% ( $p < 0.05$ ) 2d after UEC ( $57.68 \pm 19.79$ ) (**Fig. 2.13 B**). Together, these results suggest OPN and MMP act in concert during the acute immune response to signal cellular removal of debris and degenerating terminals in the early stages of reactive synaptogenesis, potentially contributing to the preparation of the ECM for synaptic sprouting during the regenerative phase.

## DISCUSSION

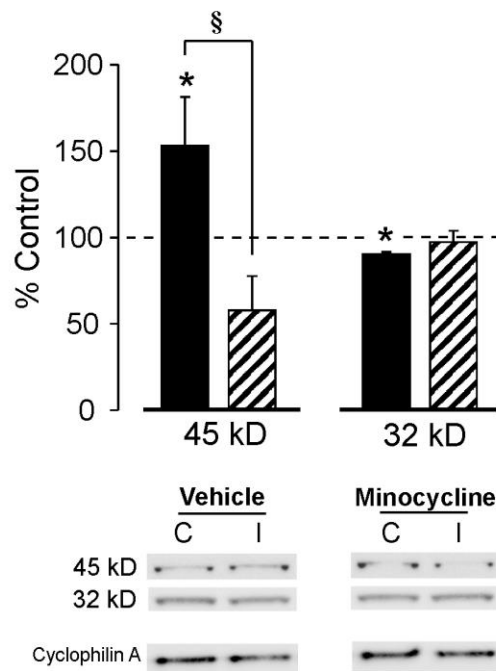
In this study, the temporal and spatial pattern of OPN response was examined in two models of synaptic deafferentation. Following adaptive UEC or maladaptive TBI+BEC insult,

**Figure 2.13 Minocycline Immunosuppression Attenuates Hippocampal MMP-9 Activity and OPN Proteolytic Fragment Generation Following UEC.** At 2d postinjury, gelatin zymography shows both MMP-2 and MMP-9 activity elevated in hippocampal extracts of the vehicle treated cases when compared with contralateral controls (A). Minocycline treatment selectively reduced MMP-9 proteolytic activity (A), which was correlated with a significant decrease in the generation of OPN 45 kD integrin receptor-binding fragment (B). By contrast, minocycline did not affect injury induced changes in either MMP-2 activity or 32 kD OPN fragment expression. Results are expressed as percent of control, with representative gel or blot images and cyclophilin A loading control shown below. \* $p < 0.05$  relative to paired contralateral controls; § $p < 0.001$ .

### A Enzyme Activity 2d UEC Hippocampus



### B OPN Lytic Fragments 2d UEC Hippocampus



hippocampal OPN protein levels were quantified and localized to cellular subtypes within the injured dentate. Traumatic injury induced a robust OPN increase compared to controls during the acute degenerative phase of reactive synaptogenesis. This significant OPN elevation occurred during early terminal degeneration (1-2d postinjury) before returning baseline during axonal sprouting and synaptic regeneration (7d postinjury). IHC staining for OPN showed granule cell staining which remained unchanged after injury as opposed to ML dendritic staining which increased following synaptic deafferentation. Microtubule associated protein MAP1B was not colocalized with OPN during axonal sprouting at 7d postinjury, however, injury-induced OPN was colocalized within both activated microglia and reactive astrocytes of the injured dentate. Interestingly, groups of activated microglia organized themselves along the dendrites at the border of deafferentation and contained OPN mRNA, indicating microglia act as synthesizers of OPN. Studies contrasting adaptive UEC and maladaptive TBI+BEC showed that level of OPN expression was similar in the two conditions, however, IHC revealed differences in OPN and glial cell distribution. Animals subjected to the combined injury paradigm showed altered glial distribution, which appeared in a random pattern within the injured ML, suggesting impaired migration of reactive glial cells toward the deafferented dendritic layer compared to the effective alignment during UEC synaptic reorganization. The presence of reactive immune cells in the injured ML is consistent with previous findings of elevated local MMP activity, and the present findings indicating an increase in a MMP-cleaved 45 kD OPN fragment at the site of deafferentation. Finally, attenuation of acute UEC immune response with minocycline dampened injury-induced OPN expression compared to controls, and altered reactive glial cell distribution generating a pattern similar to that of TBI+BEC. To further evaluate the effect of an attenuated immune response during synaptic reorganization, we probed for synapsin 1 expression

as a marker of the presynaptic terminals affected by deafferentation. During the acute inflammatory response, animals treated with minocycline exhibited persistent synapsin 1 staining in the injured dentate, suggesting impaired removal of injured presynaptic terminals.

Together, these results suggest that OPN is highly elevated over the acute postinjury period and plays an important role during the degenerative phase of reactive synaptogenesis. It has the potential to induce chemotaxis through its cytokine properties, and promote migration of reactive glia to select sites within the injured dentate, influencing phagocytosis of degenerative presynaptic terminals and the reshaping of postsynaptic spines in preparation for regrowth. Further, as the comparison of adaptive and maladaptive plasticity models shows, proper execution of terminal removal and spine morphing likely promotes correct ECM conditioning in preparation for the successful synapse regeneration.

#### Robust Osteopontin Expression Is Time-Dependent During Reactive Synaptogenesis

Our evaluation of OPN expression and distribution following hippocampal deafferentation clearly points to a time dependent relationship with the process of reactive synaptogenesis. This was an effect observed for both the UEC and TBI+BEC cases. Following deafferentation, full length hippocampal OPN protein increase was very robust, reaching as high as 80-fold over contralateral controls. This significant OPN elevation occurred as early as 24 hours postinjury, during the rapid, acute inflammatory response, before peaking within the degenerative phase at 2d postinjury. Mapping of OPN mRNA levels in hippocampal and ML extracts showed that this rise was associated with transcriptional change in OPN gene expression. Interestingly, other published studies also demonstrate a similarly robust change in OPN mRNA following traumatic insult (Morita et al., 2008; Iwanaga et al., 2008; van Velthoven

et al., 2011). The fact that OPN increase subsided by 4d, and was no longer seen at 7d, when the recovery paradigm shifts to regeneration, points to a precise inflammation-related role for OPN during the acute degenerative phase of synaptic reorganization. This pattern is consistent with prior studies of inflammatory response following TBI. For example, previous reports document rapid postinjury increases in inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  with human TBI (Helmy et al., 2011), as well as several experimental models of TBI, including impact acceleration (Shohami et al., 1994), fluid percussion injury (Vitarbo et al., 2004), controlled cortical impact (Kelso et al., 2011), and moderate blast-induced neurotrauma (Dalle Lucca et al., 2012). By contrast, the study of specific OPN response in CNS insult is limited. Nevertheless, the association of OPN signaling with immediate postinjury response is verified in several models. Acute elevation similar to that of the 24 hour period in our study was reported for models of stroke (Wang et al., 1998), ischemia (Lee et al., 1999), and SCI (Hashimoto et al., 2003; Moon et al., 2004). More recently traumatic insult models of focal TBI with hemorrhage and contusion have been examined, pointing to a rapid OPN response. These models include controlled cortical impact (von Gertten et al., 2005; Israelsson et al., 2006), cold probe cryolesion (Shin et al., 2005), and brain stab wound (Plantman, 2012). Importantly, no studies describe the acute OPN response in models of targeted synaptic deafferentation, a pathological condition present in both diffuse and focal types of TBI. Thus, the novel approach taken in the present study is valuable in that it shows an equally critical role for OPN during the specific stages of circuit reorganization following CNS insult.

## Osteopontin Role During Acute Postinjury Response Is Mediated Through Neuroglia

The fact that OPN response was greatest during the acute degenerative phase of reactive synaptogenesis suggests that its principal role may be to act as a cell surface signal for reactive neuroglia, promoting removal of degenerating presynaptic terminals and supporting subsequent synapse regeneration. Our IHC experiments mapping OPN distribution in the deafferented hippocampus showed that the molecule was present in both granule neurons and neuroglia. Importantly, deafferentation failed to notably alter the high level OPN found in neuronal cell bodies, but clearly increased OPN labeling over the deafferented ML, supporting a targeted glial interaction at the site of plasticity. Several of our findings corroborate this interaction. First, OPN was colocalized within reactive microglia and astrocytes of the deafferented zone during the 2d acute degenerative phase for UEC and TBI+BEC, returning to control levels by 7d postinjury. This reflects a similar time course as was seen with OPN expression in tissue extracts. In addition, reactive microglia, but not astroglia, express OPN mRNA at 2d after UEC, suggesting the microglia act as the principal source for subsequent signaling steps. Interestingly, OPN mRNA was similarly associated with reactive microglia at 24 hours after stroke induction, but did appear within reactive astroglia as late as 15d after the insult (Ellison et al., 1998). This result suggests that if we were to probe for OPN transcript at later time points near 15d, we may discover a second, astroglial localization of OPN mRNA and protein elevation. Unpublished IHC studies in our laboratory do indeed show evidence of elevated astroglial OPN and its targeted localization to the cell bodies of astrocytes at 15d after UEC (**Appendix E**). Future studies will more thoroughly address the possibility that these patterns represent the proposed microglial/astroglial signaling pathway. Finally, there is a significant acute elevation of the OPN cleavage fragment containing exposed integrin receptor binding sites within the deafferented

ML, providing a mechanism for local cell signaling to recruit phagocytic cells. These IHC observations raise questions as to how OPN might influence the glial response. At least three possibilities are suggested by our results: intracellular control of neuronal and glial cytoarchitecture to facilitate cell shape changes critical to plasticity, extracellular direction of glial migration and control of acute phagocytosis, and extracellular signaling between reactive glial types to coordinate the shift between degenerative and regenerative phases.

Given that recent studies demonstrate a cytosolic form of OPN which can bind to microtubule associated proteins MAP1A and MAP1B (Long et al., 2012), we reasoned that, if OPN played a role in cytoskeletal stabilization-destabilization during synaptogenesis, changes in the IHC distribution of OPN and MAP1B might map together. While we found the predicted pattern of reduced, then reemerging MAP1B dendritic staining between 2-7d postinjury, OPN colocalization with MAP1B was minimal at either postinjury time point, suggesting its role in postsynaptic dendritic retraction and spine reformation is limited. Thus, the principal role for the large elevation of OPN during acute postinjury intervals does not appear to involve the cytoarchitectural reorganization component of synaptic regeneration.

A second, more likely scenario for OPN action would be its mediation of neuroglial recruitment within the zone of deafferentation. The first phase of synaptic reorganization is the removal of degenerating severed terminals, a process known to occur by glial phagocytosis (Steward, 1989). Clearly, activated microglia can serve this role. However, previous studies also support the dual role of active microglia and reactive astrocytes to carry out the removal of degenerating terminals (Pellerin and Magistretti, 2004; Belanger and Magistretti, 2009). Absence of this process can lead to a persistent degenerative state, preventing proper axonal sprouting and subsequent neuronal and functional recovery from TBI (Sofroniew, 2005; Laird et



al., 2008; Belanger and Magistretti, 2009). In the adaptive UEC model, these reactive microglia also align themselves along the affected dendrites at the boundary between intact and deafferented zones. This pattern is consistent with the deposition of ECM proteins like agrin along this boundary to serve as border forming molecules delineating zones of repair (Falo et al., 2008). Local OPN signaling can also be chemotactic, possibly through its extracellular lysis and release of an integrin receptor binding fragment, which can then target activated microglia, driving their movement to the intact/degenerating boundary. Notably, we found both reduced glial OPN expression, and a disruption of this microglial boundary alignment with the maladaptive TBI+BEC insult. This suggests that reduced OPN expression and chemotactic stimulus in the ML under maladaptive conditions may contribute to impaired debris clearance and attenuated recovery.

A third mechanism of action for OPN could be its role as a microglia-secreted astrokine, activating pathways which alter the form, distribution, and synthetic processes of astrocytes (Ellison et al., 1999). During reactive synaptogenesis, endogenous astrocytes shape the ECM and local synaptic environment, including the regulation of growth factor signaling to guide emerging axonal sprouts and postsynaptic spines. Thus, these cells have a prominent role in facilitating the regenerative phase of synaptogenesis. We found OPN to be preferentially produced in the reactive microglia early after UEC, with only modest levels present in reactive astrocytes at the acute degenerative stage. Upon microglial secretion and lysis, OPN signaling fragments could diffuse short distances and bind to neighboring astrocytes at critical time intervals between degeneration-regeneration cycles, inducing their migration and gene expression. Evidence from the stroke literature describes a similar pattern of early microglial OPN secretion as a chemotactic astrokine, causing astrocytes to move from the penumbra to an

OPN-rich infarct core region over time (Ellison et al., 1998; Ellison et al., 1999). This profile is further supported in models of SCI (Hashimoto et al., 2003) and ischemia (Choi et al., 2007) which show acute OPN colocalization within microglia 1-3d postinjury, and delayed astrocytic colocalization between 7-15d postinjury. Given that astrocytes are known to secrete growth factors and nutrients important in the facilitation of effective axonal outgrowth (Matthews et al., 1979; John et al., 2003; Pellerin and Magistretti, 2004), our results suggest appropriate OPN response may be necessary to promote effective synaptic recovery following trauma-induced deafferentation as well. Indeed, MMPs are enzymes produced and secreted into the ECM by astrocytes after UEC, clearly capable of cleaving pro-growth factors into their active forms (Lee et al., 2001; Cunningham et al., 2005; Lu et al., 2008), flipping the local switch to axonal outgrowth and synapse regeneration (Matthews et al., 1979; John et al., 2003; Pellerin and Magistretti, 2004). Taken together, our investigation of OPN cellular distribution following hippocampal deafferentation suggests that the cytokine plays a major role in microglial signaling to promote the execution of early degenerative changes in the deafferented CNS. The results also support the activation of a subsequent microglia-astroglia OPN signaling pathway when regeneration is initiated, similar to the astrocyte cell signaling prototype described in stroke and SCI.

#### Osteopontin Fragments and Integrin Receptor Signaling During Reactive Synaptogenesis

The possibility of OPN playing a role in multiple phases of synaptic reorganization led us to examine its proposed cell signaling potential in more detail. As suggested by its biphasic expression in stroke models, OPN is a versatile molecule, capable of acting as both an acute inflammatory cytokine, and an astrocyte modulator of axonal regeneration, functions which may

be mediated by proteolytic cleavage exposing peptide sequences which bind to cell surface receptors (Senger et al., 1994; Giachelli et al., 1995; Smith et al., 1996; Yokosaki et al., 1999; Bayless and Davis, 2001). Two of the principle cell surface receptors for OPN are the  $\alpha_v\beta_3$  integrin receptor, or vitronectin (Yue et al., 1994; Yokosaki et al., 2005), and the haemopoietic cell surface receptor CD44 (Weber et al., 1996; Kang et al., 2008). Cleavage by thrombin and MMPs generates OPN fragments with fully exposed integrin receptor-binding sites (Agnihotri et al., 2001). Each of these lytic pathways can be linked to TBI related plasticity. Hemorrhagic lesion activated thrombin cleaves OPN, which, for example, induces endothelial cell  $\alpha_v\beta_3$  expression, promoting their migration to injured tissue (Ellison et al., 1998). In addition, a recent report of CNS stab wound, which also induces local MMP elevation, suggests a link between OPN and CD44-mediated neurite outgrowth (Plantman, 2012). Based on this data, we posited that OPN fragment generation is critical to its role during deafferentation induced synaptic reorganization. This view was appealing because integrin receptor upregulation has been documented during LTP (Milner and Campbell, 2002; Nikonenko et al., 2003), a functional correlate of reemerging synaptic structure. We also hypothesized that OPN-cleaved fragments modulate dendritic reorganization and cell migration during synaptic plasticity caused by TBI, an idea supported by the published integrin role following axotomy of the facial motor nucleus (Kloss et al., 1999). Our results showing correlated MMP activity and OPN expression within the injured dentate suggested that MMPs cleave the cytokine to permit greater cellular interaction, a process similar to the activation of growth factors through MMP lysis (Cunningham et al., 2005; Lee et al., 2001; Lu et al., 2008).

When UEC ML tissue extracts were probed for OPN fragments, the results only partially addressed these hypotheses. They showed that UEC increased the generation of two cleavage

products known to carry exposed  $\alpha_v\beta_3$  and CD44 binding sites, however, the two fragments were elevated at different time intervals postinjury. A 45 kD, RGD/SVVYGLR containing OPN fragment was increased over controls during the degenerative phase (1-2d postinjury), while a 32 kD OPN fragment containing the highly conserved C-terminus showed a reduction in the degenerative phase, but a subsequent elevation when regenerative sprouting begins (7d postinjury). We interpret this difference as indicating a greater role for the 45 kD integrin receptor binding fragment during acute inflammation, participating in the early microglial activation to clear damaged presynaptic terminals following UEC deafferentation. By contrast, the 32 kD OPN which contains the C-terminus known to undergo significant posttranslational modification (Kazanecki et al., 2007a; Kazanecki et al., 2007b) and bind  $\alpha_v\beta_3$  (Christensen et al., 2012), an integrin receptor important in cell migration (Brooks et al., 1994), is elevated at 7d at the onset of sprouting and synaptogenesis. This suggests the 32 kD form may direct the chemotaxis of astrocytes and their active growth factor secretion to promote successful reformation of synaptic junctions. While unexplored in this study, this chemotaxis could also affect local collateral axon sprouting which regenerates presynaptic terminals. Further, it is possible that the 2d reduced levels of the 32 kD OPN fragment may reflect a damping down of the regenerative OPN signal as the degenerative phase completes presynaptic terminal clearance. At present, the specific role(s) of these OPN lytic fragments during reactive synaptogenesis are speculative and will require additional studies which manipulate their specific expression to confirm their role in the process.

Given the potential for specific OPN fragments to modulate different cellular aspects of the synaptogenic process, mapping the receptors which bind OPN ligand sequences exposed by thrombin or MMP cleavage may help reveal fragment function. While our study did not address

the expression of these receptors, prior *in vitro* studies do show that when the  $\alpha_v\beta_3$  integrin receptor is exposed to OPN under conditions of thrombin cleavage, endothelial cell migration is increased and angiogenesis promoted (Senger et al., 1996). Further, in models of stroke and ischemia, there is an upregulation of OPN and  $\alpha_v\beta_3$  within astrocytes at the penumbra border of the glial scar, implicating a role for this pair in synaptic recovery following injury (Ellison et al., 1998). Interestingly,  $\alpha_v\beta_3$  is also expressed on the surface of microglia (Milner, 2009), and recent ischemia studies colocalized  $\alpha_v\beta_3$  and OPN within activated microglia, suggesting a role for OPN/integrin interaction facilitating the production of cytokines during acute immune response (Kang et al., 2008). Another receptor which interacts with OPN is CD44 (Weber et al., 1996), a molecule important in producing immune chemotaxis, microglial activation, and immune regulation (Pure and Cuff, 2001). Original reports of CD44 primarily focused on the receptor's role in this chemotaxis, however, later studies show the receptor upregulated in response to CNS trauma, including stroke (Wang et al., 2001) and cortical stab wound (Stylli et al., 2000). Further, TBI models using cold probe cryolesion demonstrated upregulation and colocalization of injury-induced OPN and CD44 within reactive microglia and astrocytes (Shin et al., 2005). An interesting recent study of brain stab wound goes further to link OPN with CD44-mediated neurite outgrowth (Plantman, 2012). While the present study did not probe for CD44 changes after UEC or TBI+BEC, our laboratory has found unpublished evidence that these receptors exist on reactive astrocytes within the deafferented ML, the same cells which we do show contain OPN (**Appendix E**). Taken together, the present findings, and published evidence of  $\alpha_v\beta_3$  integrin and CD44 receptor colocalization with OPN positive glia, support a role for OPN in the migration of reactive neuroglia and modulation of axonal outgrowth during trauma-induced synaptic reorganization.

## Acute Immunosuppression Attenuates Osteopontin Response and MMP-9 Activity

To further assess the relationship between the acute inflammatory response and OPN following deafferentation, we attenuated the immune response using the tricyclic antibiotic minocycline, then examined OPN expression, looking for changes in cellular response with synaptogenesis. As a tetracycline derivative, minocycline is widely used to treat bacterial infection and may provide neuroprotection in CNS injury and disease (Elewa et al., 2006). Following TBI, minocycline attenuates apoptosis (Matsukawa et al., 2009; Zhu et al., 2002), as well as free radical formation (Golub et al., 1991), and microglial activation (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999; Guasti et al., 2009; McAllister and Miller, 2010; Kobayashi et al., 2013). The drug also inhibits MMPs elevated with multiple sclerosis (MS), stroke, and cerebral hemorrhage (Brundula et al., 2002; Power et al., 2003; Machado et al., 2006; Cayabyab et al., 2013). Since many of minocycline's pathological targets are common to brain injury, we chose to evaluate its effect on synaptic plasticity using the adaptive UEC deafferentation model. Our results showed that acute dosing of minocycline does significantly attenuate postinjury OPN elevation, providing a good tool for *in vivo* manipulation of the cytokine. Using IHC methods, we were able to demonstrate that when OPN was reduced acutely, the documented elevation of OPN signal in reactive microglia was also reduced and, importantly, microglial migration to the intact/deafferented molecular boundary was disrupted. As microglia secrete OPN, it is likely that minocycline reduced their state of activation and, as a consequence, less OPN was produced and cellular mobility attenuated. This finding is consistent with previous studies in the impact acceleration model of TBI where gene microarray or cytokine array analyses demonstrate minocycline reduction in cytokines IL-1 $\beta$ , IL-6, MCP-1, and MIP-2 (Sanchez Mejia et al., 2001; Bye et al., 2007; Crack et al., 2009).

At this time, it is not entirely clear whether minocycline-like immunosuppression and its attenuation of molecules like OPN would be disruptive or beneficial in the context of synaptogenesis. Previous minocycline studies in CNS injury models show mixed results. For concussive TBI, Sanchez Mejia and colleagues originally reported that minocycline reduced lesion size and improved motor recovery (Sanchez Mejia et al., 2001), a finding also supported by improved hind limb function after SCI (Wells et al., 2003), and reduced neurobehavioral deficits after blast injury (Kovesdi et al., 2012). In addition, animals subjected impact acceleration insult plus postinjury minocycline had improved neuro-cognitive recovery, including transient neuroprotection (Bye et al., 2007), improved locomotor function (Homsy et al., 2009; Homsy et al., 2010), reduced anxiety states, and attenuated memory impairment (Siopi et al., 2012). Despite these potential benefits of minocycline therapy for TBI, a number of studies in CNS disease and injury report that minocycline may worsen related neuropathology (Diguët et al., 2004). Of most concern are clinical trial results showing that minocycline treatment actually exacerbated Parkinsonian symptoms (Ravina et al., 2003). The drug was also found to be ineffective in models of Huntington disease (Smith et al., 2003), MPTP models of Parkinson disease (Yang et al., 2003), and acute controlled cortical impact TBI (Kelso et al., 2011). Further, minocycline can worsen hypoxic-ischemic brain injury (Tsuji et al., 2004), and inhibit peripheral nerve regeneration following sciatic nerve resection (Keilhoff et al., 2007). These negative effects of minocycline are consistent with our results showing that the removal of degenerated presynaptic terminals is delayed in the UEC model after acute minocycline dosing that reduces OPN production. This delay is marked by the attenuated reduction of presynaptic terminal marker synapsin-1 over the denervated zone. Taken together with the disorganized microglial response under conditions of minocycline OPN reduction, these results mimic the

profile of disrupted degeneration seen with the maladaptive plasticity of the TBI+BEC model. It can be concluded that, during the acute postinjury intervals, inflammatory response is beneficial, including the elevation of OPN expression, which appears to influence microglial activation and facilitate the early stages of synaptogenic recovery. This observation points to further caution in the use of minocycline or other compounds with broad immune attenuation as TBI therapy. Alternative methods to affect immune response following TBI might be investigated, such as those manipulating the processing of individual cytokines to more specifically target time-dependent mechanisms underlying effective synaptic reorganization following trauma.

Lytic processing of OPN in the ECM of the deafferented zone may serve as a means to more finely tune cytokine-mediated aspects of reactive synaptogenesis. Prior studies in our laboratory have shown that the incomplete breakdown of damaged axon terminals generates a maladaptive degenerative state, marked by aberrant MMP activity. Here we extended our study to include assessment of gelatinase activity, profiling MMP-2 and 9 lysis and concurrent OPN fragment generation. Our results point to MMP-9 as a principal source of the  $\alpha_v\beta_3$  integrin binding 45 kD OPN fragment, which is consistent with published studies showing MMP-9 role in generation of functional OPN peptides (Takafuji et al., 2007). We found that minocycline reduction of OPN expression was spatially and temporally correlated with the attenuation of MMP-9 activity and OPN 45 kD fragment production. These results are in agreement with reported minocycline inhibition of MMPs (Brundula et al., 2002; Power et al., 2003), particularly the gelatinases following stroke or cerebral hemorrhage (Cayabyab et al., 2013; Machado et al., 2006). Similar to the proteolytic fragment studies mentioned above, these results support a role for 45 kD OPN during the 2d acute inflammatory period. Interestingly, minocycline did not alter the production of the 32 kD OPN fragment at 2d postinjury, a result consistent with our earlier



finding that injury-induced elevation of this peptide appears to play a more prominent role during regeneration at 7d or later, potentially interacting with integrin receptors and astrocytes to facilitate synaptic outgrowth. It is also tempting to speculate that full length OPN is not wholly responsible for its documented neuroprotective effects, but may require lytic processing by MMPs or other proteases to be truly functionally effective. Cleavage of OPN may permit the 45 kD form to signal additional immune response, while the 32 kD fragment might contribute to effective synaptic growth during the regenerative window. While attenuation of inflammatory response impairs MMP activity, OPN response, and presynaptic terminal clearance during the degenerative phase, arguing against acute immunosuppression during reactive synaptogenesis, minocycline remains a drug capable of acting on many different cellular pathways. As previously noted, these functions can produce both positive and negative effects, as minocycline negatively affects microglial function, but attenuates gelatinase induced hemorrhage in models of pial vessel disruption (Cayabyab et al., 2013). While we cannot confirm the cellular mechanism(s) underlying OPN, MMPs, and synaptic plasticity, our results show the three are interactive, and support the need for additional studies that more specifically manipulate OPN and its response after CNS trauma. One way to more directly assess OPN role in trauma-induced synaptogenesis is to probe the same outcome measures in OPN KO mice subjected to UEC deafferentation. We have generated a colony of this KO strain, and tested the effect of OPN KO on both structural and functional parameters of synaptogenesis as detailed in Chapter 3.

## **SUMMARY**

Here we have shown that OPN responds with a robust increase in expression during the acute phase of terminal degeneration induced by traumatic deafferentation. The temporal injury response of full length OPN is similar after adaptive UEC and maladaptive TBI+BEC insult,

however, the attenuated distribution of OPN, active microglia, and reactive astrocytes at the border of intact and deafferented dendrites in the maladaptive model suggests that glial OPN is critical for successful cellular migration, as well as debris phagocytosis during the acute inflammatory response. Elevated OPN mRNA in whole hippocampal extracts is greatest in deafferented ML, and transcript localized to activated ML microglia, further supporting cell directed OPN mediation of the acute immune response. Similar to studies in stroke, OPN may act as a cytokine early after injury, and later serve as an astrocyte, recruiting and interacting with astrocytes through integrin receptors to promote pre and postsynaptic outgrowth during the regenerative phase. These different OPN functions may be achieved through proteolytic processing of the cytokine, as evidenced by time dependent differences in the levels of the 45 kD and 32 kD fragments of OPN. Further, acute minocycline administration attenuated OPN response, a finding correlated with altered glial distribution similar to the random pattern in the maladaptive model. In addition, inhibition of the immune response impaired the removal of degenerating terminals, MMP activity, and production of the functional 45 kD OPN fragment. Taken together, these results suggest a mechanism of OPN, MMP, and reactive glial interaction during the acute postinjury immune response, directing effective neuroglial migration to the site of injury, and optimizing the removal of damaged terminals in preparation for effective synaptic reorganization. Further investigation of OPN role in trauma-induced synaptic plasticity will more clearly determine the extent to which this proposed mechanism contributes to beneficial and detrimental outcome.

## CHAPTER 3

### OSTEOPONTIN KNOCKOUT MICE AND SYNAPTOGENESIS

## ABSTRACT

OPN is a pleiotropic molecule capable of acting as a cytokine in the acute immune response to trauma, as well as a modulator of synaptic outgrowth. Given the results from studies in Chapter 2 showing a robust OPN elevation following trauma-induced deafferentation, and its localization within reactive glial cells at the site of reactive synaptogenesis, the studies of Chapter 3 employed OPN KO mice to determine if loss of OPN will alter this regenerative process. Our strategy was to determine if removing OPN alters molecular expression of proteins which mark three structural components critical to synaptic repair. To do this, we used MAP1B to track OPN interaction with the cytoskeleton, a critical organelle system for axon terminal growth and reshaping postsynaptic spine morphology, N-cadherin, an adhesion protein whose expression may be controlled by OPN and acts to stabilize nascent pre and postsynaptic elements, and lastly, synapsin 1, a molecule found in the membrane of transmitter-containing synaptic vesicles whose expression marks the integrity of presynaptic terminals. Both OPN KO and WT cohorts were subjected to UEC, and protein extracts obtained from whole hippocampus and enriched ML at 2d postinjury, the period when OPN expression was highest in the rat model. We probed for expression of MAP1B, N-cadherin, and synapsin 1 in these extracts, looking for strain differences which might reflect effects of OPN loss. Hippocampal tissue distribution of each protein was determined in paired cohorts with IHC methods.

In order to determine status of cognitive outcome after UEC in OPN KO, we also utilized the novel object recognition (NOR) task, comparing behavior to WT animals with UEC deafferentation. A final set of experiments tested whether lipocalin (LCN2)-mediated MMP proteolysis induced by UEC is altered in the absence of OPN. Overall, we observed reductions in the principal forms of each protein at 2d after UEC, a result predicted for the degenerative

phase of reactive synaptogenesis. MAP1B and N-cadherin were each reduced compared to controls in both hippocampal and ML extracts, however, no strain differences were observed. By contrast, synapsin 1 in OPN KO mice showed differences in response to UEC deafferentation compared with WT mice. One of its primary forms had significantly less reduction under conditions of OPN KO, a result which is consistent with the impaired removal of synapsin 1-marked degenerating terminals observed after rat UEC in Chapter 2. These synapsin 1 differences were correlated with altered microglial migration within the ML during the acute degeneration phase. Interestingly, the acute synapsin 1 changes in OPN KO mice were also associated with deficits in cognitive function as determined by poor performance in the NOR task, specifically during the subsequent 4-21d period of synapse regeneration. Evaluation of MMPs known to cleave OPN and produce integrin binding fragments showed injury-induced elevation of MMP-9 activity in WT mice, an effect significantly attenuated with OPN KO. Expression of hippocampal MMP-9-bound LCN2 was also elevated in WT 2d after UEC, but significantly reduced when OPN was absent. Notably, this KO reduction in MMP-9-bound LCN2 was not observed in the enriched ML samples, indicating that MMP-9 role in local dendritic OPN processing may be regulated by different mechanisms.

These results suggest that OPN interacts with MMP-9 during the degenerative phase of reactive synaptogenesis, potentially generating integrin receptor mediated activation and migration of local microglia within the denervated DG. This OPN-associated microglial response appears to target presynaptic terminal clearance, preparing the extracellular environment for the collateral axonal sprouting and synaptogenesis critical to the evolution of hippocampal functional recovery after traumatic insult.

## INTRODUCTION

Living with the chronic effects of TBI is a growing concern for both civilian and military populations worldwide. One critical outcome for the nearly 2 million individuals who fall victim to TBI each year are long term motor and cognitive deficits (Faul et al., 2010). These deficits not only reduce quality of life, but incur substantial cost for extensive rehabilitation adding further burden to the victims and their families (Langlois et al., 2006; Rutland-Brown et al., 2006; Finkelstein et al., 2006). Current treatment of TBI patients focuses on ameliorating the existing symptoms, a process often requiring months to years, with slow or little improvement. Unfortunately, most clinical trials of potential TBI therapies have focused on neuroprotection rather than enhancing recovery of surviving cells and circuitry. While none of these trials has identified a successful way to reverse traumatic injury and behavioral deficits, several interventions targeting acute inflammatory response show promise for enhancing the recovery process (Doppenberg et al., 1997; Chen et al., 2007; Pan et al., 2007). For example, ongoing TBI clinical trials with progesterone treatment suggest that the hormone may reduce neuroinflammation as part of its therapeutic value, potentially affecting the initial phases of synaptic reorganization (National Institute of Neurological Disorders and Stroke). Thus, it is reasonable to further evaluate how the immune system affects the development of TBI sequelae which may influence the progress of effective recovery.

During the repair of injured synapses, one of the first cellular responses is local inflammation. While this inflammation was initially regarded as an entirely deleterious following TBI, recent evidence indicates the proper evolution of the acute immune response is essential to effective synaptic reorganization, directing cytokines, and reactive glia to the injured area (Scherbel et al., 1999; Sullivan et al., 1999; Feuerstein and Wang, 2001; John et al., 2003).

For example, IL-1, a proinflammatory molecule important for the initiation of the cellular immune cascade and glial mobilization, was one of the first cytokines shown to be elevated after TBI (Rothwell, 1999; Ziebell and Morganti-Kossmann, 2010). Similarly, TNF- $\alpha$ , another proinflammatory molecule, is produced by reactive microglia in response to TBI (Shohami et al., 1994; Ziebell and Morganti-Kossmann, 2010). In order to determine if these proinflammatory cytokines act in a positive or negative role with respect to TBI neuroplasticity, researchers used both receptor inhibition and gene KO models to probe for cytokine effects on structural pathology and cognitive outcome. Interestingly, TNF KO mice had impaired neurological recovery, as well as exacerbated cortical and BBB damage (Scherbel et al., 1999; Sullivan et al., 1999), implicating a protective role for at least one cytokine following CNS trauma. While rather limited, several additional studies in SCI, neurodegenerative disease, stroke, and focal TBI also suggest that OPN may direct cellular immune response, facilitating recovery after CNS trauma. Our results in Chapter 2 suggest that the cytokine OPN may also act as a mediator of synaptic plasticity induced by brain injury, making it a promising therapeutic target for influencing recovery after TBI. Here we describe studies which manipulate OPN expression using the OPN KO mouse model, probing for effects on UEC-induced synaptic organization and recovery of cognitive behavior.

In these studies we first contrasted WT and OPN KO response to UEC at the 2d postinjury interval, the period when OPN response was the highest in the rat model (see Chapter 2 Results). The effect of OPN loss on synaptic structural components altered during the early phases of reactive synaptogenesis was assessed using molecular markers for these structures: MAP1B to track cytoskeletal reorganization, N-cadherin to monitor adhesive stability of synaptic junctions, and synapsin 1 to mark presynaptic terminals undergoing massive clearance. MAP1B,

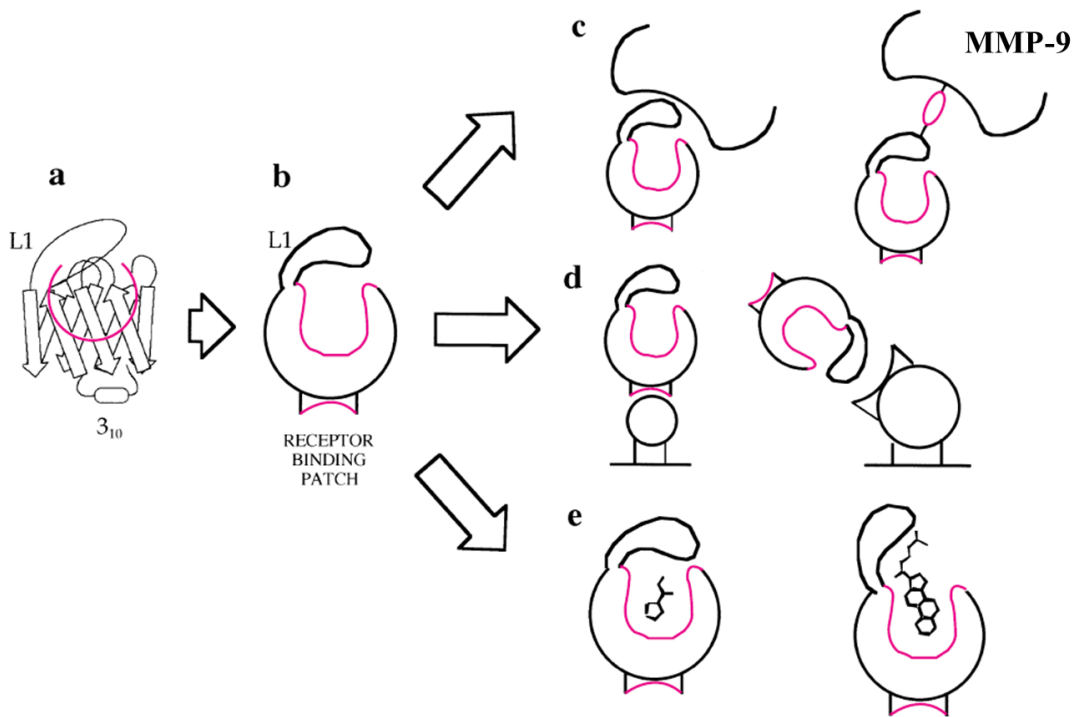
along with MAP2 and tau, regulates microtubule assembly responsible for the cytoskeletal structure of neurons and glia (Emery et al., 2003), and is part of the dynamic morphing of these cells during development (Yamanouchi, 2005; Riederer, 2007). N-cadherin is a  $Ca^{2+}$ -dependent cell adhesion molecule (CAM), often found at synapses, where it serves as a major structural protein comprising the adherens junction (Yap et al., 1997), thereby modulating synaptic plasticity in the adult brain (Huntley et al., 2002). Synapsins are abundant in presynaptic nerve terminals (Goelz et al., 1981; De Camilli et al., 1983a), serving a critical role in the binding of synaptic vesicles to cytoskeletal filaments during neurotransmitter release (Bahler et al., 1990; Sudhof, 1990; Greengard et al., 1993). Importantly, synapsin 1 mediates formation of synaptic contacts (Lohmann et al., 1978; Melloni et al., 1994; Ferreira et al., 2000) and presynaptic terminals (Han et al., 1991), as well as influences neurite elongation (Melloni et al., 1994).

Here, protein expression was used as the principal endpoint, which was measured with WB and IHC analysis. For these blot experiments we also compared whole hippocampus and ML extracts in order to investigate whether OPN KO preferentially affects the deafferented dendritic zone during the acute postinjury interval. Parallel cohorts of WT and OPN KO mice were subjected to UEC and the effect of OPN loss on cognitive function tested using the mouse novel object recognition (NOR) paradigm over 4-28d after injury. While previous OPN KO studies in SCI (Hashimoto et al., 2007) and PD models (Maetzler et al., 2010) have documented OPN-dependent alterations in neuropathology or motor function, none have assessed cognitive function following traumatic insult. For the this study, we chose the NOR task to evaluate cognitive function based on successful NOR assessment of hippocampal function in mice subjected to TBI (Baratz et al., 2011; Messier, 1997; Siopi et al., 2012). To our knowledge, this is the first study to utilize NOR in the assessment of spatial memory performance following UEC



in WT and OPN KO mice. Moreover, we demonstrate that UEC generates transient hippocampal-associated functional deficits which resolve over 28d postinjury, consistent with findings utilizing alternative hippocampal-dependent task such as Morris Water Maze (MWM), Alternation Maze, and Barnes Maze in rats (Steward et al., 1977) and mice (Hardman et al., 1997). To assess the association between our behavioral results and structural and biochemical changes, alterations in synaptic marker proteins in the degenerative phase were then compared with extent of functional recovery achieved during the subsequent regenerative phase for each mouse strain. We also further explored possible mechanisms by which secreted OPN could influence structural and functional outcome during synaptogenesis by evaluating one of the known pathways involved in processing the full length OPN protein for ECM signaling. This approach appeared feasible since we found significant elevation of OPN fragments with functionally active integrin receptor binding sites in the deafferented ML of the rat after UEC (Chapter 2). We hypothesized that, at 2d postinjury, secreted full length OPN is targeted by activated MMP-9 in the brain ECM, generating OPN peptides with exposed RGD integrin binding sequences which target receptors on reactive microglia. We further posited that this MMP-9 proteolysis of OPN is mediated through binding to the ECM siderophore lipocalin 2/neutrophil gelatinase-associated lipocalin (LCN2/NGAL), which persistently activates MMP-9 (**Fig. 3.1**). The result of this intermolecular action is fully activated glial response in the acute degenerative phase, fostering efficient debris clearance and successful regenerative sprouting for functional recovery. Here we probed for MMP-9 in WT and OPN KO mice with gelatin zymograms to determine if OPN KO affects this activity, and is correlated with altered WB expression of the MMP-9 bound form of LCN2. Our results show that under OPN KO conditions, there was a significant reduction in presynaptic synapsin 1 lysis and synapsin 1

**Figure 3.1 Lipocalin 2 Schematic Representation of Tertiary Structure and Proposed Binding Mechanisms.** LCN2 three-dimensional structure is comprised of  $\beta$ -strands surrounding an internal binding pocket with an open-end ligand-binding loop at the top, and folded receptor binding patch below (A-B). LCN2 is capable of complexing with soluble molecules through non-covalent association, and covalent disulfide linkage such as that with MMP-9 (C). Cell membrane receptor binding occurs through interaction with the receptor binding patch or the loop scaffold (D), and ligand-binding may occur with small ligands enclosed within the pocket or partly bound by the loop and pocket (E) (Flower, 1996).



presynaptic terminal removal, while ML reactive microglia were less ramified. Interestingly, hippocampus and ML samples showed similar WT and OPN KO patterns of reduced expression for each synaptic marker protein, and the overall lack of significant difference between the two regions suggests that OPN role is equally important in the cellular and dendritic zones. Parallel functional deficits in the subsequent regenerative phase were detected with NOR assessment. Interestingly, these observations were matched with reduced MMP-9 activity and LCN2 binding in OPN KO mice. Together, these experiments support OPN role in the acute inflammatory response after UEC, showing that its absence significantly alters structural plasticity and functional outcome. The results also suggest that one ECM-based mechanism for these acute deafferentation effects likely involves LCN2-mediated MMP-9 cleavage of OPN to generate peptide fragments which can activate local microglia through integrin receptor signaling.

## **METHODS**

### **Experimental Animals**

The procedures in this study met national guidelines for the care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. WT C57BL/6J mice or OPN KO mice (B6.129S6(Cg)-Spp1<sup>tm1Bih</sup>/J) (20-30 g; 8-11 weeks old; The Jackson Laboratory, Bar Harbor, ME) were used in these experiments, housed under a temperature (22°C) and humidity controlled environment with food and water *ad libitum*, and subjected to a 12 hour dark-light cycle. For molecular assessments, WT or OPN KO mice were randomly selected, subjected to UEC, and evaluated at 2d postinjury via WB, IHC, or gelatin zymography (n=40 each strain; total n= 80). For the behavioral analysis study, WT and OPN KO mice were randomly assigned to an uninjured or UEC group and evaluated via the

NOR task at 4, 7, 14, 21, and 28d postinjury before being sacrificed for either tissue extraction or IHC analysis (n=12 /group per strain; total n=48). For molecular analyses, the contralateral hemisphere served as a control for UEC lesion effects, and WT versus OPN KO compared for experimental effect. Uninjured animals served as controls for UEC lesioned animals in behavioral analyses.

### Mouse Unilateral Entorhinal Cortex Lesion

The mouse UEC protocol is a modification of that described by Hardman and colleagues (1997). Mice were anesthetized with 4% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> for 4 minutes, heads shaved, then maintained on 2.5% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> delivered via nose cone for the duration of the surgery. Body temperature was maintained at 37°C via heating pad (Harvard Apparatus, Holliston, MA), and animal heart rate (beats per minute, bpm), arterial oxygen saturation (%), breath rate (breaths per minute, brpm), pulse distention (µm), and breath distention (µm) were monitored via pulse oximeter (MouseOx; Starr Life Sciences, Oakmont, PA). While under anesthesia, mice were secured in a stereotaxic device and a midline incision exposed the cranium to permit a 2 mm wide craniectomy exposing the intact dura mater above the right EC. Electrolytic lesions were delivered via Teflon®-coated wire transmitting 2.0 mA for 15 seconds to three stereotaxic coordinates: 10° lateral to perpendicular; 0.5 mm rostral to the lamdoidal suture; 2 and 3 mm lateral to the midline at 3.5 mm ventral to the brain surface; and 3.5 mm at 3.0 mm ventral to the brain surface. After lesion completion, the electrode was removed, and the incision sutured closed and treated with topical anesthetic and antibiotic. Animals were then housed singly in a heated holding cage during the

acute surgical recovery and monitored for discomfort or distress before being returned to their home cage.

## **Molecular and Morphological Assessments**

### Protein Extraction and Analysis

Selected mice were anaesthetized with 4% isoflurane in carrier gas of 70% N<sub>2</sub>O, 30% O<sub>2</sub> for 4 minutes, then sacrificed at 2d postinjury via decapitation (n=64 each strain; total n= 128). Whole hippocampi or ML enriched fractions were dissected, homogenized on ice in 100 µl of RIPA#2 (1X Pierce RIPA Buffer, 1% Triton™ X-100, 0.1% SDS and protease inhibitor (Thermo Scientific, Rockford, IL; Roche, Mannheim, Germany), and centrifuged at 8,000 x g for 5 minutes at 4°C. Supernatant was aliquotted and stored at -80°C for protein quantification, WB, or gelatin zymography analysis. Protein concentration was determined using Thermo Scientific Pierce Protein Assay Reagent (Rockford, IL) and FLUOstar Optima plate reader (BMG Labtech, Inc., Durham, NC).

### Western Blot Analysis

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Thirty µg of protein were prepared for WB with XT sample buffer and XT reducing buffer, and denatured at 95°C for 5 minutes. Samples were electrophoresed on 4-12% Bis-Tris Criterion XT gels (200 V x 45 minutes) then protein transferred to PVDF membranes (100 V x 60 minutes). Following transfer, post-blot gels were stained 0.1% coomassie brilliant blue in destain #1 (40% MeOH, 10% HOAc x 30 minutes), then destained at room temperature (#1 x 30 minutes; #2: 7% MeOH, 10% HOAc x 30 minutes) to confirm even transfer of protein. To prevent non-specific protein

binding, membranes were first washed in nanopure H<sub>2</sub>O (2 x 5 minutes) and TBS (1 x 5 minutes), then blocked in mTBST. Blots were next cut at the 25 kD marker and the appropriate pieces incubated overnight at 4°C in mTBST with either anti-OPN (0.25 µg/ml, R&D Systems, Minneapolis, MN; or 1:300, Rockland Immunochemicals Inc., Gilbertsville, PA) or anti-cyclophilin A (1:2,500, Millipore, Billerica, MA), used as a loading control. Blots were washed with mTBST (5 x 5 minutes) then incubated in appropriate secondary in mTBST (1:10,000 goat anti-mouse; 1:20,000 bovine anti-rabbit, Santa Cruz Biotechnology Inc., Dallas, TX) at room temperature for 1 hour. Finally, membranes were washed in TBST (5 x 5 minutes) and incubated with Super Signal Dura West chemiluminescent substrate (Thermo Scientific, Rockford, IL) for signal detection. WB images were captured with Syngene G:Box and positive band signal subjected to densitometric analysis (expressed as ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data are expressed as either fold change over controls or percent change relative to paired control cases.

### Gelatin Zymography Analysis

Twenty µg of protein from 2d whole hippocampal extracts described above were prepared with 2x Tris-glycine SDS sample buffer and separated by gelatin electrophoresis at 4°C on Novex® 10% zymogram gels (Life Technologies, Grand Island, NY). Gels were then renatured in Novex® Zymogram Renaturing Buffer (LC2670, Life Technologies, Grand Island, NY) at room temperature before development in Novex® Zymogram Developing Buffer (LC2671) over 6 days at 37°C. Gelatin lysis was visualized with coomassie brilliant blue and purified enzyme run as positive control. Zymogram signal was captured as an inverted image with Syngene G:BOX, and densitometry analyzed as ROD with Gene Tools software (Syngene,

Frederick, MD). Enzyme activity data are expressed as percent change relative to paired control cases.

### Fluorescent Immunohistochemical Analysis

At 2d postinjury (mouse n=5 each C57BL/6 and OPN KO, total n=50), animals were selected for fluorescent IHC analysis. Mice were anaesthetized and sacrificed with a lethal dose of sodium pentobarbital (90 mg/kg, i.p.), followed by transcardiac perfusion with 0.9% saline (500 ml) and aldehyde fixative (500 ml 4% PFA in 0.1 M NaHPO<sub>4</sub>, pH=7.4). Perfused brains were then removed and placed in fixative for an additional 24 hours before transfer to 0.03% NaN<sub>3</sub> in 1.0 M PBS. For IHC staining, brains were blocked at the dentate gyrus, and 40 µm coronal sections sliced using the VT1000S microtome (Leica, Buffalo Grove, IL). Slices were then pre-treated in 0.5% peroxidase (30 minutes) and washed in PBS (3 x 10 minutes). To prevent non-specific binding, sections were blocked in Blotto for 30 minutes (5% Cold Water Fish Skin Gelatin, Aurion, Netherlands, 5% Triton in 1.0 M PBS) and Triton X-100 in PBS prior to overnight primary antibody incubation in Blotto (anti-OPN MPIIB10, 1:300, Iowa Hybridoma, Iowa City, IA; anti-IBA1, 1:300, Wako Chemicals, Richmond, VA; anti-GFAP, 1:300, Dako, Carpinteria, CA; anti-synapsin 1 a/b (N-19, sc-7379), 1:250, Santa Cruz Biotechnology, Inc., Dallas, TX; anti-MAP-1B (H-130, sc-25729), 1:200 Santa Cruz Biotechnology, Inc., Dallas, TX; anti-N-cadherin (L-13, sc-31029), 1:100, Santa Cruz Biotechnology, Inc., Dallas, TX; anti-NGAL (M-145, sc-50351), Santa Cruz Biotechnology, Inc., Dallas, TX). The next day, tissue sections were washed with PBS, blocked in Blotto at room temperature (30 minutes), then incubated in Blotto with the desired secondary fluorescent antibody (Alexa Fluor® 488 or 594, 1:1,000, Life Technologies, Grand Island, NY) for 1 hour at



room temperature. Free floating sections were then washed in PBS (3 x 5 minutes), equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield (Vector Laboratories, Burlingame, CA). IHC signal was visualized using the TCS-SP2 AOBS (Leica, Buffalo Grove, IL) or LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope.

## **Osteopontin Knockout Mouse Analysis**

### Genotype Verification

Confirmation of the transgenic OPN KO was assessed utilizing the PCR-verification method by The Jackson laboratory to test for presence of the neo<sup>r</sup> cassette replacing exons 4-7 of the OPN gene which creates a null mutation, and renders it incapable of producing a OPN transcript in B6.129S6(Cg)-*Spp1*<sup>tm1Blh</sup>/J mice (Liaw et al., 1998). Genomic DNA was isolated from 1 cm of tail, and genotyping confirmed utilizing the oIMR8444 primer ‘5GCC TGA AGA ACG AGA TCA GC3’ for OPN and the HotSHOT method as previously described (Truett et al., 2000; Su et al., 2010).

### Protein Expression Ablation Verification

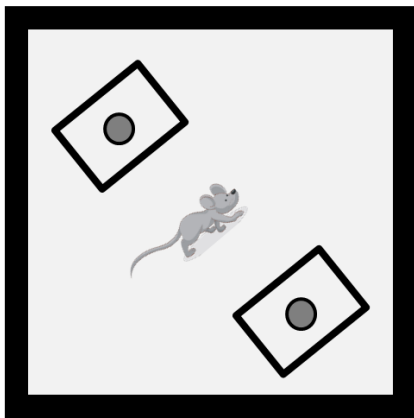
To confirm the absence of OPN protein expression in the injured hippocampus, OPN KO and WT hippocampal extracts of mice subjected to UEC were analyzed via WB analysis as described above using polyclonal anti-OPN antibody raised in goat (0.25 µg/ml, R&D Systems, Minneapolis, MN).

## Novel Object Recognition

Cognitive performance was assessed using a modified NOR test based on previous studies (Dixon et al., 1987; Ennaceur and Delacour, 1988; Messier, 1997; Han et al., 2011; Baratz et al., 2011; Siopi et al., 2012). The NOR apparatus consists of a 42 x 42 x 30 cm<sup>3</sup> open field arena under red light illumination (1 lux at mouse height) in a sound-isolated room as previously described (Fuller et al., 2012). The NOR task consists of three phases: habituation, familiarization, and testing. During habituation (3, 6, 13, 20, 27d postinjury), a single UEC (C57BL/6 n=12, OPN KO n=9; total n=21) or uninjured (C57BL/6 n=12, OPN KO n=12; total n=24) mouse was acquainted with the black opaque plastic testing chamber for two nonconsecutive 5 min intervals. Twenty four hours later, animals started the familiarization phase in the same chamber and were allowed to explore two identical objects: 50-mL conical plastic tubes affixed to the floor with Velcro® tape in symmetrical positions within the chamber. After a one hour delay, the animals entered the testing phase (4, 7, 14, 21, 28d postinjury) and returned to the testing chamber where one of the objects was replaced by a new object: a Lego® DUPLO® configuration of similar material and dimensions (height and width), but differing in shape from that of the conical tube (**Fig. 3.2**). Time spent exploring each object during both the familiarization and testing phases was recorded for each minute with an overhead video camera, and later scored. Object exploration was operationally defined as object touching, or orienting behaviors (sniffing, rearing, or head orientation) occurring within 2 cm of the object. Sitting on top of or circling was not counted. To prevent olfaction distraction or bias, the chamber and objects were thoroughly cleaned with 70% ethanol between trials. Conversely, to prevent long term memory as confounder, a different novel object configuration was presented during each progressive trial. Results of the testing phase are represented by recognition index (RI=time

**Figure 3.2 Novel Object Recognition Cognitive Assessment Task Schematic.** Animals are placed in a plastic 42 x 42 x 30 cm<sup>3</sup> box illuminated by 1 lux at animal height. During the familiarization phase (A), animals are allowed to explore two identical 50 ml conical tubes (grey circles) in symmetric locations for 5 minutes. After a 1 hour delay, animals are tested (B) for their recall of the familiar object by replacing one of the objects with a novel Lego® object (star) and recording the percentage of time spent exploring each object over a 5 minute period.

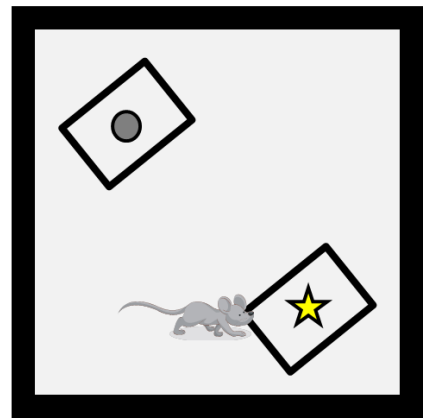
**A**



**Familiarization**



**B**



**Testing**

spent with novel object/(time spent with familiar object+time spent with novel object) x 100).

An RI score of 50 represents a chance level of performance (no object preference) and RI significantly greater than 50 indicates an intact memory capacity for this task.

### Statistical Analyses

Changes in protein levels or enzyme activity, induced by injury or associated with genetic strain, were evaluated using the GLM in SPSS v.11 (International Business Machines, Corp., Armonk, NY). These alterations were assessed using a mixed model ANOVA with hemisphere (contralateral vs. ipsilateral to injury) as a within-subjects variable, and strain as a between-subjects measure, and specific pairwise comparisons evaluated as simple main effects. The effects of strain and UEC injury on NOR behavioral performance, was evaluated with a completely randomized ANOVA design followed by Duncan post-hoc tests. Results are reported as mean +/- SEM. An alpha level of 0.05 was used in all analyses.

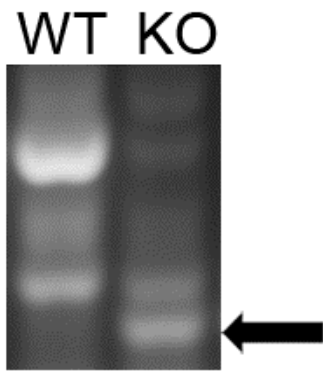
## **RESULTS**

### Osteopontin Knockout Mouse Genotype Verification

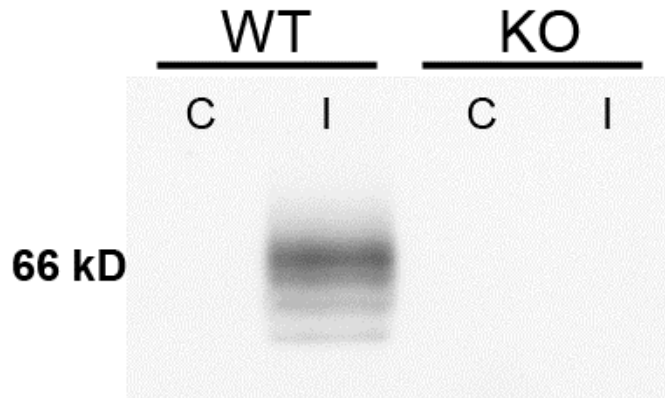
We first examined the genotype of the homozygous OPN KO mice utilizing PCR-verification, as described by The Jackson Laboratory, to confirm presence of the neo<sup>r</sup> cassette replacing exons 4-7 of the OPN gene, creating a null mutation of the OPN gene, and rendering it incapable of producing a functional OPN protein in B6.129S6(Cg)-*Spp1*<sup>tm1Blh</sup>/J mice (Liaw et al., 1998) (**Fig. 3.3 A**). In addition, we confirmed ablated OPN response by WB analysis of whole hippocampal extracts using the polyclonal goat OPN antibody (R&D Systems) at 2d following UEC, the time point where we found maximal OPN response in rat experiments of Chapter 2

**Figure 3.3 OPN KO Mouse Genotype Verification.** PCR confirmation of the neo<sup>f</sup> cassette replacing exons 4-7 of OPN (A) and representative WB of full length 66 kD OPN protein in whole hippocampal extracts (B). Similar to the response in rats, OPN increased robustly in WT C57BL/6 mice 2d after UEC, while OPN KO mice had only background signal detection, with no change between injured and contralateral control sides.

**A**  
**Spp1<sup>tm1Blh</sup>**



**B**  
**Full Length OPN**  
2d UEC Hippocampus



(see again **Fig. 2.4**). Similar to the result found when rats were subjected to UEC, WT C57BL/6 mice showed a robust OPN response within the hippocampus compared to contralateral controls ( $88.38 \pm 0.67$  fold,  $p < 0.001$ ) (**Fig. 3.3 B**). This is in contrast to a null OPN response in the hippocampi of OPN KO mice, where only background signal was detected, and which did not differ from controls ( $0.81 \pm 0.12$  fold). Together, these data confirm successful knockout of the OPN gene and lack of functional OPN protein response to trauma in our OPN KO mouse colony.

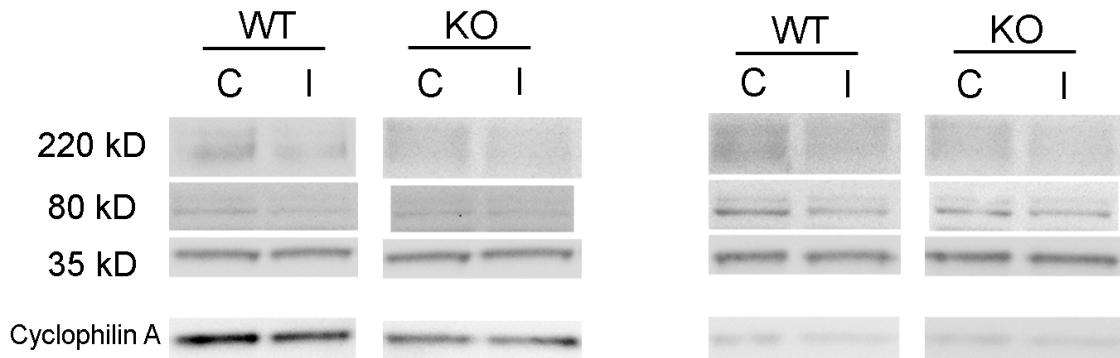
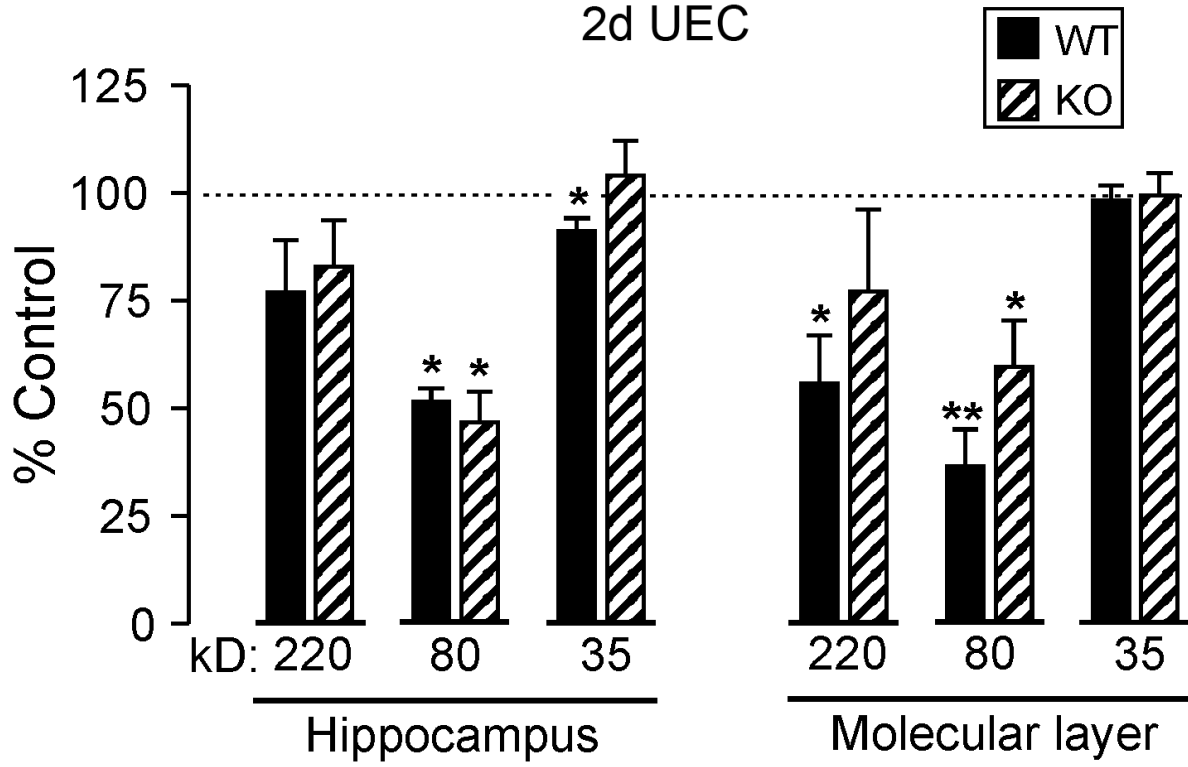
#### Osteopontin Knockout Does Not Alter MAP1B Cytoskeletal Changes Following UEC

In this experiment, similar to that of the rat, we explored the relationship between OPN and the cytoskeletal microtubule associated protein MAP1B during UEC reactive synaptogenesis in the mouse. As previously indicated, recent reports suggest that intracellular OPN may act to stabilize the cell cytoskeleton by binding with MAP1B. While MAP1B did not appear to interact significantly with OPN during the rat acute immune response, we investigated the potential for OPN to direct UEC-induced reshaping of the postsynaptic dendrites in mice, looking for differences from WT animals subjected to the same injury. Here we utilized WB to probe both whole hippocampal extracts and ML enriched fractions for MAP1B expression (**Fig. 3.4**). Goat polyclonal antibody for MAP1B detected three principal bands, at 220, 80, and 35 kD. The 220 kD signal represents full length MAP1B, while the 80 and 35 kD represent cleaved fragments, which may be generated by either calpain- or caspase-mediated cleavage, enzyme pathways known to be important in cytoskeletal reorganization after injury (Fifre et al., 2006). Results showed an overall decrease in MAP1B protein expression within the hippocampus and deafferented dentate ML 2d following UEC, consistent with the removal of injured terminals during the degradation phase of reactive synaptogenesis. Notably, no significant differences in



**Figure 3.4 MAP1B Protein Expression Following UEC.** At 2d postinjury, WB analysis of MAP1B in whole hippocampal and enriched molecular layer (ML) extracts detected three major bands: a full length 220 kD signal, and two lytic fragments at 80 and 35 kD. Overall, UEC deafferentation decreased MAP1B expression for both WT and OPN KO mice. In whole hippocampus, MAP1B showed significant reduction in the 80 kD form for both strains, and in the 35 kD form for WT animals. Within ML fractions, WT 220 and 80 kD forms were significantly reduced, while only the 80 kD fragment showed significant reduction in OPN KO animals. There was no significant difference in MAP1B expression between strains after UEC. Results are displayed as percent of control, with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$ , \*\* $p < 0.01$  relative to paired control cases.

## MAP1B 2d UEC



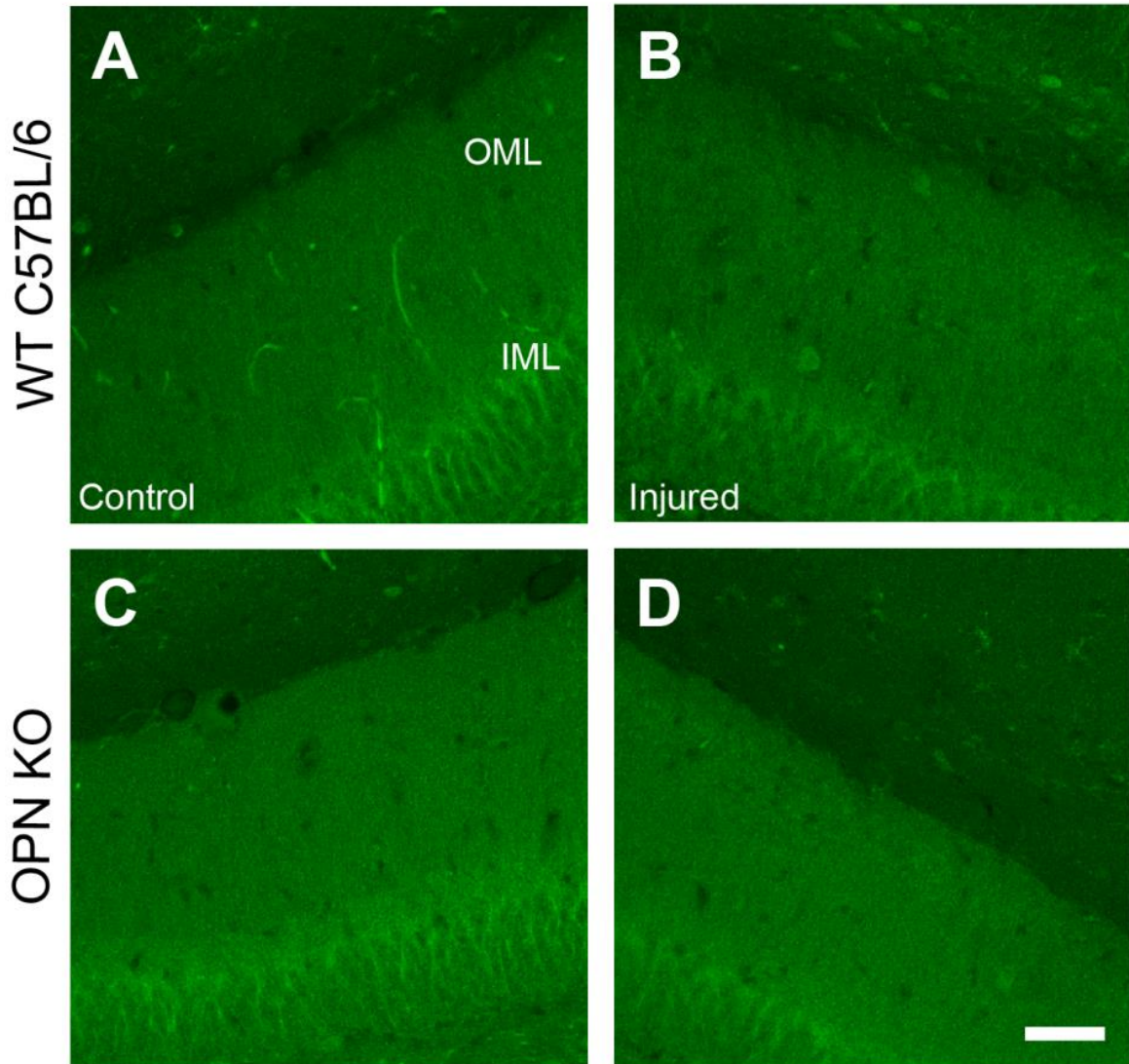
MAP1B forms were detected between the two mouse strains, or between the two regions sampled. For WT mice, the 220 kD ( $76.9 \pm 12.2\%$ ) and lysed forms were decreased in the hippocampus compared to control hemispheres, with both the 80 ( $51.6 \pm 3.1\%$ ,  $p < 0.05$ ) and 35 kD ( $91.2 \pm 2.9\%$ ,  $p < 0.05$ ) reductions reaching significance. In extracts of the WT injured ML, there was a similar overall reduction in MAP1B expression after UEC for the 220, 80, and 35 kD forms, however, change in the 35 kD did not reach significance relative to paired controls ( $98.2 \pm 3.6\%$ ). Interestingly, the reduction in the 220 kD full length form was significant in this enriched fraction ( $55.8 \pm 11.1\%$ ,  $p < 0.05$ ), and the 80 kD reduction ( $36.5 \pm 8.6\%$ ,  $p < 0.01$ ) was to lower levels in the ML, but, as noted above, failed to show statistical difference by strain or region. For OPN KO mice, hippocampal results showed changes similar to that of WT MAP1B protein. MAP1B expression of the full length 220 ( $82.9 \pm 10.7\%$ ) and 80 kD ( $46.8 \pm 7.1\%$ ,  $p < 0.05$ ) fragment were lower, however, only the 80 kD band showed significant reduction. Unlike WT, the hippocampal 35 kD trended toward increase at 2d ( $104.1 \pm 8.0\%$ ), but this difference was clearly not significant. For the OPN KO ML fractions, MAP1B appeared to go down in the 220 ( $77.1 \pm 19.1\%$ ) form and showed no change in the 35 kD ( $99.5 \pm 5.2$ ) form, but reduction in the 80 kD fragment was more consistent, reaching statistical significance ( $59.7 \pm 10.6\%$ ,  $p < 0.05$ ). Fluorescent IHC for MAP1B revealed slight decreases in staining at the IML after UEC relative to paired controls for both WT and OPN KO mice (**Fig. 3.5**). While major qualitative differences were not observed between strains, overall MAP1B signal did appear stronger in the OPN KO mice.

Together, our WB results indicate no significant differences in altered MAP1B expression between the hippocampus and ML, nor strain differences between WT and OPN KO mice. This conclusion was supported by parallel IHC staining for MAP1B in each strain. While

**Figure 3.5 IHC of MAP1B in WT and OPN KO Mice Following UEC.** Confocal MAP1B staining in WT and OPN KO mice shows a slight decrease over the IML (B, D) compared to paired contralateral controls (A, C) after UEC. Other than an overall higher signal in the OPN KO cases, no apparent differences in MAP1B signal occurred between WT and OPN KO mice. Scale bar = 50  $\mu$ m.

# MAP1B

## 2d UEC



the predicted IHC postinjury redistribution of MAP1B seen in the rat did not occur in mice subjected to UEC, the significant reduction of 80 kD MAP1B within both regions and strains suggests this form of MAP1B plays a prominent role during deafferentation-induced cytoskeletal reorganization in the mouse model.

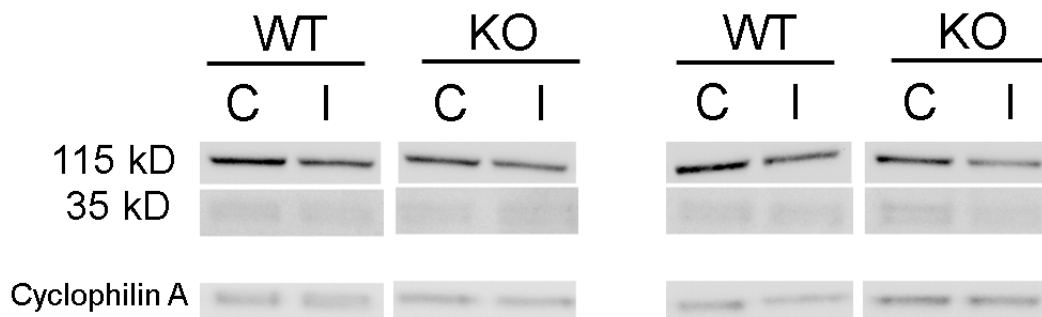
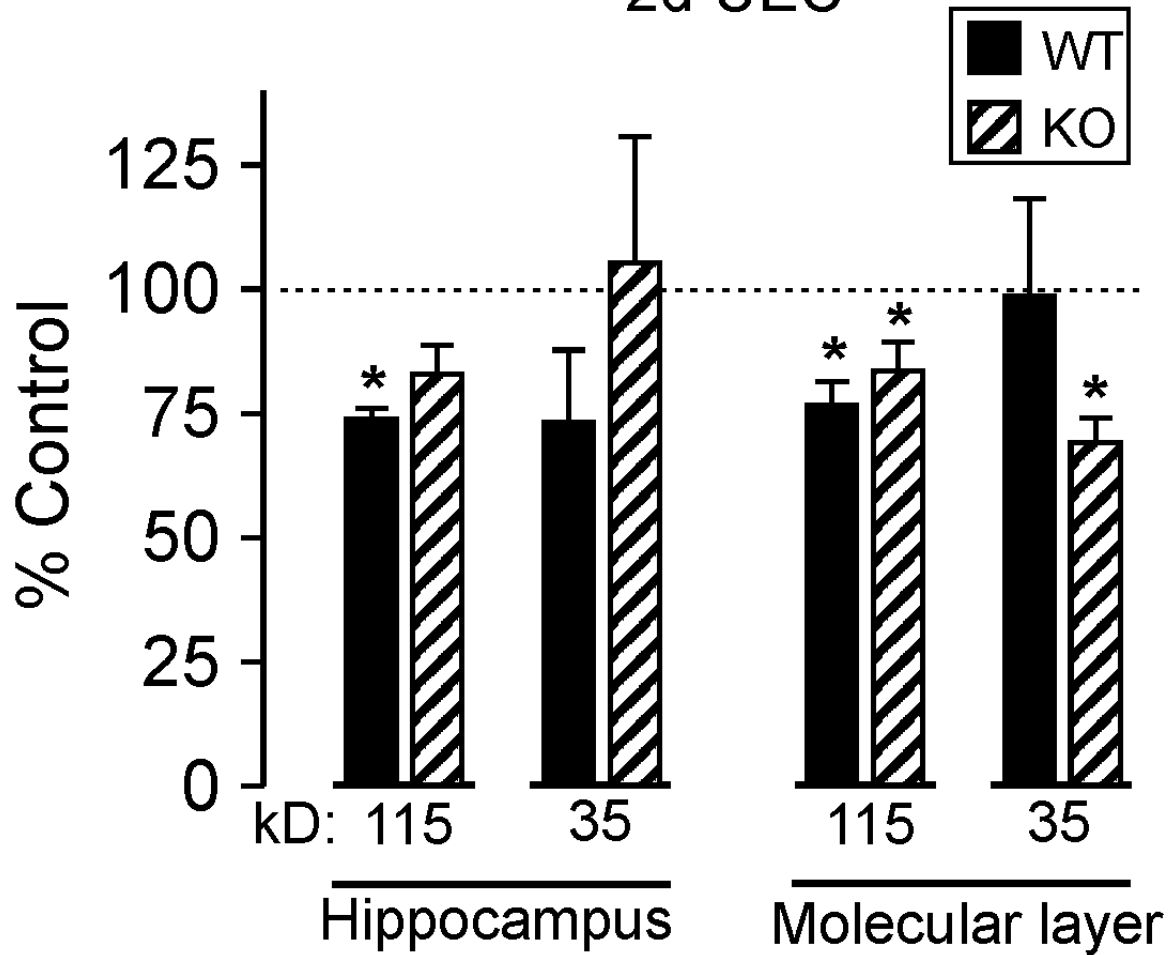
#### Osteopontin Knockout Does Not Alter N-cadherin Mediated Synaptic Stabilization after UEC

Here, we extended our evaluation of WT and OPN KO synaptic response to UEC by examining changes in N-cadherin expression. N-cadherin is a transmembrane adhesion protein which exhibits homologous binding to stabilize the synaptic junction. N-cadherin protein level was compared in WT and OPN KO mice using WB analysis of hippocampal and injured ML extracts (**Fig. 3.6**), as well as with IHC spatial localization during at the 2d degeneration phase (**Fig. 3.7**), again matching the interval of maximal OPN response for the rat studies in Chapter 2. Utilizing a goat polyclonal antibody for N-cadherin, we evaluated changes in two major bands: a full length 115 kD form, and a principal 35 kD cleaved fragment. Results showed a general decrease compared to paired controls, again predicted for the destabilization of synapses during the acute degenerative period. As for MAP1B, no regional or strain differences were significant. Nevertheless, relative to contralateral controls, WT mice exhibited lower expression of both the 115 and 35 kD forms in the hippocampus ( $73.9 \pm 2.1\%$ ,  $p < 0.05$ ;  $73.2 \pm 14.5\%$ ) and enriched ML fractions ( $76.7 \pm 4.7\%$ ,  $p < 0.05$ ;  $98.5 \pm 19.7\%$ ), however only the reduction of the full length form reached significance. For OPN KO mice, hippocampal 115 kD N-cadherin level trended toward reduction compared with controls ( $82.9 \pm 5.7\%$ ), but the 35 kD fragment showed higher variability and was actually maintained at control levels ( $105.2 \pm 25.4\%$ ). Neither of these shifts from control was statistically significant. Interestingly, although hippocampal N-cadherin

**Figure 3.6 N-cadherin Protein Expression Following UEC.** At 2d postinjury, WB analysis of N-cadherin in whole hippocampal and enriched ML extracts detected two major bands: full length 115 kD, and a cleaved 35 kD form. As for MAP1B, UEC induced a decrease in N-cadherin expression for both WT and OPN KO mice. WT hippocampal extracts exhibited significant reduction only in the 115 kD form, while OPN KO samples had higher variance, with no significant change in either the 115 or 35 kD N-cadherin. Within ML fractions, 115 and 35 kD forms were reduced in OPN KO mice, but only the 115 kD form was decreased in WT animals. There was no significant difference in N-cadherin expression between strains after UEC. Results are expressed as percent of control, with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$  relative to paired control cases.

# N-cadherin

2d UEC

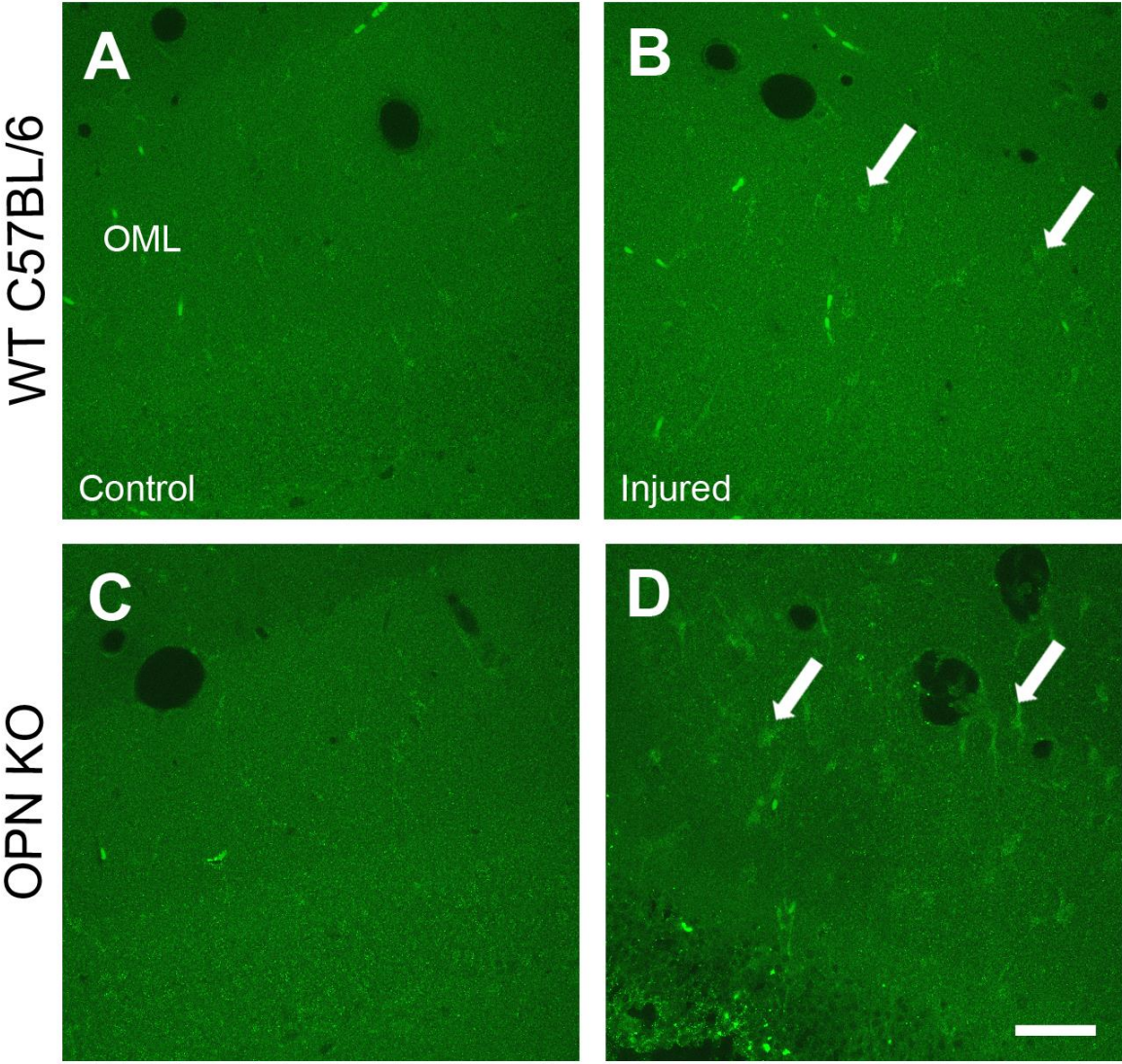




**Figure 3.7 IHC of N-cadherin in WT and OPN KO Mice Following UEC.** Confocal N-cadherin staining increased over the OML in after UEC (B, D) compared to contralateral control hemisphere (A, C). This increase was observed in non-neuronal cells distributed throughout the zone. Injury-induced cellular staining (arrows) in OPN KO mice (D) was more prominent than that of WT controls (B). Scale bar = 50  $\mu$ m.

# N-cadherin

2d UEC



change in OPN KO mice did not reach significance, reductions within the ML were significant for both the full length 115 kD form ( $83.6 \pm 5.7\%$ ,  $p < 0.05$ ) and cleaved 35 kD fragment ( $69.2 \pm 4.8\%$ ,  $p < 0.05$ ). While there were no overall region or strain differences for either WT or OPN KO mice, the shift in the 35 kD N-cadherin fragment from essentially no change in KO whole hippocampus to significant reduction in the ML supports more complete local N-cadherin proteolysis in the deafferented zone where synapse destabilization is underway. Parallel IHC of N-cadherin within the DG (**Fig. 3.7**) revealed slight increase in non-neuronal ML signal for the injured WT mice relative the paired control tissue. This N-cadherin response was even more robust within the outer ML of injured OPN KO mice. Since N-cadherin is known to localize within reactive astrocytes of the ML, these results are consistent with glial proteolysis of full length N-cadherin suggested by our WB data.

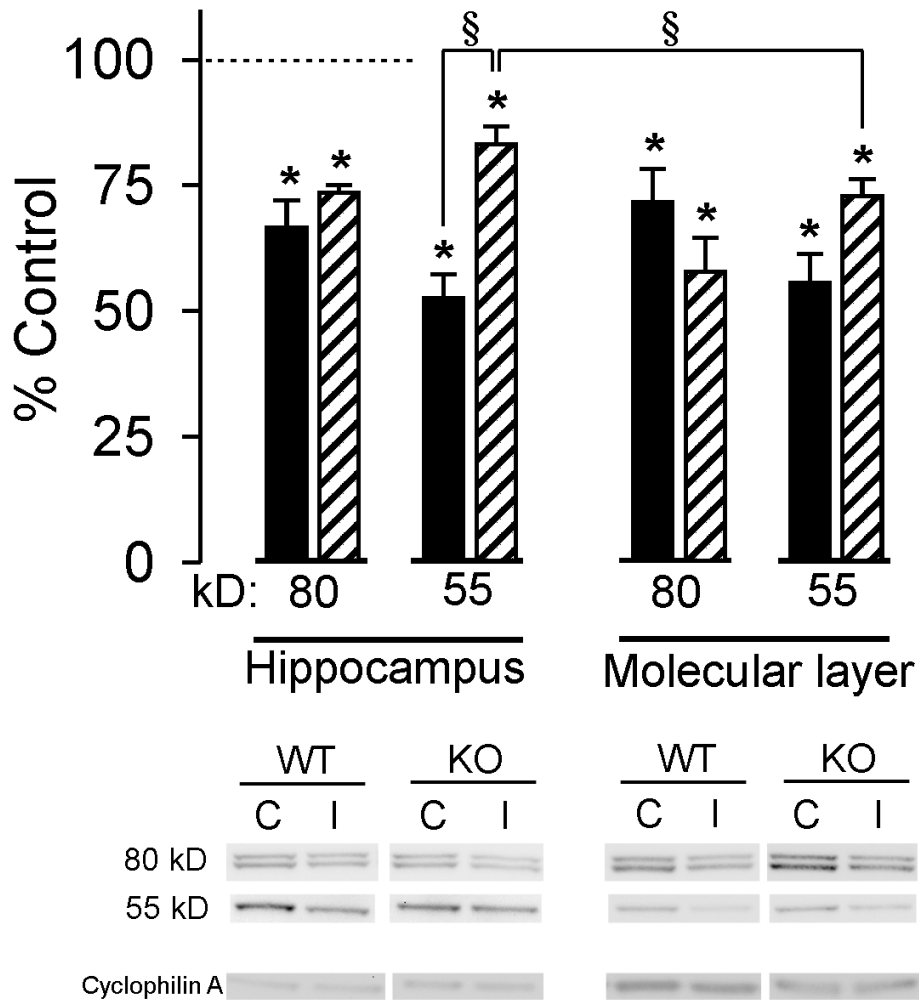
#### Osteopontin Knockout Alters Synapsin 1 and Effective Removal of Presynaptic Terminals

As for MAP1B and N-cadherin, WB analysis and fluorescent IHC were utilized to map synapsin 1 expression in order to determine the potential for OPN to direct presynaptic terminal degradation during the acute degenerative phase of reactive synaptogenesis. Utilizing a goat polyclonal antibody which recognizes the full length 80 kD form, as well as a cysteine protease-cleaved 55 kD product of synapsin 1, we evaluated the response of this protein in the hippocampus and enriched ML extracts of WT and OPN KO mice subjected to UEC (**Fig. 3.8**). As a protein linked to synaptic vesicles, we utilized this antibody as a marker of presynaptic terminals and anticipated a reduction in synapsin 1 staining, indicating removal of injured terminals, similar to that in the rat studies of Chapter 2. Results show a very similar pattern of synapsin 1 reduction in both full length and cleaved forms for WT and KO cases when compared

**Figure 3.8 Synapsin 1 Protein Expression Following UEC.** At 2d postinjury, WB analysis of synapsin 1 in whole hippocampal and enriched ML extracts detected two major bands: a 80 kD full length form, and a 55 kD fragment, representing the principal cysteine-specific cleavage product. Overall, both strains showed a similar pattern of synapsin 1 reduction after injury, each form being significantly lower than in paired control samples. The deafferentation-induced reduction of the 55 kD form was attenuated in the OPN KO mice relative to WT animals, however, this difference reached significance only for the whole hippocampus. Interestingly, the ML 55 kD synapsin 1 level was significantly lower than the corresponding hippocampal value for the OPN KO cases. Results are expressed as percent of control, with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$  relative to paired control cases; §  $p < 0.05$ .

# Synapsin 1

2d UEC

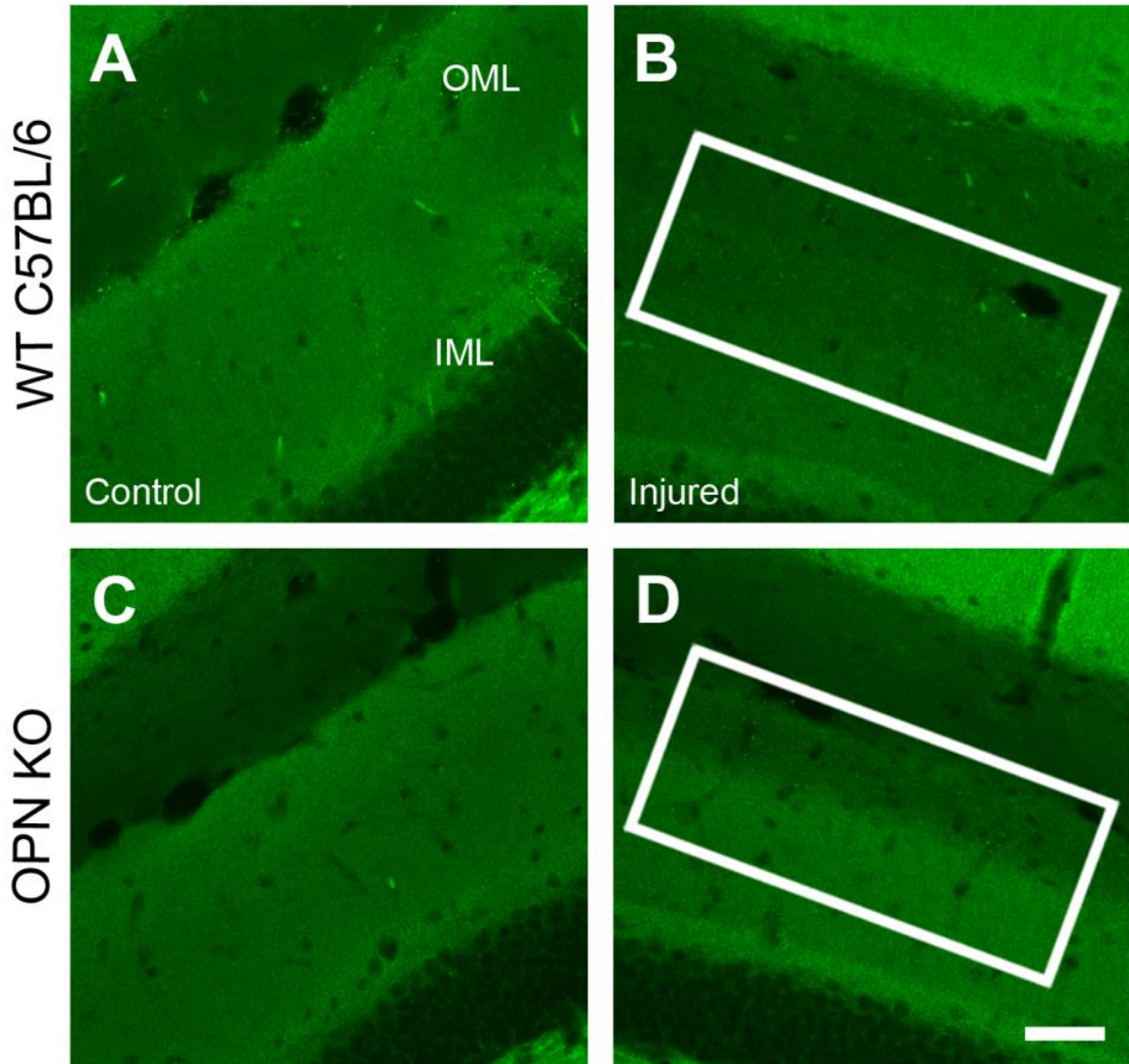


to control mice. Further, in contrast to MAP1B and N-cadherin, significant differences in synapsin 1 protein expression were found for select bands between regions and mouse strains. For WT mice, the full length 80 kD and cleaved 55 kD protein were significantly reduced at 2d after UEC in both the hippocampus ( $66.6 \pm 5.4\%$ ;  $52.6 \pm 4.7\%$ ;  $p < 0.05$ ) and ML enriched fractions ( $71.7 \pm 6.6\%$ ;  $55.6 \pm 5.7\%$ ;  $p < 0.05$ ) compared to contralateral controls, with maximal reduction occurring in the hippocampal 55 kD form. OPN KO mice showed a similar synapsin 1 response to UEC with significant decreases in both the 80 kD and 55 kD forms within the hippocampus ( $73.6 \pm 1.4\%$ ;  $83.2 \pm 3.6\%$ ;  $p < 0.05$ ) and injured ML extracts ( $57.8 \pm 6.7\%$ ,  $72.9 \pm 3.3\%$ ;  $p < 0.05$ ) relative to paired controls. WT animals did not exhibit any differences in synapsin 1 reductions between the hippocampus and ML extracts, however, OPN KO reduction in 55 kD synapsin 1 was significantly lower in the injured ML versus hippocampus ( $p < 0.05$ ). Notably, the full length 80 kD synapsin 1 response did not differ between strains, however, 55 kD synapsin 1 was significantly reduced in the hippocampus of WT mice compared to that of OPN KO mice ( $p < 0.05$ ). This difference, like the previously described lower ML levels of N-cadherin, is consistent with greater proteolysis in that zone of deafferentation. The fact that 2d regional and strain differences are apparent in the 55 kD cleaved synapsin 1 also suggests potential interaction between OPN and synapsin 1 during presynaptic terminals breakdown. Impaired removal of synapsin 1 protein in the hippocampus OPN KO mice is also supported by parallel IHC staining. As anticipated, and consistent with previous literature, synapsin 1 staining is absent in the injured dentate of WT mice compared to controls (**Fig. 3.9 A-B**), indicating the loss of entorhinal fibers which innervate the outer ML of the DG, and the successful ongoing removal of their injured terminals during the acute postinjury phase. In contrast to WT mice, ML synapsin 1 staining is persistent within the injured ML of OPN KO mice (**Fig. 3.9 C-D**),

**Figure 3.9 IHC of Synapsin 1 in WT and OPN KO Mice Following UEC.** Confocal IHC of synapsin 1 after UEC in WT mice exhibited reduced staining in the OML (boxed area, B) compared to control hemisphere (A), indicating the clearance of presynaptic terminals containing synapsin 1, similar to that seen in the rat UEC model. Clearance of axonal terminals was attenuated in OPN KO mice (boxed area, D) suggesting impaired degeneration. Scale bar = 50  $\mu$ m.

# Synapsin 1

2d UEC





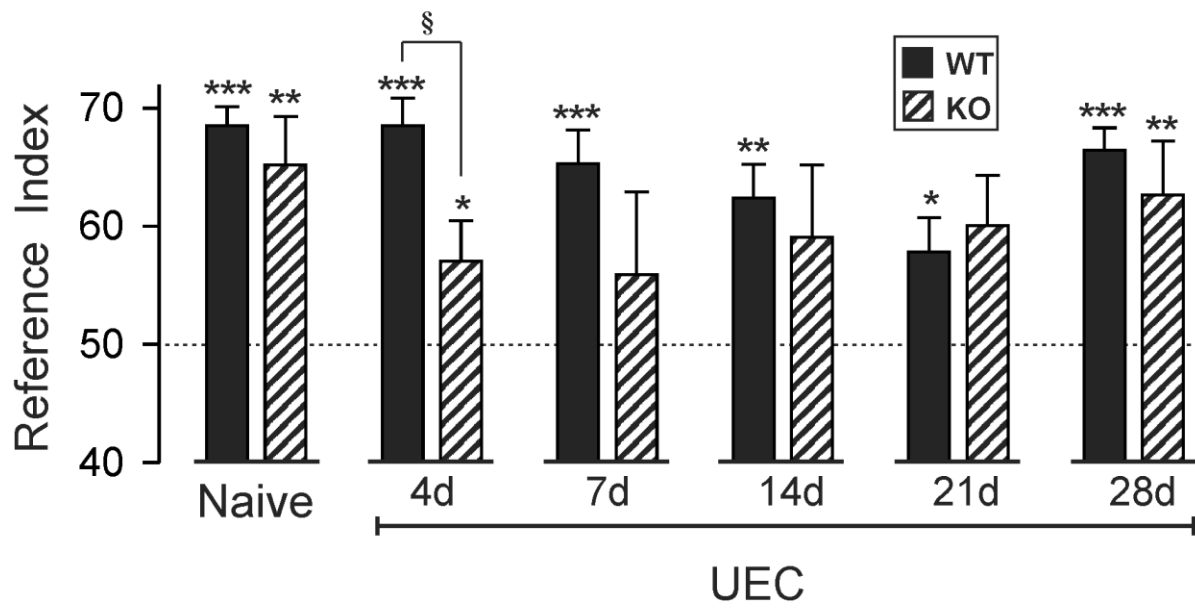
consistent with the incomplete removal of synapsin 1 in the rat UEC model when OPN expression is reduced by minocycline immunosuppression. Together, these IHC results suggest that absence of OPN impairs the removal of degenerating presynaptic terminals from the deafferented ML following UEC. This conclusion is supported by evidence of lower proteolysis of terminal marker protein synapsin 1 at 2d postinjury.

### Absence of Osteopontin Impairs Cognitive Function Following UEC

The NOR task was utilized to examine hippocampal-dependent long term memory function following UEC in mice (**Fig. 3.10**). First, uninjured naïve WT and OPN KO mice were evaluated to confirm the absence of any nascent behavioral differences between strains. Uninjured WT animals successfully recalled the familiar object, spending significantly more exploration time with the novel object compared to chance alone ( $68.5 \pm 1.59$ ,  $p < 0.001$ ). Similarly, OPN KO mice spent significantly more time with the novel object ( $65.2 \pm 4.10$ ,  $p < 0.01$ ), results that did not differ from WT performance, indicating both strains performed similarly in the absence of injury. Following UEC, mice were evaluated for 28d during the recovery window associated with sprouting (4d), synapse reformation (7d), and once a week (14, 21, 28d) during the synapse maturation and stabilization phase until 28 d postinjury, the end of the testing period. Similar to previous reports, we observed that a small percentage of mice did not explore either object, but engaged in unrelated behaviors (e.g., grooming or freezing) (Meunier et al., 2013). Because no RI can be computed for such periods, mice which exhibited this behavioral pattern for greater than 50% of the recorded time ( $n=2$  WT and  $n=5$  OPN KO) were excluded from statistical analysis. Accordingly,  $n=38$  mice were assessed on the NOR task grouped as follows: WT naïve  $n=12$ , KO naïve  $n=10$ , WT UEC  $n=10$ , KO UEC  $n=6$ . WT

**Figure 3.10 OPN KO Mice Exhibit Cognitive Deficits in the NOR Test.** NOR performance during UEC-induced synaptic reorganization (4-28d postinjury) was expressed as the RI metric (see Methods for formula details). RI values for uninjured WT and OPN KO show that mice spent significantly more time exploring the novel object compared to the familiar object. No differences were found between uninjured animals in the two strains. At 4d after UEC, WT RI values were significantly higher than that predicted by chance level behavior, while RI values for OPN KO mice were reduced by half. Although KO performance remained above chance, the percentage of time spent with the novel object at 4d was significantly less than for the WT controls. Between 7- 21d, RI values for OPN KO mice reflected significant cognitive deficits, no longer different from chance performance, while WT mice continued to successfully recall the familiar object. At these later time points, the difference between strains was no longer significant. Interestingly, by 28 days, RI values were similar between strains, with both WT and KO mice recognizing the familiar object and spending significantly more time with the novel object. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , relative to performance at the 50% chance level; § $p < 0.05$ .

# Novel Object Recognition



mice successfully recalled the familiar object after UEC, obtaining RI values significantly above chance throughout the entire testing period. At 4d postinjury, during early collateral sprouting, WT performed similarly to naïve mice ( $68.5 \pm 2.37$ ,  $p < 0.001$ ), most likely due to functional compensation by the intact contralateral hippocampal circuitry. This performance, however, declined throughout sprouting and maturation at 7d ( $65.3 \pm 2.87$ ,  $p < 0.001$ ), 14d ( $62.4 \pm 2.84$ ,  $p < 0.01$ ), and 21d postinjury ( $57.8 \pm 2.88$ ,  $p < 0.05$ ), potentially demonstrating some degree of familiarization with the task. Interestingly, WT performance rebounded 28d after UEC, with RI values similar to that of uninjured mice ( $66.4 \pm 1.89$ ,  $p < 0.001$ ), consistent with long term strengthening of synaptic connections. For the OPN KO group, animals managed to successfully recognize the familiar object 4d postinjury ( $57.1 \pm 3.39$ ,  $p < 0.05$ ), however, RI values were significantly lower than those of WT mice during this window prior to sprouting ( $p < 0.05$ ). KO animals failed to reach criterion for the task at 7d ( $55.9 \pm 7.00$ ,  $p = 0.07$ ), 14d ( $59.1 \pm 6.13$ ,  $p > 0.05$ ), and 21d ( $60.1 \pm 4.24$ ,  $p > 0.05$ ) postinjury, and their exploratory behavior was quite variable. While this variability precluded statistical difference between WT and KO performance at these three time points, KO mice clearly showed cognitive deficits, while the WT mice continued to reach criterion performance. Interestingly, OPN KO behavior trended toward improvement over the testing period, the mice spending more time with the novel object between 14 and 28d postinjury. It was also noted that over the entire testing period, WT and OPN KO showed distinct behavioral curves, with WT drifting toward a nadir at 21d, then returning to naïve performance by 28d, while the KO showed immediate postinjury reduction and persistent deficit until a rebound at 28d. Indeed, both strains showed improved NOR at this four week interval, with RI values (WT  $66.4 \pm 1.89$ ,  $p < 0.001$ ; KO  $62.6 \pm 4.56$ ,  $p < 0.01$ ) nearing those of uninjured naïve animals. Overall, WT mice subjected to trauma performed better than OPN KO

animals after UEC, although each group improved with time, suggesting that OPN loss did not prevent the eventual strengthening of new synaptic connections. This result shows that the absence of OPN impairs the progress of synaptic regeneration and delays functional hippocampal recovery.

### Osteopontin Knockout and Microglial Response to Trauma

Given the change in microglial migration following minocycline attenuation of the acute immune response of the injured rat in Chapter 2, we utilized IHC to stain for active microglia in WT and OPN KO mice and compare glial morphology (**Fig. 3.11**). Similar to the rat model, microglia responded to UEC in WT mice by displaying a reactive morphology and migrating from the outer ML toward the border of intact/deafferented dendrites (arrows, **Fig. 3.11 B**). Microglia in OPN KO mice responded similarly to deafferentation in the injured ML, however, glial morphology differed, revealing a more persistent ramified state, with many extended processes permeating the granule cell layer (arrows, **Fig. 3.11 D**). This pattern of microglial response is consistent with previous PD studies documenting abnormal reactive microglial morphology in response to degenerative insult (Maetzler et al., 2010).

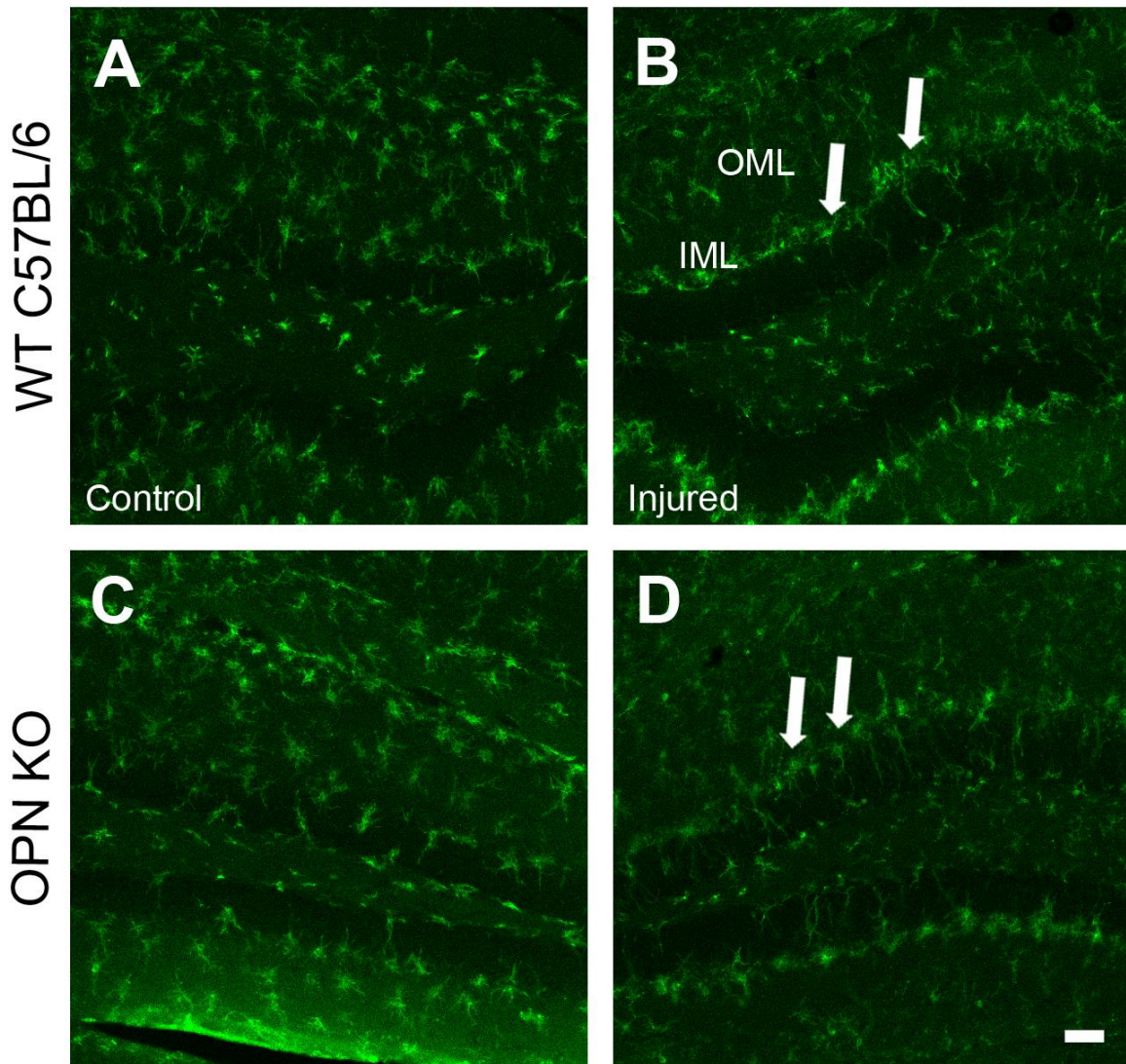
### Osteopontin Knockout, MMP Activity, and Osteopontin Proteolytic Processing

Studies in Chapter 2 indicated MMP processing may play a role exposing integrin receptor binding sites on OPN, subsequently permitting various cell signaling interactions important in reactive synaptogenesis. Given that MMP gelatinase activity increases after UEC in rats, and acute attenuation of the immune response via minocycline dampens OPN, MMP activity, and the production of MMP-cleaved OPN fragments, we further evaluated the potential

**Figure 3.11 Microglial Reactivity in WT and OPN KO Mice Following UEC.** Confocal IHC for microglia 2d postinjury shows transition from resting to activated form, and the notable migration of activated microglia to the border between the intact IML and deafferented OML in both WT and OPN KO strains (arrows). This response is similar to that observed after rat UEC. While OPN KO did not overtly alter microglial migration during the acute phase of reactive synaptogenesis, microglia of OPN KO mice exhibited different morphology, with longer, more interdigitated processes compared to WT mice. Scale bar = 50  $\mu$ m.

# IBA1

2d UEC



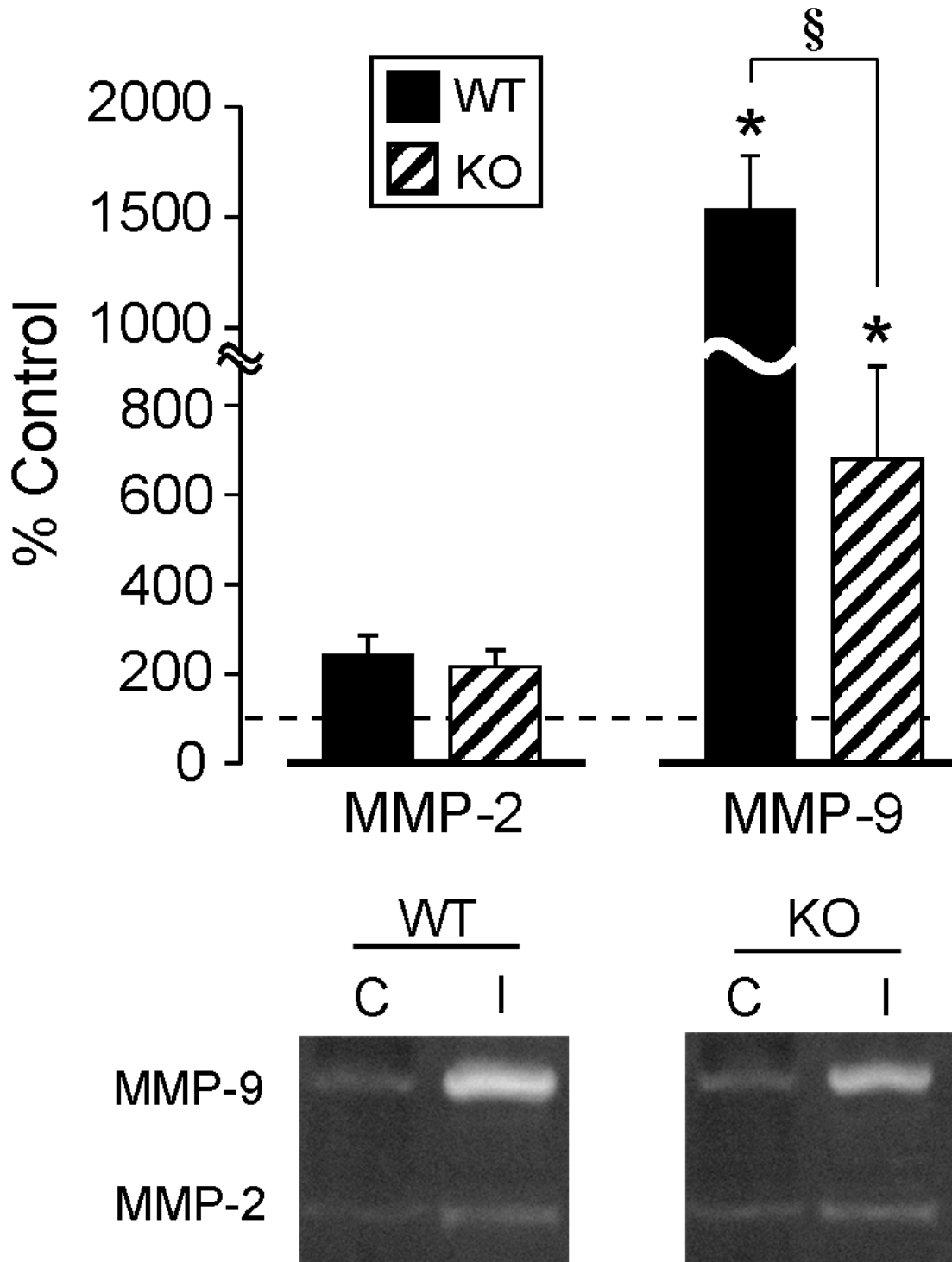
for MMP/OPN interaction as a mechanism regulating synaptic reorganization. We first examined the effect of injury on MMP activity in WT and OPN KO mice utilizing gelatin zymography to assay lytic activity of MMPs -2 and -9 (**Fig. 3.12**). We paired this data with WB analysis of LCN2, a siderophore known to bind MMP-9 and persistently activate the enzyme (**Fig. 3.13**). Following UEC, WT mice showed increased hippocampal MMP-2 ( $239.68 \pm 45.92$ ) and -9 activities ( $1534.45 \pm 244.48$ ,  $p < 0.05$ ) at 2d postinjury, however, only MMP-9 activity elevation was statistically significant compared to contralateral controls. Similarly, injured OPN KO mice showed elevated MMP-2 ( $215.94 \pm 36.33$ ) and MMP-9 ( $679.65 \pm 207.9$ ,  $p < 0.05$ ) activities, with only MMP-9 differences significant. Importantly, MMP-9 activity in OPN KO mice was significantly attenuated compared to WT animals ( $p < 0.05$ ), supporting an OPN/MMP interaction after trauma. Moreover, remaining MMP-9 activity under conditions of OPN KO indicates that MMP-9 function after UEC is dependent upon other molecular signals in addition to OPN. Parallel WB studies evaluating the WT and KO expression of LCN2 revealed two principal bands: a 130 kD form, representing MMP-9 bound LCN2 (Park et al., 2009), and a 55 kD form (**Fig. 3.13**). At 2d after UEC, both the 130 and 55 kD forms of LCN2 were significantly elevated in the injured hippocampus ( $827.88 \pm 221.87$ ,  $p < 0.01$ ;  $237.55 \pm 17.71$ ,  $p < 0.05$ ) of WT mice compared to controls. LCN2 was also elevated in the deafferented ML ( $336.7 \pm 71.3$ ,  $151.1 \pm 5.5$ ;  $p < 0.05$ ), but these changes were significantly lower than within the whole hippocampus ( $p < 0.05$ ), suggesting LCN2/MMP-9 interaction may be occurring in both cell body and dendritic zones of dentate granule cells, but to a lesser degree in the deafferented zone. For OPN KO mice, results were similar to WT animals, both LCN2 forms (130, 55 kD) were also significantly elevated compared to contralateral controls in each region (HC= $271.84 \pm 54.1$ ,  $183.39 \pm 13.50$ ; ML=  $311.1 \pm 60.8$ ,  $153.7 \pm 14.0$ ;  $p < 0.05$ ), however, there were no



**Figure 3.12 OPN KO Attenuates Hippocampal MMP-9 Activity Following UEC.** At 2d postinjury, gelatin zymography shows an elevation in MMP-9, but not MMP-2 activity within the hippocampus of WT and OPN KO mice. Injury-induced MMP-9 activity was reduced in OPN KO mice compared to WT controls. Results are displayed in ROD and expressed as percent of control, with representative gel images shown below. \* $p < 0.05$  relative to paired contralateral controls; § $p < 0.05$ .

# Enzyme Activity

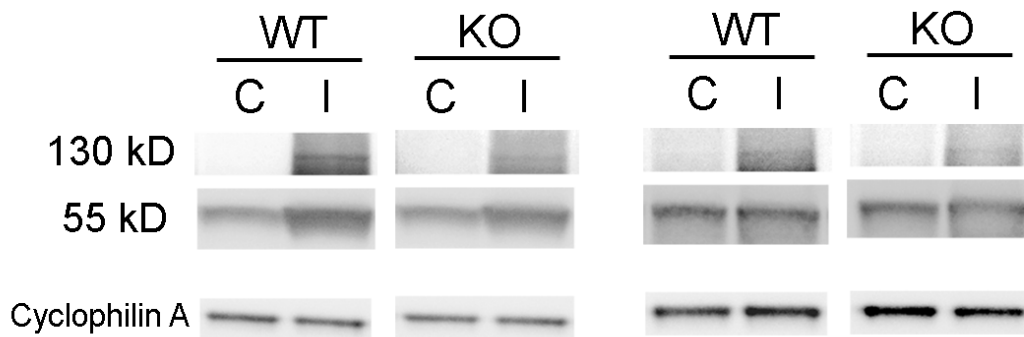
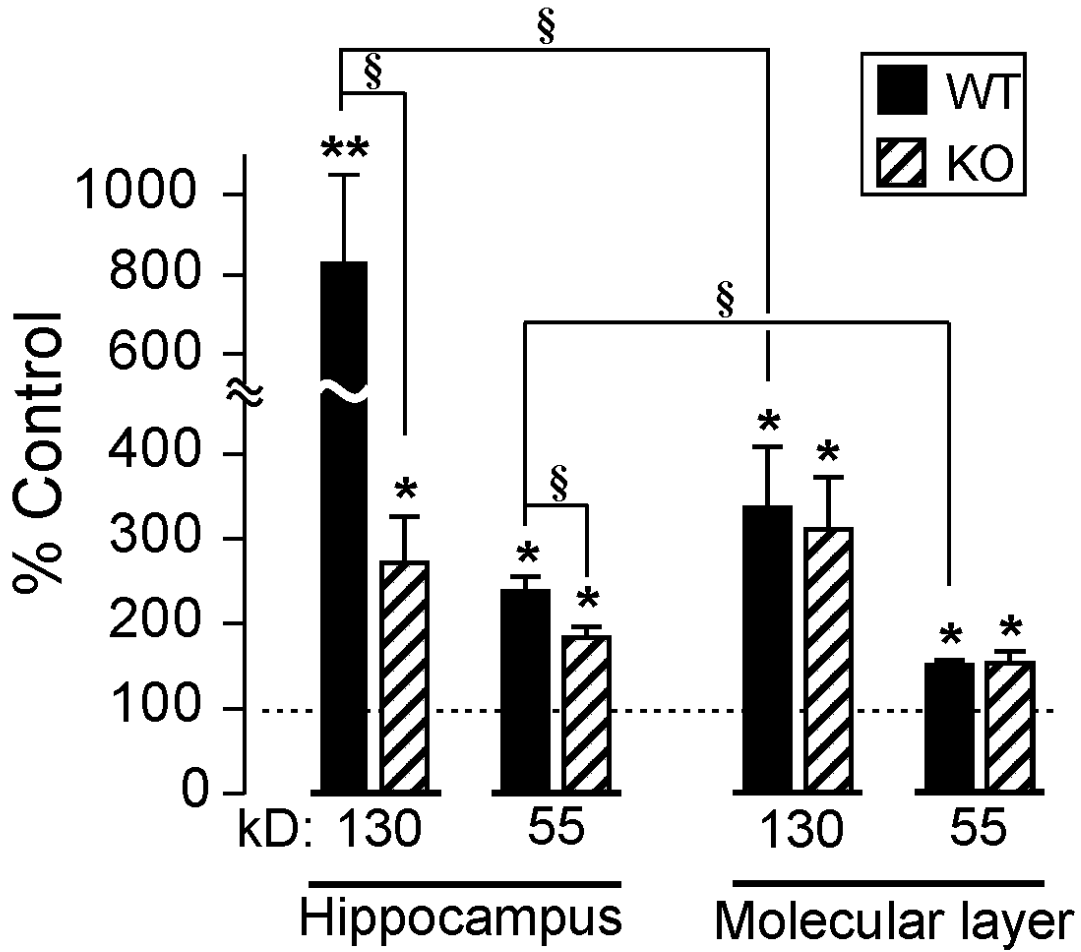
2d UEC Hippocampus



**Figure 3.13 UEC Induces Protein Expression in LCN2.** WB analysis of LCN2 protein in whole hippocampal and enriched ML extracts. UEC induced a significant increase in LCN2 compared to control hemispheres 2d postinjury in both WT and OPN KO mice. Increases in hippocampal 55 kD LCN2 were modest, while the MMP-9 bound 130 kD form increased robustly compared to controls in WT mice. Although both LCN2 bands were reduced in the KO mice, changes in the MMP-9 bound 130 kD form were much greater after OPN KO. For the ML, no strain differences were seen, however, the 55 kD LCN2 signal did show a lower overall elevation in this region versus the whole hippocampus. Results are expressed as percent of control, with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$ , \*\* $p < 0.01$ , relative to paired control cases; § $p < 0.05$ .

# Lipocalin 2

2d UEC



differences between regions. Importantly, we did observe a significant reduction in 130 kD LCN2 response within the KO hippocampal extracts compared to WT mice ( $p < 0.05$ ). This reduction parallels strain differences in MMP-9 activity seen in **Fig. 3.12**, supporting LCN2/MMP-9/OPN interaction during reactive synaptogenesis. In parallel IHC experiments, we found increased LCN2 staining within the deafferented ML of injured WT and OPN KO mice (**Fig 3.14 B, D**), where an overall qualitative reduction in the staining intensity relative to WT was apparent for the injured OPN KO animals.

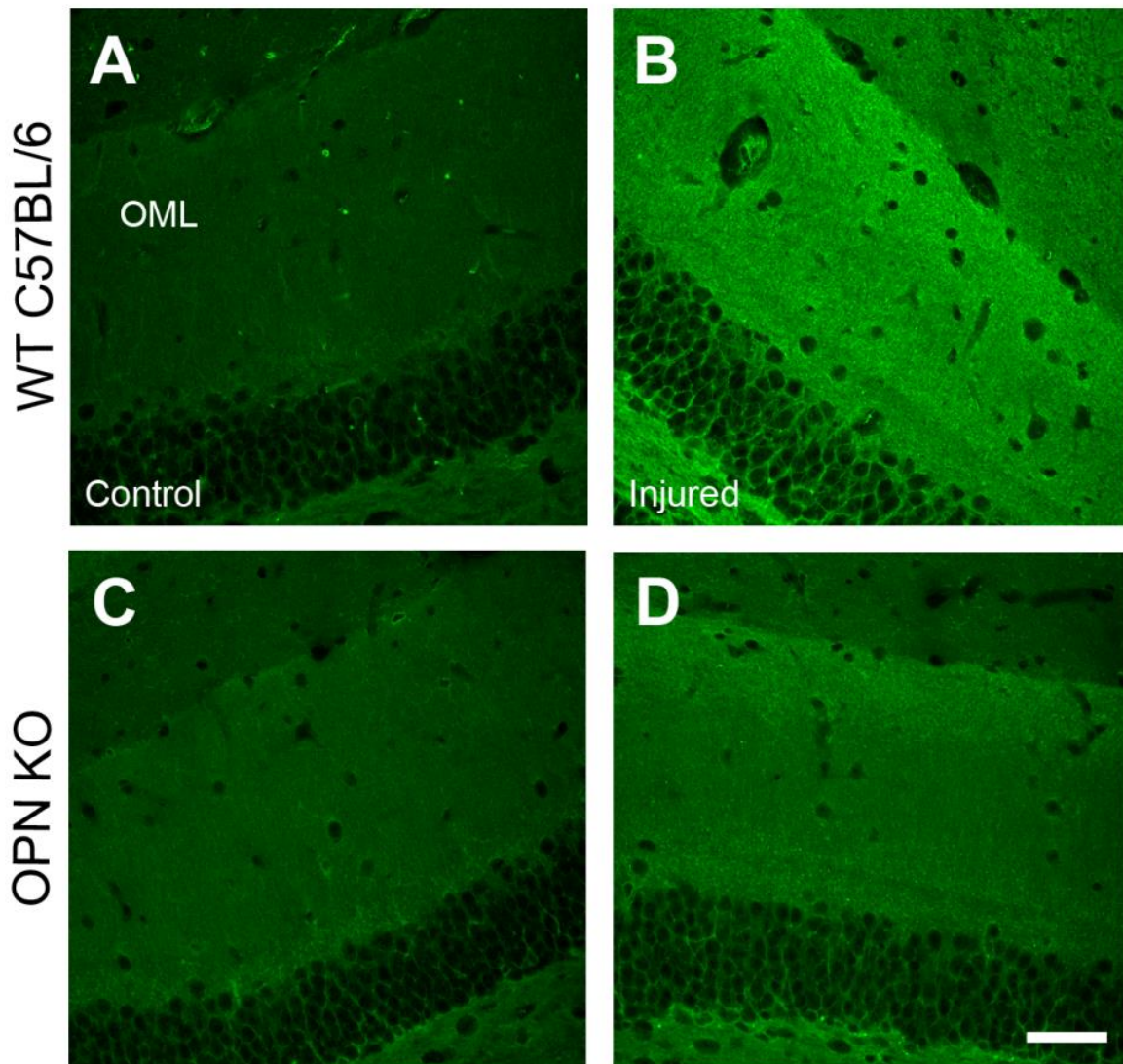
## DISCUSSION

This study utilized OPN KO mice to remove OPN and evaluate its role during adaptive reactive synaptogenesis in the UEC model. Three approaches were taken to assess how the absence of OPN alters successful regeneration. First, using antibodies for separate molecular markers of cytoskeletal organization (MAP1B), synapse stabilization (N-cadherin), and presynaptic terminal integrity (synapsin 1), changes in postinjury protein expression and tissue distribution were mapped in WT and OPN KO animals subjected to UEC. For these measures, focus was placed on the 2d postinjury interval where the rat studies of Chapter 2 showed maximal injury-induced OPN elevation. Here, both whole hippocampal and enriched ML extracts were sampled to determine if OPN KO had different effects in the cell body or dendrite-rich regions. In a second set of experiments, the effect of OPN KO on cognitive functional outcome over the time frame of reactive synaptogenesis (4-28d postinjury) was determined using the NOR task. This task has been successfully used in other mouse TBI models to assess hippocampal dysfunction as a consequence of controlled cortical impact injury or impact acceleration insult (Biegon et al., 2004; Tsenter et al., 2008). In addition, since OPN can be

**Figure 3.14 IHC of LCN2 in WT and OPN KO Mice Following UEC.** Confocal IHC of LCN2 after UEC in both WT and OPN KO mice exhibited increased staining in the ML (B, D) compared to control hemispheres (A, C), supporting protein elevations in WB profiles. LCN2 injury-induced increase was attenuated in OPN KO mice (D), consistent with lower levels of LCN2 protein change in WB data, and paralleling KO effects on MMP-9 activity see again **Fig. 3.10**). Scale bar = 50  $\mu$ m.

# Lipocalin 2

## 2d UEC



cleaved by MMPs to generate integrin binding peptides for cell signaling, the effect of OPN KO on MMP lytic activity and OPN fragment generation was tested. As a correlate to this MMP/OPN interaction, a novel MMP-9 ligand and activator, LCN2, was assayed in WT and OPN KO mice after UEC lesion. Overall, results from WB molecular marker analysis showed that MAP1B, N-cadherin, and synapsin-1 expression were reduced at 2d postinjury in each strain when compared with contralateral controls. However, of the three markers, only synapsin 1 revealed effects of OPN KO and regional sample differences. Notably, the attenuated reduction of 55 kD synapsin 1 expression in KO mice was consistent with the incomplete removal of presynaptic terminals found in the rat experiments of Chapter 2, where OPN expression was reduced with minocycline immunosuppression. In addition, activated microglia in OPN KO mice subjected to UEC presented an altered morphology compared to WT mice. These observations suggest that OPN plays a more prominent role in the presynaptic morphing during the early phases of reactive synaptogenesis. When NOR task performance was evaluated, both uninjured WT and OPN KO mice recognized the novel object at significantly higher than chance levels. Further, deficits in recognition were detected at 4, 7, 14, and 21d postinjury with OPN KO compared to injured WT cohorts. Interestingly, injured OPN KO mice also showed reduction in MMP-9 lytic activity versus WT animals subjected to UEC, and this reduced MMP-9 activity was correlated with attenuated expression of the MMP-9-bound form of LCN2. Together, these findings support OPN role in presynaptic terminal removal during the acute phase of synaptogenesis, involving integrin cell signaling through MMP-9/LCN2-mediated generation of OPN fragments. Moreover, this process is critical for adaptive outcome during the regenerative phase of synaptogenesis since the NOR deficits are detected 4-21d postinjury when



OPN is absent. From the present OPN KO results, new details have emerged regarding the importance of OPN in the acute molecular mechanisms supporting adaptive synaptic plasticity.

### Osteopontin, MAP1B, and Synaptic Cytoarchitecture

One of the first cellular responses at the onset of reactive synaptogenesis is morphological reshaping of neurons and glia to allow clearance of degenerated presynaptic terminals. Reactive glial cells become hypertrophic and extend their processes to degrade dying presynaptic terminals, while postsynaptic dendrites retract to reform their spine structure. All of these processes require significant change in cytoskeletal proteins. MAP1B belongs to a family of growth and cytoskeletal proteins, including MAP2 and tau, which regulates microtubule assembly underlying the structural integrity of glia and neurons (Emery et al., 2003). This family of proteins is found in somata, axons, and dendrites, with extensive expression in developing axons of the immature brain (Yamanouchi, 2005; Riederer, 2007). MAP1B is one of the first MAPs expressed during rat embryonic development, and can be found in a number of structures including the olfactory system, cerebellum, and spinal cord (Gonzalez-Billault et al., 2004; Tortosa et al., 2011). This intense developmental expression decreases with aging, and is nearly absent in the adult CNS, with the exception of sites which exhibit a high level of regenerative plasticity such as the hippocampus and olfactory system (Riederer and Matus, 1985; Safaei and Fischer, 1989). MAP1B is a large protein with a molecular mass of 255 kD and, like many microtubule associated proteins, undergoes phosphorylation and/or cleavage to generate additional functional forms (Riederer, 2007). Given that MAP1B can influence cell polarity and axonal outgrowth, its potential for facilitating regeneration in the adult CNS has been explored in neurological dysfunction and injury (Bates et al., 1993; Gai et al., 1996). For example, MAP1B

is localized to Amyloid- $\beta$  (A $\beta$ ) plaques and tau tangles in Alzheimer disease (Uchida, 2003), as well as visible in Lewy bodies of Parkinson disease (Gai et al., 1996). Further, *in vitro* A $\beta$ -mediated apoptosis reduces MAP1B expression through calpain and caspase cleavage (Fifre et al., 2006). By contrast, MAP1B also supports adult neuroregeneration, with documented increases in phosphorylated MAP1B during axon regrowth in trochlear neurons (Book et al., 1996), sciatic nerve lesion (Bush et al., 1996; Soares et al., 2002), and SCI (Soares et al., 2007). These examples show the active participation of MAP1B in shaping the cytoskeleton of neurons during both degenerative and regenerative processes.

The active role of MAP1B in both neurodegeneration and regeneration highlights the importance of time-dependent MAP expression in neurological disease or following traumatic insult. Rodent models of ischemia show that increased MAP1B expression does not occur until 7d after MCAO, suggesting that it plays a predominant role in the regeneration associated with recovery (Popa-Wagner et al., 1999). Other studies have also reported that reduction in MAP1B inhibits regenerative capacity, as demonstrated by delayed glial or MAP1B response as late as 14-28d postinjury (Badan et al., 2003). Together, these studies suggest that early reduction or loss of MAP1B may be linked to the structural disruption associated with early degeneration, but increased MAP1B would subsequently accompany regeneration as cytoarchitecture supports neuronal regrowth. Interestingly, a recent report has linked such cytoskeletal growth to OPN, utilizing a yeast 2 hybrid study to demonstrate that intracellular OPN binds MAP1B in the rat substantia nigra SN and striatum (Long et al., 2012). Given that MAP1B has the potential to bind OPN in the brain, is associated with glial-mediated regeneration after CNS injury, and the fact that moderate lateral fluid percussion injury induces MAP1B expression (Emery et al., 2000), we posited that MAP1B/OPN interaction may be present during the acute phases of UEC

reactive synaptogenesis. We evaluated MAP1B in its full length 220 kD, as well as cleaved 80 and 35 kD forms. In the rat studies of Chapter 2, UEC deafferentation induced cytoskeletal degradation and subsequent removal of the injured axons, a finding marked by a decrease in MAP1B expression at 2d postinjury. This result was consistent with other reported injury-induced MAP1B reductions within the hippocampus such as following gerbil forebrain ischemia (Tanay et al., 2006). A similar pattern was observed in our WT and OPN KO mice, where MAP1B expression in the hippocampal extracts was significantly reduced for both the 220 and 80 kD forms compared to controls. Interestingly, only WT showed significant reduction of the hippocampal 35 kD MAP1B. These changes in MAP1B expression are consistent with reported calpain- caspase-mediated proteolysis of MAP1B (Fischer et al., 1991; Fifre et al., 2006), and notably, these two enzymes are known to mediate neuronal damage following TBI (Saatman et al., 1996; Pike et al., 1998). For ML enriched extracts the overall effect was reduction, however, the significance of this reduction varied with respect to MAP1B form and mouse strain. Both the 220 and 80 kD forms of MAP1B tended to decrease in both the WT and KO animals, reaching significance for both forms in the WT, but only for the 80 kD in KO mice.

Given that significant reductions in 80 kD MAP1B occur for both strains, and in both sampled regions, we interpret the 80 kD MAP1B as the principal form mediating trauma-induced cytoarchitectural rearrangement within the dendrites of the mouse dentate gyrus. More importantly, regional comparisons indicated no significant differences between WT and KO strains, suggesting the intracellular form of OPN which associates with MAP1B is less likely to participate in the injury-induced structural reorganization at 2d after UEC. This result supports our rat observations in Chapter 2, which point to little or no colocalization of MAP1B and OPN, and a more prominent role for secreted, cleaved, OPN in chemokine and astrocyte signaling at

2d postinjury. Interestingly, IHC staining for MAP1B in the two mouse strains did not show notable postinjury differences, however, the OPN KO mice appeared to show stronger MAP1B signal. Since the WB results showed nearly identical strain and regional patterns for MAP1B expression, and the IHC label represents an aggregate of all three blot signals, it might be predicted that no gross staining differences would be observed. Surprisingly, the clear shift in MAP1B distribution associated with dendritic retraction seen in the rat after UEC was not found in either mouse strain. On the surface, this finding would suggest species differences in the role of MAP1B after UEC which remain to be explored. Clearly, deafferentation in the WT mouse produces presynaptic terminal loss identical to the rat as evidenced by shifts in synapsin 1 staining, but microtubule rearrangement must preferentially involve other MAPs in the mouse models. Together, these studies indicate that while secreted OPN can act as an inflammatory cytokine during the acute degenerative phase of reactive synaptogenesis, the modification of cytoskeletal structure through intracellular OPN/MAP1B binding does not appear to play a significant role in the process. Without OPN, we found no difference in the injury-induced change of MAP1B expression.

#### Osteopontin, N-cadherin, and Stabilization of the Synaptic Junction

Given that OPN did not appear to have overt effects on cytoskeletal integrity through MAP1B binding mechanisms during the degenerative phase of synaptogenesis, we next examined its potential influence on N-cadherin, a CAM important in the stabilization/destabilization of the synaptic junction. N-cadherin belongs to a larger family of cadherins which are  $Ca^{2+}$ -dependent CAMs important in stabilizing cell-cell interactions. Roughly 80 cadherins are expressed within the CNS, playing a role in development and

organization of the nervous system, including axonal outgrowth and synapse targeting (Doherty and Walsh, 1992). Cadherins are often found at synapses where they are the major structural protein comprising the adherens junction (Yap et al., 1997), and have been more recently implicated in the modulation of synaptic plasticity in the adult brain (Huntley et al., 2002). N-cadherin engages in homophilic binding on either side of synapses, forming adhesion zippers which stabilize cell-cell interactions (Shapiro et al., 1995). Synaptic plasticity during CNS development or following injury, requires the dissociation of pre and postsynaptic connections to permit the establishment of new contacts during synaptic reorganization, a process which inherently alters the expression of stabilization molecules such as N-cadherin (Shan et al., 2002). Interestingly, cadherins can be localized to specific synapse types, with N-cadherin found rather exclusively with excitatory synapses, such as those which are present on dentate granule cells of the hippocampus (Benson and Tanaka, 1998). This location makes N-cadherin a critical mediator of the stabilization between the presynaptic terminals and postsynaptic spines affected in our UEC model of adaptive plasticity. Time-dependent changes in N-cadherin expression occur following traumatic insult, being reduced during periods of acute synaptic degeneration and elevated later, when synapses regenerate (Takeichi and Abe, 2005; Warren et al., 2012). Specifically, at 2d following UEC-induced deafferentation in the rat, N-cadherin expression is significantly reduced (Warren et al., 2012), supporting its active role in synapse destabilization as degenerating terminals are removed to prepare dendritic sites for reinnervation. Interestingly, OPN expression has been inversely correlated with E-cadherin expression in the plasticity associated with hepatocellular carcinoma, potentially mediating cellular and structural growth during cancer invasion through the ECM (Iso et al., 2005; Korita et al., 2008). These findings suggest that the OPN increases we observed in Chapter 2 may be correlated with altered N-

cadherin early after injury, and the process is a part of immune system control of synapse destabilization for the removal of injured terminals during the degenerative phase of reactive synaptogenesis.

Similar to previously published studies in the rat model of UEC, N-cadherin levels were reduced in WT and OPN KO mice 2d postinjury. However, no significant strain or regional differences were found, suggesting that loss of OPN did not alter the overall acute changes in N-cadherin protein at this postinjury interval. Nevertheless, compared to contralateral controls, some differences in profile between strains and region were suggested. Both the 115 kD full length form and 35 kD cleaved fragments showed lower expression in the hippocampus and ML-enriched fractions of WT mice subjected to UEC, however only the full length 115 kD reductions in N-cadherin reached significance. For OPN KO mice, the 115 kD form showed a trend toward reduction in the hippocampus, while both forms were significantly reduced in ML. Parallel IHC for N-cadherin showed similar diffuse and cellular ML labeling as described for rat UEC at this time point, but no qualitative differences between control and injured WT mice were observed. However, KO mice did show apparent increase in N-cadherin-positive cellular staining within the ML. This elevated N-cadherin staining in the non-neuronal cells of the deafferented zone correlates with a trend toward greater reduction of N-cadherin fragment signal in the ML blot samples relative to those of the whole hippocampus, suggesting the possibility that higher IHC signal in the OPN KO may reflect an accelerated lysis of N-cadherin in reactive neuroglia. Together, these results point to only minor differences in N-cadherin expression when OPN is absent during the acute UEC-induced immune response. While additional studies will be required to explore N-cadherin processing in the OPN KO, the present findings do not support

modulation of N-cadherin and synapse stabilization as a major function of OPN during the early stages of hippocampal synaptic reorganization in mice.

### Osteopontin, Synapsin 1, and Terminal Integrity During the Acute Immune Response

A third critical component of the acute phase of reactive synaptogenesis is the clearance of degenerating presynaptic terminals. Immune-induced OPN response could play a role in facilitating this axonal clearance, a possibility supported by its localization in reactive microglia at the acute postinjury phase. To investigate this potential OPN interaction, we again probed OPN KO mice, using the expression of the presynaptic terminal marker synapsin 1 to assay whether the removal of these terminals is altered when OPN is absent.

Synapses are integral to the cell-cell transmission of information between neurons, principally through neurotransmitter release from synaptic vesicles. One protein family essential to this process are the synapsins. They are among the most abundant synaptic proteins present in the majority of nerve terminals (Goelz et al., 1981) and are specifically localized to the presynaptic compartment of synapses (De Camilli et al., 1983a; Fletcher et al., 1991; Ferreira et al., 2000). Within presynaptic terminals, synapsins interact with the cytoskeleton, as well as contribute to synapse formation as evidenced by their high level of expression during developmental synaptogenesis (Lohmann et al., 1978; De Camilli et al., 1983a; Mason, 1986; Haas and DeGennaro, 1988; Melloni et al., 1994; Bogen et al., 2009) and LTP induction in the adult brain (Lynch et al., 1994; Sato et al., 2000). Several different synapsin proteins exist, synapsin 1 being the first described, which is expressed as two nearly identical proteins in 86 and 80 kD forms (De Camilli et al., 1979; De Camilli et al., 1983b). As a protein functioning through cytoarchitectural interaction, synapsin 1 is capable of undergoing extensive

phosphorylation by protein kinase A (PKA) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaMK), posttranslational modifications which modulate its ability to bind synaptic vesicles to the cytoskeleton (De Camilli et al., 1983b; Cesca et al., 2010). Synapsin 1 binds many structural components within the presynaptic terminal, including actin (Bahler and Greengard, 1987; Petrucci and Morrow, 1987), spectrin (Bennett et al., 1985; Krebs et al., 1987), microtubules (Baines and Bennett, 1986; Goldenring et al., 1986), and neurofilaments (Steiner et al., 1987). The shift in synapsin 1 phosphorylation and dephosphorylation underlies its role bundling structural filaments during synaptic vesicle aggregation or neurotransmitter release. While synapsin 1 has historically been considered for its role in presynaptic vesicle release (Sudhof, 1990; Bahler et al., 1990; Greengard et al., 1993), evidence from recent *in vitro* and *in vivo* studies with suggest a role for synapsins in the formation of synaptic contacts (Lohmann et al., 1978; Melloni et al., 1994; Ferreira et al., 2000) and presynaptic terminal formation (Han et al., 1991). Further, synapsin 1 mediates neurite elongation in postnatal development (Melloni et al., 1994) within growth cones of cerebellar granule cells (Harada et al., 1990) and hippocampal neurons (Fletcher et al., 1991), providing support for a potential role in plasticity following CNS trauma. Our current studies provide evidence that OPN and synapsin 1 interact to influence the progress and outcome of reactive synaptogenesis.

Previous studies have documented time-dependent alterations in synapsin 1 expression following CNS trauma. The protein was reduced early following mTBI (Wu et al., 2011a) and CCI (Griesbach et al., 2009), which correlated with poor cognitive behavior assayed by MWM performance. Following UEC deafferentation, there is a loss of synapsin 1 in the dentate ML (2d postlesion), marking the removal of damaged presynaptic terminals, while its expression reemerges during synapse regeneration (7d postlesion) when new terminals are generated by



collateral sprouting. Interestingly, these early changes in synapsin 1 correlate with the acute OPN response to traumatic insult, and more recent studies have linked synapsin 1 to glutamate release in osteoblasts, a rich source of OPN, a process utilizing molecular mechanisms similar to synaptic neurotransmission (Bhangu et al., 2001). Further, synapsins are implicated in the development of schizophrenia, and recent proteomic analyses of experimental models of schizophrenia-associated elevated OPN levels with alterations in the hypothalamic cytoskeleton, providing a potential functional link between OPN and synapsin 1 during aberrant synaptic organization in the brain (Guest et al., 2012). Taken together, these published studies lend credence to the idea that OPN may interact with synapsin 1 after brain injury, mediating cytoskeletal breakdown and subsequent presynaptic terminal removal during the degenerative phase of reactive synaptogenesis.

Given that synapsin 1 plays a key role in the organization of presynaptic terminals, it's potential to interact with OPN, and the prominent presynaptic terminal degeneration at 2d after UEC, we used synapsin 1 as marker to assess how OPN affects the presynaptic component of acute reactive synaptogenesis. Following UEC, WB analysis of synapsin 1 showed significant reductions in the hippocampus and ML of both strains for the 80 kD full length form, as well as a functional 55 kD fragment, a predicted result for the 2d degenerative phase. This pattern is consistent with cysteine protease cleavage (Bahler et al., 1989) and previous studies demonstrating interaction of synapsin fragments with cytoskeletal proteins such as spectrin (Sikorski et al., 1991; Sikorski and Goodman, 1991). This overall reduction in synapsin 1 reflected the UEC-induced clearance of presynaptic terminals seen in the rat studies of Chapter 2. In contrast to the MAP1B and N-cadherin studies, which did not show strain differences after injury, we did observe a significant attenuation of hippocampal 55 kD synapsin 1 loss compared

to WT mice when OPN was absent. This result suggests that the absence of OPN impairs synapsin 1 (and axonal terminal) clearance, which is, interestingly, quite similar to the effect of minocycline induced OPN reduction on synapsin 1 IHC signal in the rat studies of Chapter 2. While WT mice did not show regional differences in UEC-induced synapsin 1 expression, OPN KO mice did. The 55 kD synapsin 1 reduction was significantly greater in the ML of OPN KO mice compared to hippocampus, consistent with a more critical OPN role in degenerating terminal removal in dendritic versus cell body enriched zones. If this OPN influence on terminal removal is mediated through synapsin 1, it would be predicted that altered microglial migration and lower OPN expression in the ML would be correlated as well. We did, in fact, see this correlation with synapsin 1-marked clearance of synaptic terminals following acute attenuation of the immune response in Chapter 2. IHC staining for synapsin 1 in the dentate gyrus of WT and KO mice further supported poor removal of degenerating axon terminals when OPN expression is altered. Loss of ML staining was incomplete in KO mice compared with paired WT animals, a finding similar to that of Chapter 2 rat studies following acute minocycline administration.

Since both OPN reduction with minocycline and OPN KO attenuated the clearance of presynaptic terminals, and this clearance is dependent, in part, upon activation of ML microglia, we predicted that OPN KO mice would also show a different microglial response than WT after UEC. As expected, IHC staining for activated microglia in uninjured hippocampus of WT and KO mice exhibited similar profiles of randomly distributed and ramified microglia. After UEC, as in the rat model, microglia switched to an activated morphology and migrated to the border of the intact and injured dendrites, however KO microglia had longer processes with greater interdigitation between the dentate granule cell bodies, morphology similar to resting microglia.

While the postinjury pattern of microglial distribution was similar between strains, this difference in morphology suggests impaired ability to transform to full reactive state following deafferentation. Similar reports of altered microglial morphology with increased process length compared to WT controls was previously reported by Maetzler and colleagues (2007) in MPTP models of PD. Attenuated synapsin 1 removal correlates with this altered microglia morphology in the KO, also supporting OPN role in regulating microglial activation in the acute phases of trauma-induced synaptic plasticity. In aggregate, our studies of synaptic markers from OPN KO mice subjected to UEC point to an instrumental role for OPN in mediating the efficacy of axon terminal removal in order to prepare the local environment for collateral sprouting and new synapse formation. OPN achieves this role, in part, through its direction of microglial migration and activation during acute postinjury intervals. The impact of these actions is to facilitate effective synaptic reorganization and return of function following CNS trauma.

#### Osteopontin Knockout Mice and Cognitive Function

In our examination of proteins marking the different components of injured synapses following UEC, synapsin 1 expression alone was found to be altered with OPN KO, while the loss of OPN failed to change postinjury expression of the cytoskeletal marker MAP1B or synaptic junction marker N-cadherin. This attenuation of synapsin 1 degradation in the KO was consistent with reduced synapsin 1 removal at the intact and deafferented dendritic boundary of the rat UEC when OPN was suppressed by minocycline. Further, OPN KO mice also showed an altered distribution of reactive neuroglia similar to that observed in Chapter 2 for the rat maladaptive TBI+BEC model, animals which have persistent cognitive deficits following deafferentation. We interpret these findings as evidence that axotomized degenerating

presynaptic terminals are inadequately removed when OPN is lacking. Since synapsin 1 is a molecule critical for both presynaptic terminal formation and neurotransmitter release, we reasoned that impaired removal of presynaptic terminals with ablation of OPN slows the necessary acute synaptic degeneration process, which delays new synapse formation, resulting in abnormal hippocampal connections and the associated cognitive deficits.

To test whether OPN KO mice exhibited impaired cognitive function, we utilized the NOR test, a hippocampal-dependent task which takes advantage of rodents' natural curiosity and exploratory behavior to test their capacity for long term memory recall. NOR was first described in 1988 by Ennaceur and Delacour as a novel one-trial test to examine memory recall in rodents (Ennaceur and Delacour, 1988). Here, animals are presented a series of 2-object sets which they are allowed to explore freely, and successful recall of an object results in a reduced exploratory time as this object is familiar and not as interesting. Performance in this task is commonly calculated by the metric of RI. This measure defines the percentage of time spent with the novel object divided by the total exploratory time. A number of studies have demonstrated successful use of this paradigm, in TBI-induced damage, including assessment of hippocampal function following impact acceleration (Baratz et al., 2011; Siopi et al., 2012) or controlled cortical contusion (Han et al., 2011; Tong et al., 2013) While OPN KO mice are considered to be developmentally normal, no studies have examined their behavioral characteristics pertaining to cognitive function, thus we first evaluated uninjured OPN KO mice to verify that their baseline behavior in the NOR task did not differ from paired WT control mice. Uninjured naïve animals were capable of recalling the familiar object, with both strains spending 65-68% of their exploration time with the novel object. While the uninjured WT animals, on average, spent more time with the novel object than the OPN KO animals, the RI values were not significantly

different, confirming that both strains had comparable hippocampal function in this test and no gross behavioral differences in attention, natural curiosity, or aversion to open field testing existed between the strains. Following UEC injury, we focused on testing between 4-28d assaying behavioral status at the end of the OPN rich degenerative phase (4d), during the initiation of collateral sprouting (7d) and three times during the subsequent interval of synapse maturation and stabilization (14, 21 and 28d).

At 4d postinjury, WT mice performed well, spending a significant amount of time with the novel object in order to perform above criterion performance at the task, and having an RI similar to WT uninjured animals. This apparent behavioral compensation after UEC might be interpreted as an indication that the task was not sensitive enough to detect cognitive deficits in these mice. An alternative, and more likely explanation, is that WT mice are able to generate sufficient activation for NOR behavior from the circuitry of the remaining intact contralateral hippocampal formation. Interestingly, this result is consistent with more rapid and efficient cognitive recovery when entorhinal lesion is performed unilaterally versus bilaterally in the rat (Steward et al., 1977). While OPN KO mice also spent more time with the novel object at 4d postinjury, RI values were significantly lower than WT animals, suggesting that the absence of OPN did alter functional status at the end of the acute degenerative phase of reactive synaptogenesis. Moreover, this result supports the importance of the events occurring during the initial 1-4d postinjury period, including presynaptic terminal clearance and establishment of degenerative boundaries, each preparing the ECM for regeneration. Importantly, we now have evidence that disruption of these events is temporally correlated with OPN loss and cognitive disruption. During the 7-21 testing period several interesting differences in NOR RI measures were found. WT mice continued to spend more than 50% of their time exploring the novel

object however, this group did show an interesting trend toward reduced RI at 21d of testing. It is possible that this reduction in NOR for the WT mice was caused by either shifts in attention to the task at this time point, or alternatively, the extensive synaptic pruning and stabilization occurring in regenerated hippocampal circuitry at this postinjury interval. The latter interpretation appears to be more likely since we found WT NOR performance at 28d had returned to a level equivalent to that of the 7d animals. Interestingly, this reduction in task performance at 21d and rebound to higher RI at later time points, is similar to that reported after WT CCI, where mice with hippocampal damage showed improved NOR during the first two weeks after injury, reduction in performance at three weeks postinjury, and a return to higher RI values at four weeks postinjury (Han et al., 2011). By contrast, OPN KO mice failed to reach criterion for NOR at 7d and, while they did show a trend toward better performance, the KO animals continued to fail at the task through the 21d testing period. There was also no significant difference between the RI values of the two strains over 7-21d, likely the result of high variance in cognitive performance for the OPN KO group. Interestingly, OPN KO mice did show recovery to above criterion performance at 28d postinjury, suggesting that the deficits in functional capacity can be compensated for by long term adjustments in hippocampal circuitry.

The present NOR results, spanning degenerative-regenerative phases of synaptogenesis, indicate that loss of OPN does alter the reemergence of cognitive function typically observed during the first three weeks after UEC (Loesche and Steward, 1977; Steward, 1981).

Performance is consistently lower than in paired WT cohorts subjected to UEC. Our NOR data also suggest that rapid, extensive rise in OPN postinjury expression is critical to the timely evolution of functional synaptic reformation after CNS deafferentation. Further, given our observations of reduced synapsin 1 degradation at 2d in the OPN KO, and the abnormal

persistence of synapsin 1 tagged presynaptic terminals in the rat when OPN is reduced, it is possible that change in OPN expression may underlie the NOR deficits seen at 4d compared to WT animals, and likely contribute to the slow, variable recovery of RI which follows. This mechanistic link is plausible since the efficacy of LTP, one of the classic cellular templates for synaptic plasticity and memory formation, can be altered by the persistence of damaged synapsin 1 containing presynaptic terminals (Sato et al., 2000). In our OPN KO model, this may manifest itself in reduced hippocampal function at 4d postinjury, prior to synaptic sprouting. The present results also show altered synapsin 1 levels in hippocampal tissue of KO mice, and associate a change in glial distribution with decreased hippocampal function in OPN KO mice. These observations suggest that OPN direction of the cell-mediated immune response fostering proper synaptic terminal removal and ECM conditioning is critical to the generation of functionally adaptive reactive synaptogenesis. Our NOR behavioral results are the first to demonstrate impaired cognitive function following CNS insult in OPN KO animals and is consistent with SCI studies which show that OPN null mice have impaired locomotor recovery compared to WT mice following crush injury (Hashimoto et al., 2007). Clearly, OPN KO mice are a good tool for further probing the role of OPN in mediating axonal and dendritic response during trauma-induced synaptic reorganization.

#### Osteopontin and LCN2-Mediated MMP-9 Lysis of Osteopontin

Previous results in Chapter 2 showed a correlation between the extent of trauma-induced MMP-9 activity and the postinjury level of the integrin binding 45 kD OPN. Notably, acute minocycline administration attenuated UEC-induced OPN expression and reduced both MMP-9 activity and 45 kD OPN. Based upon these findings, we hypothesized that injured WT mice will

express elevated MMP-9 activity and, by contrast, OPN KO mice will have reduced enzyme activity. We also posited that LCN2, a novel ECM regulator of MMPs, capable of binding MMP-9 to effect its persistent activation (Kjeldsen et al., 1993; Yan et al., 2001), will be increased in WT, and reduced along with MMP-9 activity in OPN KO mice. To determine if LCN2-mediated MMP-9 activity contributes to generation of integrin binding OPN fragments, we utilized gelatin zymography and WB analysis to determine MMP-9 activity and LCN2 expression in injured WT and OPN KO mice. We found that, similar to rats, both mouse strains had elevated MMP-9 activity 2d after UEC. While UEC-induced MMP-9 activity was significantly increased in both strains relative to contralateral controls, the elevation in KO mice was attenuated by 55% compared to that of WT mice, mimicking the effect of acute minocycline administration in Chapter 2. These results suggest that, in the absence of OPN, MMP-9 activity is reduced, and further supports a role for MMP-9 in the processing of injury induced OPN. It should be noted that, despite the significant reduction compared to WT mice, injury-induced MMP-9 changes in OPN KO mice remained significantly higher than controls, clearly indicating that MMP-9 is active in other proteolytic pathways besides that of OPN. Since MMP-9 has been associated with oligodendroglial response after CNS insult (Kim et al., 2012; Seo et al., 2013), this MMP-9 inhibition in OPN KO mice suggests that MMP-9/OPN interaction may be important to the remyelination repair of damaged axons, as well as directing successful synaptic reorganization and axonal outgrowth. While MMP-2 was elevated in both strains, it did not differ significantly from controls as was observed for rat UEC in Chapter 2, which again highlights species differences, and sample size effects.

Given these differences in MMP activity between strains, we examined LCN2 response to injury in the same hippocampus and ML extracts evaluated for synaptic markers in order to



determine if MMP-9 binding to ECM LCN2 could regulate enzyme activity. Recently, LCN2, an immune molecule which sequesters iron-containing pathogens, has been shown to contain not only a hydrophobic binding pocket, but also a ligand-binding domain for MMP-9 (see again **Fig. 3.1 C**). Specifically, LCN2 is capable of binding MMP-9 to persistently activate this protease within the ECM (Kjeldsen et al., 1993; Yan et al., 2001). In our analysis, two principal WB signals at 130 and 55 kD were detected. It is likely that the 130 kD band represents the MMP-bound form of LCN2 (Park et al., 2009), and the 55 kD, a free form of LCN2. Results showed a significant increase in both the 130 and 55 kD LCN2 within the hippocampus following UEC in WT and KO mice, however elevations in the OPN KO were significantly lower than that of WT mice. Interestingly, this LCN2 reduction in the OPN KO hippocampus was proportional to the reduction in hippocampal MMP-9 activity, implicating involvement of LCN2-mediated MMP-9 activation in the processing of OPN for effective synaptic reorganization. Within ML-enriched extracts, WB analysis showed a significant increase of both LCN2 forms following UEC in both WT and OPN KO mice compared with controls, however alterations in LCN2 did not statistically differ between strains. Interestingly, there were no regional difference in KO mice, but WT mice showed hippocampal LCN2 elevation which was significantly higher than that of the ML. This finding suggests LCN2-mediated OPN function is not concentrated within the ML, but important in heavily OPN positive cellular laminae as well. Further, IHC for LCN2 supports WB elevations within the hippocampus of WT and OPN KO mice, staining which is pronounced in the deafferented ML. Together, these results suggest a time-dependent link between OPN, the immune response, and MMP-9 activity during acute postinjury intervals. They also support LCN2-MMP-9 activation as a mechanism for processing of OPN signals which contribute to the generation of effective functional recovery.

## SUMMARY

The experiments of this chapter utilized the OPN KO mouse to test OPN involvement in the acute phase of reactive synaptogenesis, complementing the profiling of OPN elevation after hippocampal deafferentation discovered in Chapter 2. Relative to injured WT control mice, OPN KO subjected to UEC exhibited an attenuated breakdown of synapsin 1, pointing to OPN role in presynaptic terminal removal during the acute degenerative phase of reactive synaptogenesis. OPN KO IHC suggested this response is mediated through reactive microglia, and NOR behavioral testing revealed postinjury cognitive deficits when OPN is absent. A final set of experiments support LCN2 activation of MMP-9 as a novel mechanism for generating integrin binding OPN fragments to signal these changes.

## CHAPTER 4

### GENERAL DISCUSSION

## SUMMARY OF RESULTS

In Chapters 2 and 3 we presented the mRNA and protein profile of full length OPN and MMP-processed lytic fragments within the rodent hippocampus following TBI. The pattern of OPN response was contrasted between adaptive UEC, and maladaptive TBI+BEC forms of synaptic plasticity, with expression compared between whole hippocampal and enriched extracts of the denervated ML. Our results showed an elevation of OPN within 2d postinjury, having similar quantitative, but different qualitative changes in OPN for the two models. In order to explore the specific role of OPN in the degenerative phase of adaptive synaptic plasticity, we administered minocycline to suppress the immune response and OPN production in rats, and utilized OPN KO mice to determine the effect of removing OPN on synaptic proteins critical to trauma-induced structural reorganization of hippocampal synapses. Importantly, OPN deficiency altered the expression of synaptic proteins, as well as the reactivity of endogenous neuroglia and their tissue distribution, switching UEC adaptive synaptogenesis into a process similar to that of the maladaptive TBI+BEC model. Interestingly, behavioral studies utilizing a hippocampal-dependent cognitive task confirm that OPN loss contributes to poor functional outcome after brain injury, and is correlated with these structural and cellular changes. Collectively, the studies of this dissertation show that OPN response is important to the success of the acute recovery phase induced by TBI deafferentation. They specifically point to the production of OPN fragments by LCN2-mediated MMP-9 lysis, peptides that have the capacity to promote time-dependent chemotaxis and activation of both microglia and astroglia during the degenerative and regenerative phases of reactive synaptogenesis. Therefore, we conclude that OPN may be a novel target for future therapeutics designed to improve the early stages of functional recovery following TBI.

In Chapter 2, OPN protein was robustly elevated in the acute phase (1-2d) following UEC and TBI+BEC, highlighting the importance of its role as an inflammatory cytokine. Analysis of MMP-generated OPN fragments revealed precise temporal profiles associated with specific forms, suggesting multiple roles for OPN after trauma. Specifically, a 45 kD fragment was elevated early (1d) after injury, while a 32 kD form was elevated later during sprouting (7d). These results suggest this 45 kD protein may be more important in clearance of degenerating presynaptic terminals, while the 32 kD form may play a role in synapse regeneration. Further, IHC localization of OPN in reactive microglia and astroglia that demarcate the border between intact and injured dendrites, suggests that OPN/glia interaction is key for recruitment, migration, and phagocytic activation of additional glial cells. This OPN/glia interaction was further supported by transcript localized to active microglia within the injured dentate, identifying these cells as a source of acute OPN. Additional studies which showed minimal colocalization of OPN to proteins important in cytoarchitectural morphing such as MAP1B, suggests that this acute OPN response is less likely to be involved with structural reorganization during the acute phase (1-2d), but rather, indicates a more prominent role for OPN in chemotaxis and degeneration. Interestingly, while the comparison of the OPN/glia response between maladaptive TBI+BEC and adaptive UEC revealed similar OPN expression profiles, cellular analysis suggests that OPN mediates the more subtle changes of glial distribution to mark boundaries of tissue damage, a process that is disturbed with maladaptive conditions. Finally, acute minocycline administration 2d after UEC impaired trauma-induced OPN response, and altered the distribution of OPN-containing glia, again producing conditions that mimic maladaptive TBI+BEC. These OPN signals appear to involve specific MMP generated fragments since reduction of immune activation lowered MMP-9 activity and MMP-cleaved 45 kD OPN fragment production.

Taken together, this robust OPN elevation following trauma, which is localized to reactive glial cells within the injured zone, and is correlated with MMP-9 activity and generation of 45 kD integrin binding fragments, supports OPN as critical to the progress of the degenerative phase of reactive synaptogenesis. The fact that acute attenuation of the immune response impaired this potentially beneficial OPN/glial interaction led us to investigate the effect of complete OPN ablation during trauma-induced synaptic regeneration utilizing OPN KO mice. Chapter 3 results further supported the importance of OPN efficient synaptic recovery after TBI as OPN KO mice had impaired hippocampal function compared to WT mice during the window of preparation for synaptic sprouting (4d postinjury). While trauma-induced changes in cytoarchitectural (MAP1B) or junction stabilizing (N-cadherin) proteins did not differ between OPN KO and WT mice, there was a significant impairment in the breakdown of a protein marking presynaptic terminal removal during degeneration (2d) after UEC. Synapsin 1 protein clearance was inhibited in OPN KO mice, a finding localized to the OML innervated by lesioned entorhinal neurons. This impairment correlated with microglia of altered morphology, including longer cytoplasmic processes that mimicked a ramified state. OPN KO mice also had reduced trauma-induced MMP-9 activity and LCN2-bound MMP-9, supporting known roles in LCN2/MMP-9 and MMP/OPN function.

Collectively, these results suggest the following sequence of events after TBI: 1) hippocampal deafferentation induces the acute inflammatory response including activation of microglia, 2) microglia secrete extracellular OPN, 3) local activated MMPs cleave OPN into functional signaling fragments, 4) OPN signals cell migration in the acute degenerative phase to direct microglia in the removal of injured terminals, and 5) microglia signal recruitment of reactive astroglia during the initiation of sprouting, which then secrete factors important in

synaptic regeneration. Further, our initial mechanistic studies suggest the generation of various OPN functional fragments is regulated upstream through LCN2 persistent activation of MMP-9 within the local ECM, facilitating an adaptive, neuroregenerative, rather than maladaptive, attenuated recovery after CNS injury.

## **OSTEOPONTIN AND THE INFLAMMATORY RESPONSE**

These studies examining the role of OPN during synaptogenesis have widened our view of how this cytokine and the immune response as a whole may affect the recovery process following deafferentation induced by TBI. Importantly, these studies highlight the role of acute inflammation in the preparation of synaptic sprouting during effective synapse repair. UEC and TBI+BEC in rats, and UEC in mice, all resulted in acute, robust OPN response to deafferentation, which preceded molecular responses associated with the removal of injured terminals during degeneration. OPN elevation was documented 1d after injury, and peaked at 2d following UEC and TBI+BEC rats, a response considered to be an early response with regard to the molecular mechanisms which occur during synaptic reorganization. However, in other models of TBI, such as impact acceleration, cytokines such as chemokine CC ligand-2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), have been shown to increase as early as 4 hours after CHI, and peak at 12 hours postinjury, a much more acute and brief cytokine response. These examples of varied cytokine response profiles point to the importance of: 1) the model or injury condition which induces the inflammatory response, 2) the type of cytokines which respond, and 3) the temporal span of the immune system response. It is possible that focal deafferentation induces a very specific cytokine response related to synaptic repair, while more diffuse insults which induce global brain injury, including diffuse BBB disruption,

resulting in the nearly immediate cytokine release into local brain regions. As our studies are focused on synaptic changes after injury, we did not evaluate time points earlier than 24 hours postinjury, however our observations of peak OPN postinjury expression strengthen the potential for different cytokines, or in this case, distinct OPN functional fragments, to act in different roles at different time points following deafferentation. Given that results showed significant increase in the 45 kD OPN form 1-2 postinjury, it is possible that this functional fragment is specifically responsible for the cell signaling of the acute response during degeneration. Since this fragment has an exposed RGD sequence, it is capable of interacting with integrin receptors such as vitronectin ( $\alpha_v\beta_3$ ), a receptor which has been shown to modulate chemotaxis, and CD44, a receptor important in the inflammatory response. Stroke models have documented the concurrent increase in  $\alpha_v\beta_3$ , CD44, and OPN, strengthening the possibility that OPN functions to signal microglial movement to the site of deafferentation during the acute immune response (Kang et al., 2008). In addition, colocalization of these receptors with OPN also suggests that later elevations in the 32 kD form at 7d postinjury may permit OPN to function as an astrocyte, as this fragment contains the highly conserved C-terminus. This terminus has the potential for a number of functions within the ECM due to extensive posttranslational modification (Kazanecki et al., 2007a; Kazanecki et al., 2007b) and the ability to bind directly to the  $\alpha_v\beta_3$  receptor (Christensen et al., 2012).

While such results implicate a neuroprotective role for OPN following TBI deafferentation, consistent with studies in ischemia and SCI, they are in direct contrast to other studies in MS, PD, and AD which indicate OPN is a detrimental molecule, inducing neurodegeneration. A variable potentially mitigating the differences in outcome following TBI expression is the temporal window during which OPN is expressed. One characteristic shared by



AD, PD, and MS is the chronic nature of these diseases as opposed to the acute injury paradigm in ischemia, SCI, or TBI. Previous studies have demonstrated persistent microglial activation in neurodegeneration, such as occurs with the development of PD (Block and Hong, 2005), suggesting chronic OPN expression may underlie the detrimental neurodegeneration seen in MS and PD, while acute robust OPN may be responsible for facilitating repair. Given that OPN and microglia interact via integrin and CD44 receptors, it is conceivable that persistent OPN signaling leads to chronic microglial activation, and subsequent neurodegeneration seen in AD and PD. Further, clinical evidence indicates OPN expression can be coupled with microglial activation in chronic CNS diseases, including PD and HIV-induced cognitive deficits (Burdo et al., 2008; Brown et al., 2011). Together, these studies support the concept that the environment in which OPN cellular interaction occurs may determine whether OPN functions as a beneficial or detrimental molecule with respect to long term outcome following CNS injury or disease.

In contrast to the detrimental effects of OPN involved with chronic CNS disease, are models like ischemia, stroke, and SCI, which demonstrate that OPN may act in a reparative role during recovery and that OPN manipulation may promote recovery. These studies have utilized cell culture and *in vivo* models with OPN KO mice to demonstrate that the absence of OPN results in larger infarct size and poor neurological recovery, a finding similar to our studies which showed OPN KO mice had delayed clearance of the injury site and worse hippocampal function at 4-21d postinjury. Further, stroke studies have shown that postinjury administration of r-OPN can reverse the exacerbation of injury seen in KO animals, returning the injury profile to a state similar to that of WT animals. Models of SCI have examined mechanisms underlying the greater core injury and worse locomotor recovery in OPN KO mice, suggesting OPN may modulate effective axon terminal and synaptic outgrowth. Together, these models suggest OPN

may be beneficial in directing the immune response to facilitate effective synaptic recovery in acute traumatic conditions.

Further evidence that the environmental conditions play a role in OPN immune effect on the neuronal outcome is seen with its potential for shifting a neuroprotective acute response into a detrimental neurodegenerative outcome. Often TBI patients suffer polytrauma, such as that in a fall or motor vehicle accident, inducing multiple injuries including bone breaks. More recent evidence indicates that multiple peripheral injuries could have worsening effects on TBI recovery. OPN is a protein found in high levels within osteoclasts and osteoblasts during bone remodeling, and it is conceivable that TBI patients, who are often victims of polytrauma and/or skull fracture, may be subject to exacerbated CNS injury due to excessive OPN infiltration from multiple injury sites. Bone breaks could prolong OPN response due to long term infiltration from other injury locations, and induce a chronic inflammatory response consistent with neurodegeneration. For example, recent studies by Degos and colleagues indicate that when ischemic insult is compounded by tibial fracture, CNS injury is exacerbated, inducing larger infarct volumes and excessive microglia in the periinfarct region (Degos et al., 2013). As breaks far from the ischemia injury permit excessive glial cell localization to injury, local skull fracture may even further exacerbate pathology in human TBI cases, as mechanical stress in bone alone is capable of induce OPN expression in osteocytes (Denhardt and Noda, 1998; Terai et al., 1999; Nomura and Takano-Yamamoto, 2000). When considered with our present results, these studies offer support for the importance of OPN/microglial interaction during immune signaling which directs the acute postinjury recovery period, suggesting further investigation into these, and other immune molecules may provide insight into novel therapeutics to treat TBI patients.

## OSTEOPONTIN-MICROGLIAL INTERACTION DURING THE IMMUNE RESPONSE

Microglia are known to be key players during the acute CNS inflammatory response and chronic disease neuropathogenesis. In our studies, OPN mRNA was localized to active microglia, and OPN protein prominently localized to these reactive cells at the border of deafferentation. While our studies, as well as others in ischemia and stroke, have demonstrated that microglia are indeed a source of OPN, it is likely that OPN and microglia also interact in the acute immune response to effect synaptic reorganization. We have previously discussed the potential for exposed ligand binding sites on OPN permit interaction with  $\alpha_v\beta_3$  or CD44 receptors on microglia to facilitate chemotaxis and phagocytosis for debris clearance after injury. Interestingly, more recent descriptions of multiple microglial phenotype expression after CNS insult suggest that their role may be either beneficial or detrimental depending upon their specific phenotype. Two microglia phenotypes are currently known to exist: 1) M1 classic macrophages, which are produced in reaction to proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , often associated with phagocytosis in neurodegeneration and cell damage, and 2) M2 microglia, which are an alternative form, reacting to ECM molecules to secrete factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and IGF-1 in neuroreparative mechanisms (Boche et al., 2013). As an ECM molecule, it is possible that OPN promotes phenotype switching to the M2 type, directing microglia to facilitate chemotaxis and phagocytosis in repair and remodeling of the ECM as opposed to detrimental degeneration. Porcine cell culture studies have shown that exogenous OPN alters microglial state, inducing proliferation and phagocytosis (Tambuyzer et al., 2012), two functions which are supported by our IHC results showing activated microglia colocalized with OPN near injured axon terminals. In addition, these porcine studies demonstrated that OPN reduces super oxide production and NOS, supporting a switch to the M2 phenotype for

neuroprotection. Published reports have documented the presence of different subsets of microglia after TBI (Hsieh et al., 2013), and that the proportion of M1/M2 microglia may affect neurological outcome after TBI, where aged rats who expressed an increased number of M1 proinflammatory microglia exhibited poorer neurological function (Kumar et al., 2013). Other studies which indicate inflammation can be persistent for years after TBI (Smith et al., 1997; Bramlett and Dietrich, 2002) also suggest targeting the ability of OPN to facilitate expression of beneficial microglial phenotypes may be of therapeutic benefit to improve synaptic recovery after TBI.

## **OSTEOPONTIN AND SYNAPTIC PLASTICITY**

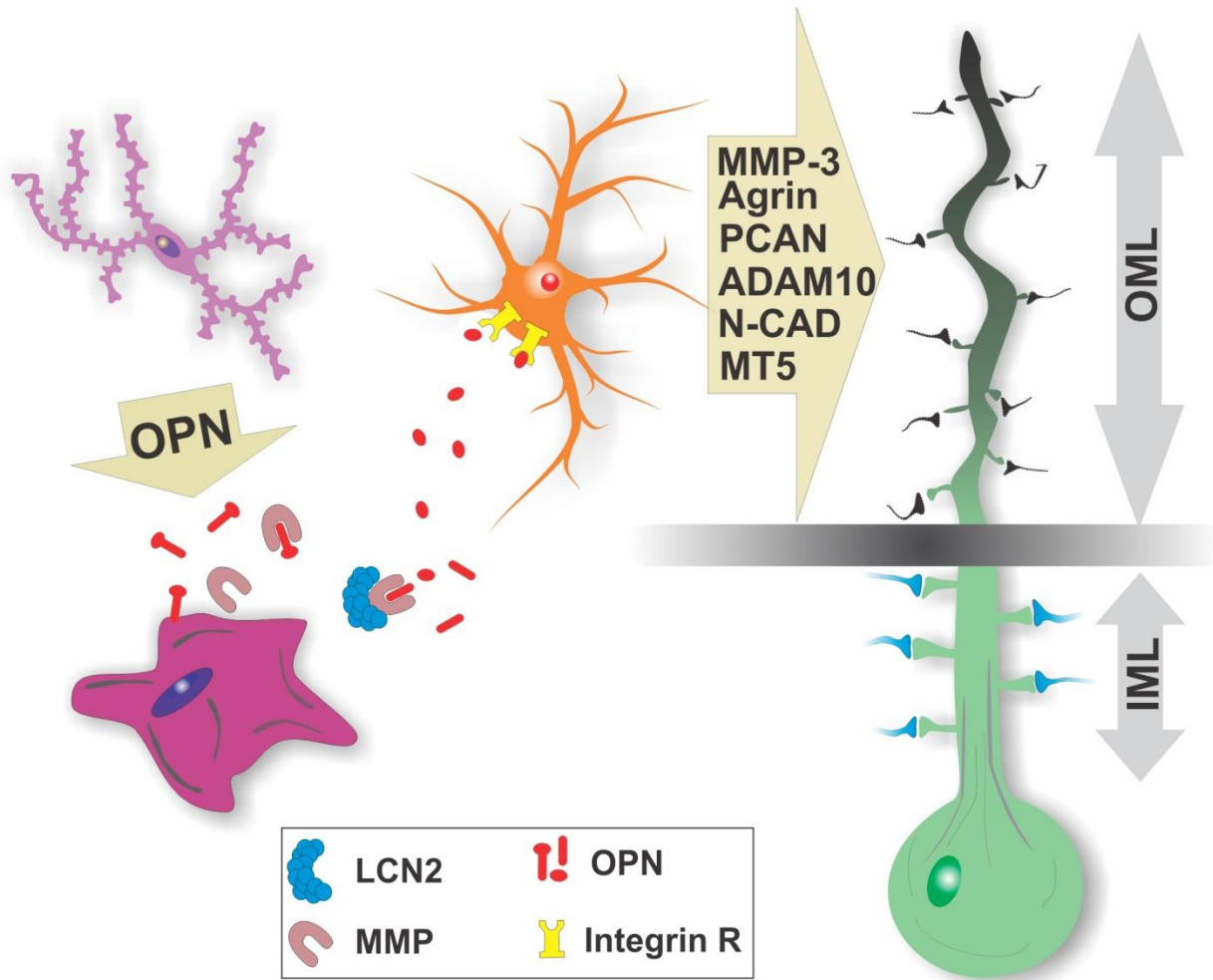
While OPN may function to facilitate a neuroprotective microglial phenotype, the exact mechanism by which this occurs is unknown, and the extent to which OPN mediates other cell types during synaptic recovery is not clear. Several models of TBI have shown that inflammation alone can alter the generation of new neurons following insults (Das and Basu, 2008; Bye et al., 2011), supporting broad effect of inflammatory molecules on regeneration. Our results in Chapters 2 and 3 indicate that at least two functional fragments of OPN, generated through proteolytic processing, may be responsible for the pleiotropic function of OPN during TBI recovery. During the acute degenerative phase, OPN can be localized in both microglia and astrocytes, however its transcription appears restricted to microglia. Further, we have demonstrated trauma-induced ECM production of a MMP-generated 45 kD OPN fragment containing the RGD binding motif, as well as a 32 kD, C-terminus-containing peptide. Given the acute (1-2d) elevation of this integrin binding 45 kD fragment, the protein may serve to selectively localize microglia at the site of injury and permit their recruitment of reactive

astrocytes to assist in matrix mediated synapse degradation (**Fig. 4.1**). It may also enter damaged neurons, interacting intracellularly with either dendritic or axonal cytoskeleton to facilitate reorganization of injured postsynaptic spines or axon terminals in preparation for sprouting and synaptogenesis. It is also possible that there is a delayed secondary OPN response as evidenced by elevation in the 32 kD form 7d postinjury. Given that MAP1B staining returns at this time in the rat, regeneration may occur by interacting with this cytoarchitectural protein, or alternatively through signaling with astroglia as suggested by the late OPN response that colocalized with reactive astrocytes in stroke models.

Contribution of OPN to microglial switching from a degenerative to a protective state may further direct a number of downstream beneficial processes. For example, microglial phenotype switching may provide a signal that permits upregulation of 32 kD OPN, which further allows OPN to act as an astrokine. Reactive astrocyte recruitment to the site of injury at later time points has been demonstrated in models of stroke and ischemia (Ellison et al., 1998; Ellison et al., 1999), and most recently in preliminary studies in our lab showing colocalization of OPN on GFAP positive cell bodies as late as 15d postinjury (see **Appendix E**). In addition, these preliminary IHC studies have shown that CD44 colocalizes with these reactive astrocytes, further supporting the potential for OPN to act as an astrokine, facilitating regenerative repair mechanisms (**Fig. 4.2**). As OPN has been correlated with neurite outgrowth in brain stab injury (Plantman, 2012), and a modulator of axonal outgrowth in sciatic nerve transection (Jander et al., 2002), it is possible that alternative forms of OPN, yet to be explicitly identified, are expressed at later time points, facilitating the plasticity supporting the improved NOR performance seen in WT mice at 28d postinjury. Astrocyte recruitment, and implementation of their functional role during recovery, may lie in the production of growth factors such as brain derived neurotrophic

**Figure 4.1 Cytokine-Astrocyte Signaling During Degeneration.** At 2d postinjury, microglia become activated, secreting OPN. Lipocalin-mediated MMP lysis of OPN exposes functional ligands to permit interaction with integrin receptors such as those on astrocytes.

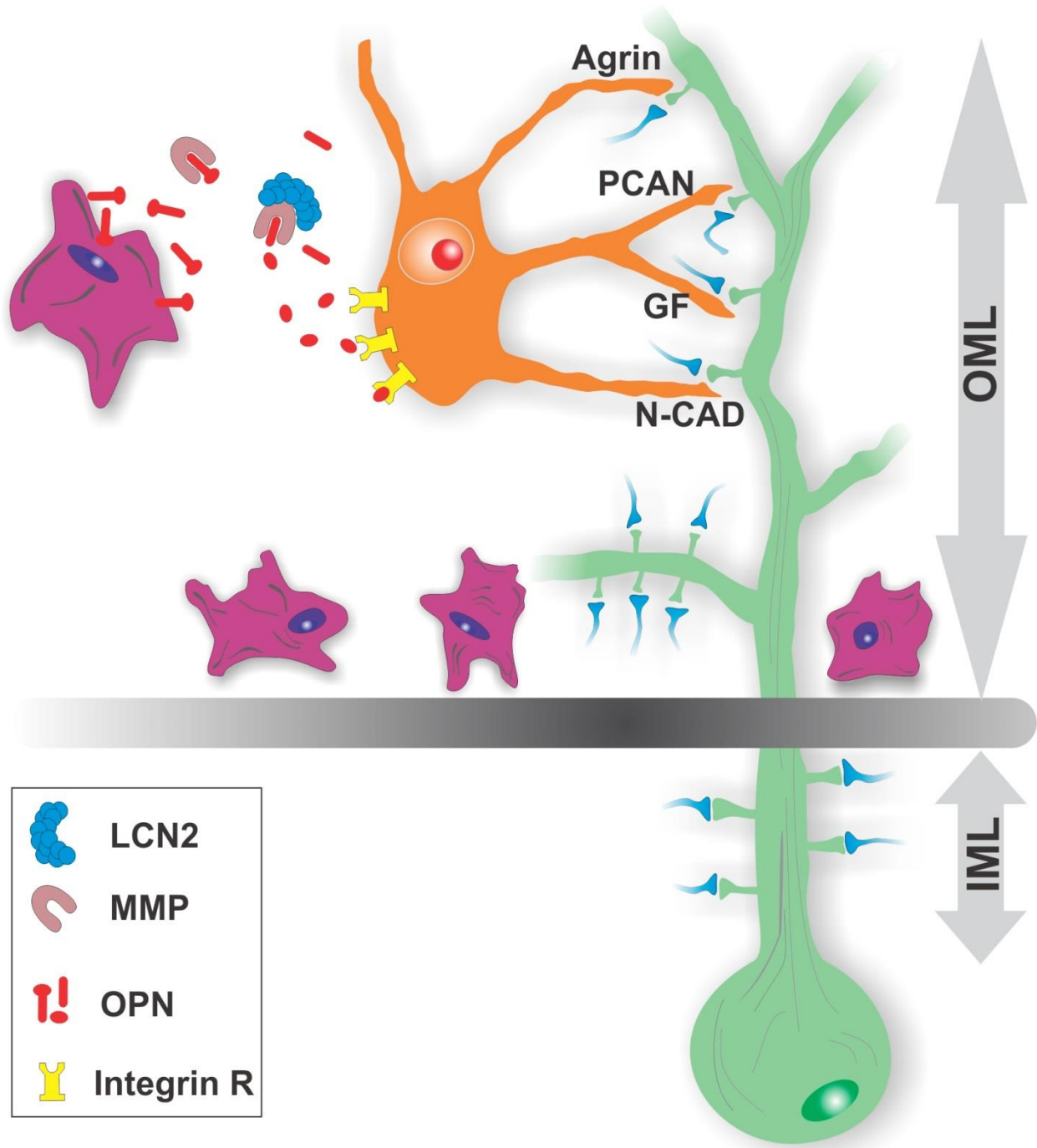
## Cytokine-Astrocyte Signaling 2d UEC







**Figure 4.2 Cytokine-Astrocyte Signaling During Regeneration.** At 2d postinjury, microglia become activated, secreting OPN. Lipocalin-mediated MMP lysis of OPN exposes functional ligands to permit interaction with integrin receptors such as those on astrocytes.



# Regenerative Phase 7 - 15d UEC



-  LCN2
-  MMP
-  OPN
-  Integrin R

factor (BDNF) (Quesseveur et al., 2013), nerve growth factor (NGF) (Goss et al., 1998), and IGF-1 (Madathil et al., 2013) shown to promote neurogenesis and neurorecovery. BDNF has been shown to increase in response to TBI (Hicks et al., 1997), and linked to improved cognitive functioning after injury as shown by performance in the MWM task (Griesbach et al., 2009). Together, these studies suggest that an alternative form of OPN may function to promote effective synaptic plasticity during sprouting 7d postinjury.

In addition to cellular signaling for astrocyte facilitation of synaptic repair, it is possible that microglial secreted OPN indirectly interacts with molecules like neuronal MAP1B as the dendritic cytoskeletal reemerges at 7d postinjury. This is supported by the fact that we found some MAP1B/OPN colocalization within microglia at this regenerative phase. However, due to the few number of microglia colocalizing with OPN, it is unlikely that this OPN pathway plays a major role regulating synaptic formation. While other studies show that intracellular OPN can bind to MAP1B (Long et al., 2012), it seems unlikely that such OPN binding is critical during reactive synaptogenesis since we did not observe OPN/MAP1B interaction with IHC during periods of neuronal growth. Nevertheless, our experiments were limited, and it remains possible that a specific OPN fragment which we do not have the tools to visualize, binds MAP1B, or that astroglia employ OPN to indirectly support cytoskeletal reorganization during sprouting and synapse stabilization. Interestingly, one structure in the adult CNS which has abundant MAP1B expression is the olfactory bulb (OB), and recent studies in our laboratory have demonstrated that rodents subjected to central fluid percussion injury show altered OPN response in their damaged OBs. While we have not yet probed MAP1B expression in this model, we do have evidence of OPN activation after injury in the OB during synaptic plasticity. Following TBI, olfactory axons may stretch as a result of acceleration and deceleration forces, causing injury

which manifests in anosmia, a common complaint in TBI patients (Reiter et al., 2004; Costanzo et al., 2012). In our mild-moderate fluid percussion injury in mice, we have demonstrated ultrastructural evidence of injured olfactory axons, as well as OB spectrin proteolysis. Interestingly, in this alternative deafferentation model, and within a different region of the CNS, OPN is also upregulated within the glomeruli and olfactory nerve layer of the injured OB at 3d after TBI. OPN also colocalizes with IBA1 at this early time point, and is found within olfactory ensheathing cells (OECs), specialized OB glial cell types important in the turnover of new synaptic connections. These data further support the importance of OPN interaction with reactive glial cells following TBI, again highlighting early glial interaction. It is possible that MAP1B directly interacts with OPN in this MAP1B-rich environment during the turnover of olfactory synapses. We also know that MMP-9 activity is elevated in the deafferented OB, from our own TBI results (**Appendix D**), as well as published studies (Bakos and Costanzo, 2011). This MMP change fits well with our working hypothesis that OPN is lysed to facilitate local cell signaling properties in different regions of the CNS undergoing neuroplasticity after TBI. Our results in the deafferented hippocampus and OB emphasize the role of the environment in OPN mediation of synaptic recovery. Mechanism of injury and location in the CNS are important factors which contribute to the time frame during which OPN is expressed and induces its subsequent effects.

Taken together, our biochemical and morphological data indicates that the cytokine OPN affects recovery processes important in memory and cognition. Interestingly, recent studies have shown that, even in the absence of trauma, immune molecules can alter cognitive function (Kipnis et al., 2012). In the case of OPN, we found that loss of this cytokine did not produce deficits in NOR performance for uninjured mice, whose RI scores did not differ from uninjured

WT animals. This result points to a less critical role for OPN in normal synapse stabilization, but rather an important role in CNS recovery following injury as suggested by studies in bone (McKee et al., 2011) and cardiovascular (Murry et al., 1994) repair. Following deafferentation in our model, NOR deficits emerged in OPN KO mice, specifically associated with the transition between degenerative and regenerative phases (4-21d), while injured WT did not show such deficits. The fact that NOR deficits evolved during this time frame further supports our posited role for OPN as a critical immune mediator of microglial and astroglial function during successful synaptic recovery. As a molecule which has the ability to recruit microglia and activate T-lymphocytes, OPN may be critical to immune-mediated synaptic reorganization as evidenced by studies which demonstrate that T-cells and microglia contribute to the acquisition of spatial memory in adult rodents (Ziv et al., 2006). Further, studies have shown that the absence of specific cytokines, such as T-cell-derived IL-4, impairs cognitive performance in the MWM task (Derecki et al., 2010). The role of the immune system in neurocognitive disease provides a potential target for therapeutic intervention in CNS injury and disease states, as various studies in patients and experimental models of SCI, neurological disease, and HIV-associated dementia (Kipnis et al., 2008; Gensel et al., 2012; Derecki et al., 2013) have demonstrated a connection between the immune system and neuroprotective function. Overall, it appears that OPN has minimal action as a baseline immune humoral defense in the rodent brain, but serves a highly important role when the brain is challenged with mounting an acute response to injury, supporting not only structural, but also functional recovery.

## **OSTEOPONTIN AS A PROMISING TARGET OF THERAPEUTIC INTERVENTION**

Given its robust elevation after CNS trauma, OPN has potential as a marker to predict outcome or a target for therapeutic treatment, the latter of which might be achieved either directly or by manipulation of its upstream OPN regulatory pathways. Several studies have proposed the use of OPN as a biomarker for head and neck cancer, and a marker of ongoing MS inflammation, but it has yet to be considered as an index of TBI outcome. While a single molecule is unlikely to have a well-defined predictability for different treatment modalities, OPN could be one of multiple cytokines or chemokines used in screening panels in order to profile patients for specific treatments. Several clinical studies in MS, PD, AD, and HIV, have correlated elevated OPN serum levels with active pathology or cognitive deficits, suggesting that those TBI patients who have long term, elevated OPN may be susceptible to excessive neurodegeneration. While many have considered targeting OPN in treatment of these chronic diseases, none have addressed the importance of the temporal window in which OPN is expressed, or the extent of its activity. OPN expression in short response windows may be beneficial, allowing the switch from M1 to M2 type microglia, promoting neuroprotection. Similarly, controlling the expression of OPN directly, or through upstream regulators such as MMPs and LCN2, may provide more precise treatment for the ongoing sequelae in TBI as opposed to globally removing or administering a molecule as a single therapy. Our studies indicate OPN/MMP interaction, and upstream LCN2 regulation of OPN proteolysis, may be alternative methods used to modulate the effects of OPN in the process of neuroplasticity. As previously described, it is not likely OPN acts alone in the neurorepair, but contributes to a complex series of molecular events which result in the smooth transition between neurodegeneration and neuroregeneration. Ultimately, combinatorial treatments which include

selective manipulation of the chronic inflammatory response, and inhibition of persistent MMP activation, may focus the OPN/MMP/LCN2 pathway in the acute phase, prohibiting detrimental neurodegeneration.

## **FUTURE DIRECTIONS**

OPN is a promising molecule for targeting in TBI therapeutics and requires further investigation regarding its potential role as a cytokine which might predict TBI outcome. OPN activation during the acute and chronic inflammatory responses can direct both degeneration and repair, clearly a complex process involving numerous cell types and ECM proteins. Due to the posited pleiotropic functions of OPN following CNS injury, additional studies utilizing OPN KO mice will be important in determining more specific details about postinjury molecular changes in the absence of OPN. First, generation of a TBI+BEC mouse model would help identify differences between adaptive and maladaptive synaptic plasticity in the context of OPN KO, providing additional structural and functional outcome data. Probing for various OPN fragments between these models may further delineate the specific OPN forms which direct different aspects of plasticity after TBI. In addition, integrin receptors likely play a role in the action of these fragments, as described by previous ischemia studies demonstrating that antagonists to the integrin-binding **GRGDS** sequence inhibited OPN function and ischemic recovery, a process which can be rescued by r-OPN administration (Suzuki et al., 2010b). Further, electron microscopy and electrophysiological analyses will provide a more precise understanding of how postinjury synaptic structure and function are affected by OPN ablation. While these studies would be effective in addressing endpoints specifically related to synaptic recovery, generation of cytokine panel assays would extend our understanding of how OPN affects other

inflammatory molecules during deafferentation-induced plasticity. Ultimately, performing additional cytokine and synaptic plasticity studies may link specific molecular components in the inflammatory response associated with neuroprotection following TBI.

Extending our postinjury time course beyond 7d over all of these endpoints may be particularly insightful, as we observed a reduction in OPN KO NOR deficits at that time point, suggesting a shift in OPN influence on synaptic recovery with the progression of time. Further characterization of OPN KO mice regarding the ultrastructural synaptic changes and tract tracing for collateral sprouting would also enhance our understanding of how OPN might operate to permit effective synaptic plasticity after injury. Electrophysiological examination of LTP during recovery intervals where OPN appears most critical will provide additional insight as to what part of the synaptic recovery process underlies behavioral deficits observed in the absence of OPN. In addition, given that other studies indicate OPN is an upstream regulator of other cytokines such as MCP-1 (Zhang 2010), cytokine assays over a more complete postinjury time course could be performed to broaden the view of how OPN affects the inflammatory process as a whole, and the subsequent downstream effects related to synaptic plasticity.

Most importantly, examination of OPN and its regulation during the later stages of synaptic plasticity, including synapse stabilization and maturation, is critical. Specifically, time points between 14 and 28d utilized in the present NOR testing would help determine if there are structural correlates which explain the WT drop in NOR performance at 21d, and rebound seen at 28d. As the similarity between TBI+BEC and UEC rat OPN profiles were only determined during acute recovery, it is possible that OPN may respond differently in the maladaptive model at the later phases of sprouting and synapse stabilization. It would also be interesting to contrast the two models in WT and OPN KO mice, particularly the investigation as to whether OPN

status would switch adaptive into maladaptive outcome or vice versa. If loss of OPN indeed induces poor synaptic recovery, we anticipate the ultrastructure of synapses of OPN KO mice would look similar to synaptic disorganization in a mouse subjected to maladaptive TBI+BEC. Likewise, electrophysiological recordings in both TBI+BEC and OPN KO UEC mice might reveal common alterations in electrical signaling, correlating abnormal structure with impaired hippocampal function. We have previously reported alterations in LTP formation following MMP inhibition, a finding which might be replicated in OPN KO UEC and TBI+BEC mice. Together, these studies would extend the temporal profile of OPN effects in synaptic reorganization following TBI, providing additional endpoints which might address structural and electrophysiological evidence of altered neuronal function in the absence of OPN.

While removing OPN may help confirm the importance of this cytokine as beneficial to synaptic recovery after injury, further studies which demonstrate rescuing impaired function would also strengthen this hypothesis. One approach would be to test r-OPN administration in OPN KO UEC or WT TBI+BEC mice as an attempt to convert a maladaptive animal into an adaptive model of synaptic plasticity. Interestingly, several studies have already demonstrated that administration of OPN prior to or following ischemic injury, reduces infarct size, stabilizes the BBB, and improves neurological scores (Suzuki et al., 2010a; Wu et al., 2011b; Suzuki et al., 2011). Unfortunately, no studies have examined the effect of this treatment on hippocampal cognitive function or ultrastructural effects on dendritic morphology and improved synaptic plasticity after injury.



## **FINAL REMARKS**

In conclusion, the results from this dissertation study highlight the potential for OPN to modulate deafferentation-induced synaptic plasticity during both the degenerative and regenerative phases of reorganization, acting in the acute inflammatory response to condition the matrix for sprouting, and subsequently influencing the cellular and molecular pathways responsible for effective synaptic recovery. Alterations in structural and functional recovery in rodents implicate that this cytokine has the ability to modify the ECM of the hippocampus to promote regeneration. While previous studies have suggested that immune molecules may serve as future therapeutic targets in the treatment of CNS injury, this study is the first to provide evidence that a specific cytokine has the potential to direct the immune response within reactive neuroglia during neuroplasticity, as well as facilitate structural reorganization and cognitive recovery under conditions of TBI-deafferentation. Overall, these results give greater insight into the relationship between the immune response and effective synaptic recovery following CNS injury. Further characterization of OPN and its interaction with immune cells and matrix molecules following trauma may ultimately assist in the development of novel treatments which reduce persistent impairment and bolster effective recovery in TBI patients.

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## List of References

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## Appendix A

### Affymetrix Rat Genome Microarray Screening

**Table A-1 7d Rat OPN (SPP1) Microarray Values: Fold Change Over Paired Control**

	<b>UEC</b>	<b>TBI+BEC</b>
<b>Hippocampus</b>	13.6	40.5
<b>Molecular layer</b>	21.1	5.5



## Appendix B

### Novel Object Recognition Paradigm

### Specific Novel Object Recognition Apparatus Setup

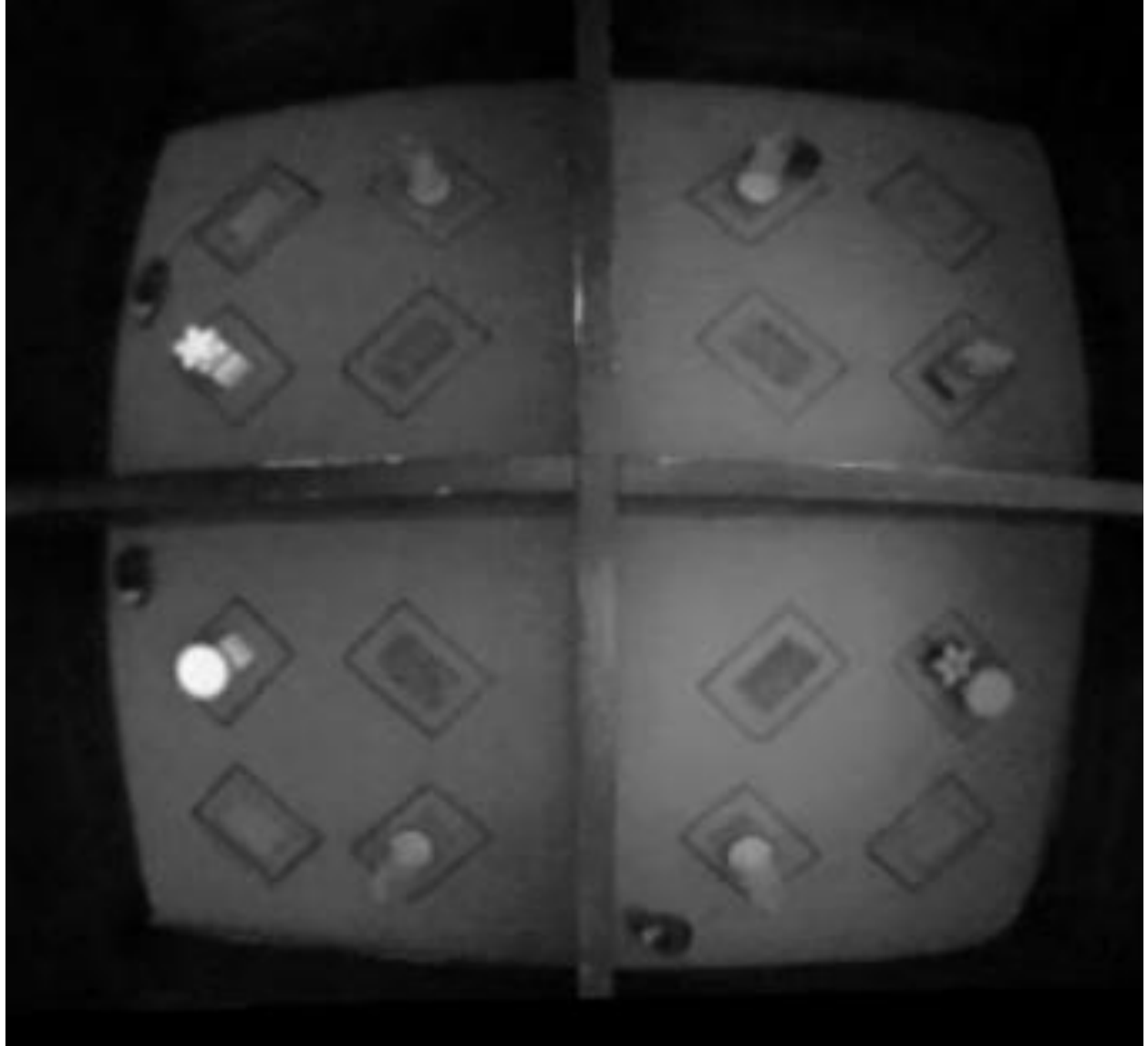
For the NOR paradigm, we utilized a black Plexiglas box (DIMENSIONS 90 x 90 x 45 cm<sup>3</sup>) divided into 4 identical quadrants (42 x 42 x 30 cm<sup>3</sup>) (**Fig. B-1**). Each square served as an individual habituation/familiarization/testing chamber for a single animal at a single postinjury time point, allowing four animals to be run at once, and maximizing efficiency. As described in Chapter 3, four groups were compared: naïve WT, naïve OPN KO, WT subjected to UEC, and OPN KO subjected to UEC. On the first habituation day, animals were randomly assigned to a quadrant, and habituated to the environment as described in Chapter 3. This same quadrant was used for the animal in both familiarization and testing phases as described in Chapter 3 until the task was completed for the specific time point. Starting with the upper left quadrant, and rotating clockwise, animals were placed in their designated quadrant, and five minutes of exploration recorded following a one minute delay to standardize data recording. For each subsequent time point, animal placement was rotated clockwise to the next quadrant to avoid any inherent bias regarding the quadrant itself, or placement within the room. To prevent bias induced by long term memory recall, animals were exposed to a different novel Lego® object at each time point by assigning each quadrant to specific sets of novel objects. Ultimately, each animal was exposed to unique single quadrant-novel-object paradigm at a different time point, preventing any potential bias due to the novel object itself, quadrant placement, or time-point-specific quadrant-object paradigm.

### 63d Novel Object Recognition Study

Prior to the 28d NOR study, a longer 63d study comparing the hippocampal function of WT C57BL/6 and OPN KO mice was performed. In this task, the parameters and methods used

were the same as the 28d study described above, with the exception that the familiar object consisted of Lego® shapes with alternative configurations as opposed to a 50 ml conical tube. Thus, the animals were challenged to discriminate between two Lego® configurations in performing the NOR task. Throughout the 63d study, the RI of WT and KO animals was not significantly different from chance alone, nor were the RI measures between strains with or without injury. The null results of this prior 63d study, using only Lego configurations, may not be surprising given that previous behavioral studies in mice show that these rodents utilize a number of cues, including vision and whisker-mediated tactile response, as well as olfactory input, during typical exploratory behavior (Brown and Wong, 2007). It is likely that the similarity of texture and composition outweighed the visually detected differences in the objects, resulting in a failure to detect experimental effect. In fact, a recent comprehensive study of mouse behavioral testing shows that the extent of sensory utilization can vary considerably between mouse strains (Wong and Brown, 2006). In our case, the C57BL/6 background strain was found to be efficient at utilizing the visual cues for which the NOR task relies, but still failed to show UEC related deficits, suggesting that object novelty was not adequate. Together, our two NOR studies highlight the importance of choosing an appropriate task during behavioral analysis, particularly when evaluating transgenic mice for the first time.

**Figure B-1 Specific NOR Apparatus.** Screenshot of NOR apparatus with four identical quadrants and symmetrically placed familiar and novel objects.



## Appendix C

### Osteopontin Knockout Mouse Characterization

## Osteopontin Knockout Mouse Phenotypic Behavior

OPN KO mice appeared phenotypically normal, with the exception of a slightly lower weight around weaning age (21d). To prevent differences in weight/growth which could induce surgical complications between strains, both WT C57BL/6 and OPN KO mice were given a special semi-soft DietGel® 76A (ClearH<sub>2</sub>O®, Portland, ME) ad libitum for 10 days following weaning (P21-31). By 8 weeks of age, both strains average weight was within the normal range (25 g). Physically, OPN KO mouse appearance includes a slightly greyer coat and thicker body compared to WT mice, which have a very sleek black coat and leaner form. OPN KO and WT breeding rates appeared similar, however, OPN KO mothers did appear to give birth slightly less often than WT mice. This may be due to a somewhat longer gestational time (which would support initial observations of impaired growth/development rates compared to WT mice), nonviable embryos, or a delay in conception due to behavioral characteristics. The precise mechanism for the observed differences in litter birth rate was not investigated, as it posed no deleterious implications for our studies.

Slight differences in behavior were observed between WT and OPN KO mice. In general, male WT mice appeared more interested in huddling together when sleeping, while OPN KO mice showed no preference for solitary or group sleeping arrangements. This territorial mentality is also supported by observation that male OPN KO mice would fight with other siblings when returned to their home cage following open field events during NOR. Very few WT mice exhibited this characteristic while almost all OPN KO mice had these aggressive tendencies after undergoing the NOR task assessment. It is possible that absence of OPN alters limbic and/or hippocampal circuitry, affecting mood or emotion, as suggested by studies demonstrating the importance of immune molecules in synaptic plasticity and maintenance.

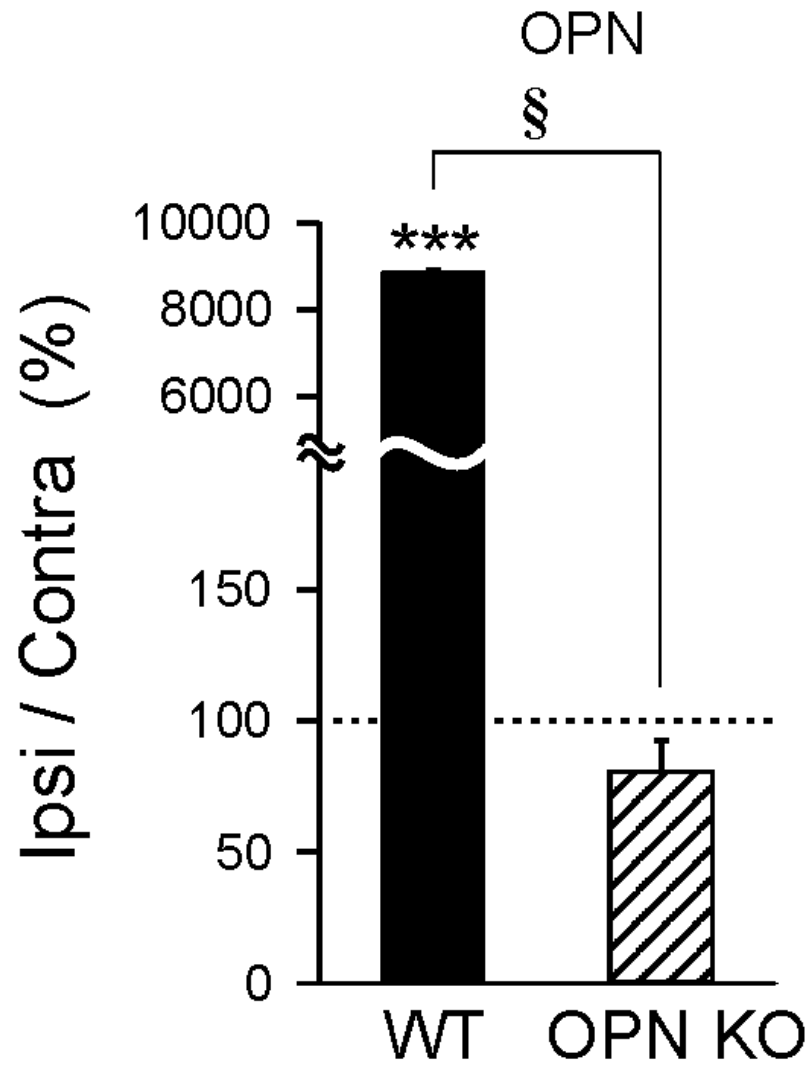
Despite these slight differences in strains, none of these observations appears to have affected the outcome of our studies.

### Western Blot Characterization

To ensure that OPN KO mice did not produce OPN protein, we compared WT and KO hippocampal extracts, probing for full length OPN in animals subjected to UEC lesion. Here we show the graphical representation of this OPN WB analysis (**Fig. C-1**). WT mice subjected to UEC showed a robust increase in hippocampal OPN expression ipsilateral to the lesion, which was significantly elevated over contralateral control at 2d postinjury, a result similar to that of rats in Chapter 2. As anticipated, we did not detect full length OPN protein response in OPN KO mice subjected to UEC (for representative blot images, see again **Fig. 3.3 B**). With Gene Tools software, we measured relative optical density of probed signal and blot background for each lane. In this case, OPN KO samples subjected to UEC showed essentially no difference from their contralateral controls, each achieving only background non-specific signal density. Thus, no protein signal for OPN was detected in OPN KO mice, with or without UEC lesion. Clearly, the eighty fold elevation in OPN for lesioned WT was significantly greater than the absence of signal in the OPN KO after injury. These quantitative measures further confirm the efficient KO of OPN in our mouse strain.



**Figure C-1 Hippocampal OPN Protein Expression Following UEC in WT and OPN KO Mice .** WB analysis of full length 66 kD OPN protein in whole hippocampal extracts. UEC induced an acute robust increase in OPN protein in mice, similar to rats. No OPN protein response was detected in OPN KO mice, confirming proper expression of the SPP1 transgene and null OPN protein mutation. Results are displayed as percent change over control. \*\*\* $p < 0.001$  relative to paired control cases; § $p < 0.05$ .



## Appendix D

### Fluid Percussion Injury of Olfactory Bulb

## Olfactory Bulb

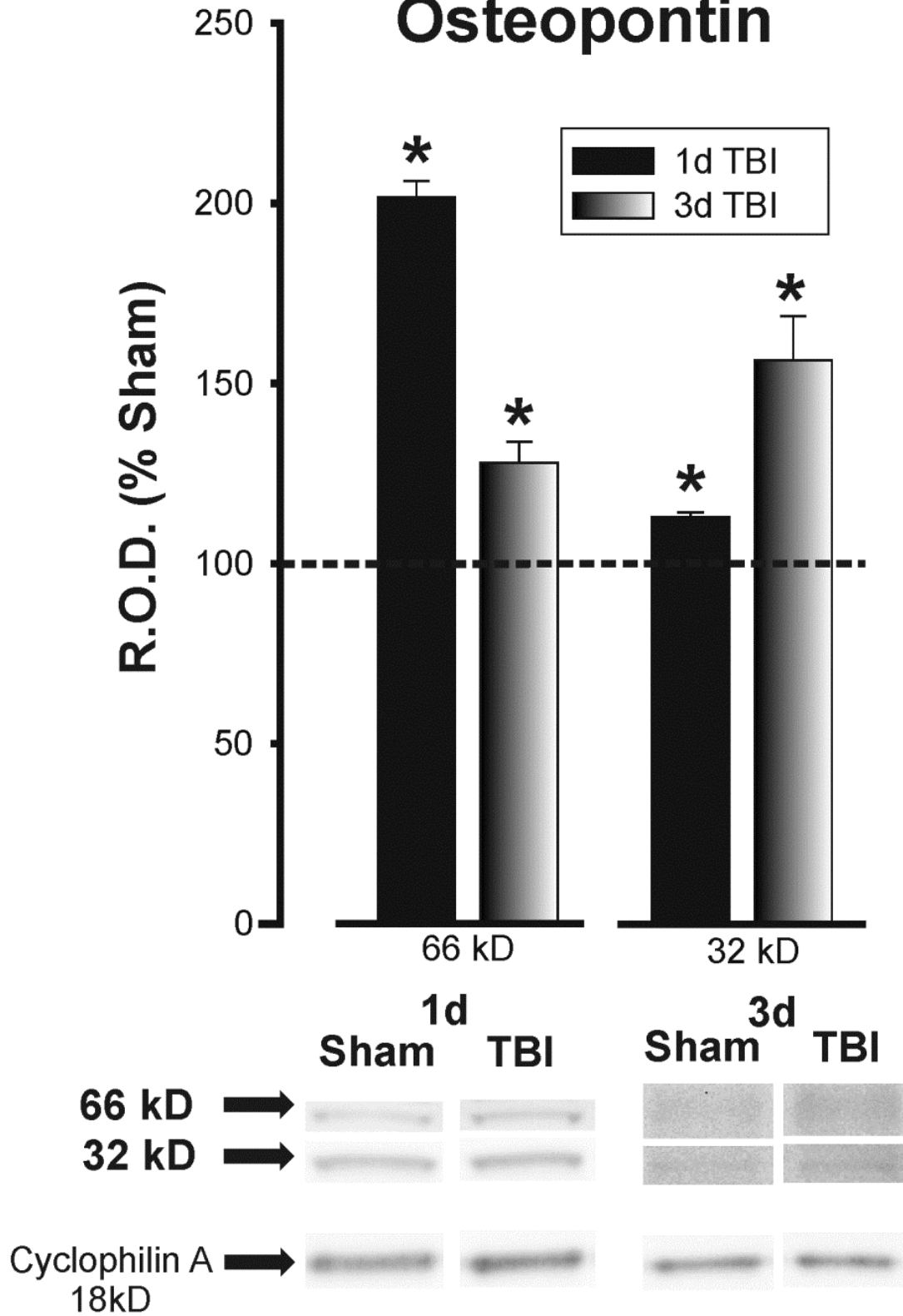
Given the potential for OPN to mediate overall CNS synaptic plasticity following TBI-induced deafferentation, we performed parallel pilot studies to investigate the role of OPN during reactive synaptogenesis in the injured olfactory bulb (OB). Olfactory disturbances are common after human brain injury, with anosmia occurring in up to 30 % of victims (Costanzo and Zasler, 1992) and correlating with MRI detected damage in olfactory bulbs and tracts (Yousem et al., 1996). Here we examined the effects of diffuse fluid percussion TBI on OB OPN response. WB analysis showed significant elevation for both full length OPN and its 32 kD MMP-cleaved fragment 1 and 3 days postinjury, during the acute degenerative phase of reactive synaptogenesis (**Fig. D-1**). Interestingly, we discovered a time-dependent difference in the pattern of these two OPN bands, with the full length form peaking early at 1d, and the MMP fragment peaking at 3d postinjury. These differences, when compared with the OPN postinjury protein expression in deafferented hippocampus (see again, Chapter 2), support model and regional variation of OPN response during reactive synaptogenesis.

Parallel gelatin zymography experiments revealed a concurrent increase in OB MMP-9 activity after TBI (**Fig. D-2**), pointing to prominent role for this enzyme in processing OPN within the injured olfactory bulb. Finally, similar to hippocampal results in the of Chapter 2, confocal IHC showed OPN response at 3d postinjury associated with reactive microglia and the highly plastic OEC population in the glomerular synaptic regions (**Fig. D-3**). The 3d postinjury astroglial response did not appear to involve detectable OPN expression, but likely influence synaptic plasticity given the interdigitation of their reactive processes among synaptophysin-positive synapses (**Fig. D-4**). Taken together, these WB and IHC results suggest that glial

OPN/MMP-9 interaction plays a role in ECM conditioning to facilitate effective synaptic recovery in the olfactory bulb following diffuse TBI.

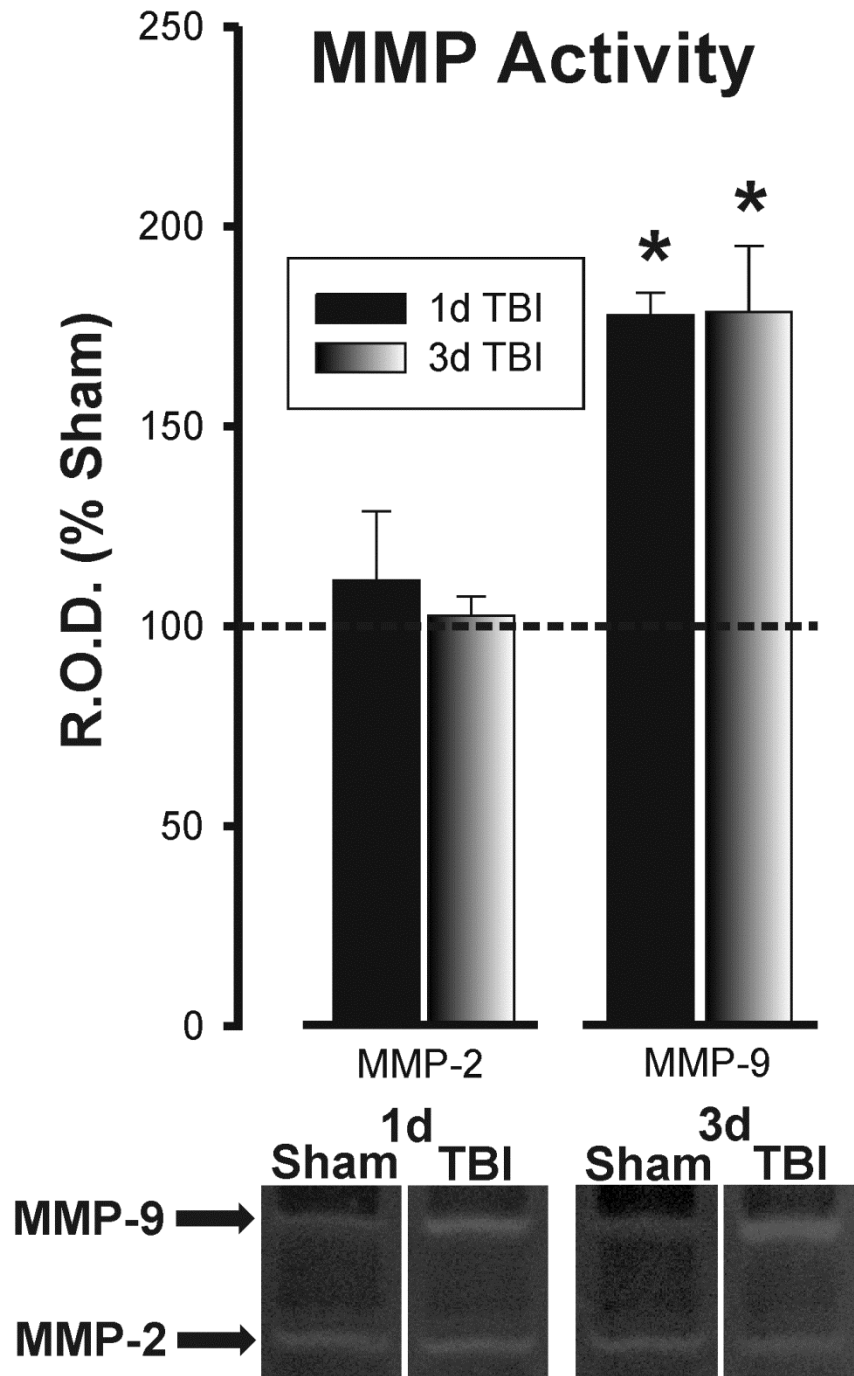
**Figure D-1. Olfactory Bulb OPN Protein Expression Following Central Fluid Percussion Injury.** WB analysis of full length 66 kD OPN and its MMP-cleaved 32 kD fragment in olfactory bulb extracts. Mild-moderate central fluid percussion injury (TBI) induced an increase in both full length and MMP-cleaved OPN at 1 and 3d postinjury. The most robust change was in the full length OPN during the acute 1d postinjury time period. Results are displayed as percent change over control with representative blot images and cyclophilin A loading controls shown below. \* $p < 0.05$  relative to paired control cases.

# Osteopontin



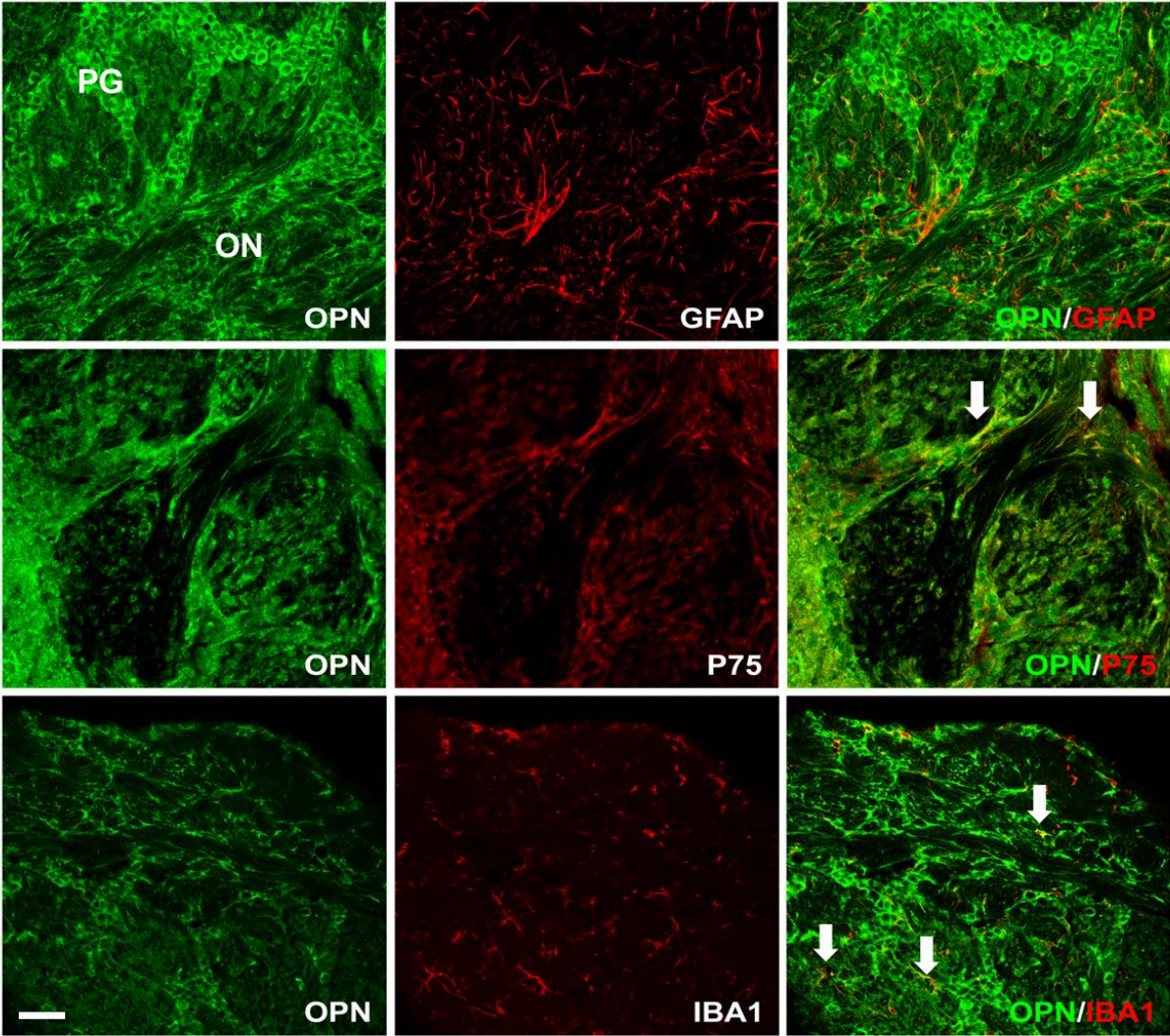
**Figure D-2. Olfactory Bulb MMP Activity Following Central Fluid Percussion Injury.** At 1 and 3d after central fluid percussion injury, gelatin zymography shows injury-induced elevation in MMP-9, but not MMP-2 activity in the olfactory bulb relative to sham controls. Results are expressed as percent of control, with representative gel images shown below. \* $p < 0.05$  relative to paired contralateral controls.



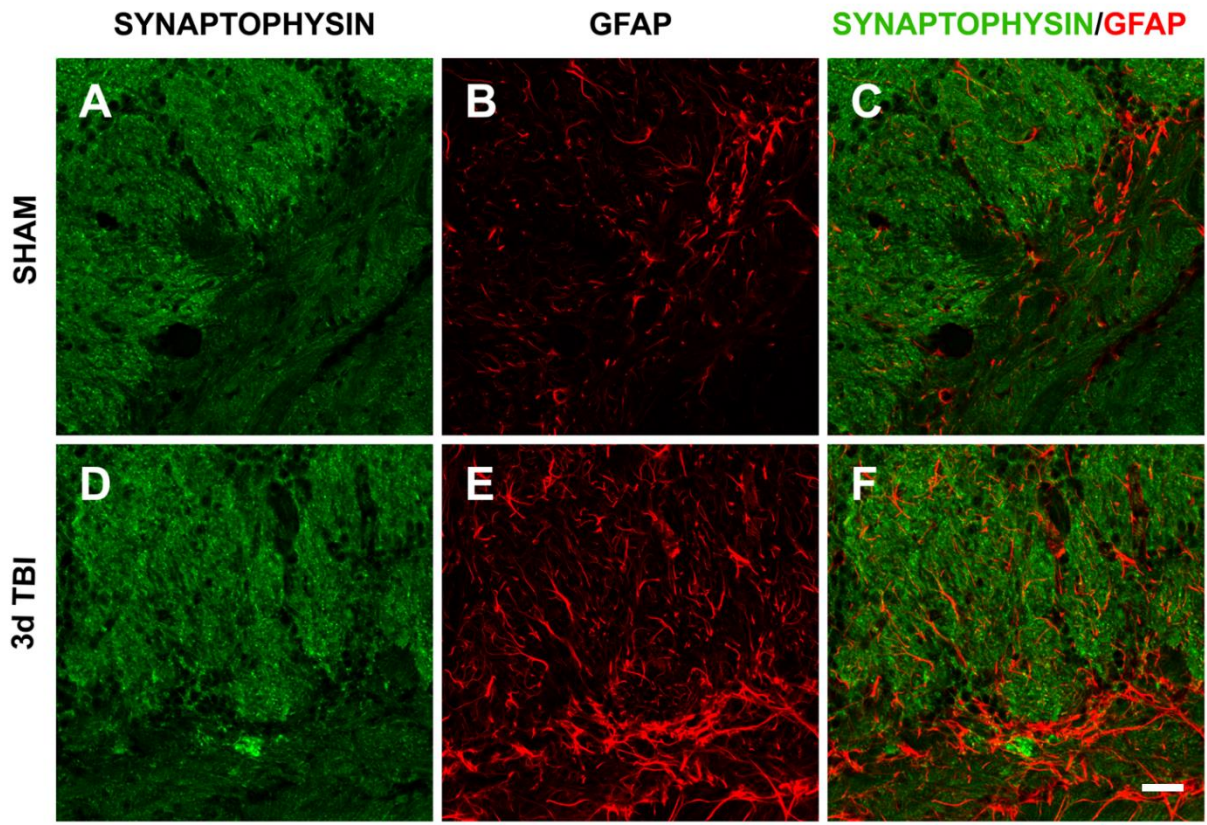


**Figure D-3. IHC of OPN and Glial Cell Types in the Olfactory Bulb at 3d Following Central Fluid Percussion Injury.** Strong confocal OPN signal (green in left panels) is present in olfactory nerve layer (ON) and in periglomerular cell layers (PG). OPN shows colocalization (arrows) with NgR-p75, a marker for OECs, and with IBA1, a marker for microglia, but fails to show colocalization with GFAP. Scale bar = 30  $\mu$ m.

# 3d TBI



**Figure D-4 IHC of Synaptophysin and GFAP 3d Following Central Fluid Percussion Injury.** Confocal imaging of synaptophysin staining (green), a synaptic terminal marker, shows increased aggregation of signal with TBI relative to sham controls (D, A). Astroglia (GFAP positive) labeled in red show an injury-induced increase in 3d TBI animals (E) compared to sham controls (B). Confocal overlays demonstrate the increased number of astroglial processes interdigitated among synaptophysin positive synapses within the injured olfactory bulb after TBI (C). Scale bar = 20  $\mu$ m.

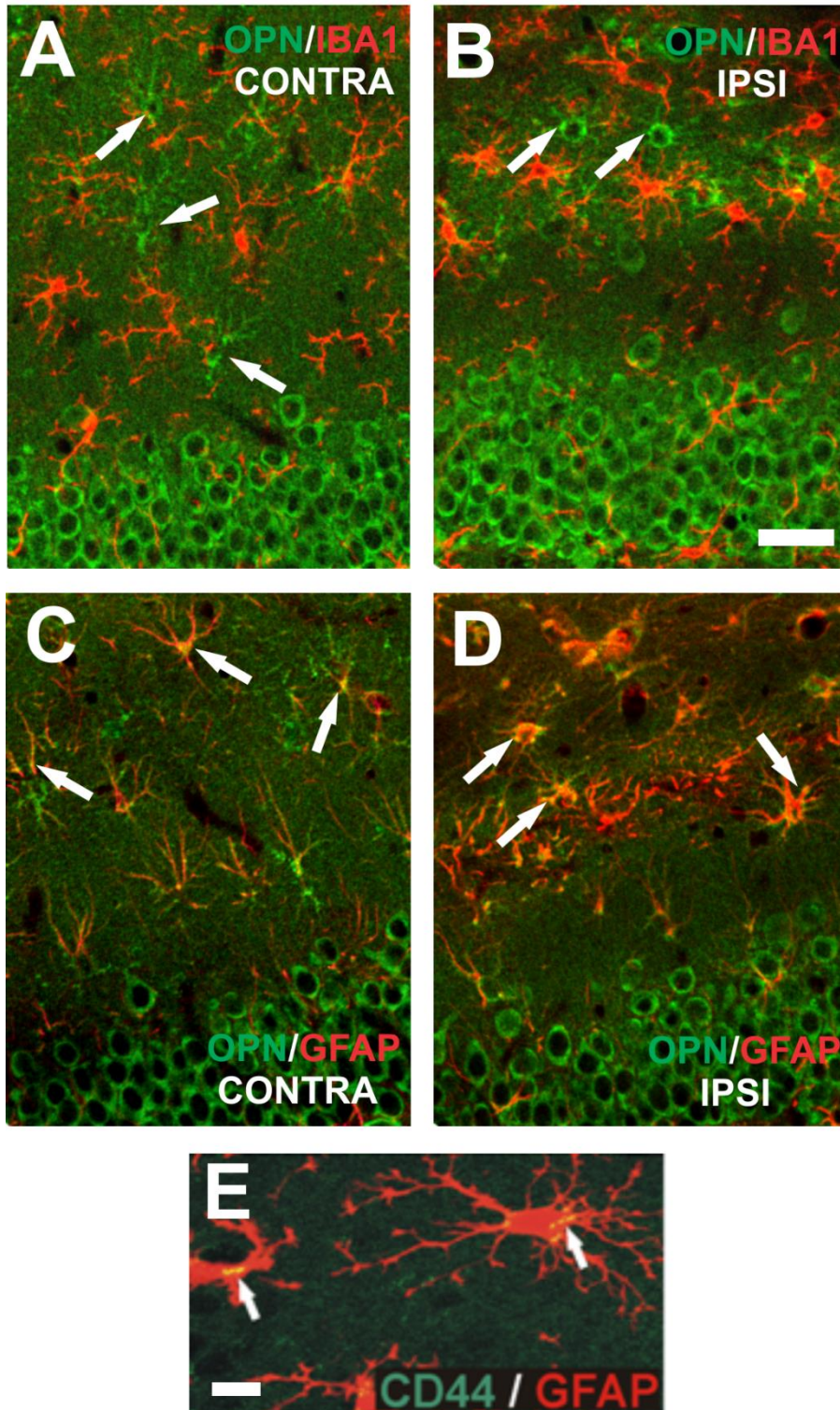


## Appendix E

### Delayed Osteopontin and CD44 Localization to Reactive Astrocytes

**Figure E-1 IHC of OPN Colocalized with IBA1 or GFAP 15d Following UEC.** Confocal imaging of OPN (green) in combination with either IBA1 (red) or GFAP (red) is shown for the dentate ML during the stabilization and maturation phase of reactive synaptogenesis. Low level of OPN signal is seen in reactive microglia of both control and injured sides (A, B), but non IBA1 positive cells contain significant OPN in processes and along the surface of their cell bodies (arrows in A, B). When OPN is colocalized with GFAP, the cells with significant OPN signal are identified as reactive astrocytes (arrows in C, D). Notably, similar GFAP positive reactive astrocytes have high density signal for OPN along their cell surfaces (D), regions where the OPN receptor CD44 can be localized (arrows in E). Scale bar = 30  $\mu\text{m}$  in A-D; 10  $\mu\text{m}$  in E.

# 15d UEC





## Vita

Julie Lynn Chan was born on November 10, 1983 in Santa Clara County, California, and is citizen of the United States of America. She graduated from Sacred Heart Preparatory in Atherton, California in 2001. She received her B.S. in Neurobiology from the University of California, Irvine in 2005 where she continued research in the laboratory of molecular pathogenesis under the guidance of Frank LaFerla through 2006. She then entered the M.D.-Ph.D. program at Virginia Commonwealth University in 2007, and anticipates a graduation date of May 2015.