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Induction of cerebral hyperexcitability by peripheral viral challenge: Role of CXCL10 chemokine

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Induction of cerebral hyperexcitability by peripheral viral challenge: Role of CXCL10 chemokine

Tiffany J. Petrisko

Dissertation
submitted to the School of Medicine
at West Virginia University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Neuroscience

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2019

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Abstract

Induction of Cerebral Hyperexcitability by Peripheral Viral Infection: Role of CXCL10 Chemokine

Tiffany J. Petrisko

Peripheral viral infections are potent comorbid factors that exacerbate neurodegeneration. Although the underlying mechanisms have not been defined, neuronal hyperexcitability has been established as an underlying feature. Our lab has developed a preclinical model in which a viral mimetic, polyinosinic-polycytidylic acid (PIC) is injected intraperitoneally to induce an anti-viral acute phase response (APR). APR in turn elicits robust neuronal hyperexcitability. The present study was undertaken to characterize molecular mechanisms that mediate the development of hyperexcitability in response to PIC challenge. The analysis of brain tissue after PIC challenge revealed a robust elevation of CXCL10 chemokine, indicating its putative role in the process. We have further shown that CXCL10 is overwhelmingly produced by neurons. Neurons also express the cognate receptor, CXCR3, indicating that the CXCL10/CXCR3 axis acts in an autocrine/paracrine manner. Intracerebroventricular injection of CXCR3 inhibitor, AMG-487, abrogates PIC challenge-induced development of hyperexcitability, indicating that the CXCL10/CXCR3 axis drives this process. Moreover, CXCR3 signaling activates microglia. Because microglia are potent modulators of synaptic activity, they likely mediate the development of hyperexcitability. Accordingly, we posit that CXCL10/CXCR3 axis induces the generation of neuronal factors that activate microglia. Activated microglia in turn, impede the activity of inhibitory synapses. This de-inhibition results in the hyperexcitability of neuronal networks. Our results provide a springboard for the full elucidation of the underlying mechanisms that would facilitate the development of novel therapeutic modalities to combat the co-morbidity of peripheral viral infections in neurodegenerative diseases.

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Glossary of Abbreviations

^3H	tritium
ACSF	artificial cerebral spinal fluid
AD	Alzheimer's disease
AKT	Protein Kinase B
AMG	AMG-487
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APR	acute phase response
BBB	blood brain barrier
BDNF	bone derived neurotrophic factor
C(2,3,...)	complement component
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CFB	complement factor b
CNS	central nervous system
CPM	counts per minute
CSF	cerebral spinal fluid
CSS	cumulative seizure score
CTX	cortex
CVO	circumventricular organ
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DAMP	damage associated molecular pattern
DEG	differentially expressed gene
DG	dentate gyrus
DMSO	dimethyl sulfoxide
EAAT	excitatory amino acid transporter
EAE	experimental autoimmune encephalomyelitis

ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
fEPSP	field excitatory post synaptic potential
GABA	gamma-aminobutyric acid
GABA _A R α 2	GABA _A receptor subunit alpha 2
GABA _B R1	GABA _B receptor subunit 1
GABRE	GABA receptor subunit epsilon
GAD	glutamate decarboxylase
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IEG	immediate-early gene
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	CXCL10
IP3	inositol triphosphate
ITAC	CXCL11
KA	kainic acid
LPS	lipopolysaccharide
LTD	long-term depression
LTP	long-term potentiation
MAC	membrane attack complex
MDA	melanoma differentiation-associated protein
mGLUR2/3	metabotropic glutamate receptor 2/3
MIG	CXCL9
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MS	multiple sclerosis
NMDA	N-methyl-D-aspartate
NMDAR1	NMDA receptor type 1
PAMP	pathogen associated molecular pattern
PD	Parkinson's disease
PIC	polyinosinic:polycytidylic acid
PKB	protein kinase B

PKC	protein kinase C
PKR	protein kinase R
PPF	paired pulse facilitation
PRR	pattern recognition receptor
PTx	pertussis toxin
qRT-PCR	quantitative real-time polymerase chain reaction
RIG	retinoic acid inducible gene
RMANOVA	repeated measures analysis of variance
RNA	ribonucleic acid
s.c.	subcutaneous
SAL	saline
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SXC	system xc cystine/glutamate antiporter
TBOA	dl-threo-beta-benzyloxyaspartate
Th(1,2..)	T-helper
TIR	Toll-interleukin-1 (IL-1) receptor
TLR	toll like receptor
TNF	tumor necrosis factor
TRIF	TIR domain-containing adaptor protein inducing interferon- β
WNV	West Nile Virus

CHAPTER 1: INTRODUCTION

1.1. IMMUNE-TO-BRAIN COMMUNICATION

The existence of a bi-directional communication between the peripheral immune system and the brain has been recognized for many years. One of the most well-known examples of immune-to-brain communication can be observed following activation of the innate immune system by a pathogen. Recognition of the pathogen initiates systemic production of pro-inflammatory factors, the process referred to as the acute phase response (APR). Blood-borne APR factors, in turn, induces a “mirror” inflammatory response in the central nervous system (CNS) (McCusker and Kelley, 2013). This cerebral response then elicits alterations in neurochemical and neuroendocrine signaling, resulting in the induction of a group of symptoms collectively known as “sickness behavior”. Sickness behavior represents an adaptive behavioral response consisting of fever, depression, anorexia, malaise, anxiety, cognitive dysfunction, and fatigue consistently observed across animal species (Hart, 1988; Quan and Banks, 2007; Dantzer, 2009).

The induction of a cerebral response by peripheral APR can occur by different mechanisms. Blood-borne inflammatory mediators can be directly transported from the circulation by the endothelial cells that form the blood brain barrier (BBB). Inflammatory mediators can also be transduced by the endothelial cells releasing secondary mediators into the brain parenchyma. Blood-borne mediators can also enter the brain through circumventricular organs (CVOs). In contrast to the most areas of the brain containing continuous capillaries, CVOs contain fenestrated capillaries, and thus provide no BBB (Ganong, 2000). Once inflammatory mediators enter the brain, they are able to activate the innate immune cells of the brain, mainly astrocytes and microglia, which in response generate additional inflammatory mediators. In addition, neurons express a multitude of cytokine receptors, and therefore can also generate additional mediators. Overall, cerebral cells are exposed to a combination of peripherally and centrally generated inflammatory mediators which modulate neurotransmitter and neuroendocrine systems, and ultimately alter brain function resulting in the sickness behavior (Konat, 2016). Finally, peripherally-generated cytokines may activate afferents of the vagal nerve which conveys the stimulus to multiple nuclei located in the medulla oblongata, and induce several symptoms of sickness behavior, including fever, taste aversion, anorexia and hyperalgesia (Fleshner et al., 1995; Watkins et al., 1995).

Despite resulting in uncomfortable side effects, sickness behavior is a survival adaption that alters organisms' priorities to eliminate pathogens by conserving metabolic resources and preventing the spread of the pathogen within the population (Adelman and Martin, 2009; Shakhar and Shakhar, 2015). Additionally, the cerebral inflammatory response is neuroprotective, likely by priming the brain against plausible future insults. Indeed, multiple studies have shown that systemic pretreatments of animals with bacterial endotoxin, lipopolysaccharide (LPS), or viral mimetic polyinosinic: polycytidylic acid (PIC) reduce stroke volume, white matter damage following traumatic brain injury, and nitric oxide toxicity (Huang et al., 2008; Packard et al., 2012; Turner et al., 2017; Lv et al., 2018; Yu et al., 2018).

While APR is neuroprotective to the healthy brain, it is detrimental to the pathological brain. Peripheral infections have long been known to exacerbate symptoms in patients suffering from neurological disorders. Examples of this exacerbation can be observed in patients with seizure syndromes, such as epilepsy, in which both the incidence and severity of seizure, is increased (Tellez-Zenteno et al., 2005; Vezzani and Granata, 2005; Verrotti et al., 2009). In patients with multiple sclerosis (MS), bacterial urinary tract infections as well as upper respiratory infections, such as influenza, have been correlated with an increased incidence of relapses (Andersen et al., 1993; Panitch, 1994; Edwards et al., 1998; Buljevac et al., 2002; Libbey and Fujinami, 2010). Viral infections have been demonstrated to hasten cognitive decline and induce delirium in Alzheimer's disease (AD) patients (Murray et al., 1993; Nee and Lippa, 1999; Holmes et al., 2003; Holmes, 2013). In Parkinson's disease (PD), respiratory infections have been found to be the most frequent cause of increased motor impairments (George et al., 1997; Zheng et al., 2012; Brugger et al., 2015). Additionally, post-stroke systemic infections increase disability and mortality of the victims (Westendorp et al., 2011; Learoyd et al., 2017).

Although the correlation between peripheral infections and neurological exacerbations has been well documented, the mechanisms underlying induction of these exacerbations remain poorly understood. However, there is a compelling body of evidence that the cerebral response is mediated by the initial response of the innate immune system to infection, i.e., the APR (Dantzer, 2006; Cunningham et al., 2007; McCusker and Kelley, 2013; Mardiguian et al., 2017).

1.2. INNATE IMMUNE ACTIVATION

The innate immune system is the body's first line of defense against infectious and/or other insults. The innate immune system relies on the activation of pattern recognition receptors (PRRs) expressed predominantly on innate immune cells, including dendritic cells, leukocytes and tissue macrophages, as well as many types of somatic cells (Medzhitov, 2001). PRRs have evolved to recognize common patterns of the molecular structures that are found in pathogens, called pathogen-associated molecular patterns (PAMPs), as well as endogenous molecules released from damaged and/or dying cells, called damage associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). An important family of PRRs are toll-like receptors (TLRs), which are found on the plasma membrane, endosomes, and lysosomes, permitting them to recognize extra-cellular and intra-cellular PAMPs and DAMPs. Ligation of a specific TLR with its corresponding PAMP/DAMP results in the activation of downstream signaling pathways, and subsequently, the production and secretion of cytokines, chemokines and other inflammatory mediators at the site of infection (Baumann and Gauldie, 1994; Kushner and Rzewnicki, 1994; Cray et al., 2009; Jain et al., 2011). In addition to their local action, the inflammatory mediators quickly enter the blood stream to induce the APR. Of note, APR entails the activation of other tissues and organs, notably, the liver that propagates the inflammatory reaction by generating additional factors. The resultant cocktail of circulating inflammatory factors includes chiefly cytokines, chemokines and complement proteins.

1.2.1. CYTOKINES

Cytokines are a broad category of small peptides, typically less than 80 kDa in size, which are secreted by a variety of cell types to regulate a range of biological functions, including the innate immune system, inflammation, and hematopoiesis. These extracellular signaling proteins can function in autocrine, paracrine, and endocrine manners to exert effects on both nearby cells and long-range targets. Cytokines can be further categorized based on the secreting cell type and function (Zhang and An, 2007; Holdsworth and Gan, 2015). For example, lymphokines and monokines are secreted by lymphocytes and monocytes, respectively. Interleukins are cytokines that are secreted by leukocytes and act upon other leukocytes. Interferons are antiviral cytokines that block the replication of viruses within infected cells. Cytokines can exert pro-inflammatory or anti-inflammatory functions, depending on their concentration, the activation signal, and the target cell (Cavaillon, 2001). Due to the diverse nature of cytokines, their receptors vary greatly in both structure and mechanisms of signal transduction (Wang et al., 2009).

1.2.2. CHEMOKINES

Chemokines are a subclass of cytokines which are classically thought to function as chemoattractant signals that recruit immune cells to the site of infection (Turner et al., 2014). They are small, 8-10 kDa proteins characterized by a conserved amino acid structure. Most chemokines contain four cysteines that interact with one another through intramolecular disulfide bonds. Based on the structure and the spacing of their first cysteine residues, chemokines can be divided into four groups (Asensio and Campbell, 1999; Reaux-Le Goazigo et al., 2013). *C chemokines* contain only two cysteines, one near its N terminus and another located downstream. *CC chemokines* contain two adjacent cysteines near their amino terminus while *CXC chemokines* contain two N-terminal cysteines separated by a single amino acid. Finally, *CX₃C chemokines* have three amino acids between their two cysteine residues.

Chemokines primarily signal through 7-transmembrane G-protein coupled receptors (GPCRs). Receptor ligation results in homo or heterodimerization (Colvin et al., 2004) resulting in the dissociation of G protein subunits. This in turn, increases intracellular calcium, and activates downstream signaling pathways (Bajova et al., 2008). These pathways include the extracellular signal-regulated kinases (Ras/ERK), protein kinase B (PI3K/AKT), and protein kinase C (PKC) signaling pathways. Their activation induces cytoskeleton rearrangements, cell proliferation, migration, and adhesion of immune cells (Curnock et al., 2002; Legler and Thelen, 2018).

1.2.3. COMPLEMENT PROTEINS

Complement proteins are distinct plasma proteins that aid in the clearance of invading microbes and infected/damaged host cells. The complement proteins react with one another to generate activated complement proteins that bind covalently to pathogens and tag them for phagocytosis. The activation process also generates small fragments called anaphylatoxins that act as chemoattractants and propagate inflammatory responses. Finally, the terminal complement components assemble into the membrane attack complex (MAC), which forms transmembrane pores on the invading microbes or infected cells resulting in cell lysis and death.

1.3 PIC MODEL

To model exacerbation of neurological disorders by peripheral viral infections, the Konat lab established a periclinical model utilizing intraperitoneal (i.p.) injection of the viral mimetic, PIC. PIC is a synthetic double stranded RNA (dsRNA), a PAMP of viral infections, as most viruses either consist of dsRNA or generate dsRNA during their replication cycles (Jacobs and Langland, 1996; Weber et al., 2006). Multiple PRRs are present on mammalian cells that detect both intracellular and extracellular dsRNA, including the endosomal toll-like receptor 3 (TLR3), and the cytoplasmic receptors, i.e., retinoic acid inducible gene-1 (RIG-1), melanoma differentiation-associated protein 5 (MDA-5), and protein kinase R (PRK) (Berke et al., 2013; Konat, 2016).

PIC preferentially binds to TLR3, which results in dimerization of the receptor and recruitment of the adaptor protein Toll-interleukin-1 (IL-1) receptor (TIR) domain-containing adaptor protein interferon- β (TRIF) to the TIR domain of TLR3 (Perales-Linares and Navas-Martin, 2013). Activation of TLR3 initiates the antiviral-APR, characterized by the generation of a plethora of cytokines and additional inflammatory mediators, in particular the type 1 interferons $INF\alpha$ and $INF\beta$ (Guha-Thakurta and Majde, 1997). This APR instigates neurochemical changes within the brain, and ultimately sickness behavior.

Additionally, one needs to be cognizant that the peripheral PIC injection does not model viral infections. Rather, it models the initial response to viral infections, i.e., the APR. Directly studying the antiviral APR, as opposed to a viral infection provides multiple benefits. Firstly, the PIC-induced antiviral APR is an innate immune response and is produced regardless of the specifics of a virus, allowing us to more broadly understand the immune response to viral infections. Secondly, the APR has been repeatedly shown to underly the exacerbation of a patient's neurological symptoms.

As such, the PIC model offers a variety of advantages to study immune-to-brain communication. Thus, a single bolus injection of PIC into the peritoneal cavity results in a rapid production of inflammatory mediators that are quickly relayed to the brain induce the cerebral inflammatory response. PIC injected into the intraperitoneal cavity is rapidly degraded by RNases (Krasowska-Zoladek et al., 2007) and does not reach the circulation (Fil et al., 2011). Thus, the brain is exposed to a bolus stimulation solely by APR inflammatory mediators and is not

compounded by viral particles in the circulation. Consequently, these features allow detailed kinetic analyses of both the systemic and cerebral responses.

1.3.1. BLOOD CYTOKINES

Peripheral PIC challenge induces significant yet transient elevation of serum levels of several key proinflammatory cytokines: interferon-beta (INF- β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF α), and interleukin-1 beta (IL-1 β) (Cunningham et al., 2007; Michalovicz and Konat, 2014). Serum levels rapidly peak at 3 hours post-PIC challenge, and promptly decline back to basal levels. All these cytokines are known to modulate neuronal activity (Schneider et al., 1998; Viviani et al., 2003; Schafers and Sorkin, 2008; Olmos and Llado, 2014), and to contribute to the induction of sickness behavior (Murray et al., 2015). Of note, the injection of blood plasma from PIC-challenged animals into naïve mice mimics the cerebral response to PIC challenge (Fil et al., 2011), indicating that the response is mediated by blood-borne factors.

1.3.2. BEHAVIOR

As described above, intraperitoneal injection of PIC into mice results in sickness behavior (Guha-Thakurta and Majde, 1997; Cunningham et al., 2007; Michalovicz and Konat, 2014), which is consistent with the symptoms of viral infections in humans (Loftis et al., 2008; Nelligan et al., 2008). In mice, sickness behavior peaks at 6 hours following PIC injection, and subsides within 48 hours. These results are consistently seen across motivational tests including burrowing test, and locomotor activity examined by the open field and rearing tests (Cunningham et al., 2007; Konat et al., 2009; Michalovicz and Konat, 2014). Notably, the rearing test developed in the Konat lab provides the most sensitive assessment of PIC challenge-induced sickness behavior (Michalovicz and Konat, 2014). This test was routinely used in the present studies.

1.3.3. HIPPOCAMPAL HYPEREXCITABILITY

Peripheral PIC challenge exerts profound functional effects on neuronal circuitry in the brain. A stellar example of this is demonstrated by Kirschman et al. (2011) in which pretreatment with PIC exacerbated chemically induced seizures. As mentioned previously, peripheral viral infections increase the risk of seizures in the susceptible population (Tellez-Zenteno et al., 2005; Vezzani and Granata, 2005). In this experimental paradigm, PIC challenge 24 hours prior to seizure induction with the glutamate agonist, kainic acid (KA), dramatically augmented seizure severity and duration. This seizure hypersusceptibility persisted up to 72 hours post PIC

challenge, indicating that a single PIC challenge results in a protracted hyperexcitability of neuronal networks in the brain.

Seizures result from an imbalance of excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission resulting in an overall hyperexcitable network. This imbalance can result from an increase in excitatory transmission, a decrease in inhibitory transmission, or both (During and Spencer, 1993; Bradford, 1995; Barker-Haliski and White, 2015). In line with enhanced neuronal activity following PIC challenge, our electrophysiological studies of the hippocampus, the ictal site of KA induced seizures, 24 hours after PIC demonstrated enhanced basal synaptic activity and plasticity (long term potentiation, LTP) compared to saline injected controls, two indicators of neuronal hyperexcitability (Hunsberger et al., 2016).

1.3.4. EXTRACELLULAR GLUTAMATE

Studies in anesthetized mice have demonstrated a profound increase in extracellular glutamate levels in the hippocampus 24 hour after peripheral PIC challenge (Hunsberger et al., 2016; Hunsberger et al., 2017)). The largest increase of 11-fold was observed in the dentate gyrus (DG), closely followed by 9.8-fold increase in the cornu ammonis 1 (CA1) and 5.8-fold increase in the cornu ammonis 3 (CA3)-Similar increase in the extracellular glutamate was observed in awake animals with implanted glutamate-sensing electrodes (Hunsberger et al., 2017). Moreover, increased extracellular glutamate positively correlated with seizure severity and duration.

Although there was no differences in synaptic capacity to release glutamate as seen from potassium evoked release, PIC challenge decreased the paired-pulse facilitation (PPF), indicating increased probability of glutamate release (Hunsberger et al., 2016)It is plausible that this discrepancy is due to potassium evoked release measuring the ceiling capacity for glutamate release rather than the more physiological levels demonstrated by PPF. Astrocytes are responsible for the uptake and recycling of glutamate via the excitatory amino acid transporters (EAATs). Astrocytes can also release glutamate through the cystine-glutamate antiporter (the system x_c^- ; Sxc), which exchanges extracellular cystine for intracellular glutamate. PIC challenge decreased the uptake of exogenously applied glutamate, and increased efflux of intracellular glutamate, indicating that both EAAT and Sxc might be responsible for the augmentation of extracellular glutamate.

1.3.5. CEREBRAL GENE EXPRESSION

Genome-wide transcriptome analysis of the hippocampus in response to PIC challenge revealed a total of 625 differentially expressed genes (DEGs) identified as dysregulated genes during 48 hours after PIC challenge (Michalovicz et al., 2015a). Unsurprisingly, this genomic reprogramming entails mostly genes related to the innate immune response, in particular cytokine, chemokine, and complement genes, as well as several genes related to neurotransmission.

1.3.5.1. Cytokines. The levels of cerebral messenger RNA (mRNA) encoding the pro-inflammatory cytokines $\text{INF}\beta$, IL-6, IL-1 β , and $\text{TNF}\alpha$ peaked within 3-6 hours post PIC challenge and returned to baseline at 48 hours (Cunningham et al., 2007; Fil et al., 2011; Michalovicz and Konat, 2014). Moreover, similar upregulation of cytokine mRNA was observed across major brain regions (Cunningham et al., 2007; Konat et al., 2009), indicating that the upregulation is global and not restricted to a specific anatomical structures.

Cytokines play an important role in the central nervous system by modulating neuronal transmission. Thus, IL-1 β decreases calcium influx from cultured hippocampal neurons, and reduces presynaptic glutamate release (Murray et al., 1997) that creates functional deficit in LTP (Cunningham et al., 1996; MacManus et al., 2000; Zhou et al., 2006b; Zhou et al., 2006a). At high IL-1 β concentrations, this impairment is further compounded post-synaptically by decreasing both spontaneous and evoked N-methyl-D-aspartate (NMDA) post-synaptic currents (Yang et al., 2005). $\text{TNF}\alpha$, which is constitutively expressed at low levels in both neurons and glial cells *in vitro*, also exhibits multiple neuromodulatory functions (Gahring et al., 1996; Stellwagen et al., 2005). Increased $\text{TNF}\alpha$ expression enhances excitatory neurotransmission via an exocytosis of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors while simultaneously diminishing inhibitory neurotransmission by removing gamma-aminobutyric acid (GABA) receptors from neuronal membranes (Stellwagen et al., 2005). Additionally, the constative nature of low levels of the cytokine allow for glial $\text{TNF}\alpha$ to modulate synaptic scaling (Beattie et al., 2002; Stellwagen et al., 2005). Short term application of $\text{TNF}\alpha$ to cultured astrocytes impairs glutamate uptake by downregulating glutamate transporters (Zou and Crews, 2005; Olmos and Llado, 2014), or by damaging these transporters via the generation of reactive oxygen species (Mir et al., 2009; Sandoval et al., 2018).

1.3.5.2. Chemokines. The mRNA of a plethora of chemokines and their receptors are also significantly upregulated in the brain following PIC challenge (Fil et al., 2011; Michalovicz and Konat, 2014). CXCL1, CXCL9, CXCL10, and CXCL11 feature the highest upregulation by several thousand-folds over control (Fil et al., 2011; Michalovicz and Konat, 2014; Konat, 2016). The CCL2, CCL5, CXCL1 and CXCL2 genes are also upregulated, however only by several hundred-folds. Most chemokine mRNAs peak rapidly, within 3-6 hours, and several remain elevated even 72 hours post PIC challenge.

While the neuromodulatory functions of chemokines has not been thoroughly researched, there is mounting evidence that chemokines play an important role in both maintaining synaptic networks and altering them in disease. CXCL10 (inducible protein 10/IP-10) along with CXCL9, (monokine induced by gamma interferon/MIG) and CXCL11 (interferon-inducible T-cell alpha chemoattractant/TAC) bind to their cognate receptor, CXCR3. In C57BL/6 mice, however, CXCL11 is non-functional due to a point mutation (Groom and Luster, 2011a). Astrocytic production of CXCL10 enhanced long-term potentiation of the hippocampal networks (Vikolinsky et al., 2004). *In vitro* studies examining the effects of exogenous CXCL10 found its application to increase both spontaneous and evoked electrical activity as well as intracellular calcium levels (Nelson and Gruol, 2004; Sui et al., 2006). This increase in electrical activity was paralleled by decreased GABAergic and increased glutamatergic signaling (Cho et al., 2009; Tyagarajan et al., 2013; Zacchi et al., 2014; Battaglia et al., 2018).

CCL2, which signals through its cognate receptor CCR2, also increases neuronal activity. Acute exogenous CCL2 application to hippocampal slices increased postsynaptic excitatory potentials although no changes to LTP were observed (Gruol, 2016). Additionally, mice engineered to overexpress CCL2 in astrocytes exhibit similar results, with enhancements in short-term but not long-term potentiation (Nelson et al., 2011). Furthermore, CCL2 elicits a transient calcium influx in cultured neurons (Banisadr et al., 2005) and significantly reduces GABA-induced neuronal currents in spinal neurons (Gosselin et al., 2005).

1.3.5.3. Complement proteins. Transcriptomics analysis identified the complement pathway as the primary pathway upregulated by peripheral PIC challenge in the hippocampus (Michalovicz et al 2015). Quantitative real time polymerase chain reaction (qRT-PCR) revealed several complement genes, to be significantly upregulated following PIC challenge. These genes exhibited a rapid increase in expression, peaking at 24 hours post-PIC. The mRNAs encoding

complement factor b (Cfb), C6, and C3 featured the highest peak upregulation, of 53-, 40-, and 12-fold over control, respectively.

Recent studies have revealed a critical role of the complement system during brain development by tagging overabundant synapses for phagocytosis by microglia (Stevens et al., 2007; Schafer et al., 2012; Stephan et al., 2012). Additionally, aberrant activation of the complement system in neurological disorders, such as epilepsy and AD has been hypothesized to be responsible for synapse loss (Zhou et al., 2008; Fonseca et al., 2011; Hong et al., 2016b).

1.4. DISSERTATION AIMS

As discussed above, activation of the innate immune system in the periphery induces profound molecular and functional changes in the brain. In particular, the antiviral APR exacerbates neurological conditions resulting likely from a protracted neuronal hyperexcitability. However, no mediators responsible for the induction of hyperexcitability have been identified. The robust upregulation of inflammatory genes in the brain discussed above suggests that their protein products may mediate the development of hyperexcitability in response to PIC challenge. The aim of this dissertation was to test this possibility by characterizing expression of these genes at the protein level. The ultimate long-term goal of this research endeavor is to provide a springboard for the development of novel therapeutics that would target cerebral pathways to prevent/ameliorate the exacerbation of neurological symptoms without hampering the resolution of systemic infections.

1.5 REFERENCES

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CHAPTER 2: PERIPHERAL VIRAL CHALLENGE TRIGGERS HIPPOCAMPAL PRODUCTION OF INFLAMMATORY PROTEINS

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2.1. ABSTRACT

Peripheral viral infections increase seizure propensity and intensity in susceptible individuals. We have modeled this comorbidity by demonstrating that intraperitoneal (ip) injection of the conventional viral mimetic, polyinosinic-polycytidylic acid (PIC), renders the brain hypersusceptible to seizures induced by kainic acid (KA). At the molecular level, the hippocampus, which is the ictal site of KA-induced seizures, exhibits upregulated expression of messages encoding several inflammatory genes. Here, we profiled temporal expression of these genes at the protein level. Briefly, eight-week old female C57BL/6 mice were ip injected with 12 mg/kg of PIC and inflammatory proteins were quantified in the hippocampus and blood by ELISA. We found a robust but transient increase in blood concentration of IL-6, CXCL10, CCL2, CXCL9, CCL7 and CCL12 six hours after PIC challenge. CXCL1, IL1 β , TNF α and CXCL2 featured a moderate increase. However, only four chemokines were increased in the hippocampus. CXCL10 showed the highest increase 6-12 h after PIC challenge, and its level dwindled to the baseline by 48 h. CXCL1, CXCL9 and CXCL2 were also transiently elevated but their maximal values were by an order of magnitude lower than the values for CXCL10. These results indicate that CXCL10 is the primary inflammatory protein generated in the hippocampus in response to PIC challenge, and that this chemokine may drive the development of seizure hypersusceptibility. In addition, the hippocampus featured a protracted increase in the levels of anaphylatoxins C3a and C5a, indicating the activation of the complement cascades.

2.2. INTRODUCTION

Peripheral microbial infections have been recognized as important comorbid factors that increase the frequency and severity of seizures in susceptible individuals (Tellez-Zenteno et al., 2005; Scheid and Teich, 2007; Verrotti et al., 2009). Peripherally-instigated seizure hypersusceptibility is believed to be triggered by the acute phase reaction (APR), i.e., the initial response of the host aimed at containing the spread of infection. APR entails a fulminant generation of cytokines and other inflammatory mediators often referred to as “cytokine storm”. These blood-borne mediators are relayed to the brain and induce inflammatory response in the cerebral parenchyma. The resultant neuroinflammatory milieu modifies the activity of neuronal networks and alters cerebral function (Dantzer, 2006; Dantzer and Kelley, 2007; Quan and Banks, 2007). In particular, the inflammatory agents may decrease inhibitory inputs, increase excitatory inputs, or both, resulting in hyperexcitability of neuronal networks, a hallmark of seizures. However, the pertinent agents and the underlying cellular/molecular mechanisms have not been explored in detail.

We have developed a preclinical murine model of seizure hypersusceptibility induced by peripheral viral challenge. In this model, APR induced by intraperitoneal (i.p.) injection of a viral mimetic, polyinosinic-polycytidylic acid (PIC), renders the brain hypersusceptible to kainic acid (KA)-induced seizures as seen from a robust increase in the intensity and duration of status epilepticus (Kirschman et al., 2011; Hunsberger et al., 2017). This hypersusceptibility is protracted for three days after PIC challenge (Michalovicz and Konat, 2014). This behavioral phenotype is underscored by profound hyperexcitability of neuronal networks in the hippocampus (Hunsberger et al., 2016), the ictal site of KA-induced seizures (Ben-Ari and Cossart, 2000). In a quest to identify molecular substrates of seizure hypersusceptibility, we profiled hippocampal gene expression. We found PIC challenge to induce an extensive genomic reprogramming as seen from the dysregulated expression of 625 genes at the mRNA level (Michalovicz and Konat, 2014; Michalovicz et al., 2015b). The upregulated genes include genes encoding several chemokines and cytokines. Generally, the upregulation of respective mRNAs peaks between three and six hours following the PIC challenge. The *Cxcl11*, *Cxcl10* and *Cxcl9* genes feature the highest upregulation reaching the maxima of several thousand over the control values before gradually subsiding to the basal level. Other chemokine genes in descending order of upregulation are: *Cxcl1*, *Cxcl2*, *Ccl12*, *Ccl2*, *Ccl7* and *Ccl5*. Also, the genes encoding cytokines with known ictogenic activity, i.e., IL-1 β , IL-6 and TNF α are modestly

upregulated. In addition, PIC challenge significantly upregulates several genes within the complement pathway.

The present study was undertaken to parse a plausible involvement of inflammatory genes in the development of seizure hypersusceptible phenotype induced by PIC challenge. We selected hippocampal genes that feature upregulation at the message level, and profiled their expression at the protein level over the time period of seizure hypersusceptibility. We also determined temporal fluctuations of the respective proteins in the blood.

2.3. METHODS

2.3.1. ANIMALS

Eight-week old female C57BL/6J mice obtained from Charles River Laboratories (Wilmington, MA) were housed under 12-h light/dark conditions (lights on at 6 am) and fed ad libitum. Peripheral APR was induced by a single i.p. injection of 12 mg/kg of ultrapure PIC (Invivogen, San Diego, CA) in 100 μ l of saline. Mice injected with equivolume saline served as controls. To verify successful PIC injection, the development of sickness behavior was assessed after 6 h by the rearing test (Michalovicz and Konat, 2014). All procedures were approved by the West Virginia University Animal Care and Use Committee and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

2.3.2. ELISA

At different time points after PIC challenge, mice were deeply anesthetized by i.p. injection of 65 mg/ml of pentobarbital (Beauthanasia, Patterson Veterinary, Devens, MA), and sacrificed by pneumothorax. Blood was quickly collected by heart puncture, and the animals were perfused transaortally with saline. Blood was allowed to coagulate overnight at 4°C, and centrifuged at 3,000 rpm for 10 minutes. Serum was aspirated and stored at -20°C. Both hippocampi were dissected and disintegrated in the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40 and a protease inhibitor, pH 7.5) by sonication. Complete Protease Inhibitor Cocktail was procured from Roche Diagnostics (Mannheim, Germany), and all other chemicals were from Sigma-Aldrich (St. Louis, MO). The homogenates were spun at 14,000 rpm for 20 min and the supernatants were aspirated and stored at -20°C. Inflammatory proteins were quantified by ELISA according to manufacturers' instructions. CXCL1, CXCL2, CXCL9, CXCL10, TNF- α , and IL-1 β ELISA kits were obtained from R&D Systems, Inc. (Minneapolis,

MN), while CCL2, CCL5, CCL7, CCL12, Cfb, C3, C3a, C8b, and C9 ELISA Kits were from LifeSpan Biosciences (Seattle, WA). ELISA kits for C5a and IL-6 were purchased from Abcam (Cambridge, MA).

2.3.3. PROTEIN DETERMINATION

Total protein was measured in serum and tissue homogenates by ABC method (BioRad, Hercules, CA) using bovine serum albumin as a standard. The levels of inflammatory proteins determined by ELISA were normalized to the amount of total protein in samples.

2.3.4. STATISTICAL ANALYSIS

The values from PIC-injected vs. saline-injected mice were analyzed by one-way ANOVA followed by Bonferroni post-hoc test and presented as means $\pm$ SE. Differences between groups were considered significant at $P \leq 0.05$.

2.4. RESULTS

APR induced by peripheral PIC challenge transiently upregulates hippocampal expression of messages encoding several chemokines (Michalovicz and Konat, 2014). Most of these chemokines surged transiently in the blood with a maximum attained 6 h after PIC injection (Fig. 1). CXCL10 reached the highest values [F(5, 14)=38.74, $p<0.001$], and was followed by CCL2 [F(6, 15)=21.67, $p<0.001$], CXCL9 [F(6, 21)=34.59, $p<0.001$], CCL7 [F(6, 10)=12.76, $p<0.001$], CCL12 [F(6, 14)=27.82, $p<0.001$], CXCL1 [F(5, 14)=39.52, $p<0.001$] and CXCL2 [F(5, 14)=25.65, $p<0.001$]. The concentration of all these species returned to control level by 48 h or sooner. Blood concentration of CCL5 was not affected by PIC challenge [F(6, 12)=1.30, $p=0.326$]. CXCL11 is inactive in C57BL/6 mice due to a null mutation (PubMed accession numbers NT_109320 and AK050012.1), and was not measured.

As shown in Fig. 1, CXCL10 also featured the highest generation in the hippocampus 12 h after PIC challenge [F(6, 32)=44.96, $p<0.001$]. Its levels dropped by 45% at 24 h, and returned to the baseline at 48 h. The levels of CXCL1 [F(6, 33)=38.50, $p<0.001$], CXCL9 [F(6, 32)=20.79, $p<0.001$] and CXCL2 [F(6, 29)=28.85, $p<0.001$] were also increased, although to a lesser extent. Unlike CXCL10, the levels of CXCL1, CXCL9 and CXCL2 rapidly declined to the baseline by 24 h after PIC challenge. Hippocampal concentrations of CCL2 [F(6, 13)=1.86,

$p=0.165$], CCL5 [$F(6, 12)=0.59$, $p=0.733$], CCL7 [$F(6, 12)=1.12$, $p=0.406$] and CCL12 [$F(6, 14)=1.08$, $p=0.42$] were not altered by PIC challenge.

Figure 1. Production of chemokines induced by peripheral PIC challenge. Mice were intraperitoneally injected with 12 mg/kg of PIC. Saline-injected mice served as respective controls. At different time points, blood (blue triangles) and hippocampal (red circles) levels of the chemokines were quantified by ELISA. The results are expressed as pg of

chemokines per mg of total tissue protein. Symbols represent means $\pm$ SEM from 4-7 animals. Asterisks denote significant differences from saline-injected controls (** $p \leq 0.01$; *** $p \leq 0.001$).

In concordance with previous studies (Cunningham et al., 2007; Michalovicz and Konat, 2014), the three ietogenic cytokines, IL-1 β , IL-6 and TNF α , surged in the blood following PIC challenge (Fig. 2). IL-6 [$F(6, 20)=28.53$, $p<0.001$] reached concentrations comparable to that of CXCL10 at 6 h, whereas IL-1 β [$F(6, 19)=5.69$, $p=0.002$] and TNF α [$F(5, 12)=8.14$, $p<0.001$] featured modest peaks. Like in the case of chemokines, blood concentration of these cytokines rapidly returned to the basal levels by 12 or 24 h. Hippocampal level of IL-6 showed a slight, approximately 2-fold increase 6 h after PIC challenge [$F(6, 27)=4.83$, $p<0.005$], whereas, the levels of IL-1 β [$F(6, 17)=0.90$, $p=0.516$] and TNF α [$F(5, 14)=2.05$, $p=0.134$] were unchanged.

Figure 2. Production of IL-1 β , IL-6, and TNF α induced by peripheral PIC challenge. Mice were intraperitoneally injected with 12 mg/kg of PIC. Saline-injected mice served as respective controls. At different time points, blood (blue triangles) and hippocampal (red circles) levels of the cytokines were quantified by ELISA. The results are expressed as pg of chemokines per mg of total tissue protein. Symbols represent means $\pm$ SEM from 4-7 animals. Asterisks denote significant differences from saline-injected controls (*** $p \leq 0.001$).

Based on our previous study (Michalovicz et al., 2015b) showing that PIC challenge upregulates the expression of the complement system in the hippocampus, we profiled temporal changes in the levels of select complement proteins (Fig. 3). All proteins tested were present in the control hippocampus. C3 featured the highest basal level followed by C8b, C3a CfB (complement factor B), C5a and C9. C3 [F(6, 23)=5.32, $p=0.001$] and C8b [F(6, 33)=2.85, $p=0.024$] were transiently increased at 72 and 12 h post PIC, respectively. The level of C3a anaphylatoxin rapidly increased within the initial 24 h and approached a plateau at approximately 48 h after PIC challenge [F(6, 32)=6.88, $p<0.001$]. C5a anaphylatoxin displayed a more complex kinetics, as its levels increased rapidly at 12 h, declined at 48 h, and then increased again at 72-96 h [F(6, 32)=9.97, $p<0.001$]. C9 [F(6, 32)=1.71, $p=0.151$] and CfB [F(6, 11)=3.07, $p=0.051$] were not affected by PIC challenge at any time point.

Figure 3. Hippocampal production of the complement proteins induced by peripheral PIC challenge. Mice were intraperitoneally injected with 12 mg/kg of PIC. Saline-injected mice served as respective controls. At different time points, hippocampal levels of the complement proteins were quantified by ELISA and expressed as pg of complement proteins per mg of total tissue protein. Symbols represent means $\pm$ SEM from 4-7 animals. Asterisks denote significant differences from saline-injected controls (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

2.5. DISCUSSION

PIC has been commonly used to induce antiviral APR that features a surge in blood-borne cytokines including IFN β , IL-6, IL-12, TNF α and IL-1 β (Pruett et al., 2004; Cunningham et al., 2007; Michalovicz and Konat, 2014). Herein, we found that PIC challenge also engenders an increase in circulating chemokines, i.e., CXCL10, CCL2, CXCL9, CCL7, CCL12, CXCL1 and CXCL2. Like the cytokines, the chemokines revealed an early, but transient elevation.

This blood cytokine/chemokine surge induces a robust generation of CXCL10, and to a lesser extent the generation of CXCL9, in the hippocampus. CXCL10 and CXCL9 are ligands of the common receptor, CXCR3. CXCL10 has been shown to modulate neuronal function in diverse in vitro systems. For example, in cell cultures, CXCL10 enhanced electrical activity of neurons (Nelson and Gruol, 2004), reduced the activity of GABAergic synapses and increased synaptic network activity (Cho et al., 2009). The exposure of hippocampal slices to exogenous CXCL10

inhibited long-term potentiation (LTP) and altered presynaptic transmitter release through CXCR3 signaling (Vlkolinsky et al., 2004). Hippocampal levels of two ligands of CXCR2, CXCL1 and CXCL2, were also increased by PIC challenge. Signaling through CXCR2, the receptor for CXCL1 and CXCL2, has also been shown to increase neuronal excitability (Ragozzino et al., 1998; Lax et al., 2002; Wang et al., 2008). Consequently, hippocampal elevation of the four chemokines found in the present study (Fig. 1) strongly implicates them as candidate molecules that mediate the development of hyperexcitability, and thus, seizure hypersusceptibility instigated by peripheral PIC challenge. However, the combined maximal concentration of CXCL9 and CXCL10 was approximately 9-fold higher than the combined maximal concentration of CXCL1 and CXCL2. Consequently, CXCR3 activation is most likely the primary signaling pathway.

In addition to the direct effects on neuronal excitability discussed above, chemokines may induce hyperexcitability through alternative mechanisms. For example, CXCL10 is a potent activator and chemoattractant of microglia (Rappert et al., 2004; Clarner et al., 2015), and microglia are well known for their ability to disassemble neuronal synapses (Ji et al., 2013; Kettenmann et al., 2013). Therefore, activated microglia may displace presynaptic terminals from GABAergic synapses leading to disinhibition, and thus, hyperexcitability and seizure hypersusceptibility. In support of such a mechanism, peripheral challenge with a bacterial mimetic, lipopolysaccharide (LPS) has been shown to induce hyperexcitability of cortical neurons through microglia-mediated displacement of presynaptic GABAergic terminals (Chen et al., 2014).

IL-1 β , IL-6, and TNF α have been shown to be icogenic in a variety of experimental systems (Li et al., 2011). However, in spite of the significant increase in their blood concentrations instigated by PIC challenge, only IL-6 was found minimally (~2-fold) increased over basal level in the hippocampus (Fig. 2). This is in contrast to the robust elevation of the chemokines (Fig. 1). Therefore, IL-6, IL-1 β and TNF α are not likely to be involved in the induction of hyperexcitable phenotype following PIC challenge.

The complement system has been implicated in the modulation of neuronal network function through synaptic scaling (Schafer et al., 2012). Previously, we reported the upregulation of hippocampal mRNA encoding several complement components following PIC challenge (Michalovicz et al., 2015b). In concordance with the increased abundance of the C3 mRNA, we

found the elevation of C3 protein (Fig. 3). However, while the mRNA peaked at 24 h, C3 protein peaked at 72 h indicating the possibility of delayed translation of the message. Also, there was no upregulation of the C8 gene expression of the message level, but a significant upregulation at the protein level was evident at 12 h post PIC. This discrepancy indicates a posttranscriptional regulation of these genes. The most notable is the divergence in the expression of the *Cfb* gene. The *Cfb* message peaked to 53-fold over control 24 h after PIC challenge (Michalovicz et al., 2015b). Also, an increase in the CfB protein was evident in the tissue by immunofluorescence. However, no significant elevation of hippocampal CfB was detected by ELISA (Fig. 3). CfB is a single chain polypeptide that upon activation is proteolytically cleaved to Ba and Bb fragments. Bb binds to membrane-linked C3b subunit, and subsequently to C5 to form the alternative pathway convertase C3 and convertase C5, respectively. It can be envisaged that PIC challenge increases the synthesis of CfB that is rapidly activated by cleavage. Therefore, the cleavage of the CfB polypeptide could abolish antibody binding in ELISA, but the fragments were still detected by the antibody used for immunofluorescence.

The generation of anaphylatoxins, C3a and C5a, the products of C3 and C5 convertases, substantiates the activation of the complement cascades in the hippocampus. Interestingly, the elevated concentration of C3a and C5a did not dropped even 96 h after PIC challenge (Fig. 3). This kinetics is in contrast with the chemokines that dwindle to the control level by 48 h, or sooner. Also, seizure hypersusceptibility decreases gradually after 24 h and ultimately disappears 96 h after PIC challenge (Michalovicz and Konat, 2014). Consequently, the anaphylatoxins are not likely to mediate the increase in seizure susceptibility. In summary, the generation of CXCL10 in the hippocampus induced by peripheral PIC challenge is likely the primary molecular event that drives the development of neuronal hyperexcitability and ensuing seizure hypersusceptibility.

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Conflict of interest

The authors declare no conflicts of interest.

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CHAPTER 3: NEURONAL CXCL10/CXCR3 AXIS MEDIATES THE INDUCTION OF CEREBRAL HYPEREXCITABILITY BY PERIPHERAL VIRAL CHALLENGE

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3.1. ABSTRACT

Peripheral infections can potentially exacerbate neuropathological conditions, though the underlying mechanisms are poorly understood. We have previously demonstrated that intraperitoneal (i.p.) injection of a viral mimetic, polyinosinic-polycytidylic acid (PIC) induces a robust generation of CXCL10 chemokine in the hippocampus. The hippocampus also features hyperexcitability of neuronal circuits following PIC challenge. The present study was undertaken to determine the role of CXCL10 in mediating the development of hyperexcitability in response to PIC challenge. Briefly, young female C57BL/6 mice were i.p. injected with PIC, and after 24 h, the brains were analyzed by confocal microscopy. CXCL10 staining of neuronal perikarya and a less intense staining of the neuropil was observed in the hippocampus and cortex. CXCL10 staining was also evident in a subpopulation of astrocytes, whereas microglia were CXCL10 negative. CXCR3, the cognate receptor of CXCL10 was present exclusively on neurons, indicating that the CXCL10/CXCR3 axis operates through an autocrine/paracrine neuronal signaling. Blocking cerebral CXCR3 through intracerebroventricular injection of a specific inhibitor, AMG487, abrogated PIC challenge-induced increase in basal synaptic transmission and long-term potentiation (LTP), as well as the reduction of paired-pulse facilitation (PPF). The PIC-mediated abolishment of long-term depression (LTD) was also restored after application of AMG487. Moreover, CXCR3 inhibition attenuated seizure hypersensitivity induced by PIC challenge. The efficacy of AMG487 strongly strengthens the notion that CXCL10/CXCR3 axis mediates the induction of cerebral hyperexcitability by PIC challenge.

3.2. INTRODUCTION

Peripheral infections are important comorbid factors for the major neuropathological conditions. For example, peripheral infections exacerbate dementia in Alzheimer's disease (AD) (Murray et al., 1993; George et al., 1997; Nee and Lippa, 1999; Holmes et al., 2003; Holmes, 2013), relapses in multiple sclerosis (MS) (Andersen et al., 1993; Edwards et al., 1998; Buljevac et al., 2002; Libbey and Fujinami, 2010) and seizures (Tellez-Zenteno et al., 2005; Scheid and Teich, 2007; Verrotti et al., 2009). It is generally believed that inflammatory agents generated during the initial innate immune response to the invading microbes, i.e., the acute phase response (APR), are relayed to the brain, and by augmenting the ongoing neuropathology exacerbate disease symptoms. However, the underlying cellular/molecular mechanisms have not been defined.

We have developed a preclinical murine model to study mechanisms by which APR exerts its effects on the brain. In this model, APR is induced by intraperitoneal injection of the epitomic viral mimetic, polyinosinic-polycytidylic acid (PIC). We have demonstrated that PIC challenge induces hyperexcitability of neuronal networks as seen from a profound increase in the basal synaptic transmission and long term potentiation (LTP) in hippocampal slices (Hunsberger et al., 2016), as well as from hypersusceptibility to kainic acid (KA)-induced status epilepticus (Kirschman et al., 2011; Michalovicz and Konat, 2014; Hunsberger et al., 2017). Because neuronal hyperexcitability is an invariable feature of the major neuropathologies (Esclapez et al., 1999; Lehmann et al., 2000; Buljevac et al., 2002; Holmes et al., 2003; Caramia et al., 2004; Palasik et al., 2005; Tellez-Zenteno et al., 2005; Scheid and Teich, 2007; Verrotti et al., 2009; Khedr et al., 2011; Penzes et al., 2011; Rossi et al., 2012; Scharfman, 2012; Huynh et al., 2013; Yener and Basar, 2013; Eikermann-Haerter, 2014), it might provide a mechanistic link for the exacerbating effects of peripheral inflammation on disease progression.

At the molecular level, PIC challenge induces a fulminant but transient increase of several inflammatory cytokines (IFN β , IL-6, IL1 β and TNF α) and chemokines (CXCL10, CCL2, CXCL9, CCL7 and CCL12) in the blood (Michalovicz and Konat, 2014; Petrisko and Konat, 2017). This “cytokine storm” in turn, leads to a robust generation of CXCL10 in the hippocampus, whereas other major inflammatory mediators are either only slightly elevated (CXCL1, CXCL2, CXCL9, IL-6), or unchanged (IL-1 β , TNF α). The expression of the *Cxcl10* mRNA is also massively upregulated in the hippocampus following PIC challenge (Michalovicz and Konat, 2014), indicating that CXCL10 is produced in situ in the brain.

Because CXCL10 is a potent modulator of neuronal activity (Nelson and Gruol, 2004; Vlkolinsky et al., 2004; Cho et al., 2009), it seems plausible that it might be a putative molecule, which acting through its cognate receptor, CXCR3, drives the development of hyperexcitability. This is congruent with the emerging role of CXCL10 as an important player in diverse neuroinflammatory and neurodegenerative diseases (Michlmayr and McKimmie, 2014b).

The present study was undertaken to identify cellular origin of cerebral CXCL10 production instigated by PIC challenge. We also appraised the cellular origin of CXCR3, and its involvement in the development of neuronal hyperexcitability.

3.3. MATERIALS AND METHODS

3.3.1. ANIMALS

Eight-week old female C57BL/6J mice obtained from Charles River Laboratories (Wilmington, MA) were housed with free access to food and water in a humidity- and temperature-controlled rooms under a 12:12 h light-dark cycle. Mice in all experimental groups were matched by weight prior to treatments. All experimental procedures were approved by the West Virginia University and Auburn University Animal Care and Use Committee and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

3.3.2. DRUG ADMINISTRATION

Peripheral APR was induced by a single i.p. injection of 12 mg/kg of ultrapure PIC (Invivogen, San Diego, CA) in 100 μ L of saline. Mice injected with equivolume saline served as controls. To verify successful PIC injection, the development of sickness behavior was assessed after 6 h by the rearing test (Michalovicz and Konat, 2014).

To block CXCR3, a specific inhibitor, AMG-487 (Tocris), was administered by intracerebroventricular (i.c.v.) injection. Briefly, mice were anesthetized with isoflurane (1.9%-3.4% inhalation; continuous) and immobilized on a stereotaxic frame (Kopf, Tujunga CA). Three mg/kg of AMG-487 (Tocris, Bristol, UK) in 5 μ L of artificial cerebrospinal fluid containing 20% of DMSO (ACSF) was delivered gradually (0.5 μ L/min) into the cerebral ventricles through a 26s-gauge needle as a bilateral injection of 2.5 μ L per side. The coordinates from bregma were: anteroposterior: -0.45mm, mediolateral: +/-0.95mm, and dorsoventral: -2.6mm (Paxinos and Franklin 2001). The needle was left in place for 5 min to minimize back-flux of the injectate. After 2 h, the animals were PIC-challenged as above.

3.3.3. INDUCTION OF SEIZURES

Twenty-four hours after PIC injection, seizures were induced via subcutaneous (s.c.) injection of 12 mg/kg of kainic acid (KA; Sigma Chemical Co., St Louis, MO), and seizure severity was graded as previously described (Kirschman et al., 2011; Hunsberger et al., 2017) using the 6-step scale (Morrison et al., 1996). Seizure cessation was defined as two consecutive seizure severity scores of 0. Cumulative seizure scores were calculated as the summation of all scores over the entire period of status epilepticus.

3.3.4. EXTRACELLULAR FIELD POTENTIAL RECORDING

Twenty-four hours after PIC injection, animals were euthanized with carbon dioxide, and 350- μ m thick coronal slices through the dorsal hippocampus were prepared using a Leica VT1200S Vibratome (Leica Microsystems, Wetzlar, Germany). Electrophysiological measurements, i.e., basal synaptic transmission, paired-pulse facilitation (PPF), long-term potentiation (LTP) and long-term depression (LTD), were performed as previously described (Parameshwaran et al., 2013; Bhattacharya et al., 2015; Hunsberger et al., 2016; Bhattacharya et al., 2017).

3.3.5. CONFOCAL MICROSCOPY

24 h after PIC challenge, mice were deeply anesthetized by i.p. injection of 65 mg/kg of pentobarbital (Beauthanasia, Patterson Veterinary, Devens, MA), sacrificed by pneumothorax, and transaortically perfused with saline followed by 4% paraformaldehyde. The brains were dissected, cryoprotected, and cut into 30 μ m coronal sections using the ThermoFisher Scientific HM450 Sliding Microtome (Thermo Fisher Scientific, Waltham, MA USA). Free-floating immunofluorescent staining of CXCL10, GFAP and Iba1 was performed as previously described (Michalovicz et al., 2015b). For CXCR3 localization, Triton X-100 concentration was increased to 0.4%. Additionally, for microglia co-staining, sections were incubated with anti-CD11b antibody for 96 h at 4°C with rat-anti-CXCR3 being added for the last 12 h. Sections were mounted to slides with Prolong Gold (Thermo Fisher Scientific, Waltham, MA USA), and imaged using the Nikon A1R Confocal microscope (Nikon Instruments, Melville, NY). Z-stacks were taken with a 60X objective every 0.125 μ m, and 3D projections were rendered using NIS Elements Advanced Research imaging software (Nikon Instruments, Melville, NY). For the co-localization of CXCL10 the primary antibodies were: goat-anti-CXCL10 (R&D Systems, Minneapolis, MN), mouse-anti-NeuN (Millipore, Burlington, MA), rabbit-anti-GFAP (Dako, Santa Clara, CA) and rabbit-anti-Iba1 (Wako Chemicals, Richmond, VA). Secondary antibodies were: anti-goat conjugated with Alexa Fluor 555, and anti-mouse and anti-rabbit conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA USA). For the co-localization of CXCR3 the primary antibodies were: rabbit-anti-CXCR3 (Novus Biologicals, Centennial, CO), mouse-anti-NeuN (Millipore, Burlington, MA), mouse-anti-GFAP (Millipore, Burlington, MA) and rat-anti-CD11b conjugated to BD Horizon BV480 (BD Biosciences, Franklin Lakes, NJ). Secondary antibodies were: anti-rabbit conjugated with Alexa Fluor 555, and anti-mouse conjugated with Alexa Fluor 555 (Thermo Fisher Scientific, Waltham, MA USA).

3.3.6. STATISTICAL ANALYSIS

The data were analyzed by ANOVA, expressed as means $\pm$ SEM. Statistical comparisons between groups were performed using Tukey post hoc tests. Differences between groups were considered significant at $P < 0.05$.

3.4. RESULTS

Immunofluorescent analysis performed 24 h after PIC challenge revealed intense CXCL10 staining throughout the brain, whereas a negligible staining was observed in the brain of saline-injected mice. Representative confocal images of the CA1 hippocampal region and the motor cortex are shown in the upper panels of Fig. 1. Most of CXCL10 staining was localized to the cytoplasm of neuronal perikarya as revealed by neuron-specific NeuN antibody (Fig. 1, lower panels). The staining extended into the proximal parts of neuronal processes. However, the intensity of CXCL10 staining among NeuN-positive neurons varied. This variation was particularly well demonstrable in the cortex where individual neurons were better separated. Also, a subpopulation of astrocytes expressed CXCL10. The staining was restricted to the cell bodies. In contrast, no CXCL10 staining co-localized with Iba1-positive microglia. The same cell-specific co-localization of CXCL10 was observed at shorter time intervals after PIC challenge, i.e., 6 and 12 h (not shown).

Figure 1. Cellular origin of cerebral CXCL10 production following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC or saline (SAL), and after 24 h, analyzed by immunohistochemistry. Upper panels The expression of CXCL10 (red) in the hippocampal CA1 region (A) and in the cortex (B). Lower panels Cell-specific expression of CXCL10 (red) in the CA1 and in the cortex of PIC-challenged mice. Anti-NeuN, anti-GFAP and anti-Iba1 antibodies (green) were used to identify neurons, astrocytes and microglia, respectively. Confocal images were captured at 60X magnification. Scale bars represent 50 μ m.

As depicted in Fig. 2, neurons also expressed the cognate receptor of CXCL10, CXCR3. In congruence with the previously observed lack of upregulation of the *Cxcr3* mRNA expression by PIC challenge (Fil et al., 2011), there was no detectable difference in CXCR3 staining intensity between brain tissue from PIC-injected and saline injected mice (upper panels). The expression was confined to the cell surface of neuronal perikarya. Although, CXCR3 staining was also evident in neuronal processes, particularly, in the cortex. CXCR3 expression was evident throughout the brain. No apparent CXCR3 staining was detectable in either astrocytes or microglia.

Figure 2. Cellular expression of CXCR3. Mice were i.p. injected with 12 mg/kg of PIC or saline (SAL), and after 24 h, analyzed by immunohistochemistry. Upper panels The expression of CXCR3 (red) in the hippocampal CA1 region (A) and in the cortex (B). Lower panels; cell-specific expression of CXCR3 (red) in the CA1 and in the cortex of PIC-challenged mice. Anti-NeuN, anti-GFAP and anti-CD11b antibodies (green) were used to identify neurons, astrocytes and microglia, respectively. Confocal images were captured at 60X magnification. Scale bars represent 50 μ m.

To assess the role of the CXCL10/CXCR3 axis in the induction of neuronal hyperexcitability, we used i.c.v. injection of a specific inhibitor, AMG487, to block CXCR3 signaling before challenging the animals with PIC. To determine whether the inhibitor alters the PIC-induced sickness behavior, we measured the loss of body weight 24 h after PIC challenge. As previously observed (Cunningham et al., 2007), the injection of saline and PIC induced approximately 1.5 and 5.9% body loss as assessed 24 h later (Fig. 3). No additional increase in the weight loss was found in mice injected with PIC and AMG487.

Figure 3. Weight loss in PIC challenged mice. Mice received a bolus of i.c.v. injection of AMG487 (3 mg/kg) or vehicle (VEH). Two hours later, mice were weighed prior to induction of the acute antiviral response via an i.p. injection of 12 mg/kg of PIC in saline (a). Mice injected with 100 μ L of saline served as vehicle controls. Twenty-four hours after PIC injection, mice were weighed again, and the amount of weight lost was determined (b). Bars represent means $\pm$ SEM from 5 to 6 mice per group. (ANOVA followed by Tukey's multiple comparisons test; ** $p \leq 0.01$, *** $p \leq 0.001$; ns - not significantly different).

Basal synaptic transmission was evaluated in hippocampal slices 24 h after PIC challenge (Fig. 4). At stimulus intensities from 40 to 100 μ A, basal synaptic transmission increased by approximately 85% in the slices from PIC-challenged compared to vehicle-injected mice. CXCR3 blockade abrogated this increase (Fig. 4A). To determine whether there is a relationship between pre- and post-synaptic responses, fiber volley, which represents the presynaptic action potential in response to stimulus, was compared to postsynaptic responses represented as field excitatory postsynaptic potentials (fEPSPs) using linear regression analysis. PIC challenged mice exhibited increased postsynaptic responses to the same fiber volley amplitude compared to the vehicle-treated mice, whereas blockade of CXCR3 mitigated this increase (Fig. 4B). To determine if the hyperexcitability of the neurons following PIC challenge are due to presynaptic modifications, the probability of neurotransmitter release was examined by

measuring paired pulse facilitation (PPF). There was a significant reduction in PPF at the short stimulus intervals in slices from PIC-challenged vs. vehicle-injected mice (Fig. 4C), indicating an increase in pre-synaptic release probability. The drop in $EPSP_2/EPSP_1$ was 35 and 25% at 25 and 50 ms stimulus intervals, respectively. No significant changes were evident at longer stimulus intervals. The PIC challenge-induced decrease in PPF was attenuated by the pretreatment of mice with AMG487. To test if the changes in PPF in PIC-challenged mice are due to presynaptic axonal recruitment, fiber volley amplitude versus stimulus intensities was compared. PIC challenge had no effect on the fiber volley amplitude across various stimulus intensities (Fig. 4D), which indicates no changes in presynaptic axon recruitment.

Figure 4. Synaptic transmission in hippocampal slices. (A) Basal synaptic transmission represented by the fEPSP slope measured at increasing stimulus intensities. (B) Fiber volley (FV) analysis represented by the FV amplitude measured at increasing stimulus intensities. (C) Paired-pulse facilitation expressed as the change of ratio of the second stimulus fEPSP to the first stimulus fEPSP slope plotted as a function of interstimulus interval. (D) Basal synaptic transmission represented by the slope of the linear regression between fEPSP slope (Y axis) and FV amplitude (X axis). Symbols represent means $\pm$ SEM from 5 to 6 mice per group. (ANOVA followed by Tukey's multiple comparisons test; *represents significant difference between Veh-Veh and Veh-PIC; #represents significant difference between Veh-PIC and AMG-PIC; *# $p \leq 0.05$, **/### $p \leq 0.01$, ***/#### $p \leq 0.001$; ns = not significantly different).

To determine if these alterations in basal synaptic transmission could result in altered synaptic plasticity, LTP and LTD were assessed. PIC challenge significantly enhanced LTP (Fig. 5A,B). When expressed as average fEPSP slope during 50-60 minutes following LTP induction, the enhancement amounted to 32% over the control value. AMG487 pretreatment negated this enhancement. PIC-challenged mice failed to exhibit LTD (Fig. 5C,D), whereas CXCR3 inhibition with AMG487 restored LTD to levels similar to vehicle-treated mice.

Figure 5. Synaptic plasticity in hippocampal slices. (A) Long term potentiation (LTP) represented by percent change in fEPSP slope over time. (B) LTP as represented by fEPSP slope during 50-60 minutes following LTP induction. (C) Long term depression (LTD) represented by percent change in fEPSP slope over time. (D) LTD as represented by fEPSP slope during 50-60 minutes following LTD induction. Symbols represent means $\pm$ SEM from 5 to 6 mice per group. (ANOVA followed by Tukey's multiple comparisons test; *represents significant difference between Veh-Veh and Veh-PIC; #represents significant difference between Veh-PIC and AMG-PIC; *# $p \leq 0.05$, **## $p \leq 0.01$, ***### $p \leq 0.001$; ns = not significantly different).

As previously demonstrated (Kirschman et al., 2011; Michalovicz and Konat, 2014; Hunsberger et al., 2017), PIC-challenge increased the susceptibility of mice to KA-induced seizures, as indicated by a 47% increase in cumulative seizure score (Fig. 6). CXCR3 blockade with AMG-487 attenuated this PIC-mediated increase.

Figure 6. CXCR3 inhibition attenuates seizure hypersensitivity induced by PIC challenge. Mice received a bolus of i.c.v. injection of AMG 487 (3 mg/kg) or vehicle (VEH). Two hours later, the acute antiviral response was induced by i.p. injection of 12 mg/kg of PIC in saline. Mice injected with 100 μ L of saline served as vehicle controls. Twenty-four hours after PIC injection, status epilepticus (SE) was induced by subcutaneous injection of 12 mg/kg of KA. Mice injected with saline in lieu of PIC served as

controls (VEH). Seizures were expressed as cumulative seizure score. Symbols represent means $\pm$ SEM from 3 to 6 mice per group. (ANOVA followed by Tukey's multiple comparisons test; * $p \leq 0.05$, ** $p \leq 0.01$; ns = not significantly different).

3.5. DISCUSSION

We have previously shown that PIC challenge robustly upregulates cerebral expression of the Cxcl10 gene at the message (Fil et al., 2011; Michalovicz and Konat, 2014) and protein (Petrisko and Konat, 2017) levels. Here, we found neurons to be the predominant cell type responsible for CXCL10 generation in the brain. Interestingly, neuronal CXCL10 production has been shown to be induced by viral encephalitides (Rappert et al., 2004; Klein et al., 2005; Chai et al., 2015), indicating a commonality of neuronal response to systemic and central viral challenge. In concordance with previous study (Xia et al., 2000), only a subpopulation of astrocytes expressed CXCL10, although the contribution of these cells to the global production of the chemokine seems to be negligible. Resting microglia do not express CXCL10, but the expression is induced in microglia activated by viral infection of the brain (Chai et al., 2015). PIC challenge did not elicit microglial expression of CXCL10, indicating no development of proinflammatory phenotype of microglia. Our previous studies showing no effect of PIC challenge on the expression of cerebral iNOS (Konat et al., 2009) and CX3CL1 (Fil et al., 2011), the indices of neuroinflammation, buttress this contention.

CXCR3 is expressed constitutively on neurons and neuronal processes in the brain (Xia et al., 2000). Here, we found a similar pattern of expression of the receptor. Neuronal expression of both the receptor and its ligand indicates that the CXCL10/CXCR3 axis operates through an

autocrine/paracrine neuronal signaling. Moreover, we found CXCR3 to be restricted to neurons, as both astrocytes and microglia were CXCR3-negative. Previously, CXCR3 expression was found in reactive, but not quiescent astrocytes and microglia in a variety of CNS pathologies (Goldberg et al., 2001; Tanuma et al., 2006). Thus, the lack of glial CXCR3 expression provides further support for the notion that PIC challenge does not promote proinflammatory transformation of astrocytes and microglia.

In concordance with our previous electrophysiological study (Hunsberger et al., 2016), PIC challenge increased basal synaptic transmission, resulting in hyperexcitability of hippocampal circuits. The increased basal synaptic transmission likely occurred due to both increased presynaptic release and enhanced postsynaptic activity. The increase in presynaptic release in PIC-challenged mice possibly resulted from altered release mechanisms at the synapse because the data suggests no changes in presynaptic axon recruitment. Future studies will assess whether the enhanced postsynaptic activity is due to increased recruitment of glutamate receptors at the synapse or increased ion channel activity, or possibly both. Of note, we showed that alterations in both basal synaptic transmission and synaptic plasticity, as well as PIC-induced seizure hypersensitivity, are abolished by the inhibition of CXCR3. These findings strongly indicate that the CXCL10/CXCR3 axis governs the induction of neuronal hyperexcitability. These results dovetail with previous *in vitro* studies that found CXCL10 to increase electrical activity of neurons in culture (Nelson and Gruol, 2004; Cho et al., 2009). Several possible mechanisms can be envisaged. For example, increased neuronal activity might result from the suppression of inhibitory GABAergic transmission, as a consequence of the downregulation of GAD65/67 and two GABAergic receptors, GABA_BR1 and GABA_AR α 2 (Cho et al., 2009). Interestingly, we found PIC challenge to downregulate the GABA receptor subunit ϵ (GABRE) (Michalovicz and Konat, 2014). Moreover, the ligation of neuronal CXCR3 activates the ERK1/2 pathway (Xia et al., 2000), which phosphorylates GPH leading to the disruption of GABAergic synapses and their decreased transmission (Tyagarajan et al., 2013). Increased neuronal activity might also be attributed to the enhancement of excitatory glutamatergic transmission, as CXCL10 upregulates the expression of two glutamatergic receptors, NMDAR1 and mGluR2/3 (Cho et al., 2009). It is likely that both the attenuation of GABAergic and the enhancement of excitatory transmission contribute to shifting the neuronal balance toward excitation. Furthermore, additional, as yet unexplored, mechanisms might also mediate the development of hyperexcitability triggered by the neuronal CXCL10/CXCR3 axis signaling.

In conclusion, we have demonstrated that anti-viral APR activates neuronal CXCL10/CXCR3 axis in the brain, and that this activation elicits hyperexcitability of neuronal circuits. Our results strongly suggest that the CXCL10/CXCR3 axis may play a critical role in the comorbid effect of peripheral viral infections on the progression of major neuropathological diseases.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

MR and GK conceived and designed the experiments and wrote the manuscript. VS aided in experimental design. TP performed immunohistochemical and imaging experiments, analyzed the data, and edited the manuscript. JB, SS, SS, PP, YD, HH and RH performed the AMG inhibition experiments, analyzed the data, or edited the manuscript.

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CHAPTER 4: UNPUBLISHED DATA

4.1. SPATIOTEMPORAL CXCL10 EXPRESSION INDUCED BY PIC CHALLENGE

Supplemental Data to Chapter 3

Fig. 1 in Chapter 3 depicts a robustly increased generation of neuronal CXCL10 in hippocampal CA1 region and the motor cortex (CTX), 24 hr after PIC challenge. This supplemental study was designed to determine time course and regional expression of neuronal CXCL10. As shown in Fig. 1, low levels of neuronal CXCL10 were observed in CA1, CA3, DG and CTX in saline injected mice. In the hippocampal subregions, CXCL10 staining increased with time reaching maximum at 12 h, decreased slightly at 24 hr, and dropped to the basal level at 96 hr after PIC challenge. This temporal expression of the chemokine is congruent with the generation of hippocampal CXCL10 determined by ELISA (Chapter 2). The cortex featured a similar kinetics, although the peak intensity of CXCL10 staining was maintained between 12 and 24 hr post PIC challenge. Moreover, the upregulation of CXCL10 expression was not restricted to the four regions studied, but was evident throughout the entire brain (Fig. 2).

Figure 1. Time course of neuronal CXCL10 expression in different brain regions following PIC challenge. The animal treatment and microscopic analysis were performed as described in Chapter 3. CXCL10 is stained red, while neurons are stained green. CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus; CTX, motor cortex. Confocal images were captured at 60X magnification. Scale bars represent 50 μ m.

Figure 2. Cerebral generation of CXCL10 induced by PIC challenge. The animal treatment and immunohistochemistry were as described in Fig. 1. Epifluorescent images of coronal brain sections were captured at 10X magnification. Scale bars represent 500 μ m.

These results indicate that peripheral PIC challenge induces transient generation of CXCL10 by neurons, and that this neuronal response is global, i.e., entails all brain regions. However, kinetics of this response may differ between brain regions as exemplified by the hippocampus vs. cortex regarding the chemokine generation at 12 vs. 24 hr post PIC.

4.2 NEURONAL C-FOS EXPRESSION AS AN INDEX OF HYPEREXCITABILITY

Manuscript in Preparation

4.2.1. INTRODUCTION

C-fos is an immediate-early gene (IEG) that undergoes a dramatic, yet transient, upregulation in neurons in response to a variety of depolarizing stimuli including neurotransmitters (Kawasaki et al., 2009; Shevelkin et al., 2012), neurotrophic factors (Baille-Le Crom et al., 1996; Joo et al., 2016), cytokines (Srinivasan et al., 2004), and direct stimulation (Sheng et al., 1990; Thompson et al., 1995; Fields et al., 1997). Neuronal c-fos expression profoundly increases in the hippocampus and cortex during seizures (Morgan et al., 1987; Baille-Le Crom et al., 1996; Herrera and Robertson, 1996; Zhang et al., 2002; Dudek, 2006; Barros et al., 2015). As such, the use of c-fos immunoreactivity has become increasingly common, because it allows for much faster and more cost-effective measure of neuronal activity than electrophysiology (Bullitt, 1990; Kovacs, 2008; Kawashima et al., 2014; Malhi et al., 2014)

This study was undertaken to determine whether c-fos immunoreactivity is a viable technique to examine PIC challenge-induced hyperexcitability.

4.2.2. METHODS

4.2.2.1 Drug Administration

Cerebral CXCR3 inhibition in mice was achieved as described in Chapter 3 with some modifications. In particular, we used unilateral i.c.v. injection AMG-487, and the coordinates from bregma were anteroposterior 0.45 mm, mediolateral +1.0mm, and dorsoventral -3.6mm. Because AMG-487 solution was prepared in ACSF containing 10% of DMSO, respective controls were injected with equivolume amounts of 10% DMSO in ASCF. After 2 hours, the animals were i.p. injected with PIC in saline, or saline alone (controls) as described in Chapters 2 and 3. Six hours after PIC challenge, the rearing test was performed as in Chapters 2 and 3. Additionally, mice were weighed at 0 and 24 hr after PIC challenge.

4.2.2.2. Microscopy

Immunohistochemistry was performed 24 hr post PIC challenge as previously described (Chapter 3) using mouse-anti-NeuN (1:500; Millipore) and rabbit-anti-c-fos (1:10,000 Abcam)

primary antibodies. Confocal images were obtained using a 40X objective with a Nyquist value of 0.23 μ m at a resolution of 512x512 pixels. 3D projections were rendered using NIS Elements Advanced Research imaging software (Nikon Instruments, Melville, NY) unless otherwise indicated. The same LUT settings were applied to all images.

4.2.2.3. Neuronal Expression of c-fos

C-fos positive neurons were quantified using Nikon Elements General Analysis 3 (GA3). Briefly, de-identified confocal z-stacks were imported into Nikon Elements and GA3. Background subtraction and gaussian smoothing were applied prior to 3D thresholding for neurons and c-fos modeling. Additional background staining was eliminated by a setting minimum diameter requirement of $\sim 7\ \mu$ m for neurons and $\sim 3\ \mu$ m for c-fos in 3D. Parameters were adjusted on an image-by-image basis to maximize the accuracy of the modeling with the researcher blinded to the conditions. The total number of neurons, and the percentage of c-fos containing neurons within the image were then counted. The intensity of c-fos within individual neurons was calculated using the original, unmodified fluorescent data.

4.2.2.4. Statistical Analysis

The data was analyzed by one-way ANOVA followed by the Bonferroni post-hoc test. Differences between groups were considered significant at $p < 0.05$.

4.2.3. RESULTS AND DISCUSSION

Because the high density of neurons within the hippocampus prevents accurate quantitation of neurons, we performed these experiments in the motor cortex. Fig 1. depicts c-fos-expression in neurons at different time points after PIC challenge. Only a few c-fos+ neurons were present at 0 hr. At 24 hr, the population of c-fos+ neurons and the intensity of c-fos staining dramatically increased. A slight increase was also evident at 6 hr post PIC challenge. Quantitative analysis of c-fos+ neurons by General Analysis 3 and one-way ANOVA showed the effect of time was significant [$F(3,12) = 20.582$, $p < 0.001$]. Post-hoc analysis (Fig. 2A) revealed that the percentages of c-fos positive neurons did not significantly differ between 0 ($4.48\% \pm 3.19$), 6 ($17.53\% \pm 3.68$) and 12 ($11.84\% \pm 4.08$) hr after PIC challenge. However, the percentage of c-fos+ neurons at 24 hr ($48.25\% \pm 4.62$) was significantly higher than the value at 0 ($p < 0.001$), 6 ($p < 0.004$) and 12 hr ($p < 0.001$). The increase at 24 vs. 0 h was approximately 10-fold. This increase was concomitant with an augmented intensity of c-fos staining in individual neurons Fig. 2B. Total number of neurons per image did not differ between groups.

As previously shown by electrophysiology in Chapter 3, neuronal hyperexcitability induced by PIC challenge is profoundly attenuated by the inhibition of CXCR3 receptor. Therefore, we tested whether neuronal c-fos expression is also CXCR3-dependent.

Initially, we determined whether the i.c.v. injections of the CXCR3 inhibitor, AMG-487, alters PIC-induced sickness behavior by measuring the loss of body weight, and by the rearing test. No difference in body weight loss was observed between DMSO-SAL and AMG-SAL injected mice (Fig. 1). Consistent with previous study (Cunningham et al., 2007), PIC challenge resulted in an approximately 10% weight body loss during 24 hours. No significant difference was observed between DMSO-PIC and AMG-PIC treated animals.

Figure 1. Effect of CXCR3 Inhibition on body weight. Mice received i.c.v. injection of AMG-487 or DMSO. Two hours later, mice were i.p. injected with PIC or saline (SAL) as described in Methods. Mice were weighed at 0 and 24 hr after PIC challenge to calculate body weight loss. Bars represent means $\pm$ SEM from 3 mice per group. * p <0.05, ** p <0.01, *** p <0.001.

Our highly sensitive rearing test (Michalovicz and Konat, 2014) quantifies the loss of locomotor activity, motivation, and exploratory behavior by measuring the number of times animals stand on their hind limbs and reach a height of at least 8 cm during a 15-minute period. This test was performed six hr after PIC injection, as this time point corresponds to the peak of PIC challenge-induced sickness behavior. Significant differences between groups was observed [F (3,11) =

13.346, $p=0.002$]. DMSO+PIC (16.0 ± 5.69) animals reared significantly less than DMSO+SAL (53.33 ± 10.71 ; $p=0.008$) animals (Fig. 2). There was no difference in the number of rears between DMSO+PIC and AMG+PIC (5.00 ± 1.53) animals nor between AMG+SAL and AMG+PIC treatments. Surprisingly, AMG-SAL (26.33 ± 3.93) treated animals reared significantly less compared to DMSO-SAL animals ($p=0.008$).

Figure 2. Effect of CXCR3 Inhibition on rearing

behavior. Mice received i.c.v. injection of AMG-487 or DMSO. Two hours later, mice were i.p. injected with PIC or saline (SAL) as described in Methods. Six hours after PIC challenge, the animals were examined to the rearing test. The number of rears in a 15-minute period were recorded. Bars represent means $\pm$ SEM from 3 mice per group. * $p<0.05$, ** $p<0.01$

Together, these results show that i.c.v. injected AMG-487 does not impair somatic functions as seen from the lack of effect of the body weight. However, the inhibitor has a profound effect on the CNS function as seen from the reduction of rearing events. Interestingly, CXCR3-deficient mice were previously reported not to develop depressive behavior following PIC challenge (Blank et al., 2016). The discrepancy between this and our result might be due to the different models used, i.e., transgenic vs. pharmacological reduction of CXCR3 activity. Additionally, we are unable to determine the exact amount of AMG-487 relative to the extracellular CXCL10 concentration, as our previous study profiling chemokine protein levels (Chapter 2) examined whole hippocampi consisting of both extracellular and intracellular CXCL10. As immunohistochemistry studies have shown the majority of CXCL10 expression is intracellular (Chapters, 3 and 4.1), we are unable to know exactly how much CXCL10 is present in the extracellular fluid throughout the brain. However, it must be noted that while the dose of AMG-

487 utilized throughout this study may result in off target effects on additional receptors that may account for the observed results.

Representative images presented in Fig. 3 demonstrate an overt reduction of the c-fos+ neuronal population brought about by AMG-487. Quantitative analysis revealed a significant effect of group on the percentage of c-fos positive neurons [F (3,14) = 43.700, $p < 0.001$] (Fig. 4A). DMSO-PIC ($28.454\% \pm 1.61$) animals had significantly more c-fos positive neurons compared to DMSO-SAL (14.019 ± 1.38 ; $p < 0.001$), AMG-SAL ($8.474\% \pm 2.11$; $p < 0.001$) and AMG-PIC ($4.373\% \pm 0.26$; $p < 0.001$). There was no difference between DMSO-SAL and AMG-SAL, indicating that AMG pretreatment by itself did not significantly alter c-fos expression. Additionally, AMG pretreatment returned AMG-PIC c-fos expression back to AMG-SAL levels ($p = 0.423$). Total number of neurons per image did not differ between groups. The point plot displaying the intensity of c-fos staining in individual neurons is shown in Fig. 4B.

Figure 3. Time course of neuronal c-fos expression following PIC challenge. At various time points after PIC challenge, brains were analyzed by confocal microscopy (see Methods). Images were captured with a 40X objective. Scale bars represent 40 μ m.

Figure 4. Temporal expression of neuronal c-fos instigated by PIC challenge. 3D modeling of neurons and c-fos was performed using General Analysis 3 in Nikon Elements. A) The percentage of neurons expressing c-fos at each time point. Bars represent means $\pm$ SEM from 3 mice per group. ***p < 0.001 compared to 0 HR. ##p < 0.01 between pairwise comparison between non-zero HR time points B) Box and whisker displaying the range of mean c-fos intensities within individual c-fos positive neurons.

While c-fos is typically expressed within minutes of increased neuronal activity, and in seizure models sustained for approximately 12 hours (Baille-Le Crom et al., 1996; Barros et al., 2015), here, we found that the increase in the number of c-fos+ neurons is profoundly delayed by 24 hr after PIC challenge. This apparent discrepancy can be explained by the fact that the aforementioned studies induced neuronal hyperactivity, while we induce neuronal hyperexcitability. PIC challenge does not result in seizures but renders the neuronal circuits more prone to the induction of seizures. Thus, the neuronal activity is not, but the propensity of neurons to fire is increased. The delayed increase in c-fos+ neurons indicates a complex transcriptional, translational and posttranslational process that ultimately lead to hyperexcitability. These processes should be addressed in future studies.

The increased expression of neuronal c-fos is concordant with our electrophysiological experiments (Chapter 3) showing neuronal hyperexcitability (Michalovicz, 2013; Hunsberger et al., 2016; Hunsberger et al., 2017). Moreover, the attenuation of neuronal c-fos expression by CXCR3 inhibition further confirms our electrophysiological results. Overall, these results indicate

that cortical c-fos immunoreactivity is a viable technique to examine neuronal hyperexcitability induced by PIC challenge, and possibly in other experimental and clinical conditions.

Figure 5. Effect of CXCR3 inhibition on PIC challenge – induced neuronal c-fos expression. Mice received a i.c.v. injection of CXCR3 inhibitor, AMG-487 (AMG), or the vehicle (DMSO). Two hours later, mice were i.p. injected with PIC (PIC), or the vehicle (SAL). After 24

hr, the cortex was analyzed by immunohistochemistry using antibodies for c-fos (red) and neurons (green). Confocal images of the cortex were captured with a 40x objective. Scale bars represent 40 μ m.

Figure 6. Effect of CXCR3 inhibition on c-fos expression. 3D modeling of neurons and c-fos was performed using General Analysis 3 in Nikon Elements to determine A) the percentage of neurons expressing c-fos following i.c.v injections of AMG-487 or DMSO 2 hours prior to i.p. injections of saline or PIC. A) The percentage of neurons expressing c-fos at each time point. *** $p < 0.001$ compared to DMSO-PIC. ## $p < 0.01$ B) Box and whisker displaying the range of mean c-fos intensities within individual c-fos positive neurons.

4.3. RESPONSE OF MICROGLIA TO PIC CHALLENGE

Manuscript in Preparation

4.3.1. INTRODUCTION

Microglia are integral components of the central nervous system. Their primary function entails the surveillance of tissue environment (Nimmerjahn et al., 2005), and the initiation of immune responses following insult or injury (Aldskogius et al., 1999; Cunningham, 2013; Franco and Fernandez-Suarez, 2015; Morrison et al., 2017). They are also critical for proper neurodevelopment (Cunningham et al., 2013; Schafer et al., 2013), and the maintenance of synapses (Beattie et al., 2002; Hong et al., 2016a; Akiyoshi et al., 2018) either via physical contact (Chen et al., 2014; Akiyoshi et al., 2018), or by secreting modulatory factors (Beattie et al., 2002; Ji et al., 2013). When activated, microglia can take on a variety of morphological shapes and functions, ranging from pro-inflammatory states, which promote inflammation, to anti-inflammatory states, which assist in the repair of damaged tissue. Due to their importance as the main resident immune cell of the brain, as well as their ability to modulate neuronal activity, understanding how microglia react to peripheral PIC-challenge can provide insight into mechanisms underlying the induction of hyperexcitability.

The goal of this study was to determine the response of microglia to peripheral PIC injection. We examined the activation and proliferation of microglia, as well as their interaction with neurons. We also examined the role of CXCR3 in microglial responses to PIC challenge.

4.3.2. METHODS

4.3.2.1. *Drug Administration*

Peripheral PIC challenge was induced in C57BL/6 J mice as previously described (Chapters 2, 3). CXCR3 inhibition was achieved by intracerebroventricular (i.c.v.) injection of 3.0 mg/kg of AMG-487 (Amgen, Thousand Oaks, CA). Briefly, mice were anesthetized with isoflurane (5% inhalation, 2-4% inhalation, continuous) and placed in a stereotaxic frame (Kopf, Tujunga, CA). AMG-487 was freshly prepared in artificial cerebral spinal fluid (ACSF) containing 10% of DMSO. 5 μ L of AMG-487 solution was gradually delivered to the right ventricle through a 26s-gauge needle. The coordinates from bregma were anteroposterior 0.45 mm, mediolateral +1.0mm, and dorsoventral -3.6mm. The needle remained in place for 30 seconds to minimize

back-flux of the injectate. After 2 hours, animals were PIC-challenged and tested for sickness behavior, as in Chapter 2, 3.

4.3.2.2. *Microscopy*

Immunohistochemistry and confocal microscopy were performed as described in Chapter 3. Z-stacks were taken using the 60X or 40X objective through the middle of Cornu Ammonis 1 (CA1) and layers 2/3 of the cortex, approximately 1mm from midline every 0.23 μm at a resolution of 512x512 pixels. 3D projections were then rendered using NIS Elements Advanced Research imaging software (Nikon Instruments, Melville, NY)

Cross sectional 10X images were obtained with the Olympus VS120 Slide Scanner as described in Chapter 4.

To assess microglia proliferation, microglial cell bodies were identified as Iba1 positive perikarya containing DAPI positive nuclei. We used maximum intensity projections, and counted microglia blinded by hand using the Photoshop counter tool.

4.3.2.3. *Neuron-Microglia Surface Area Contact*

Neuronal surface area in direct contact with microglial processes was quantified using IMARIS (Bitplane Inc, Concord, MA). Briefly, 40X Z-stacks of the motor cortex, stained for neurons (NeuN) and microglia (Iba1) were imported to IMARIS, and surface renderings for both neurons and microglia were created. A smoothing value of 0.6 μm was utilized for neuronal cell bodies to eliminate dendritic and axonal arborization and background subtraction was applied. The minimum diameter of 5.00 μm for neurons was determined by selecting the “slice view” and using the line tool to measure the diameter of the smallest neuronal cell body. A manual threshold of 40 was applied to all images, a value which was determined to best cover the cell body of neurons without extending into axonal segments or covering negative area. Seed points, to separate touching neurons, was applied at a quality above 40 with a threshold growing diameter of 9.00 μm . Finally, neuronal surfaces were required to have an area above 250 μm^2 to eliminate any partial neuronal surfaces or background fluorescence.

Microglial surfaces were rendered with a smoothing value of 0.4 μm , a value decided upon by selecting the “slice view” and using the line tool to measure the diameter of the average microglial processes. Background elimination was once again applied and the largest diameter to be remodeled was maximized at 1.00 μm , a value which ensured fully coverage of microglial

cell bodies. A manual threshold of 50 was selected to ensure complete microglial process coverage in both control and PIC conditions. Finally, surfaces above 20 voxels and above the automatic threshold for the channel were selected to ensure removal of small microglial processes from non-visualized microglia as well as any background fluoresce.

The settings were applied to all samples and all neurons and microglia within the visual field of the z-stack. Once rendered, the surface-surface contact area X-Tension was applied to determine the percentage of neuronal surface area directly in contact with a microglial.

4.3.2.5. Statistical Analysis

Differences between groups were compared using Student's t- test and considered significant at $P \leq 0.05$.

4.3.3. RESULTS AND DISCUSSION

Throughout this study, we used the 24-hour post PIC challenge, because neuronal hyperexcitability peaks at this time point (Kirschman et al., 2011; Hunsberger et al., 2016). Microglia activation was observed throughout the brain following induction of peripheral PIC challenge. Fig. 1 shows representative images of Iba1 staining of the hippocampal CA1 subregion and the motor cortex. In both regions, PIC challenge lead to a profound hypertrophy of microglia and retraction of their processes.

Figure 1. PIC challenge induces microglial activation. Mice were i.p. injected PIC or saline (SAL) were analyzed by immunohistochemistry after 24 hours. Microglial expression (red) co-stained with NeuN (green) in the CA1 subregion of the hippocampus [A] and cortex [B]. Confocal images were captured at 60X magnification. Scale bars represent 50 μ m.

As activated microglia often proliferate in response to stimuli, we counted the number of microglial cell bodies within the visual field (101.25 mm^2 area) of the CA1, CA3, and DG at 24 hours after saline (SAL) or PIC injection. No difference in the number of cell bodies was observed in the CA1, CA3, or DG subregions of the hippocampus. No difference in the number of cell bodies was observed within the CA1 [SAL= 9.5 ± 0.80 , PIC= 10.0 ± 0.81 ; $t(24) = -0.437$, $p=0.666$], the CA3 [SAL= 7.5 ± 0.59 , PIC= 8.4 ± 0.87 ; $t(22) = -0.888$, $p=0.384$] or the DG [SAL= 9.67 , PIC= 11.42 ; $t(22) = -1.207$, $p=0.240$], indicating that despite microglial activation at 24 hours, microglia are not proliferating.

As microglia can interact with axo-somatic synapses to modulate neuronal activity, we determine the covering of neuronal cell bodies by microglial processes in PIC challenged vs. control brains using the IMARIS system. The accuracy of IMARIS modeling of neurons and microglia, as well as the representative results of surface-surface contact on each surface is depicted in Fig. 2. PIC challenge seemed to increase the percentage of cortical neuronal surface covered by microglial by approximately 30%. However, the difference was not statistically significant between saline treated (5.39 ± 1.90) and PIC challenged animals (7.93 ± 0.85) [$t(7) = 1.442$, $p=0.193$]. Moreover, no difference in either the number of neuronal perikarya or neuronal surface area was evident (data not shown).

A

SAL

B

PIC

Figure 2. IMARIS modeling of neuron-microglia interactions. Mice were injected with saline [A] or PIC [B] and the cortices were analyzed by immunohistochemistry 24 hours later. Microglia were stained red and neurons were stained green. Confocal images were captured at 40X magnification. Images were imported into IMARIS, and the surface models for glia and neurons were created (see Methods). Top panels represent original microscopy images in 3D. Middle panels demonstrate the accuracy of the neuron and glial models. Lower panels highlight the contacts (in yellow) between neurons and microglia. Scale bars represent 20 μm .

Figure 3. Interaction of microglia with cortical neurons following PIC challenge. After rendering neuron and microglia models, the surface-surface contact area X -tension was applied to determine the percentage of total neuronal surface directly in contact with microglia. (Figure 2). Data are presented as means $\pm$ SEM from 3-4 cortical images (approximate volume of each image was 1518.75mm^3). The difference between SAL and PIC groups was assessed by Student's t -test. $p=0.193$.

These results indicate that in response to PIC challenge, microglia undergo activation that entails hypertrophy and retraction of processes, but not proliferation. Moreover, this activation does not involve increased neuronal contacts. Nevertheless, it remains plausible that microglia are affecting neuronal synapses. For example, microglia may secrete factors that disrupt the extracellular matrix (Chen et al., 2014; Akiyoshi et al., 2018), or factors that directly alter synaptic activity (Beattie et al., 2002; Ji et al., 2013).

However, signals that activate microglia are not known. CXCL10, which is robustly produced in response to PIC challenge (Chapters 2 and 3), is a plausible candidate. However, microglia do not express its receptor, CXCR3 (Chapter 3). Therefore, we consider three possible mechanisms. First, microglial activation is elicited indirectly by factors release from other cells in response to CXCR3 ligation with CXCL10. Second, microglial activation is mediated by TLR4 signaling, because CXCL10 can efficiently activate TLR4 (Schulthess et al., 2009) and microglia strongly express TLR4 (Olson and Miller, 2004). Third, the minor chemokines generated by PIC challenge, e.g., CXCL1 or CXCL2 (Chapter 2), mediate microglial activation (Fan et al., 2017)

To address these mechanisms, we blocked cerebral CXCR3 using i.c.v. injection of AMG-487, the non-competitive inhibitor of the receptor. Because the inhibitor was delivered in 10% DMSO, respective controls were i.c.v. injected with DMSO solution alone. Similarly, mice i.p. injected with saline served as controls for PIC challenged mice.

As shown in Figure 4, microglia from mice injected with DMSO + SAL displayed a resting, ramified morphology that was identical to SAL-injected mice (Fig. 1). In mice injected with DMSO + PIC, microglia were robustly activated, as evidenced by their hypertrophy and retraction of fine processes. Blockage of CXCR3 attenuated microglial activation. The injection of AMG-487 alone induced microglia activation, but to a lesser extent than PIC challenge. Similar results were observed throughout all brain regions (not shown).

Based on these results, we postulate the indirect mechanism of microglial activation. Accordingly, CXCL10 binds to neuronal CXCR3 leading to the release of secondary factors. These factors, in turn, elicit microglial activation.

Figure 4. CXCR3 Inhibition attenuates microglia activation induced by PIC. Mice were *i.c.v* injected with AMG-487 or DMSO. Two hours later, mice were *i.p.* injected with PIC or saline (see Methods). Twenty-four hours after PIC injection brains were analyzed by immunohistochemistry using anti-Iba1 antibody for microglia (red) and anti-NeuN antibody for neurons (green). Left panel shows 20X epifluorescent images of the hippocampus. Right panel shows 40X confocal images of the CA1 subregion of the hippocampus.

4.4. IN VITRO EFFECT OF PIC CHALLENGE ON GLUTAMATE UPTAKE

4.4.1. INTRODUCTION

We have previously shown that PIC challenge-induced hyperexcitability of hippocampal neuronal networks is concomitant with a robust increase in the level of extracellular glutamate in the hippocampus (Hunsberger et al., 2016). This is important finding because increased extracellular glutamate leads to neuronal hyperexcitability (Campbell and Hablitz, 2004, 2008; Featherstone and Shippy, 2008; Campbell et al., 2012; Campbell et al., 2014; Putatunda et al., 2014), and thus, may reveal molecular mechanisms by which PIC challenge enhances excitatory transmission. We have also shown that hippocampi of PIC-challenged mice had impaired uptake of glutamate that might underscore the increased extracellular glutamate, and thus, neuronal hyperexcitability. However, mechanistic studies of glutamate uptake in living animals are laborious, timely, and require a great number of animals. Thus, the present study was undertaken to test whether glutamate uptake can be analyzed in an in vitro model system of hippocampal slices to increase the efficiency of such studies.

4.4.2. METHODS

4.4.2.1. *Preparation of hippocampal slices*

C57BL/6 J mice were PIC-challenged as previously described (Chapters 2) and analyzed 24 hr later. Mice were anesthetized with isoflurane, and brains were removed and immediately placed in ice-cold phosphate buffered saline (PBS). Hippocampi were dissected and cut into 350 μ m slices using a McIlwain Tissue Chopper (Campden Instruments Ltd., Lafayette, IN). The slices were pre-incubated for 30 minutes at room temperature in an incubation chamber containing circulating artificial cerebral spinal fluid (ACSF). ACSF consisted of 124 mM NaCl, 26 mM, NaHCO₃, 4.4 mM KCl, 1 mM of NaH₂PO₄, 1.3 mM of MgSO₄, 2.5 mM CaCl₂, 10 mM glucose, and was saturated with 5% CO₂/95% O₂.

4.4.2.2. *[³H]-glutamate uptake*

Intracellular uptake of glutamate was determined as described by Griffin et al. (2015). Briefly, following pre-incubation, hippocampal slices were transferred into glass vials, and incubated in ACSF containing 250 nM [³H]-glutamate (51 Ci/mM; Perkin Elmer, Waltham, MA) and 100 μ M unlabeled glutamate. The slices were incubated for seven min at 37°C with shaking. The uptake of glutamate was stopped by placing the vials on ice and aspirating the incubation medium.

Slices were washed five times with ice-cold saline containing 100 μ M glutamate. The slices were solubilized with 5% SDS overnight at room temperature followed by the addition of the scintillation cocktail Ultima Gold (Perkin Elmer). The radioactivity was determined in the LS 6500 Multi-Purpose scintillation counter (Beckman Coulter, Brea, CA), and expressed as counts per minute (CPM).

4.4.2.3. Protein Determination

Total protein was measured in SDS-solubilized homogenates by ABC method (BioRad, Hercules, CA) using bovine serum albumin as a standard. The protein content was used to normalize the radioactivity to CPM per μ g of protein.

4.4.2.4. Statistical Analysis

Differences between groups were compared using Student's t- test and considered significant at $P \leq 0.05$.

4.4.3. RESULTS AND DISCUSSION

In concordance to previous study in slices of the nucleus accumbens (Griffin et al., 2015), hippocampal slices avidly incorporated [3 H]-glutamate (Fig. 1). The incorporation in slices from saline-injected and PIC-injected mice averaged 844 and 969 CPM/ μ g of protein, respectively. The difference between these two values was non-significant [$t(18)=1.310$, $p=0.207$].

Figure 1. Hippocampal glutamate uptake following PIC challenge. Mice were i.p. injected with 12mg/kg PIC or equivolume saline. After 24 hours, the uptake of [3 H]-glutamate was determined in hippocampal slices and expressed as CPM/ μ g of protein. Bars represent averages $\pm$ SDs from 5 animals.

In contrast to our previous in vivo study revealing a robust (approximately 4-8-fold) impairment of exogenous glutamate uptake instigated by PIC challenge (Hunsberger et al 2016), no

impairment of glutamate uptake was evident between hippocampal slices from saline-injected vs. PIC-injected mice. This discrepancy is likely due to the artifacts of the in vitro system. For instance, our transcriptome analysis (Michalovicz, 2015) revealed no change in the expression of the message encoding the excitatory amino acid transporter 2 (EAAT2), the astrocytic protein responsible for ~90% of extracellular glutamate uptake in the brain (Kim et al., 2011). Therefore, the impairment of glutamate uptake is likely due to a modification of EAAT2 activity. For example, a binding of a protein or other factors that regulate EAAT2 activity in vivo can be envisaged. These regulatory factors might have been lost in our in vitro system.

In conclusion, the hippocampal slice system is not applicable for studies of alterations of glutamate uptake instigated by PIC challenge. Such studies must be performed in vivo, using EAAT2 inhibitors (e.g., TBOA), activators (e.g., sulbactam), and/or transgenic techniques (e.g., knock-in and knock-out).

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CHAPTER 5: PERIPHERAL VIRAL CHALLENGE EXACERBATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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5.1. ABSTRACT

Peripheral viral infections are potent triggers of exacerbation in multiple sclerosis (MS). Here, we used a preclinical model of MS, the experimental autoimmune encephalomyelitis (EAE) to corroborate this comorbidity in an experimental setting. EAE was induced by immunization of mice with MOG peptide, and paralysis was scored using a 5-point scale. At the onset of the chronic phase of the disease (Days 42-58 after MOG injection) the animals were divided into low responders (LR) and high responders (HR) with the mean score of 1.5 and 2.5, respectively. The acute phase response (APR) was induced by intraperitoneal injections of a viral mimetic, polyinosinic-polycytidylic acid (PIC). Two daily injections were performed on Days 42 and 44 (PIC_{42,44} challenge) and on Days 54, 55 and 56 (PIC_{54,55,56} challenge). PIC_{42,44} challenge had no effect of EAE disease, whereas PIC_{54,55,56} challenge rapidly increased paralysis but only in HR group. This exacerbation ultimately led to animal death by Day 58. These results demonstrate that antiviral APR is a potent exacerbator of EAE, and that this activity directly correlates with the severity of the disease. This in turn, indicates that antiviral APR might play a pivot role in linking peripheral viral infections with MS exacerbations.

5.2. INTRODUCTION

Multiple sclerosis (MS) is characterized by unpredicted occurrence of exacerbations that detrimentally affect the long-term disability. Epidemiological studies strongly implicate viral infections in the periphery as triggers of the exacerbations (Sibley et al., 1985; Andersen et al., 1993; Panitch, 1994; Edwards et al., 1998; Buljevac et al., 2002; Correale et al., 2006; Libbey and Fujinami, 2010). Importantly, viral infection-instigated exacerbations result in more persistent neural deteriorations than other exacerbations. Although the mechanisms have not been defined, several pathways have been suggested. For example, the infections may decrease the ability of the CNS to protect itself against autoimmune attack by leukocytes

(Matullo et al., 2011), enhance the autoimmune responsiveness of the host leukocytes (Correale et al., 2006), or induce re-activation of a CNS-resident virus (Borkosky et al., 2012).

Because MS exacerbations are induced by various, and often unrelated viruses (Libbey and Fujinami, 2010; Kakalacheva et al., 2011), the triggering process is likely to involve a common viral signature. A good candidate is a double stranded RNA (dsRNA) that is generated by most viruses during their replication cycle (Jacobs and Langland, 1996; Weber et al., 2006). dsRNA is recognized by the major antiviral receptors of the host cells, i.e., toll-like receptor 3 (TLR3), retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA-5) and protein kinase R (PKR). The ligation of these receptors elicits the acute phase response (APR), the first line of antiviral defense characterized by the fulminant generation of interferons, cytokines, chemokines and other inflammatory mediators that curtail the spread of the infection. However, these inflammatory mediators are also conveyed via circulation to the CNS whereby they induce a “mirror inflammatory response” (Dantzer and Kelley, 2007). The inflammatory factors generated peripherally and/or centrally during antiviral APR are expected to exert a profound effect on the progression of MS.

A synthetic dsRNA, polyinosinic-polycytidylic acid (PIC), is a commonly used tool to induce antiviral APR [reviewed in (Hunsberger et al., 2016)]. APR induced by intraperitoneal PIC injection involves a robust but transient surge of blood cytokines, such as IL1 β , IL6, TNF α , CXCL9, CXCL10, CXCL1, CXCL2, CCL2, CCL7, CCL12 and IFN β (Michalovicz and Konat, 2014; Petrisko and Konat, 2017). The brain response to this surge entails the production of CXCL10, and to a lesser extent the production of CXCL1, CXCL2 and CXCL9. In addition, the cerebral response includes the production of complement proteins and the activation of anaphylatoxin cascades (Michalovicz and Konat, 2014; Petrisko and Konat, 2017). Most of these inflammatory factors have been implicated in the pathology of MS (Cheng and Chen, 2014; Pranzatelli, 2018).

We hypothesize that the inflammatory factors generated during antiviral APR augment the ongoing MS pathology resulting in the exacerbation of the disease. The present study was undertaken to test this hypothesis using the autoimmune model of MS, the experimental autoimmune encephalomyelitis (EAE) in mice. During the chronic phase of EAE, mice were challenged with PIC, and progression of the disease was monitored and compared to that in EAE animals without PIC challenge.

5.3. MATERIALS AND METHODS

5.3.1. ANIMALS

Ten-week old female C57BL/6 mice obtained from Charles River Laboratories (Wilmington, MA) were housed on Diamond Soft bedding under 12-h light/dark cycle with ad libitum access to food and water. All procedures were approved by the West Virginia University Animal Care and Use Committee and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

5.3.2. EAE INDUCTION

The Hooke Kits™ for EAE Induction (Hooke Laboratories, Lawrence, MA) was used according to the manufacturer's protocol. Briefly, on Day 0, mice were lightly anesthetized with isoflurane and subcutaneously (s.c.) injected with the MOG₃₅₋₅₅ emulsion in CFA into four sites of the back (50 µL per site), followed by an intraperitoneal (i.p.) injection of 300 ng of pertussis toxin (PTx) in 100 µL of saline. The PTx injection was repeated the next day (Day 1). The animals were scored daily for signs of EAE using a 5 point scale: 0, unaffected; 1, loss of tail tone; 2, hind limb weakness; 3, complete paralysis of hind limbs; 4, quadriplegia; and 5, moribund. If an animal appeared to be between two scores, an average score was assigned.

5.3.3. PIC CHALLENGE

On Day 42, mice were divided into high responders (HR) with scores >2 (mean score of 2.5 ± 0.1) and low responders (LR) with scores ≤ 2 (mean score of 1.5 ± 0.1). To induce antiviral APR, half of the animals in each group received two daily i.p. injections of 12 mg/kg of ultrapure PIC (Invivogen, San Diego, CA) in 100 µL of saline. These injections were spaced two hours apart. The first PIC challenge was performed on Days 42 and 44. The second PIC challenge was executed on Days 54, 55 and 56. The other half of the animals were injected with an equivolume saline in lieu of PIC. Thus, four groups were generated: LR-PIC, LR-Sal, HR-PIC and HR-Sal. The animals were scored and weighted daily. Severely affected mice were housed with animals of similar severity in cages containing HydroGel (Westbrook, ME). Animals that scored a 4.0 for two consecutive days or lost more than 30% of body weight were euthanized. A score of 5.0 was assigned to animals that were euthanized or died.

5.3.4. STATISTICAL ANALYSIS

Statistical comparisons were performed by two-way repeated measures ANOVA (RMANOVA) followed by the Holm-Sidak post hoc test. A Kaplan-Meier plot was generated and the logrank test was used to test differences in survival time. Differences between groups were considered significant at $P \leq 0.05$.

5.4. RESULTS

The initial wave of paralysis commenced on Day 10, peaked on Day 16 and partially resolved around Day 25 after MOG immunization (Fig. 1). This was followed by a second slower relapse that plateaued around Day 40 indicating a chronic phase of the disease. There was no mortality at any time point.

We tested the comorbidity of antiviral APR induced by PIC injection during the chronic EAE phase. Although the peak scores during the initial relapse (Fig. 1) were tightly clustered (e.g., 2.4 ± 0.1 at Day 16), the variability of sickness intensity increased substantially thereafter. Consequently, on Day 42, we divided the animals into two subpopulations based on the disease severity, i.e., high responder (HR) group with scores of 2.5 ± 0.1 , and low responder (LR) group with scores of 1.5 ± 0.1 .

PIC is rapidly deactivated within the peritoneal cavity, and consequently a single PIC injection results in a bolus stimulation of the innate immune system (Hunsberger et al., 2016). Because viral replication, and thus dsRNA generation in the host are protracted, we performed two consecutive PIC injections two hours apart to better simulate APR instigated by viral infection. To assess the effect of PIC challenge severity, we used two paradigms. Initially, the animals were challenged with PIC on Days 42 and 44 (PIC_{42,44} challenge). After ten days of recovery, the animals were injected for three consecutive days, i.e., Days 54, 55 and 56 (PIC_{54,55,56} challenge).

Figure 1. Time course of MOG-induced EAE. Ten-week old female C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide (Day 0) and the progression of paralysis was scored daily (for details see Materials and Methods). Points represent averages $\pm$ SE from 20 (Days 0-42) and 11 (Days 43-59) animals.

The response of animals during the chronic phase of EAE to PIC challenge is shown in Fig. 2. RMANOVA on Days 42-59 showed a significant effect of group [$F(3, 238)=21.48$, $p<0.001$], and time [$F(14, 238)=4.38$, $p<0.001$]. The interaction between groups and time was also significant [$F(42, 238)=2.80$, $p<0.001$]. In HR group, PIC_{42,44} challenge tended to temporarily increase EAE scores as compared to EAE mice injected with saline, but the differences were not statistically significant. PIC_{54,55,56} challenge profoundly exacerbated paralysis, and the difference in EAE scores between HR-PIC and HR-Sal groups reached high statistical significance ($p<0.001$) on Days 56, 57 and 58. Moreover, the exacerbation of EAE symptoms resulted in animal death. The first animal in HR-PIC group died on Day 56, and no animal survived beyond Day 58. The difference from HR-Sal group assessed by the logrank test was highly significant ($p\leq 0.001$).

Figure 2. Comorbidity of PIC-induced APR during chronic phase of EAE. Upper panels show the effect of PIC challenges on neurological disability. Arrows indicate PIC injections during the first (PIC_{42,44}) and second (PIC_{54,55,56}) challenge (for details see text). Red circles; PIC-injected EAE mice. Blue triangles; saline-injected EAE mice. Symbols represent averages $\pm$ SE. LR-PIC ($n=5$), LR-Sal ($n=5$), HR-PIC ($n=5$) and HR-Sal ($n=6$). $*P\leq 0.001$. Lower panels show mortality after the second PIC challenge. Broken red lines represent PIC-challenged EAE mice. Solid blue lines represent saline-injected controls.

In contrast, no change in sickness scores was observed in LR-PIC group as compared to LR-Sal group after either PIC_{42,44} or PIC_{54,55,56} challenges (Fig. 2). Although PIC_{42,44} challenge tended to ameliorate the disease in LR-PIC, the decrease of EAE scores was not statistically significant. Following PIC_{54,55,56} challenge, one LR-PIC mouse died (20% mortality) in on Day 56, albeit the survival of this group did not significantly differ from LR-Sal group.

5.5. DISCUSSION

Previous preclinical studies addressed the comorbidity of peripheral viral infections and MS by focusing on their involvement in the induction phase of EAE (Peacock et al., 2003; Verbeek et al., 2007). These experiments have revealed that preceding peripheral viral infections increase the susceptibility of animals to develop EAE. However, the effect of viral insult during the effector phase of EAE has not been studied. In the present study, we demonstrated comorbid effect of viral challenge in mice with established EAE. Consequently, our experimental paradigm models viral infection-induced exacerbations in MS patients (Sibley et al., 1985; Andersen et al., 1993; Panitch, 1994; Edwards et al., 1998; Buljevac et al., 2002; Correale et al., 2006; Libbey and Fujinami, 2010).

The effect of PIC-induced exacerbation of EAE disease depended on the strength of PIC challenge. Thus, no effect was evident in EAE mice following challenge on two alternate days (PIC_{42,44}). In contrast, PIC challenge for three consecutive days (PIC_{54,55,56}) induced a fulminant relapse of the disease as seen from the rapid exacerbation of paralysis and ultimate death. Although the animals were allowed to recover for ten days after the first PIC challenge, a possibility for a priming of the immune system for a heightened response to the second PIC challenge could also be considered.

The exacerbating effect of PIC challenge was also strongly dependent on the severity of EAE disease. Thus, the exacerbation of EAE symptoms occurred only in HR group, while no exacerbation was evident in LR group. It is expected that HR group with the initial score of 2.5 has more intense inflammation than LR group with the score of 1.5. These results might indicate the existence of a threshold in the inflammatory status required for the fulminant response to occur. Alternatively, the response might be more gradually dependent on disease severity. For example, although LR mice were evidently spared, a tendency to increase paralysis score and the death of one animals occurred on Day 58. Thus, the exacerbation in LR mice may be delayed beyond Day 58. This dependence on EAE severity is reminiscent of MS patient

response to inflammatory mediators that is depend on the disease subtype (Nikfar et al., 2010). The effects of EAE severity as well as the strength of PIC challenge on EAE exacerbation will be delineated in future studies.

PIC is a potent inducer of generic APR in a manner independent of the viral type (Guha-Thakurta and Majde, 1997; Traynor et al., 2004). The induction of antiviral APR is restricted to the peritoneal cavity, because PIC is swiftly degraded within this compartment, and does not reach the circulation (Konat, 2016). PIC stimulates peritoneal macrophages and mesothelial cells, but does not cause tissue damage, and thus does not elicit the confounding effects of systemic response to tissue injury. Consequently, the present study provides the proof-of-concept that APR per se is a principal component of the innate immune response to viral challenge responsible for the exacerbation of EAE, and by extension, for MS exacerbations.

The inflammatory factors generated during PIC-induced APR are likely responsible for the augmentation of EAE pathology. For example, IL6 that is profoundly increased by PIC challenge in the blood (Michalovicz and Konat, 2014) has been demonstrated to enhance the recruitment of patrolling leukocytes through the neuroendothelium (Richard et al., 2011). CXCL10 that is also robustly increased by PIC challenge in both the blood and CNS (Petrisko and Konat, 2017) is a potent activator and chemoattractant of circulating leukocytes (Liu et al., 2001) and resident microglia (Rappert et al., 2004; Clarner et al., 2015). The increase of leukocytic infiltration promoted by IL6 and/or CXCL10 is likely to augment the neuroinflammatory milieu in the CNS parenchyma leading to exacerbation of the disease. Moreover, PIC challenge upregulates the generation and activation of the complement proteins in the CNS (Michalovicz et al., 2015b; Petrisko and Konat, 2017), and the complement system activation is an intrinsic feature of MS pathology (Storch et al., 1998). PIC-induced APR may also act at the periphery by enhancing the activity of lymphocytes. For example, PIC is a potent promoter of expansion and differentiation of CD8⁺ T cells (Ngoi et al., 2008), which are primarily responsible for CNS damage during MS relapses (Malmestrom et al., 2008). Finally, one should also be cognizant of the possibility that EAE animals with ongoing autoimmune pathology may generate different profiles of inflammatory factors in response to PIC challenge that differ from those induced in naïve animals used in the aforementioned studies. The identification of these factors would open the way for the development of therapeutic strategies to control the progression of MS.

In conclusion, this is the first study that translates the clinical correlation between peripheral viral infections and MS pathology to experimental exploration by focusing on the role of APR as the hub that links infections by unrelated viruses to MS exacerbations. We have developed a preclinical paradigm to examine this comorbidity, and demonstrated that antiviral APR exacerbates EAE in mice, an autoimmune model of MS. The PIC paradigm provides a unique model to identify the components of antiviral APR responsible for the disease exacerbation.

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Conflict of interest

The authors declare no conflicts of interest.

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CHAPTER 6: DISCUSSION

This dissertation research is based on and expands previous study from our laboratory by addressing molecular mechanisms, by which peripheral PIC challenge induces neuronal hyperexcitability. In this chapter, we discuss the major findings and their impact on elucidating these mechanisms.

6.1. CXCL10/CXCR3 AXIS

The primary discovery of this research is that cerebral CXCL10/CXCR3 axis mediates the development of neuronal hyperexcitability in response to anti-viral APR. This axis is an important component of pathological processes. For example, CXCL10 is a chemoattractant responsible for the migration of leukocytes to the site of infection, and polarizes T cells towards a proinflammatory, Th1/Th17 phenotype via ligation to CXCR3 (Dufour et al., 2002; Groom and Luster, 2011b; Kariya et al., 2016). CXCL10 can either inhibit or promote proliferation of endothelial cells, depending on the subtype of CXCR3 (Bodnar et al., 2006; Hueso et al., 2018). CXCL10 is also a key mediator of several peripheral immune conditions, including psoriasis, Chron's disease, and rheumatoid arthritis (Berry, 2004; Lee et al., 2017). The importance of the CXCL10/CXCR3 axis has led to the development of targeted therapeutic strategies. Thus, numerous anti-CXCL10 antibodies have shown success in phase II clinical trials (Angiolillo et al., 1995; Wijtmans et al., 2008; Yellin et al., 2012; Mayer et al., 2014), indicating therapeutic effect of reducing CXCL10 signaling. Also, a plethora of CXCR3 inhibitors have been developed (Andrews and Cox, 2016), and made their way to clinical trials (Floren LC, 2003; Berry, 2004) (Fig. 1).

Figure 1. Signaling pathways activated following neuronal CXCR3 activation. Ligand of CXCL10 to CXCR3 results in dissociation of $G\alpha$ from the catalytic $G\beta\gamma$ subunit. In turn, these subunits activate multiple pathways, including Akt, Ras, and PLC β , with the latter resulting in increases of intracellular calcium. Additionally, activation of the Ras pathway promotes production of c-fos. As a transcription factor itself, c-fos along with c-Jun and AP-1 result in the production of additional cytokines and chemokines. Adapted from Dabiri et al. (2016)

While the specific role of the CXCL10/CXCR3 axis in brain pathology is not yet understood, many studies have established a negative impact of increased CXCL10 and CXCR3 expression on the progression of neurological diseases (Sorensen et al., 2002; Klein, 2004; Michlmayr and McKimmie, 2014a; Vazirinejad et al., 2014; Krauthausen et al., 2015; Roberts et al., 2015; Iwanowski et al., 2017). For example, elevated CXCL10 and CXCR3 levels have been demonstrated in the cerebrospinal fluid (CSF) of MS patients, and these elevations are

correlated with clinical relapses. CXCL10 is produced in active demyelinating lesions by macrophages and astrocytes, and likely contributes to the tissue distraction. Alzheimer's disease (AD) patients also exhibit increased levels of CXCL10 and CXCR3, and animal models have implicated the CXCR3 signaling in the induction of plaque formation and the promotion of cognitive and behavioral deficits.

Genetic knockout models have been created for both CXCL10 and CXCR3 and applied to neuroinflammatory paradigms. For example, CXCL10^{-/-} mice infected with West Nile virus (WNV) have increased viral burden within the brain as well as increased mortality due to the inability of infected neurons to properly recruit peripheral immune cells (Klein et al., 2005). In experimental autoimmune encephalomyelitis (EAE), astrocytic CXCL10^{-/-} mice demonstrated delayed onset of experimental autoimmune encephalomyelitis, but did not prevent progressive axonal loss (Mills Ko et al., 2014) while global deletion of CXCR3 worsened disease outcome in EAE mice (Müller et al., 2007). However, caution must be used when interpreting the results of knockout studies as C57BL/6 mice, which were utilized throughout the above studies. As mentioned before, C57BL/6 mice are deficient for CXCL11, which also binds to CXCR3, due to a point mutation. As CXCL10 deficient mice were generated using 129 embryonic stem cells that express the wild-type CXCL11, these mice as regained a functioning CXCL11 protein and compensatory mechanisms of this protein cannot be discounted (Groom and Luster, 2011a). Additionally, CXCR3 deficient mice have a deficiency of natural killer cells suggesting a regulatory role of CXCR3 (Christensen et al., 2004). Additionally, the neuroinflammatory conditions studied with CXCL10/CXCR3 knockout mice exhibit profound breakdown of the blood-brain barrier (BBB) and the severity of the disease model is mediated by recruitment of peripheral immune cells to the CNS, while our model does not exhibit breakdown of the BBB (unpublished data) nor the recruitment of peripheral immune cells (confirmed by lack of ameboid macrophage morphology in Iba1 or Cd11b stained glia). As such, an non-neuronal process, such as T cell recruitment or generation of effector cells could impact what appears to be a CNS mediated effect (Michlmayr and McKimmie, 2014b).

On the other hand, these (Andersen et al., 1993; Murray et al., 1993; Panitch, 1994; George et al., 1997; Edwards et al., 1998; Nee and Lipka, 1999; Buljevac et al., 2002; Holmes et al., 2003; Libbey and Fujinami, 2010; Holmes, 2013), and other neuropathological conditions, i.e., epilepsy (Tellez-Zenteno et al., 2005; Vezzani and Granata, 2005; Verrotti et al., 2009), Parkinson's disease (Zheng et al., 2012; Brugger et al., 2015) and stroke (Westendorp et al.,

2011; Learoyd et al., 2017) are exacerbated by peripheral viral infections. Consequently, our finding that CXCL10 is the primary protein generated in the brain in response to peripheral viral challenge (Chapters 2 and 3) provides a compelling evidence that this chemokine is a putative mediator of the deleterious effects of peripheral viral infections on ongoing neuropathologies.

Another key finding of this dissertation is that neurons are the principal generators of CXCL10. This cellular specificity cannot be overstated. In particular, neurons are highly differentiated cells specialized in electrical impuls conduction, and therefore, are not expected to be the main contributors to immune response that is typically attributed to glial cells, i.e., microglia and astrocytes. Yet, neurons, but not glial cells, generate the overwhelming amounts of CXCL10 in response to PIC challenge. This finding is reminiscent of our previous observation of neurons being the primary cells generating the complement factor B (CfB) following PIC challenge (Michalovicz & Konat, 2015). Consequently, our findings indicate that neurons can carry on important innate immune functions.

Similarly to CXCL10, CXCR3 expression is also restricted to neurons in both naïve and PIC challenged brains (Chapter 3). Neuronal expression of CXCR3 is not surprising, as changes of synaptic function in response to exogenous application of CXCL10 have been demonstrated (Nelson and Gruol, 2004; Bajova et al., 2008; Cho et al., 2009; Kodangattil et al., 2012). Nevertheless, the cellular expression of the chemokine and its cognate receptor demonstrates that the CXCL10/CXCR3 axis operates through autocrine and/or paracrine neuronal networks, where CXCL10 being secreted by a neuron binds to CXCR3 on either the same or a nearby neuron. While neuronal autocrine/paracrine signaling is repeatedly utilized during neurodevelopment (Herrmann and Broihier, 2018), in the adult CNS, this activity is restricted to the synaptic transmission. Also, neurons are not thought as being the main cells to respond to environmental challenges in such a dramatic manner as secreting and reacting to the same factor. Although neurons respond to inflammatory signals, these signals are typically produced by neighboring glial cells (Beattie et al., 2002; Allen, 2014). As such, peripheral PIC-induced CXCL10-CXCR3 signaling provides a unique platform to understand a broader role of neurons beyond the electrical activity.

This study is limited by the use of only female animals, as we are unable to determine if there is a sex difference in PIC-induced antiviral APR and CXCL10/CXCR3 signaling. The decision to use only females stems from the original work by Colm Cunningham examining the induction of

sickness behavior following peripheral PIC challenge (Jain et al., 2011). The authors made the decision to exclude males from their study due to fighting between male cage mates throughout this and future studies (Fields et al., 1997; Murray et al., 2015) examining peripheral and cerebral inflammatory responses. As such, we continued to add to this literature; however, future studies should utilize both males and females.

6.2. NEURONAL HYPEREXCITABILITY

As presented Chapters 3 and 4, CXCR3 mediates the induction of cerebral hyperexcitability following peripheral PIC challenge. The CXCL10-CXCR3 is also responsible for the activation of microglia. Because microglia are potent regulators of synaptic activity, we propose the following mechanism (Fig. 2).

Blood borne cytokines, chemokines, and/or other APR factors, bind to receptors on brain endothelial cells, resulting in the secretion of an unknown factor (X), into the brain parenchyma. Factor X induces neuronal production of CXCL10. Previous studies showing that transfer of plasma from PIC challenged mice into naïve animals induces upregulation of cerebral cytokine and chemokine genes (Fil et al., 2011) strongly supports this contention. Neuronally secreted CXCL10 binds to CXCR3 on the surface of neighboring neurons. The activation of CXCR3 results in the secretion of factor Y, that in turn, activates microglia.

Activated microglia impede inhibitory transmission by decoupling GABAergic synapses. Studies from the Trapp lab showing processes of activated microglia invading the synaptic cleft and physically stripping the presynaptic terminals, strongly buttress this mechanism (Chen et al., 2014). However, microglia may hinder inhibitory transmission by secreting soluble factors (Z). For example, activated microglia have been shown to secrete bone derived neurotrophic factor (BDNF). BDNF can reverse the chloride anion gradient, resulting in neuronal depolarization following ligation of GABA receptors, turning GABA into an excitatory neurotransmitter (Coull et al., 2005).

Alternatively, microglia may also strengthen excitatory transmission. For example, microglial processes may contact excitatory synapses, strengthen their activity, and thus enhance excitatory transmission (Tremblay et al., 2010; Akiyoshi et al., 2018).

Figure 2. Proposed mechanism of CXCL10/CXCR3-mediated induction of hyperexcitability. Peripheral blood borne cytokines (circles) activate endothelial cells to release an unknown Factor X (triangles) into the CNS parenchyma. Factor X induces neuronal generation and secretion of CXCL10. CXCL10 then acts in an autocrine/paracrine fashion by binding to its receptor, CXCR3, on neurons. CXCR3 activation induces neuronal secretion of Factor Y (diamonds), which in turn, activate microglia. Activated microglia home onto axosomatic synapses of inhibitory neuron (orange) and displace the presynaptic terminals. Alternatively, activated microglia may secrete a soluble Factor Z (squares), disrupting the activity of inhibitory synapses. This de-inhibition renders the pink neuron hyperexcitable.

6.3. NEURONAL C-FOS EXPRESSION

The expression of c-fos has often been utilized as a measure of neuronal activity. For example, seizures, which represent neuronal hyperactivity, profoundly upregulate c-fos expression (Baille-Le Crom et al., 1996; Barros et al., 2015). We also observe a dramatic upregulation of neuronal c-fos following PIC challenge. However, PIC challenge does not elicit seizure, and thus does not result in neuronal hyperactivity. Instead, we have shown that PIC challenge renders the neuronal circuits more prone to kainic acid induced seizures (Michalovicz and Konat, 2014; Michalovicz et al., 2015a; Hunsberger et al., 2017). This indicates that the neurons are not firing more but are more likely to fire. This elevated propensity of neurons to fire is defined as hyperexcitability.

Consequently, PIC challenge-increased c-fos expression indicates that c-fos Immunohistochemistry, in addition to assessing neuronal hyperactivity, can also be utilized to examine neuronal hyperexcitability.

Of note, neuronal production of c-fos has been implicated in multiple cellular processes, e.g., the expression of the pro-survival factor, BDNF (Zhang et al., 2002). Ergo, neuronal production of c-fos following PIC challenge may not only serve as an index of neuronal hyperexcitability, but may also provide an insight to the underlying mechanisms.

6.4. COMORBIDITY OF PIC CHALLENGE IN EAE

Peripheral viral infections have been demonstrated to be responsible for exacerbations of many neurological disorders (refer to Chapter 1). In particular, infections are a known co-morbidity for MS relapse (Andersen et al., 1993; Panitch, 1994; Edwards et al., 1998; Buljevac et al., 2002; Libbey and Fujinami, 2010). Here, we have verified this comorbidity by showing that PIC challenge has a detrimental effect on mice during the chronic phase of EAE, an animal model of MS (Chapter 5). However, due to a fulminant mortality, we were unable to examine the molecular changes in the brains of these mice. Nevertheless, based on our previous results, CXCL10-CXCR3 signaling is likely a key mediator of this exacerbation. As discussed above, CXCL10 and CXCR3 are both increased in the CSF of MS patients and CXCL10 is expressed by macrophages/microglia and astrocytes in active lesions (Sorensen et al., 2002; Vazirinejad et al., 2014; Iwanowski et al., 2017). As such, further studies are required to verify the role of CXCL10/CXCR3 axis in the EAE model.

6.5. CONCLUSIONS

This study addresses molecular mechanisms by which peripheral PIC challenge induces hyperexcitability of cerebral neurons. We have demonstrated that cerebrally produced CXCL10 chemokine mediates this process. CXCL10 and its cognate receptor CXCR3 are chiefly expressed by neurons, indicating that neuronal autocrine/paracrine loops govern the development of neuronal hyperexcitability. These neuronal loops also activate microglia, potent modulators of synaptic activity. Consequently, we propose that activated microglia impede inhibitory synapses leading to hyperexcitability of neuronal networks.

Furthermore, we have demonstrated that neuronal hyperexcitability can be accurately assessed by quantifying the expression of neuronal c-fos.

In addition, we have developed a preclinical system in which PIC challenge exacerbates the progression of EAE, a murine model of MS. This finding validates detrimental effects of peripheral viral infections in MS patients.

Further elucidation of CXCL10-CXCR3-mediated pathways in future studies will provide a springboard for the development of novel therapeutic approaches to prevent exacerbations of neuropathological diseases.

6.6. Future Directions

This dissertation established that the induction of neuronal CXCL10 is a putative mediator of PIC challenge-induced neuronal hyperexcitability. However, the underlying mechanisms have to be elucidated. Specifically:

1. **DELINEATE PATHWAY BETWEEN BLOOD BORNE INFLAMMATORY MEDIATORS AND THE NEURONAL GENERATION OF CXCL10**

Previous studies by the Konat lab have shown that transfer of plasma from PIC challenged animals into naïve animals upregulates cerebral cytokine and chemokine genes (Fil et al., 2011). This finding provides an experimental for the determination of transduction pathways of blood cytokines by endothelial cells. Elucidation of these pathways will provide additional opportunities for therapeutics.

2. DELINEATE NEURONAL SIGNALING PATHWAYS ACTIVATED BY CXCR3

While several studies have shown the effect of CXCL10 on neurons, the signaling pathways have not been determined (Nelson and Gruol, 2004; Cho et al., 2009; Kodangattil et al., 2012; Gruol, 2016). The identification of the signaling pathways would provide additional therapeutic targets.

3. INVOLVEMENT OF MICROGLIA IN THE DEVELOPMENT OF HYPEREXCITABILITY

The results from Chapter 4.3 indicate that microglia, potent regulators of synaptic activity, are involved in the development of CXCL10-mediated hyperexcitability. We posit that microglia are activated by neuronal factors released in response to CXCL10 ligation. Activated microglia in turn, impede inhibitory synapses, resulting in hyperexcitability. However, mechanisms of the synaptic impairment are not known and also, the enhancement of excitatory synapses is a valid alternative. These mechanisms are very important for therapeutic purposes and therefore should be deciphered in future studies.

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