THE BENEFICIAL EFFECTS OF AN IMMUNOTHERAPEUTIC ON PRECLINICAL GULF WAR ILLNESS NEUROLOGICAL ABNORMALITIES ALONG THE HIPPOCAMPAL DORSOVENTRAL AXIS

by

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(Under the Direction of John J. Wagner)

ABSTRACT

Gulf War Illness (GWI) is a chronic, progressive, multisymptom disease that affects one-third of the 700,000 U.S. military personnel deployed to 1990-1991 Gulf War. Currently, there is a paucity of available information regarding the acute and prolonged effects of GWI on synaptic plasticity and transmission. Further, GWI therapeutics are limited, leading to a demand for developing efficacious treatments for the disease. We assessed the circuit-level mechanisms underlying the detrimental acute impact of GWI-related chemicals on synaptic transmission along the hippocampal dorsoventral axis in *ex vivo* slice preparations. The efficacy of the immunotherapeutic lacto-N-fucopentaose-III (LNFPIII) in ameliorating GWI-specific neurological impairments in two well-established GWI animal models was also evaluated. Acute exposure to a nerve agent surrogate used in GWI animal models, diisopropylfluorophosphate (DFP), inhibited excitatory transmission via distinct cholinergic and noncholinergic mechanisms in the dH compared to the vH whereas disinhibition of inhibitory transmission in both hippocampal sectors was largely mediated by an N-methyl-D-aspartate receptor-specific mechanism. A GWI animal model comprised of pyridostigmine bromide (PB) and permethrin (PM) exposures produced long-term deficits in motor, mood, and cognitive function, acute and persisting deficits in synaptic responses along the hippocampal dorsoventral axis, and delayed neuroinflammation. Coadministration of LNFPIII during PB-PM exposure enhanced

hippocampal synaptic plasticity and transmission, ameliorated PB-PM-induced deficits in dH synaptic transmission, and enhanced trophic factor expression. Delayed LNFPIII treatment beginning four months after PB-PM exposure was beneficial for behavioral responses in the motor and cognitive domains, ameliorated or enhanced hippocampal synaptic responses uniquely along the dorsoventral axis, and rebalanced neuroinflammation. In a different GWI model, an initial enhancement followed by a delayed reduction in hippocampal synaptic transmission as well as a prolonged impairment in synaptic plasticity was detected in animals exposed to N,N-diethyl-meta-toluamide, PB, DFP, and corticosterone, aberrations that became more pronounced in the dH over time. Importantly, GWI-specific synaptic abnormalities in this model were ameliorated by delayed LNFPIII treatment, ameliorations that coincided with beneficial effects on trophic factor expression in animals that received the immunotherapeutic. Collectively, these findings addressed considerable knowledge gaps in GWI research and underscore the potential for LNFPIII as an efficacious treatment for the disease state.

INDEX WORDS: Dorsal hippocampus, Gulf War Illness, LNFPIII, Synaptic plasticity, Synaptic transmission, Ventral hippocampus, Working memory

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DEDICATION

My friends, family, and the city of Athens, GA

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Hippocampus: A Historical Perspective

During 300 BC – 300 AD, ancient scholars of the Alexandria school of medicine noticed intricate curvature of a ram horn-shaped formation in the medial temporal lobe of the brain. Accordingly, these intellectuals named this novel structure after the Latin phrase for horn of the ram, *cornu ammonis* (Andersen et al., 2007). This ventricular protrusion continued to fascinate researchers over the following years, leading to the 16th century Bolognese anatomist Giulio Cesare Aranzi designating it the hippocampus as it more closely resembles a seahorse-shaped structure. Early opinions on the functional significance of the hippocampus were varied until the 1930s when consensus began to form around a fundamental olfactory role for the structure (Ferrier, 1876; Rose, 1935; Penfield and Erickson, 1941). While contemporary evidence associates the hippocampus with a minor, but noteworthy, role in olfaction, the veracity of the proposition that the hippocampus was primarily involved in olfaction was diminished by Brodal (1947) where evidence that was inconsistent with this putative role of the hippocampus was reviewed. For instance, one report indicated that temporal lobe lesions did not impair the performance of dogs in olfactory discrimination (Allen, 1940). Hypotheses based on hippocampal neural circuitry such as the Papez circuit (Papez, 1937), as well as the discovery of functional oscillatory activity in the hippocampus known as “theta” (Jung and Kornmüller, 1938) continued to cast doubts on the postulation that the hippocampus served as a principal player in olfaction. Revitalized interest in the role of the hippocampus in cognition began to subsequently burgeon. For instance, an earlier report by Brown and Sharpey-Schafer (1888) was one of the first studies associating the hippocampus with learning and memory. Following

other researchers in the 1880s, such as Carl Wernicke and Sergei S. Korsakov describing amnesia induced by thiamine deficiency, Bekhterev (1900) found that patients presenting comparable diseases exhibited substantial memory deficits and notable softening of the hippocampus (Wernicke, 1881; Korsakoff, 1889). Later, Scoville and Milner (1957) conducted a bilateral medial temporal lobectomy in a patient (H.M.) exhibiting severe epilepsy, an experiment that yielded devastating amnesia. Notably, following the procedure H.M. exhibited impaired ability to develop new episodic memories whereas his procedural and working memory function were unaffected. This lobectomy and the subsequent findings prompted the accelerated development of animal models to study learning and memory (Kimble, 1963; Douglas and Isaacson, 1964; Kimble, 1968) as well as robust and sensitive behavioral tasks to study learning and memory deficits (Kaada et al., 1961; Kimble, 1963; Kveim et al., 1964; Mishkin and Delacour, 1975; Olton et al., 1977; Morris, 1984). This preclinical research facilitated the discovery of multiple forms of hippocampal-dependent and hippocampal-independent memory such as declarative (Tulving, 1972) and nondeclarative memory (Winograd, 1975; Cohen and Squire, 1980), respectively. Importantly, work by O'Keefe and Dostrovsky (1971) demonstrated an association between hippocampal cellular activity and the relative location of an animal in space, suggesting that hippocampal place cells enable the hippocampus to serve as a cognitive map. These findings cemented the hippocampus as a pivotal structure involved in learning and memory, leading to decades of fruitful research inquiring into the circuitry-level mechanisms underlying the basis of hippocampal-dependent learning and memory.

Hippocampal studies revealed foundational principles of neural science such as excitatory (Kandel et al., 1961; Andersen et al., 1966a; Andersen et al., 1966b) and inhibitory synapses (Kandel et al., 1961; Andersen et al., 1964a; Andersen et al., 1964b; Curtis et al., 1970), long-term potentiation (Lømo, 1966; Bliss and Lømo, 1973; Bliss and Collingridge, 1993), and the functional role of oscillatory activity like theta rhythm (Vanderwolf, 1969; Kramis et al.,

1975). Unique anatomical and synaptic features of the hippocampus enabled the discovery of the aforementioned and many more cardinal features of the nervous system. For instance, the single-cell laminar structure of the hippocampus enabled a relatively easy investigation of circuit-level changes in the tissue compared to neocortical structures. This is due to the predominate unidirectional connectivity of the hippocampus, making the study of homogeneous principal cells in a particular layer relatively straightforward. Moreover, unidirectional connectivity in the hippocampus allowed for the assessment of heterogenous circuits such as the trisynaptic pathway. The first reports of axonal sprouting and reactive synaptogenesis in the brain were described in the hippocampus (Raisman, 1969; Matthews et al., 1976), revealing an inherent plasticity of this structure that has facilitated studies of neurogenesis. Further, synaptic properties can be assessed in long-living hippocampal neurons grown in culture. While early studies employed hippocampal cell cultures that lacked the laminar structural integrity exhibited in vivo (Banker and Cowan, 1977; Banker and Goslin, 1991), a later group developed the organotypic hippocampal slice culture (Gähwiler, 1981; Zimmer and Gähwiler, 1984; Gähwiler and Brown, 1985; Gähwiler, 1988), facilitating a variety of experiments such as the evaluation of neuron development before or after xenobiotic insult. The establishment of the transverse acute hippocampal slice experimental paradigm enabled the study of wide-ranging physiologically-relevant synaptic plasticity and transmission responses in vitro (Li and McIlwain, 1957; Bliss and Richards, 1971; Skrede and Westgaard, 1971). Properties such as the lack of a blood-brain barrier and sustained health of the preparation permitted in-depth investigation of translational hippocampal slice synaptic properties with extracellular and intracellular recording approaches. For instance, continued refinement of hippocampal electrophysiology techniques and hippocampal-dependent behavioral tasks led Whitlock et al. (2006) to observe an association between a synaptic response, long-term potentiation, and the capacity for learning and memory. As electrophysiological approaches such as multielectrode arrays and in vivo unit recordings as well as imaging technology like functional magnetic resonance imaging continue to be

improved, the story of the hippocampus will evolve. The following subsections will survey the neuroanatomy of the hippocampal formation, cellular properties that underlie the crucial functions of the hippocampus in synaptic transmission, forms and phases of hippocampal synaptic plasticity, and the pivotal role of the hippocampus in learning and memory.

1.2 Hippocampal Anatomy and Circuitry

1.2.1 General anatomy of the hippocampus

The hippocampus is a bilateral structure located in the mammalian medial temporal lobe (Fig. 1.1). Decades of research has been conducted on the intricate laminar configuration of the hippocampus that shapes its complex role in synaptic transmission. Recent studies have led to an increasing appreciation of the differential functionality along the hippocampal dorsoventral axis. Notably, this unique activity may be attributed to diverging extrinsic projections and distinct cellular properties throughout the hippocampal longitudinal axis. The following subsection will briefly review the anatomy and circuitry of the hippocampal formation.

A broad neuroanatomical characterization of this temporal lobe structure is the "hippocampal formation", which consists of the entorhinal cortex, parasubiculum, presubiculum, subiculum, dentate gyrus, and hippocampus proper (Fig. 1.2; Andersen et al., 2007). The hippocampus proper refers to the CA1-CA4 subfields and is generally defined as the unambiguous hippocampal structure. Regions within the hippocampal formation exhibit distinct laminar cytoarchitecture. Specifically, the subiculum, dentate gyrus, and hippocampus proper are distributed into laminar subregions defined as the *alveus*, *stratum oriens* (str. oriens), *stratum pyramidale* (str. pyr.), *stratum lucidum* (str. luc.), *stratum radiatum* (str. rad.), *stratum lacunosum-moleculare* (str. lac-mol.), *stratum granulosum* (str. gr.), and the hippocampal fissure (Andersen et al., 2007). The alveus is a thin fiber consisting of efferent CA1 fibers located on the ventricular surface of the hippocampus, bundling to form projections from hippocampal pyramidal cells to the subiculum and subsequently to the fornix. The str. oriens is positioned deep relative to the pyramidal cell layer and can be defined as an infrapyramidal region that

contains CA3-CA1 Schaffer collateral projections in addition to CA3-CA3 associational projections. The str. oriens also consists of basket cells, horizontal trilaminar cell somata, and basal dendrites of pyramidal cells. The str. pyr. is the principal cell layer of the hippocampus proper, comprising tightly packed pyramidal cell somata in the CA1 subfield and less condensed pyramidal cells in the CA2 and CA3 subfields. Cell bodies of interneurons such as radial trilaminar cells, axo-axonic cells, and bistratified cells, are also found in the str. pyr. A very thin, acellular, CA3-specific lamina including mossy fibers known as the str. luc. is positioned above the str. pyr. Directly above the str. pyr. in the CA1 and CA2 subfields but above the str. luc. in the CA3 subfield is the str. rad., a suprapyramidal lamina containing CA3-CA1 Schaffer collateral projections and CA3-CA3 associational connections (Fig. 1.2). A litany of interneurons (i.e., basket cells and radial trilaminar cells) as well as the apical dendrites of pyramidal cells are located within the str. rad. The subsequent layer, the str. lac-mol., is the most superficial lamina of the hippocampus, consisting of far-traveling distal apical dendrites of pyramidal cells and a myriad of interneurons. Dendritic branches of dentate gyrus granule cells are also found in the str. lac-mol. Notably, the str. lac-mol. is the target site of the perforant pathway, a significant entorhinal cortex projection. The str. gr. is a U/V-shaped layer consisting of dentate gyrus granule cell somata, where in between this lamina are dentate gyrus mossy cells and several other polymorphic cells that make up the hilus. The hippocampal fissure is a cell-free region involved in oscillatory activity and anatomically separates the dentate gyrus from the CA1 subfield of the hippocampus proper (Fig. 1.2).

1.2.2 Fundamental hippocampal circuitry

The structurally distinct and well-defined laminar configuration of the hippocampus formation facilitated the investigation of local circuitry within the structure, revealing two critical unidirectional glutamatergic projections that originate in the entorhinal cortex: the perforant pathway and the alvear pathway (Neves et al., 2008). The entorhinal cortex receives significantly processed innervation from brain regions responsible for integrating sensory

information, resulting in distinct subregions and layers within the entorhinal cortex serving as an interface between neocortical and hippocampal connectivity (Fyhn et al., 2004). The initial synapse of the perforant pathway, or trisynaptic pathway, is produced by excitatory projections from layers II and III of the entorhinal cortex that form a synapse at the dendritic branches of dentate gyrus granule cells. Axons of granule cells known as mossy fibers then carry excitatory projections to the apical dendrites of CA3 pyramidal cells, forming the second synapse in this trisynaptic pathway. Excitatory Schaffer collaterals originating in the CA3 subfield then form the third synapse at CA1 pyramidal cell apical dendrites in the str. rad., where the signal ultimately perforates the subiculum or feeds back to the entorhinal cortex. Additionally, the alvear pathway originates in layers II and III of the entorhinal cortex where the pathway turns toward the alveus before perforating CA1/CA3 pyramidal cells via termination in the str. lac-mol. (Clark and Squire, 2013). These two well-studied pathways underscore the efficiency of synaptic transmission mediated by the distinct subregion, subfield, and laminar organization of the hippocampal formation.

1.2.3 Functional differentiation along the hippocampal dorsoventral axis

There has been increased appreciation for differential functionality of the posterior [dorsal (dH), septal] and anterior [ventral (vH), temporal] hippocampal sectors over the past decade (Bannerman et al., 2004; Fanselow and Dong, 2010). Functional differences in efferent and afferent projections along the hippocampal dorsoventral axis were initially suggested in the 1970s (Siegel and Tassoni, 1971; Swanson and Cowan, 1977), followed by later findings by Moser et al. (1995), which revealed the unique roles the two sectors play in behavior. Overall, hippocampal-dependent behavior is regulated by dorsoventral-specific synaptic plasticity and transmission, resulting in the dH being predominately linked to spatial memory whereas the vH is primarily associated with affective and stress-related activity (Bannerman et al., 2004). Unique local circuitry along the hippocampal dorsoventral axis contributes to the differing properties of the dH and the vH (Fig. 1.3). For example, the majority of entorhinal cortex

projections reach the vH CA1 subfield via the perforant pathway; however, entorhinal cortex fibers increasingly follow the alvear pathway at more septal portions of the hippocampal longitudinal axis (Andersen et al., 2007). Therefore, entorhinal cortex innervation primarily moves along the alvear pathway to reach the dH CA1 subfield. Diverging efferent and afferent projections along the hippocampal longitudinal axis is another rudimental factor for this distinct behavioral functionality (Risold and Swanson, 1996; Fanselow and Dong, 2010; Poppenk et al., 2013). For instance, visuospatial sensory efferents from respective associational cortices reach the dH via caudolateral fibers of the entorhinal cortex whereas olfactory, gustatory, and visceral sensory-related efferents project to the vH through rostromedial entorhinal cortex fibers (Burwell, 2000). Neocortical efferents from regions such as the retrosplenial cortex selectively target the dH, resulting in this sector of the hippocampus playing a unique role in integrating spatial and cognitive information (Naber and Witter, 1998). Additionally, distinctive, direct projections from the olfactory bulb and other primary olfactory cortical regions to the vH CA1 lead to a vH-specific influence over sensory input (Roberts et al., 2007).

1.2.4 Dorsal hippocampal-specific circuitry

The dH CA1 and dorsal subicular complex send significant afferent projections to the retrosplenial and anterior cingulated cortices (Risold et al., 1997; Roberts et al., 2007), leading to further influence in spatial navigation, memory processing, and integration of visuospatial input (Jones and Wilson, 2005). The dH CA1 and CA3 subfields also innervate portions of the lateral septal nucleus (Risold and Swanson, 1996), a structure that projects to brain regions involved in the production and modulation of hippocampal theta rhythm during locomotion such as the supramammillary nucleus and medial septal complex (Stewart and Fox, 1990). The dH CA3 subfield projection to the lateral septum also leads to indirect innervation of the ventral tegmental area, impacting motivation and locomotor activity (Luo et al., 2011). The dorsal subiculum further shapes locomotor activity through direct projections to the rostrolateral nucleus accumbens and rostral caudoputamen (Groenewegen et al., 1996; Naber and Witter,

1998), resulting in subsequent indirect innervation of the ventral tegmental area and substantia nigra via the ventral pallidum or globus pallidus (Groenewegen et al., 1996). Additionally, the dorsal subiculum complex innervates the anterior thalamic complex and mammillary nuclei via the postcommissural fornix (Kishi et al., 2000), a pathway that reciprocally innervates the dH to generate and finetune a cognitive map during navigation and exploration (Risold et al., 1997).

1.2.5 Ventral hippocampal-specific circuitry

On the other hand, the vH CA1 and ventral subiculum exhibit unique bidirectional projections between the infralimbic, prelimbic, and agranular insular cortices of the medial prefrontal cortex as well as a myriad of amygdalar nuclei, resulting in the vH being well-positioned to influence affective states and emotional responses (Fanselow and Dong, 2010). Direct and indirect innervations from this bidirectional vH circuit project to the medial and central nuclei of the amygdala as well as the bed nucleus stria terminalis, ultimately terminating in the periventricular and medial zones of the hypothalamus to impact fundamental emotional and motivational behaviors such as defense, reproduction, and ingestion (Kishi et al., 2000). A monosynaptic projection to the nucleus accumbens shell from the vH CA1 as well as ventral subiculum further impact motivational states such as feeding and reward processing (Groenewegen et al., 1996; Naber and Witter, 1998). Additionally, polysynaptic projections from the ventral subiculum to the ventral tegmental area shape behavior related to motivation, further highlighting distinctions between extrinsic circuitry along the dorsoventral axis (Lisman and Grace, 2005; Kahn and Shohamy, 2013).

1.2.6 Cellular and molecular differences between the dorsal hippocampus and the ventral hippocampus

Additionally, differential cellular characteristics such as greater density and selectivity of place cells in the dH compared to the vH (Jung et al., 1994) contribute to unique functionality along the hippocampal dorsoventral axis (Fig. 1.4). A litany of other receptor-related differences influences distinct physiological activity between the dH and the vH (Table 1.1). Notably,

divergent glutamatergic, γ-amino butyric (GABA)-ergic, and neuromodulatory receptor density in the dH compared to the vH results in divergent synaptic properties. Specifically, the dH exhibits greater α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor density than the vH (Pandis et al., 2006). The vH has a greater GluN2A:GluN2B density ratio compared to the dH, contributing to discrete cation conduction that influences alterations in synaptic plasticity (Pandis et al., 2006). Further, studies suggest differential expression of metabotropic glutamate receptors (mGluR) such as greater mGluR5 density in the dH compared to the vH promotes divergent synaptic properties between the sectors (Kouvaros and Papatheodoropoulos, 2016a). Unique density of GABA receptors has also been described along the hippocampal dorsoventral axis. For example, a greater density of the α_1 subunit of the $GABA_A$ receptor was reported in the dH whereas elevated quantities of the α_2 subunit and α_5 subunit $GABA_A$ receptor were described in the vH (Sotiriou et al., 2005). Further, studies demonstrated that the $GABA_B$ receptor is found at greater densities in the dH compared to the vH (Papatheodoropoulos, 2015b; Papatheodoropoulos, 2015a; Kouvaros and Papatheodoropoulos, 2016b). Receptors involved in modulatory neurotransmission are also differentially expressed in the dH compared to the vH. For instance, the cannabinoid receptor type 1 was observed at greater densities in the dH compared to the vH (Kouvaros and Papatheodoropoulos, 2016a). Further, distinct expression of the muscarinic acetylcholine receptor (mAChR) and the nicotinic acetylcholine receptor (nAChR) were reported along the dorsoventral axis (Ruiz et al., 1993; Jung et al., 1994; Huang and Winzer-Serhan, 2006; Kenney et al., 2012). Receptors implicated in the regulation of intracellular Ca^{2+} signaling like the ryanodine receptor (RyR) were also shown to be differentially expressed along the dorsoventral axis where the RyR1 was found at greater densities in the vH compared to the dH (Grigoryan et al., 2012). Additionally, membrane receptors involved in stress-related responses such as the glucocorticoid (GR) and mineralocorticoid (MR) receptors are dissimilarly expressed along the

hippocampal dorsoventral axis where the GR was found at greater densities in the dH whereas the MR was expressed at higher levels in the vH (Robertson et al., 2005).

1.2.7 Differential synaptic plasticity and transmission along the hippocampal dorsoventral axis

These unique cellular properties contribute to divergent synaptic transmission in the dH compared to the vH. For instance, the vH exhibits enhanced CA1 pyramidal cell excitability compared to the dH (Papatheodoropoulos, 2018). The vH also exhibits weaker overall synaptic inhibition (Papatheodoropoulos et al., 2002) and recurrent inhibition in the CA1 subfield (Petrides et al., 2007) relative to the dH, responses that are reflected in a greater fast and slow inhibitory postsynaptic potential amplitude in the dH compared to the vH (Papatheodoropoulos et al., 2002). Dorsoventral-specific differences in synaptic transmission also manifest in responses related to synaptic plasticity. For instance, dorsoventral-specific short-term alterations in synaptic plasticity were reported in a study that described the dH exhibiting greater paired-pulse facilitation magnitude whereas the vH displayed an elevated probability of neurotransmitter release (Papatheodoropoulos and Kostopoulos, 2000b). Further, the dH has a greater capacity for persisting synaptic potentiation, which is reflected by elevated long-term potentiation (LTP) magnitude and LTP half-life in the dH compared to the vH (Papatheodoropoulos and Kostopoulos, 2000a; Maggio and Segal, 2007b). Evidence of unique oscillatory activity in the dH compared to the vH has also been described. For example, the vH is the origination site of sharp-wave ripples (Patel et al., 2013; Kouvaros and Papatheodoropoulos, 2017) and exhibits greater spontaneous network activity (Brazier, 1970; Papatheodoropoulos, 2018) than the dH. These types of oscillatory responses and a weaker overall inhibitory tone in the vH contributes to the initiation of epileptogenesis in the temporal sector of the hippocampus that propagates toward the septal sector (Gilbert et al., 1985; Bragdon et al., 1986; Traub et al., 1993; Derchansky et al., 2006; Häussler et al., 2012; Mikroulis and Psarropoulou, 2012; Papatheodoropoulos, 2018). On the contrary, the dH is more responsive to oscillations in the theta range (5 Hz) and theta entrainment initially occurs in the

septal hippocampal sector and propagates towards the temporal sector (Goutagny et al., 2009; Lubenov and Siapas, 2009; Royer et al., 2010; Hinman et al., 2011; Long et al., 2015). Nonetheless, some physiological responses are sector-independent. For example, basal synaptic activity such as baseline input/output field excitatory postsynaptic potential (Papatheodoropoulos and Kostopoulos, 2000b; Papatheodoropoulos and Kostopoulos, 2000a; Pofantis and Papatheodoropoulos, 2014; Kouvaros and Papatheodoropoulos, 2016a; Kouvaros and Papatheodoropoulos, 2016b; Papatheodoropoulos, 2018) and presynaptic fiber volley (Papatheodoropoulos and Kostopoulos, 2000a; Maggio and Segal, 2007a; Grigoryan et al., 2012; Pofantis and Papatheodoropoulos, 2014; Kouvaros and Papatheodoropoulos, 2016b) responses, as well as synaptic plasticity measurements like long-term depression (LTD) (Maggio and Segal, 2009), are similar between the sectors. It is also presently unclear if there are any significant differences between dH and vH pyramidal cell resting membrane potential (Papatheodoropoulos, 2018). Exposure of the hippocampus to abnormal conditions underscores the profound functional impact of unique properties in the dH and the vH. For instance, the vH exhibits a greater capacity for metaplasticity, which is reflected in elevated vH, but not dH, LTP magnitude following in vitro exposure to a mGluR1 (Maggio and Segal, 2007b) or RyR agonist (Grigoryan et al., 2012) as well as pre-tetanic low-frequency stimulation priming (Maggio and Segal, 2007b). Further, a metaplastic stress effect resulted in the vH exhibiting greater LTP magnitude than the dH following exposure to stressful stimuli or bath application of corticosterone (Maggio and Segal, 2007a; Maggio and Segal, 2011; Grigoryan et al., 2015). An additional example of the metaplastic stress effect includes the substantial dorsoventral-specific disparities in LTD magnitude observed following exposure to stress or corticosterone where dH LTD was enhanced while vH LTD was reduced before being converted to slow-onset LTP (Maggio and Segal, 2009; Maggio and Segal, 2011). These distinct stress-induced responses in synaptic plasticity along the dorsoventral axis are related to the uniquely elevated expression of the GR and the MR in the dH and the vH, respectively (Maggio and Segal, 2010; Maggio and

Segal, 2012). Specifically, corticosterone-induced activation of the GR in the dH led to enhanced inhibitory postsynaptic current (IPSC) amplitude, resulting in hyperpolarized membrane potential and inactivation NMDA receptors. This consequently led to reduced dH LTP and elevated LTD. In the vH, corticosterone-mediated stimulation of the MR decreased the frequency of IPSCs, facilitating pyramidal cell excitability. This change in excitability enabled the activation of voltage-gated Ca^{2+} channels (VGCC), increasing vH LTP magnitude. Additionally, increased vH pyramidal cell excitability stimulated the mGluR1, decreasing GABA production to ultimately shift LTD to slow-onset LTP. Overall, this example of differential stress-induced metaplasticity in the dH and the vH highlights the intricate functional segregation of the hippocampal dorsoventral axis which allows these structures to respond and adapt to a myriad of exogenous stimuli (Thiagarajan et al., 2007).

Cumulatively, these studies demonstrate how the elaborate yet well-organized cytoarchitectural and laminar structure of the hippocampal formation contributes to its activity in physiological responses. Functional segregation along the hippocampal dorsoventral axis may be attributed to distinct local and extrahippocampal afferent and efferent projections as well as dissimilar cellular properties within the sectors. Cellular properties that differentially manifest across the dH and the vH contribute to critical and unique roles these sectors play in synaptic plasticity and transmission.

1.3 Hippocampal Synaptic Transmission: Cellular Mechanisms

1.3.1 Overview

The action potential facilitates neural responses via neuronal signal transduction, resulting in the release of synaptic vesicles that enable synaptic transmission. Hippocampal synaptic transmission is predominately driven by excitatory glutamatergic activity and shaped by inhibitory GABA-related signaling. Neurotransmission is also modulated by neurotransmitters such as acetylcholine (ACh). The neurotransmitter responsible for facilitating excitatory transmission of principal cells, glutamate, primarily interacts with fast-acting AMPA and slow-

acting NMDA ionotropic receptors. The ionotropic $GABA_A$ receptor is responsible for rapid inhibition of hippocampal neurotransmission whereas the metabotropic $GABA_B$ receptor mediates slow inhibition of synaptic activity. ACh interacts with the ionotropic nAChR as well as the metabotropic mAChR, resulting in a variety of neuromodulatory effects on hippocampal synaptic transmission. The following subsection will provide a brief overview of a fundamental response involved in cell-to-cell communication throughout the nervous system, the action potential, that results in synaptic vesicle release. Additionally, fundamental receptors involved in glutamatergic, GABAergic, and cholinergic hippocampal synaptic transmission will be discussed.

1.3.2 Presynaptic mechanisms of synaptic transmission

The firing of an action potential is one of the most important decisions a neuron will make (Bean, 2007). Starting at a standard resting membrane potential of -70 mV, the action potential is initiated due to spatiotemporal-dependent graded depolarization of the membrane potential (V_m). Once a sufficient membrane depolarization threshold is met, the upstroke of the action potential begins via a substantial electrochemical gradient-driven influx of Na^+ through opening of the activation gate of voltage-gated Na^+ (Na_V) channels. As the V_m approaches the Na^+ equilibrium potential (E_{Na}), the driving forces of the electrochemical gradient are reduced, leading to a reduced influx of Na^+. Additionally, while the V_m approaches E_{Na}, spontaneous inactivation of Na_V channels begins to occur due to the closing of the inactivation gate within the channel, ultimately producing the top of the action potential upstroke. At about the same time as inactivation of Na_V channels, the activation gate of delayed rectifying voltage-gated K^+ (K_V) channels begins to open, allowing a massive efflux of K^+ and the downstroke of the action potential. As Na_V channels are still inactivated and the cell is predominately permeable to only K^+, the electrochemical driving force of K^+ efflux and K^+ leak channels enable the V_m to become hyperpolarized relative to the resting membrane potential, resulting in a hyperpolarized afterpotential that is very similar to E_K. As the inactivation gate of Na_V channels and activation

gate of K_V channels close, a refractory period begins and the V_m of the cell reverses back to the resting membrane potential, resetting the cell for another action potential. At the subcellular level, the described action potential was generated at the axon hillock and travels down the axon via saltatory conduction to result in synaptic vesicle release in a process that consists of stages known as docking, priming, fusion-pore opening, and fusion (Ramakrishnan et al., 2012; Südhof, 2013). Docking is initiated as neuronal depolarization occurs following action potential invasion of the presynaptic bouton, resulting in altered V_m. This shift in V_m enables steric alterations in membrane-bound receptors such as the VGCC, facilitating Ca^{2+} influx. Intracellular Ca^{2+} then interacts with calmodulin, an interaction that results in the synapsin protein being released from the actin cytoskeleton. Synapsin releasing from the actin cytoskeleton initiates mobilization of the synaptic vesicle to its docking position in the active zone of the presynaptic terminal. Once the synaptic vesicle is docked, priming begins via synaptic vesicle-associated Rab3 interaction with active zone-associated proteins Munc13 and Rab3 interacting molecule (RIM). Following the occurrence of the Rab3-Munc13 interaction, the formation of the soluble N-ethylmaleimide-sensitive factor (NSF) attachment receptor (SNARE) complex is initiated. The SNARE complex consists of synaptic vesicle-associated protein synaptobrevin (VAMP-1/2) and active zone-associated proteins syntaxin-1 and synaptosome-associated protein 25 (SNAP25). These three SNARE proteins physically interact via their relatively similar SNARE binding motif to assemble the SNARE complex. Other proteins interact with SNARE proteins to enable the priming process. For instance, Munc18 binds to the Habc domain of syntaxin-1 to facilitate stability of the SNARE complex. Sec1 also interacts with the SNARE complex to enable stabilization. The RIM-Rab3-Munc13 protein complex interacts with the active zone-associated RIM-binding protein (RIM-BP) to ensure that the synaptic vesicle is physically close enough to the active zone to encounter the trigger for vesicular fusion: Ca^{2+}. Specifically, RIM-BP simultaneously interacts with VGCC proximal to the active zone and RIM-Rab3-Munc13 synaptic vesicle complex to ensure sufficient Ca^{2+} will be present at the active zone to trigger

fusion. A synaptic-vesicle-associated protein, synaptotagmin, acts as a Ca^{2+} sensor for fusion. Synaptotagmin functions as the Ca^{2+} sensor as a result of its two C2 Ca^{2+} binding domains that facilitate a physical interaction between the synaptic-vesicle-associated protein and the divalent cation. Once the priming steps are completed and sufficient Ca^{2+} is in the active zone, the fusion-pore opening will initiate for the readily releasable pool of synaptic vesicles as a result of Ca^{2+} binding to synaptotagmin. A phospholipid-binding motif of the Ca^{2+} sensor protein will then fuse with the cell membrane, allowing synaptotagmin to interact with the SNARE complex. Other proteins such as complexins facilitate this synaptotagmin-SNARE complex interaction. Synaptic vesicle fusion will occur next as the content of the vesicle is released into the synapse. Compounds such as SNAPs, NSF, and ATP will subsequently aid in clathrin-mediated or clathrin-independent endocytosis, vesicular recycling, and disassembly of the SNARE complex. Synaptic vesicles contain neurotransmitters such as glutamate, GABA, and ACh, chemical messengers that travel throughout the synapse following vesicular release and induce physiological responses by interacting with membrane-bound receptors such as the AMPA, NMDA, GABA, mACh, and nACh receptors.

1.3.3 Receptor-specific roles in hippocampal synaptic transmission

1.3.3.1 Glutamatergic receptors: AMPA and NMDA receptors

The AMPA receptor is a ligand-gated ion channel and a heterotetramer consisting of GluR1-GluR4 subunits capable of conducting cation (i.e., primarily Na^+ and K^+) at almost all hippocampal excitatory synapses (Hollmann et al., 1989; Keinanen et al., 1990; Hollmann and Heinemann, 1994; Nakanishi et al., 1998; Ozawa et al., 1998). The subunits contain M1, M2, M3, and M4 segments, each responsible for unique functions (Bleakman and Lodge, 1998; Andersen et al., 2007). The M1 and M3 subunit segments interact with the pore-loop region of the M2 segment to form the ion channel pore of the AMPA receptor. The M2 segment determines the ion selectivity of the AMPA receptor channel. The S1 region of the N-terminal domain and the S2 region of the extracellular loop between M3 and M4 subunit segments

generates the binding site for extracellular ligands. The binding site for intracellular proteins that facilitate AMPA receptor trafficking and signaling is found at the C-terminal domain of each subunit within the cell. Distinct amino acid compositions of the GluR subunits result in diverse channel properties. Interestingly, elevated divalent cation (i.e., Ca^{2+}) conduction occurs in a voltage-dependent manner as polyamine molecules are cleared from the GluA2-lacking AMPA receptor (Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Kamboj et al., 1995; Koh et al., 1995), facilitating a non-linear, rectifying I/V relationship and unique alterations in synaptic plasticity (Geiger et al., 1995; Swanson et al., 1997); however, the AMPA receptor generally conducts Na^+ current at resting membrane potential, although low-levels of K^+ conduction does occur at depolarized membrane potentials. Compared to other glutamate receptors like the NMDA receptor, the AMPA receptor exhibits very fast glutamate binding kinetics, a rapid rise time, and high opening probability following exposure to a pulse of glutamate (Jonas et al., 1993). AMPA receptors deactivate quickly (Colquhoun et al., 1992) and are susceptible to desensitization upon prolonged exposure to aberrant concentrations of glutamate, a response that exhibits a time course determined by AMPA receptor subunit composition (Mosbacher et al., 1994). While all four AMPA receptor subunits are found in the hippocampus, a litany of studies report cell-specific expression of AMPA receptor subunits throughout the hippocampus (Jensen et al., 2003). This differential expression pattern results in unique cellular properties and subregion-specific responsibilities of AMPA receptors throughout the hippocampus. For instance, AMPA receptors found in pyramidal cells predominately consist of GluR1 and GluR2 subunits (Keinanen et al., 1990) whereas interneurons express GluR1 and GluR4-containing AMPA receptors (Andersen et al., 2007). While some GluR2/GluR3 and homomeric GluR1 AMPA receptors are expressed in principal cells (Keinanen et al., 1990; Wenthold et al., 1996), the consistent presence of a GluR2 subunit facilitates non-rectifying monovalent cation conduction with low divalent cation permeability. On the contrary, the GluR1/GluR4 heterotetramer AMPA receptors commonly expressed by interneurons permit distinct cellular

activity of these GABAergic cells compared to their principal cell counterparts. For example, GluR1/GluR4-containing AMPA receptors expressed in interneurons are highly permeable to divalent cation and exhibit a rectifying I/V relationship (Geiger et al., 1995). Evidence also suggests a subcellular-specific distribution of hippocampal AMPA receptors where AMPA receptors are expressed at greater densities in the distal portion of dendrites compared to the proximal segment, suggesting that subcellular-specific expression of AMPA receptors may contribute to integration of synaptic activity (Magee and Cook, 2000).

Another classic ionotropic glutamate receptor is the NMDA receptor, a heterotetramer that is comprised of GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, or GluN3B subunits (McBain and Mayer, 1994). Each subunit consists of M1-M4 segments, serving functions that are generally similar to those mentioned in the previous AMPA receptor subsection. NMDA receptors commonly incorporate two GluN1 subunits and two GluN2 subunits (Dingledine et al., 1999). Current nomenclature rules define NMDA receptors containing two GluN1 and two GluN2A subunits as GluN2A receptor subtypes whereas NMDA receptors comprised of two GluN1 and two GluN2B subunits are defined as GluN2B receptor subtypes, and so on for the remaining GluN2 and GluN3 receptor subtypes. While the GluN2A and GluN2B receptor subtypes are the most commonly expressed forms of the NMDA receptor in the hippocampus, all NMDA receptor subtypes except the GluN3B receptor subtype are expressed in the temporal lobe structure (Andersen et al., 2007). Activation of the NMDA receptor requires glycine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988) or D-serine (Schell et al., 1995; Barañano et al., 2001) binding in addition to a physical interaction with glutamate, and the GluN1 subunit contains a binding site for these two co-agonists. GluN1 knockout experiments demonstrated that this subunit is essential for channel function of the NMDA receptor (Forrest et al., 1994). GluN2 subunits contain a binding pocket for glutamate and are primarily responsible for regulating electrophysiological as well as pharmacological properties of channels within the NMDA receptor (Laube et al., 1997). Specifically, GluN2 subunits regulate the NMDA receptor

conductance profile, duration of opening, and Mg^{2+} sensitivity; however, different GluN2 subunits provide unique physiological properties for NMDA receptors (Andersen et al., 2007). Specifically, the GluN2B receptor subtype conducts substantially more cation current than the GluN2A receptor subtype, leading to the former being essential for learning and memory-related effects of the NMDA receptor. Further, Mg^{2+} voltage sensitivity, as well as the activation/deactivation kinetic activity of GluN2D receptor subtypes, are substantially reduced compared to GluN2A and GluN2B receptor subtypes, providing cell-specific differences in hippocampal synaptic transmission. GluN3 subunits contain a glycine binding site, resulting in GluN3 NMDA receptor subtypes playing an essential role in glycine conduction (Dingledine et al., 1999). Specifically, heterotetramers comprised of GluN1 and GluN3 subunits act as glycine-gated ion channels (Chatterton et al., 2002). While both the AMPA and NMDA receptors play a fundamental role in hippocampal excitatory synaptic transmission, the latter receptor exhibits slower overall kinetics (Dingledine et al., 1999). Altered kinetics of the NMDA receptor also leads to decrease probability of the receptor being in an open state (Jahr, 1992; Rosenmund et al., 1995) in addition to exhibiting a greater affinity for the endogenous ligand glutamate (Patneau and Mayer, 1990) compared to its AMPA counterpart, facilitating differential roles for these two receptors in glutamatergic transmission. Further, the NMDA receptor functions as a “coincidence detector” during unique moments of synaptic activity (Wigström and Gustafsson, 1986). Specifically, a voltage-dependent Mg^{2+} channel pore in the NMDA receptor is blocked under resting and slightly depolarized membrane potential, preventing the conduction of divalent cation (i.e., Ca^{2+}); however, upon glutamate binding to the NMDA receptor and sufficient postsynaptic membrane depolarization, the Mg^{2+} block is expelled and Ca^{2+} conduction occurs. Subsequent downstream Ca^{2+}-dependent signaling mechanisms activated by NMDA receptor-induced Ca^{2+} conduction facilitates processes of synaptic plasticity that have been associated with hippocampal-dependent learning and memory such as the induction of LTP (Emptage et al., 1999; Lüscher and Malenka, 2012). NMDA receptor subunits are differentially expressed

throughout the hippocampus in particular cell types, resulting in cell-specific physiological roles within hippocampal subregions (Monyer et al., 1994). For example, GluN1 subunits are expressed in all pyramidal cells, dentate granule cells, and many interneurons whereas GluN2A and GluN2B are expressed in principal cells as well as interneurons throughout all hippocampal lamina (Laurie and Seeburg, 1994; Monyer et al., 1994). The GluN3A receptor subtype is primarily expressed by CA1 pyramidal cells in the adult hippocampus (Laurie and Seeburg, 1994; Monyer et al., 1994), further underscoring region and cell-specific expression of NMDA receptor subunits. NMDA receptor subtypes are also differentially expressed at synapses. For instance, GluN2A receptor subtypes are preferentially expressed on commissural/associational synapses in the CA3 subfield whereas GluN2B receptor subtypes are commonly found at fimbrial-CA3 synapses (Ito et al., 1997). Subcellular-specific distribution of NMDA receptor subtypes within the hippocampus is also unique where GluN2B receptor subtypes are preferentially expressed on apical dendrites rather than basal dendritic compartments of neurons in the CA1 subfield that receive innervation from the CA3 subfield (Kawakami et al., 2003). Collectively, these reports briefly illustrate the complex synaptic, cellular, and region-specific expression profile of dissimilar NMDA receptor subtypes that result in diverse physiological functionality of this essential glutamatergic receptor.

1.3.3.2 Inhibitory receptors: GABA receptors

Excitatory glutamatergic transmission and network oscillatory activity in the hippocampus is shaped by GABAergic interneurons that express presynaptic, postsynaptic, extrasynaptic, or perisynaptic $GABA_A$ and $GABA_B$ receptors (Watkins and Evans, 1981; Kullmann et al., 2005). The composition of ionotropic $GABA_A$ receptors is distinct from metabotropic $GABA_B$ receptors, resulting in unique physiological functions of the former monovalent anion (i.e., Cl^-) conducting receptor compared to the latter G-protein coupled receptor (GPCR) (Chebib and Johnston, 1999). $GABA_A$ receptors are from the Cys-loop ligand-gated ion channel superfamily and are heteropentameric transmembrane (TM) receptors

comprised of either α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, or θ subunits (Mehta and Ticku, 1999). The subunits α_1, α_2, α_5, β_3, and γ_2 are notably expressed in the hippocampus proper whereas α_3, α_4, β_1, β_2, γ_1, γ_3, and δ are found at low levels in the hippocampus proper (Sperk et al., 1997). Studies suggest that other subunits are differentially distributed throughout the rodent hippocampus while the subunits α_6, ε, π, and θ are not found in the rodent hippocampus (Sperk et al., 1997). Hippocampal $GABA_A$ receptors predominately consist of two α and two β subunits with either an individual γ or δ subunit (Chang et al., 1996; Farrar et al., 1999; Whiting et al., 1999). Each $GABA_A$ receptor subunit serves a unique role in channel functionality, leading to diverse physiological properties of $GABA_A$ synapses such as altering decay rate and conductance of IPSCs. For instance, the α subunit regulates receptor affinity for the endogenous GABA ligand, exogenous ligands such as benzodiazepines and anesthetics, and neuromodulatory agents like ethanol and Zn^{2+} (Barnard et al., 1998). $GABA_A$ receptors are fast-acting (milliseconds) ligand-gated ion channels, allowing for hyperpolarization of membrane potential following rapid Cl^- conduction that results in regulation of action potential frequency and elevation of secondary signaling molecules like Ca^{2+} (Andersen et al., 2007). While activation of $GABA_A$ receptors instigates point-to-point inhibition of synaptic activity (Isaacson et al., 1993), the reversal potential of Cl^- ions is hyperpolarized relative to the resting membrane potential of many neurons in the hippocampus, resulting in additional influence on the resting membrane potential due to Cl^- shunting (Vida et al., 2006). The location of $GABA_A$ receptors relative to the synapse leads to differential hyperpolarization effects due to varying spatial effects of Cl^- conduction (Mody, 2001). Differential subcellular distribution of $GABA_A$ receptors in pyramidal cells and interneurons also significantly influences synaptic activity. While evidence suggests that an individual hippocampal pyramidal cell exhibits approximately 1700 GABAergic synapses (Megıas et al., 2001), the apical dendritic shaft displays the greatest $GABA_A$ receptor density relative to the rest of the pyramidal cell dendrite (Papp et al., 2001). The high density of GABAergic synapses at this axon hillock region of pyramidal cells permits substantial regulation

of action potential initiation while distal GABAergic synapses on dendritic compartments monitors Ca^{2+} spike propagation. Additionally, differential density of $GABA_A$ receptor subtypes expressed by hippocampal pyramidal cells permits distinct physiological function throughout the temporal lobe structure. For instance, unique kinetics of IPSCs are observed at various positions throughout pyramidal neurons, influencing neuronal electrotonics (Banks et al., 1998; Maccaferri et al., 2000). Overall expression of $GABA_A$ receptors in interneurons is particularly concentrated at the perisomatic region as well as on the proximal segment of dendrites (Gulyás et al., 1999). Heavy $GABA_A$ receptor density is also found at distal dendritic sites of interneurons but at a relatively lower density compared to the concentration at proximal dendritic sites. The functional consequence of this subcellular distribution as well as interneuron-specific $GABA_A$ receptor expression results in a unique interneuron-selective interneuron impact on network oscillations in the hippocampus (Bartos et al., 2002).

Metabotropic $GABA_B$ receptors in the hippocampus are heterodimer GPCRs consisting of a pair of seven TM domain protein subunits, $GABA_BR_1$ and $GABA_BR_2$ (Bettler et al., 2004). The $GABA_BR_1$ subunit contains a binding site for the endogenous ligand GABA as well as all competitive $GABA_B$ ligands. The $GABA_BR_1$ subunit is essential for the function of $GABA_B$ receptors as inactivation of the $GABA_BR_1$ gene results in the absence of biochemical and electrophysiological-related $GABA_B$ activity (Andersen et al., 2007). The $GABA_BR_2$ subunit is involved in the structural activity of the $GABA_B$ receptor such as mobilization of the heterodimer to dendritic compartments and translocation of $GABA_BR_1$ subunits to the cell surface (Pagano et al., 2001; Gassmann et al., 2004). Further, $GABA_BR_2$ knockout animals exhibit $GABA_B$ responses in electrophysiological experiments, suggesting that the $GABA_BR_2$ subunit facilitates GPCR activity but is not essential to $GABA_B$ functionality (Gassmann et al., 2004). Differential distribution of $GABA_B$ receptor subtypes throughout synapses of principal cells and interneurons influences the receptor's diverse functional roles in the hippocampus. For example, activation of $GABA_B$ receptors induces slow (500-2000 ms) physiological effects as a result of presynaptic,

postsynaptic, and extrasynaptic expression (Bettler et al., 2004). Presynaptic $GABA_B$ receptors are typically localized to GABAergic and glutamatergic axon terminals (Kulik et al., 2003) and act through G_i/G_o α-coupled mechanisms (Anwyl, 1991; Mintz and Bean, 1993; Poncer et al., 2000). Postsynaptic $GABA_B$ receptors are heavily enriched on CA1 and CA3 pyramidal cell spines (Kulik et al., 2003) and facilitate K^+ conduction (Andrade et al., 1986; Misgeld et al., 1995). Studies also suggest that activation of the $GABA_B$ receptor induces the release of transcription factors such as cAMP-response element-binding (CREB) protein 2 and ATFx, potentially producing trophic factors like brain-derived neurotrophic factor (BDNF; Nehring et al., 2000; White et al., 2000). Sufficient GABA spillover at the synapse following repetitive stimulation during events of widespread interneuron activation like network oscillations results in activation of extrasynaptic $GABA_B$ receptors, leading to diffuse inhibitory transmission that may play a role in particular learning and memory states (Andersen et al., 2007). The functional role of $GABA_B$ receptors is further complicated by their distinct concentration in subcellular compartments of hippocampal neurons. For instance, $GABA_B$ receptors are predominately expressed on dendrites of hippocampal cells; however, subtypes of the $GABA_BR_1$ subunit are differentially expressed in subcellular locations of hippocampal pyramidal cells, particularly in the soma and distal dendritic compartments (Kulik et al., 2003). Subtypes of the $GABA_BR_2$ subunit localize in subcellular regions of pyramidal cells in a similar manner to subtypes of the $GABA_BR_1$ subunit (Kulik et al., 2003).

1.3.3.3 Neuromodulatory cholinergic receptors

Modulatory neurotransmitters such as ACh influence excitatory and inhibitory synaptic transmission in the hippocampus (Benardo and Prince, 1982a; Benardo and Prince, 1982b; Madison et al., 1987). Functional projections from the basal forebrain such as the medial septum/diagonal band complex and the nucleus basalis provide substantial extrahippocampal ACh to the temporal lobe structure (Stewart and Fox, 1990; Vertes and Kocsis, 1997; Hasselmo, 2006). Some hippocampal interneurons also produce low levels of ACh locally

(Andersen et al., 2007). These extrahippocampal and local sources of ACh allow the nAChR, a ligand-gated cation channel, and the mAChR, a metabotropic GPCR, to modulate hippocampal synaptic transmission. The nAChR is a member of the Cys-loop ligand-gated ion channel superfamily and is a pentameric structure (Albuquerque et al., 2009). Neuronal nAChRs consist of α_2-α_{10} and β_2-β_4, resulting in a litany of distinct nAChR subtypes. While a myriad of nAChR subtypes are expressed throughout the nervous system, the majority of hippocampal nAChRs include the α_7 nAChR, the $\alpha_4\beta_2$ nAChR, and the $\alpha_3\beta_4$ nAChR subtypes (Zoli et al., 1998; Fabian-Fine et al., 2001). Each nAChR subunit contains four TM domains that facilitate specialized properties for each nAChR subtype. Importantly, the Cys-loop extracellular amino-terminal domain contains an α subunit that binds with the endogenous ligand, ACh, while TM2 acts as a rotating ion selectivity pore (Albuquerque et al., 2009). The α_7 nAChR rapidly inactivates following nonselective cation channel conduction and exhibits the unique property of being highly permeable to Ca^{2+}. The $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nAChR subtypes are predominately permeable to monovalent cations while the former is the primary target for nicotine. The α_7, the $\alpha_4\beta_2$, and the $\alpha_3\beta_4$ nAChRs are expressed in a wide-variety of subcellular locations in principal cells and interneurons including the cell soma, dendrites, preterminal axon regions, axon terminals, and myelinated neuronal axons (Albuquerque et al., 1995; Wonnacott, 1997; MacDermott et al., 1999; Hogg and Bertrand, 2003; Alkondon and Albuquerque, 2004; Gotti et al., 2006; Albuquerque et al., 2009). Increasing evidence suggests that α_7 nAChRs play a substantial role in synaptic plasticity as a result of their high Ca^{2+} permeability, modulating the probability of inducing hippocampal LTP due to increased neurotransmitter release (Dajas-Bailador and Wonnacott, 2004). Additionally, α_7 nAChR Ca^{2+} conduction induces intracellular Ca^{2+}-dependent signaling cascades that lead to persisting alterations in synaptic structure and plasticity such as activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), phosphorylation of the downstream extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling cascade, subsequent prolonged phosphorylation of the transcription factor CREB, and

production of trophic factors like BDNF (Khiroug et al., 2003; Albuquerque et al., 2009). These synaptic modulations mediated by the α_7 nAChR suggest that the receptor is involved in overall cognitive function as well as hippocampal-dependent learning and memory, which is supported in Alzheimer's disease studies (Liu et al., 2001). Further, a consistent function for $\alpha_4\beta_2$ and the $\alpha_3\beta_4$ nAChRs in the hippocampus is currently elusive. For instance, aged, but not young, β_2 knockout mice exhibited impaired performance in the Morris water maze, a deficit that was attributed to hippocampal neurodegeneration rather than the effect of β_2 knockout (Champtiaux and Changeux, 2002). Collectively, these data indicate that nAChRs play an intricate, heterogenous, modulatory role in cognition that likely involves substantial crosstalk within the cholinergic network as well as between glutamatergic and GABAergic signaling.

The counterpart to the nAChR in cholinergic transmission is the mAChR: a seven TM domain-containing receptor that is a member of the metabotropic GPCR superfamily. The mAChR is currently known to be expressed in five subtypes, the M1-M5 mAChR (Wess, 2004). Emerging evidence continues to underscore the intricate machinery that functionally differentiates these receptor subtypes from one another (Maeda et al., 2019). For instance, a report found that the antagonist binding pocket is deep within the TM3-TM7 of the M2 mAChR (Haga et al., 2012) and a recent study outlined structural distinctions between the M1 mAChR and the M2 mAChR (Maeda et al., 2019). The heterotrimeric G-proteins that are coupled to the M1, M3, and M5 mAChRs also functionally distinguish them from the M2 and M4 mAChRs. Specifically, the M1, M3, and M5 mAChRs are coupled to phospholipase C and downstream Ca^{2+}-dependent signaling mechanisms via G_q/G_{11} G proteins whereas the M2 and M4 mAChRs are associated with negatively regulating adenylate cyclase activity and subsequent cyclic adenosine monophosphate (cAMP) through the G_i/G_o pathway (Lanzafame et al., 2003). The M1 mAChR is the most abundantly expressed mAChR in the hippocampus and is predominately found in the soma as well as dendrites of CA1 and CA3 principal cells in addition to dentate granule cells; however, studies indicate that M1 mAChRs are expressed in

interneurons to a substantially lesser extent than principal cells (Buckley et al., 1988; Levey et al., 1995; Volpicelli and Levey, 2004). The M1 mAChR is primarily found in postsynaptic regions of the synapse although some expression has been observed on axonal and terminal regions (Drever et al., 2011). The M3 mAChR subtype is concentrated at significantly lower levels than the M1 mAChR in the hippocampus, resulting in the latter being the predominate driver of G_q/G_{11}-coupled modulatory activity in the temporal lobe structure (Buckley et al., 1988; Levey et al., 1995). Very low quantities of M5 mAChR mRNA have been observed in CA1 pyramidal cells whereas few reports have identified M5 mAChR protein in the hippocampus (Vilaró et al., 1990). Similar to the M1 mAChR, M3 and M5 mAChRs are predominately found on the postsynaptic compartment of pyramidal cells (Drever et al., 2011). Although at a relatively decreased density compared to the M1 mAChR, M2 mAChR expression is abundant in the hippocampus, particularly in GABAergic interneurons (Levey et al., 1995). Evidence suggests that M2 mAChRs are concentrated in pyramidal cells as well (Drever et al., 2011). The M2 mAChR is commonly expressed as a presynaptic autoreceptor at interneuron-pyramidal cell synapses but can also be found on postsynaptic pyramidal cells (Drever et al., 2011). M4 mAChRs are detected at relatively lower densities in the hippocampus compared to M2 mAChRs and are primarily expressed on postsynaptic CA1 and CA3 pyramidal neurons (Levey et al., 1995; Andersen et al., 2007). Activation of M1 mAChRs contributes to hippocampal neuronal excitability via decreased K^+ conductance and modulation of intracellular Ca^{2+} signaling (Dutar et al., 1995), leading to enhancement of synaptic plasticity through augmentation of NMDA receptor currents and alteration of VGCC currents (Marino et al., 1998; Giessel and Sabatini, 2010). Additionally, hippocampal M1 mAChRs play a substantial role in the induction of unique forms of LTP such as muscarinic LTP (Segal and Auerbach, 1997; Shinoe et al., 2005). These synaptic data coupled with reports of M1 mAChR knockout mice exhibiting no impairments in contextual fear conditioning or Morris water maze task performance yet displaying deficits in a non-matching-to-sample working memory and consolidation task suggest that M1 mAChRs are

involved in intricate learning and memory processes such as hippocampal reactivation (Anagnostaras et al., 2003). Studies have demonstrated that M2 mAChRs found on septo-hippocampal terminals as well as local hippocampal M2 mAChR autoreceptors shape excitatory and inhibitory hippocampal synaptic transmission via increased K^+ conductance and decreased Ca^{2+} conductance (Quirion et al., 1995; Kitaichi et al., 1999; Zhang et al., 2002), leading to hyperpolarized membrane potential that decreases the probability of releasing neurotransmitters such as ACh, GABA, and glutamate (Raiteri et al., 1984; Raiteri et al., 1990). Furthermore, knockout of the M2 mAChR resulted in impaired performance in passive avoidance tasks, suggesting that the M2 mAChR regulates synaptic mechanisms that impact hippocampal-dependent learning and memory (Tzavara et al., 2003).

Collectively, the current subsection illustrated the elegant machinery involved in the generation of the action potential, resulting in diverse forms of cellular communication. The subsequent release of chemical messengers facilitates the activation of excitatory, inhibitory, and modulatory receptors in the hippocampus, initiating the stimulation of intracellular signaling cascades that shape alterations in synaptic plasticity as well as crucial cognitive processes such as learning and memory.

1.4 Hippocampal Synaptic Plasticity

1.4.1 Overview

The capacity for synapses to undergo experience or activity-related alterations in efficacy is defined as synaptic plasticity. Alterations in hippocampal synaptic plasticity influence mammalian behavioral responses such as learning and memory; however, shifts in synaptic plasticity are particularly heterogenous, facilitating the capacity of synaptic mechanisms to impact wide-ranging forms of behavior. For instance, variations of short-term plasticity shape the integration of somatosensory stimuli. Additionally, the induction, expression, and maintenance of a form of long-term hippocampal synaptic plasticity that has been established as a synaptic substrate for learning and memory, LTP, results from crosstalk between the

hippocampal formation and neocortical structures, enabling the recollection of memories (Kandel et al., 2014). The current subsection will briefly review different types of short-term and long-term hippocampal synaptic plasticity.

1.4.2 Essential concepts in synaptic plasticity

Modulations in synaptic plasticity are mediated by neural modifications at the cellular level. Importantly, research suggests that changes in synaptic plasticity may be related to alterations in behavior. This hypothesis is particularly pertinent to synaptic plasticity in the hippocampus where increasing evidence indicates that changes in synaptic efficacy in this temporal lobe structure may facilitate or impair learning and memory. Repetitive, contextual, and affective-related properties of a particular learning experience impact the ability for persisting changes to occur in hippocampal synaptic plasticity, changes that are especially influenced by spatiotemporal factors. For instance, a fundamental concept of learning and memory known as Hebbian/associative learning is related to specific hippocampal synaptic activity. Specifically, Hebb (1949) proposed that pairing of repeated and persistent presynaptic stimuli with postsynaptic membrane potential depolarization results in the modulation of synaptic efficacy and the production of experience-dependent learning. Later work validated this proposal and also predicted that spike-timing-dependent plasticity regulated Hebbian learning (Caporale and Dan, 2008). Hippocampal-related behavioral paradigms that utilize Pavlovian classical conditioning provide relatively simple examples of Hebbian learning. For instance, inhibitory avoidance learning results in modulated hippocampal synaptic efficacy after an animal learns to associate a conditioned stimulus (e.g., contextual location) with an aversive, unconditioned stimulus (e.g., footshock; Whitlock et al., 2006). Following repeated pairing of the conditioned and unconditioned stimuli, responses indicative of modulated synaptic efficacy were recorded in the hippocampus. Therefore, this alteration in synaptic efficacy is indicative of Hebbian learning, underscoring the association between hippocampal-dependent behavior with modulations in hippocampal synaptic plasticity. The Schaffer-commissural projection that synapses at

hippocampal CA1 pyramidal cells is the canonical Hebbian synapse, particularly due to its role in facilitating LTP; however, there are exceptions to Hebbian plasticity such as heterosynaptic LTP and LTD, mGluR-dependent LTD, and mossy fiber LTP (Andersen et al., 2007).

1.4.3 Short-term plasticity

Hebbian plasticity is just one of many fundamental hippocampal synaptic mechanisms investigated over the past few decades. Presynaptic and postsynaptic alterations in short-term and long-term synaptic plasticity have been intensely studied since the mid-20th century, resulting in a considerable debate over mechanisms that underlie essential synaptic processes involved in learning and memory such as LTP (Nicoll, 2017). For instance, forms of short-term plasticity known as post-tetanic potentiation (PTP), facilitation, and depression have been observed (Fig. 1.5; Regehr, 2012). These forms of short-term plasticity are generally dependent on presynaptic function and last as briefly as ten milliseconds or as long as a few min. PTP is a transient elevation in synaptic strength lasting up to a few min after prolonged presynaptic activation with high-frequency stimulation. Increasing the duration of tetanic stimulation generally induces longer-lasting PTP (Magleby, 1987). While the precise mechanism of PTP is uncertain, it is clear that presynaptic Ca^{2+} plays a significant role in this form of short-term plasticity. Specifically, tetanic stimulation leads to a buildup of presynaptic residual Ca^{2+}, which decays with slow and rapid components. Regehr (2012) summarizes two mechanisms of slow residual Ca^{2+} decay that may account for the PTP phenomenon. The first proposal suggests that presynaptic mitochondria are filled with Ca^{2+} immediately following tetanic stimulation, Ca^{2+} that subsequently leaks out to facilitate a prolonged enhancement of presynaptic Ca^{2+} levels. The second hypothesis suggests that rapid removal of Ca^{2+} via the Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger during and immediately after tetanic stimulation is slowed when presynaptic Na^{+} concentrations approach E_{Na}, resulting in interplay between the Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger that leads to substantially reduced residual Ca^{2+} decay. Synaptic facilitation is commonly observed in a phenomenon known as paired-pulse facilitation (PPF) that occurs

when the release probability of a particular neurotransmitter at a synapse is low. Specifically, when two sequential stimuli separated by a brief interpulse interval reach the presynaptic bouton, the second postsynaptic response elicited by the paired stimulation is substantially larger than the first response. On the contrary, when the probability of neurotransmitter release at a synapse is high, the second paired-pulse stimulation elicits a postsynaptic response that is significantly reduced compared to the first pulse, producing a phenomenon known as paired-pulse depression (PPD). Responses indicative of short-term plasticity such as PPF are commonly observed at Schaffer Collateral-CA1 pyramidal cell synapses in the str. rad. Interestingly, most synapses in the hippocampus can exhibit PPF and PPD as these responses are fundamentally dependent upon presynaptic activity patterns, basal neurotransmitter release probability, size of the readily releasable pool of synaptic vesicles, and residual Ca^{2+} in the presynaptic bouton. For instance, as the duration of the interpulse interval is modulated, the magnitude of the readily releasable pool of synaptic vesicles and residual Ca^{2+} levels shift, allowing for a myriad of approaches to interrogate hippocampal synapses (Jackman and Regehr, 2017). One of the most prominent hypotheses that aim to explain PPF and PPD suggests that the quantity of residual Ca^{2+} remaining in the presynaptic bouton before the second paired-pulse stimulation determines whether facilitation or depression is observed. Specifically, the residual Ca^{2+} hypothesis proposes that Ca^{2+} can remain in the presynaptic terminal following the first of two paired-pulse stimuli, facilitating an even larger Ca^{2+} signal and greater Ca^{2}+ release during the second stimulation. This is supported by reports indicating that small elevations in presynaptic Ca^{2+} levels enhance synaptic strength (Dodge Jr and Rahamimoff, 1967) whereas the addition of a photolabile Ca^{2+} chelator to whole-cell recordings blocks the facilitation of vesicular release as well as increases in postsynaptic responses (Kamiya and Zucker, 1994).

1.4.3.1 Short-term plasticity in vivo

Current evidence suggests the functional role of short-term plasticity in vivo is complex and not entirely clear; however, some studies illustrate the imperative responsibility this form of synaptic plasticity plays in physiological activity. For instance, Chung et al. (2002) demonstrated that somatosensory cortex neurons responded to initial, but not repetitive, whisker stimuli, a sensory adaption that was mediated by synaptic depression of thalamocortical synapses. Another study suggested that transient, rather than absolute, depression of synaptic activity occurs when excitatory firing frequency is suddenly elevated, allowing for percent shifts in encoding response to sensory input (Abbott et al., 1997). Functional regulation of hippocampal circuit activity via short-term plasticity was also described by Pouille and Scanziani (2004) where prolonged activation of CA3 pyramidal cells resulted in a temporary shift of synaptic inhibition from the soma to dendrites receiving the excessive stimulation. Overall, short-term plasticity plays a significant functional role in spatiotemporal-dependent regulation of synaptic computation as well as circuit-level neuronal signaling and coding (Abbott and Regehr, 2004; Grande and Spain, 2005).

1.4.4 Long-term plasticity

More than 60 years ago researchers observed rudimental indicators of long-term plasticity such as notable potentiation of synaptic activity following high-frequency stimulation (Cragg and Hamlyn, 1955; Kandel and Spencer, 1961; Gloor et al., 1964), leading to Andersen (1960) showing that commissural responses of CA3 and CA1 cells exhibited elevated excitability for up to eight min following brief 10-20 Hz stimulation. Subsequent studies demonstrated that repeated, high-frequency stimulation greater than 20 Hz resulted in significantly longer-lasting enhancement of synaptic efficacy (Lømo, 1966), laying the foundation for experiments that detected LTP as well as the growth of synaptic potentials for hours to weeks after tetanus (Bliss and Lømo, 1970; Bliss and Lømo, 1973). While LTP has been recorded in regions such as the amygdala, cerebellum, and prefrontal cortex (Laroche et

al., 1990; Armano et al., 2000; Sigurdsson et al., 2007), the majority of LTP research has been conducted in the hippocampus. Therefore, this review will focus on hippocampal long-term plasticity with a particular emphasis on NMDA receptor-dependent LTP in the hippocampus proper.

The phases and mechanisms responsible for hippocampal LTP in the CA1 subfield puzzled researchers for decades (Nicoll, 2017). There are currently three recognized phases of hippocampal LTP in the CA1 subfield (Herring and Nicoll, 2016). Phase one contains three subphases where the first subphase, PTP, is mediated via presynaptic activity and endures for up to a few min after high-frequency stimulation. The second subphase of phase one is known as short-term potentiation (STP), lasting up to 30 min post-tetanus. STP transitions to the third subphase, early-LTP (E-LTP), one h post-high frequency stimulation. Phase two is recognized as intermediate form of LTP, consisting of de novo protein synthesis from preexisting mRNA. Phase three results in the transcription of novel mRNA and is defined as late-LTP (L-LTP). LTP may also be classified into three definite phases known as induction, expression, and maintenance, phases that will be explored in particular detail in this subsection.

1.4.4.1 General classifications and properties of long-term plasticity

Notably, NMDA-receptor-dependent LTP exhibits three distinct properties: associativity, cooperativity, and input specificity (Andersen et al., 2007). Specifically, associativity denotes the observation that if individual, weak stimulation of a single pathway is insufficient to induce LTP, concurrent, strong stimulation of another pathway will induce LTP along both tracts. Working in parallel with associativity, the cooperativity property suggests that LTP may be induced at a synapse via weaker stimulation of a myriad of pathways that converge at the aforementioned synapse. Input specificity is regulated by the associativity and cooperativity properties where LTP is propagated only along a pathway that was initially stimulated; however, induction of LTP at an individual synapse may sensitize adjacent synapses to experience induction, a phenomenon known as synaptic tag and capture (Frey and Morris, 1997). The presynaptic and

postsynaptic mechanisms underlying the induction, expression, and maintenance of hippocampal LTP in the CA1 subfield generated decades of consternation, but it is currently recognized that induction requires the transient action of postsynaptic kinases following NMDA receptor-mediated Ca^{2+} conduction, expression involves persistent activation of postsynaptic kinases, and maintenance necessitates sustained protein synthesis (Fig. 1.5; Lüscher and Malenka, 2012). LTP researchers generally agree that AMPA and NMDA receptors play a critical role in the molecular mechanisms responsible for LTP induction at glutamatergic hippocampal synapses. Specifically, sufficient depolarization of presynaptic terminals results in glutamate release, a neurotransmitter that interacts with postsynaptic AMPA receptors to facilitate cation conduction and persistent depolarization of the postsynaptic membrane potential. Acting as a coincidence detector, NMDA receptors bind to glutamate and also sense the sufficient AMPA receptor-mediated depolarization, resulting in the expulsion of an Mg^{2+} block in the ion pore of the former receptor. Removal of the Mg^{2+} block upon sufficient postsynaptic membrane depolarization and binding of the glutamate ligand to the NMDA receptor results in NMDA receptor-mediated influx of Ca^{2+}. This influx of Ca^{2+} initiates a litany of intracellular mechanisms that underlie the expression of hippocampal LTP. This is supported by research demonstrating activation of postsynaptic AMPA receptors is insufficient to induce LTP whereas Ca^{2+} conduction via NMDA receptors is sufficient to induce LTP (Malenka et al., 1988); however, the interplay between the AMPA receptor and NMDA receptor underscores the essential nature of the associativity property in LTP. Therefore, transient facilitation of presynaptic glutamate release and elevated postsynaptic intracellular Ca^{2+} concentration are fundamental components of LTP induction (Malenka and Bear, 2004), with the latter being necessary and sufficient for the induction of LTP in CA1 pyramidal cells (Malenka et al., 1988). Interestingly, CA1 pyramidal neurons and many other brain regions exhibit NMDA receptor-dependent LTP induction (Malenka and Bear, 2004) whereas some synapses such as the CA3 neuron-mossy fiber synapse display NMDA receptor-independent LTP induction (Zalutsky and

Nicoll, 1990; Bortolotto et al., 1999; Schmitz et al., 2001). The latter, NMDA receptor-independent mechanism requires presynaptic and postsynaptic kainate receptor activation for LTP induction, which caused substantial confusion in the field of LTP research decades ago. The present review from herein will provide an overview of many molecular mechanisms that are currently recognized as being responsible for NMDA receptor-dependent LTP induction, expression, and maintenance.

1.4.4.2 NMDA receptor-dependent LTP induction

Beginning from the point at which Ca^{2+} enters the postsynaptic cell via the NMDA receptor, at least one s but no more than 2.5 s of increased intracellular Ca^{2+} levels (Malenka et al., 1992) results in the induction of LTP and physical interaction between Ca^{2+} and calmodulin (Ca^{2+}/calmodulin) (Herring and Nicoll, 2016). When calmodulin interacts with Ca^{2+}, the Ca^{2+}/calmodulin complex mobilizes and physically interacts with a serine/threonine-protein kinase that is responsible for the initiation of kinase-dependent LTP expression, CaMKII. For instance, a high-frequency stimulation-insensitive enhancement of CA1 pyramidal cell excitatory postsynaptic potentials (EPSPs) that resembled the potentiated EPSPs observed after a tetanus protocol was reported following viral transduction of constitutively active CaMKII into hippocampal slices (Pettit et al., 1994) or CaMKII injection into CA1 principal cells (Lledo et al., 1995). Ca^{2+}/calmodulin or CaMKII chelator-mediated blockade (Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992), as well as CaMKII-specific inhibition (Malinow et al., 1989; Ito et al., 1991; Wang and Kelly, 1995; Otmakhov et al., 1997) or mutation (Silva et al., 1992a; Silva et al., 1992b), also prevented the induction of LTP in addition to hippocampal-dependent learning and memory function. Additionally, CaMKII is involved in synaptic tag and capture following LTP induction, resulting in sensitization of adjacent synapses to undergo LTP induction (Redondo et al., 2010). Overall, these data suggest that Ca^{2+}/calmodulin interaction and subsequent CaMKII activation are essential in the induction and expression stages of LTP.

1.4.4.3 NMDA receptor-dependent LTP expression

The initiation of LTP expression is mediated by CaMKII activation (Herring and Nicoll, 2016). Generally, when a sufficiently large influx of Ca^{2+} occurs following NMDAR-mediated postsynaptic membrane depolarization, there is a subsequent, strong Ca^{2+}/calmodulin-mediated activation of CaMKII; however, simultaneous, transient Ca^{2+}/calmodulin-induced activation of calcineurin also occurs. Activated calcineurin subsequently dephosphorylates postsynaptic membrane AMPA receptors, leading to a reduced influx of cation. As Ca^{2+} is removed from the cytoplasm and Ca^{2+}/calmodulin diffuses off of calcineurin and CaMKII, calcineurin-mediated dephosphorylation of AMPA receptors is terminated whereas CaMKII undergoes autophosphorylation. Persistently activated CaMKII then phosphorylates other intracellular proteins to sustain the LTP expression phase. Interestingly, low-level Ca^{2+} influx results in transient activation of both calcineurin and CaMKII, resulting in significantly greater calcineurin activity than CaMKII, resulting in LTD expression. The molecular mechanism of CaMKII-mediated autophosphorylation and subsequent protein phosphorylation in LTP expression is as follows. CaMKII is a pinwheel-shaped protein that is composed of 12 subunits, each of which contains an autoinhibitory subdomain and a catalytic subdomain. The autoinhibitory subdomain consists of a Ca^{2+}/calmodulin site as well as an autophosphorylation site whereas the catalytic subdomain contains a T-site and an S-site. The Ca^{2+}/calmodulin binding site regulates the catalytic subdomain, and when no Ca^{2+}/calmodulin is interacting with the binding site, the autoinhibitory subdomain remains closed. The T-site of the catalytic subdomain keeps the subunit closed by binding to the autoinhibitory subdomain whereas the S-site binds to and phosphorylates substrates whenever the subunit opens. Upon binding of Ca^{2+}/calmodulin to its respective binding site, the autoinhibitory subdomain and autophosphorylation site open, allowing for autophosphorylation of Thr286 (Giese et al., 1998) and Thr287 (Miller et al., 1988) on the α and β subunits, respectively. Several S-sites subsequently phosphorylate the autophosphorylation site on neighboring subunits, allowing for successive protein

phosphorylation. Notably, the phosphorylation status of Thr305 and Thr306 facilitates the expression of either LTP or LTD (Pi et al., 2010). Following activation, CaMKII dissociates from F-actin, and microtubule-associated mechanisms result in the translocation of the kinase to the postsynaptic density (PSD; Shen and Meyer, 1999; Lemieux et al., 2012; Lisman et al., 2012). CaMKII mediates numerous activities at the PSD including complex formation with NMDA receptors (Gardoni et al., 1998; Sanhueza et al., 2011; Lisman et al., 2012) and phosphorylation of AMPA receptors (Barria et al., 1997; Mammen et al., 1997). CaMKII binds to the GluN2A and GluN2B NMDA receptor subunits (Bayer et al., 2001; Barria and Malinow, 2005), resulting in the docking of the kinase Thr286 residue into the catalytic subdomain of adjacent subunits and preventing CaMKII dephosphorylation (Bayer et al., 2001; Lisman and Raghavachari, 2015). Notably, CaMKII positions itself within the Ca^{2+} microdomains of the NMDA receptor such that kinase activity is further enhanced as well as strategically located proximal to synaptic AMPA receptors (Merrill et al., 2005). CaMKII subsequently phosphorylates the Ser831 residue at the C-terminal tail of the AMPA receptor GluR1 subunit (Barria et al., 1997; Mammen et al., 1997). This phosphorylation results in a variety of changes in physiological properties of the AMPA receptor including elevated opening frequency and unitary conductance (Benke et al., 1998; Kristensen et al., 2011).

Beyond CaMKII, short-term elevations in postsynaptic Ca^{2+} facilitate changes in synaptic plasticity via modulation of AMPA receptors in the synaptic field. For instance, GluR1-containing AMPA receptors are the principal AMPA receptor subtype that undergo fast upregulation during LTP induction before being slowly exchanged for GluR2/3-containing AMPA receptors, where the latter play a larger role as LTP progresses from induction to maintenance via enabling the recycling of synaptic membrane-bound AMPA receptors (Passafaro et al., 2001; Shi et al., 2001). Alterations in the quantity and composition of membrane-bound AMPA receptors are accomplished through lateral diffusion of extrasynaptic AMPA receptors and the insertion of intracellular AMPA receptors into the extrasynaptic membrane (Makino and Malinow, 2009),

resulting in upregulation of AMPA receptor density in the PSD. For instance, a report demonstrated that NMDA receptor-dependent activity induces fast and slow lateral diffusion as well as the incorporation of extrasynaptic AMPA receptors to the synaptic field whereas AMPA receptors already positioned within the synaptic field are immobile (Shi et al., 1999; Lu et al., 2001; Pickard et al., 2001; Borgdorff and Choquet, 2002; Tardin et al., 2003). Several different proteins such as stargazin, protein kinase C (PKC), and CaMKII promote the incorporation of these extrasynaptic AMPA receptors into the synaptic field. Specifically, GluR1-associated stargazin is a transmembrane AMPA receptor-associated protein that is the primary regulator of AMPA receptor membrane trafficking (Chen et al., 2000; Schnell et al., 2002; Tomita et al., 2005a; Sumioka et al., 2010). CaMKII and other kinases like PKC phosphorylate the PDZ binding domain of stargazin (Tomita et al., 2005b), leading to a stargazin-postsynaptic density 95 (PSD-95) interaction that facilitates the incorporation, stabilization, and anchoring of AMPA receptors into the synaptic field (Chen et al., 2000; Chetkovich et al., 2002; Schnell et al., 2002; Bats et al., 2007). PKC-mediated phosphorylation of GluR1 Ser818 and Thr840 residues (Boehm et al., 2006; Lee et al., 2007) also enables incorporation of AMPA receptors into the PSD while subsequent phosphorylation of the AMPA receptor Ser831 residue via PKC and CaMKII shape AMPA receptor conduction properties (Kristensen et al., 2011; Jenkins and Traynelis, 2012). Exocytosis of endosomes filled with AMPA receptors is another effect of transient enhancements in postsynaptic Ca^{2+}, resulting in the insertion of intracellular AMPA receptors into the synaptic field (Makino and Malinow, 2009). Specifically, GluR1 phosphorylation via protein kinase A (PKA) (Roche et al., 1996; Esteban et al., 2003; Diering et al., 2014) and CaMKII-mediated phosphorylation of a protein involved in anchoring AMPA receptors to the actin cytoskeleton, synaptic associated protein 97 (SAP97) (Leonard et al., 1998; Mauceri et al., 2004; Nikandrova et al., 2010), promote AMPA receptor containing endosome trafficking to the extrasynaptic membrane. Actin motor protein myosin VI subsequently interacts with SAP97 to facilitate docking of the trafficking AMPA receptors with

the extrasynaptic membrane, culminating in SNARE-mediated fusion at the perisynaptic exocytic domains (Lu et al., 2001; Wu et al., 2002).

1.4.4.4 NMDA receptor-dependent LTP maintenance

The maintenance of LTP occurs during the intermediate LTP (two-three h post-tetanus) and L-LTP (greater than three h post-tetanus) phases and is dependent upon postsynaptic protein synthesis that yields persistent synaptic structural changes (Herring and Nicoll, 2016). This is evidenced by reports demonstrating that the application of protein synthesis inhibitors blocked LTP one h-post tetanus (Frey et al., 1989; Vickers et al., 2005). Additionally, mutation of kinases involved in LTP maintenance-related immediate early gene expression (Bourtchuladze et al., 1994) as well as the blockade of immediate early gene mRNA (Guzowski et al., 2000) yields impaired long-term, but not short-term, hippocampal-dependent learning and memory. A fundamental protein that is involved in the persistence of LTP maintenance is CREB, a transcription factor activated via a litany of converging Ca^{2+}-dependent pathways that results in alterations in the density of synapses (Lee and Silva, 2009). One of the most well-established mechanisms that ultimately lead to CREB activation is initiated via NMDA receptor-mediated assembly of Ca^{2+}/calmodulin. Specifically, Ca^{2+}/calmodulin activates adenylate cyclase to elevate the production of cAMP (Chetkovich and Sweatt, 1993), resulting in stimulation of kinases like PKA (Nguyen and Kandel, 1997). PKA activation then promotes ERK/MAPK-induced phosphorylation of CREB at Ser133, removing steric hindrances that prevent CREB-related activities while initiating the recruitment of CREB-binding protein (Ofir et al., 1991). Ca^{2+}/calmodulin also influences the stimulation of CREB via interactions with other kinases like CaMKII and CaMKIV, where the former may positively or negatively regulate CREB-dependent transcription (Sun et al., 1994). Once phosphorylated, CREB then binds to the cAMP response element, beginning the production of immediate early genes that play a substantial role in LTP maintenance such as *BDNF*, *Arc*, and c-*fos* as well as other transcription factors that facilitate the transcription of synaptic plasticity-related late response genes (Lee and Silva, 2009).

Transcription of these genes generally occurs within the soma for axonal-somatic synapses or locally at the dendritic spine via shuttling of mRNA-containing granules (Hirokawa, 2006; Shepherd and Huganir, 2007). Transcribed mRNA is then translated to protein, allowing for persistent structural changes of hippocampal synapses such as dendritic spine density enhancement as well as synaptogenesis (Herring and Nicoll, 2016).

Activity located within the dendrites also contributes to components of LTP maintenance such as structural alterations of synapses as well as prolonged modulation of AMPA and NMDA receptor density. Persistently activated kinases (Herring and Nicoll, 2016), previously transcribed mRNA (Banko et al., 2004; Kelleher et al., 2004; Klann and Dever, 2004; Sutton and Schuman, 2005), and organelles (Hirokawa and Takemura, 2005; Shepherd and Huganir, 2007; Cui-Wang et al., 2012) required for the production of synaptic plasticity-related proteins are localized to dendritic compartments, contributing to soma-independent structural and receptor-related modulations occurring throughout LTP maintenance. For instance, PKC and PKA stimulation enable dendritic ERK activity, resulting in phosphorylation of downstream kinases like MAP kinase-interacting serine/threonine-protein kinase 1 (Mnk1; Banko et al., 2004). Studies suggest that Mnk1 facilitates the translation of previously transcribed mRNA that is localized within dendrites such as F-actin, PSD-95, CaMKII, PKC, GluR1, and GluR2, allowing for rapid remodeling of dendritic structural features as well as synthesis and phosphorylation of glutamatergic receptors (Toni et al., 1999; Yuste and Bonhoeffer, 2001; Ju et al., 2004; Shepherd and Huganir, 2007). Additionally, delayed trafficking of NMDA receptors occurs during LTP maintenance (Hirokawa and Takemura, 2005; Lau and Zukin, 2007). Immediately after LTP induction and during E-LTP, substantial upregulation of PSD-associated AMPA receptors ensues, which is reflected by a notable potentiation of the AMPA/NMDA ratio (Watt et al., 2004). At least two h post-tetanus, primarily extrasynaptic GluN2B-containing NMDA receptors that were introduced to the synaptic field in the earlier stages of LTP are replaced by more stable but less conductive GluN2A-containing NMDA receptors, reshifting the AMPA/NMDA receptor ratio

(Barria and Malinow, 2002; Groc et al., 2006). Research suggests that the lengthy GluN2 cytoplasmic tail that contains multiple regulatory binding sites results in this particular subunit being primarily responsible for the trafficking of NMDA receptors during LTP maintenance (Chung et al., 2004). Further, crosstalk between kinases such as CaMKII, PKC, and MAPK (Lan et al., 2001; Kim et al., 2005; Yan et al., 2011), as well as metabotropic receptors like mGluR1/5 (Lan et al., 2001b; Matta et al., 2011), regulate the shuttling of NMDA receptors during this terminal phase of LTP.

Collectively, these studies illuminate some of the intricate mechanisms involved in hippocampal synaptic plasticity (Fig. 1.5). An array of molecular mechanisms underlie modulations in heterogeneous forms of short-term and long-term hippocampal synaptic plasticity, leading to highly adaptive and elegant neuronal activity following unique experiences. Transient and persisting cellular responses to novel experiences influence crosstalk between hippocampal and neocortical structures, resulting in the ability to store and shape memory traces for a lifetime.

1.5 Hippocampal-dependent Learning and Memory

1.5.1 Overview

The profound capacity to learn and remember is arguably one of the fundamental characteristics that distinguish humans from other mammals. Memory may be generally defined as the ability to recall previous experiences to shape responses to current and future tasks. Classifying different forms of learning and memory as neural substrates has been historically challenging. The challenge in the categorization of learning and memory is related to the rare situation of a particular form of learning being related to a straightforward, one-to-one relationship with a specific task. That is, upstream sensory input shapes downstream motor processing during a learning and memory task, suggesting that altered responses as a result of a particular behavioral experience may be related to impairments in hippocampal-related function or in tissues that are responsible for sensorimotor regulation. Over time, a consensus

has formed around definitive types of learning and memory with the broadest classifications being short-term memory, working memory, and long-term memory (Andersen et al., 2007). Distinct phases of learning and memory known as encoding, consolidation, and retrieval were also revealed after decades of investigation (McGaugh, 2000). The discovery of unique forms and phases of learning and memory was facilitated by the establishment of highly sensitive and robust behavioral tasks such as the Morris water maze and radial arm maze. The following subsection will provide a brief overview of forms and phases of hippocampal-dependent learning and memory. Common tasks utilized to investigate hippocampal-dependent learning and memory will also be surveyed.

1.5.2 Short-term memory and working memory

Short-term memory is hippocampal-dependent and unstable, rapidly decaying a few min following the learning and memory experience (Fig 1.6; Cowan, 2008; Aben et al., 2012). Short-term memory may be defined as the temporary capacity to store limited, but highly accessible, quantities of information. Differential auditory or visual cortices also regulate short-term memory activity depending upon the particular experiential circumstance (D'Esposito and Postle, 2015). Working memory is a distinct form of memory that is commonly, but erroneously, used interchangeably with short-term memory (Aben et al., 2012). A common definition of working memory is the preservation and regulated manipulation of a defined quantity of information before recall (Baddeley, 1992). Some researchers suggest that working memory has supplanted the older term of short-term memory (Gray, 2007) whereas other scholars recognize that working memory is a theory of short-term memory (Nairne and Neath, 2012), or that short-term memory and working memory coexist (Gathercole and Alloway, 2006; Nadel and Hardt, 2011). Working memory was initially defined as memory that is used to plan and carry out behavior such as retaining ideas in an extended oral debate (Miller et al., 1960). The utilization of the phrase “working memory” was enhanced following the seminal model of Baddeley and Hitch (1974), a model that was ultimately developed to suggest a central executive manages multiple

modules to complete complex, working memory-related tasks (Baddeley, 2012). Four distinct components consist of the classic working memory model: a central executive of attention-related processes, two domain-specific submissive systems that contain information known as the phonological loop and visuospatial sketch pad, and an episodic buffer. The central executive plays a substantial role in task-specific localization by coordinating the response to sensory stimuli as well as the retrieval of long-term memories throughout a cognitive task. The phonological loop manages auditory input whereas the visuospatial sketch pad responds to visual information. Short-term memory data is subsequently processed or reprocessed by the episodic buffer, transferring the information to the central executive for further management. Accordingly, working memory is currently recognized as a type of memory that is dependent upon coordination between multiple components. Contemporary research suggests the central executive is not a sole entity but stems from crosstalk between a myriad of structures found in the frontal lobe such as the medial (Euston et al., 2012), ventrolateral (Wolf et al., 2006), and dorsolateral (Barbey et al., 2013) prefrontal cortices. Communication between the medial temporal lobe, prefrontal cortex, and parietal cortex also contributes to central executive function throughout working memory tasks (Stern et al., 2001; Collette and Van der Linden, 2002; Axmacher et al., 2007). Research suggests the phonological loop is located at the boundary of the inferior parietal lobe and superior posterior temporal lobe (Salmon et al., 1996; Prabhakaran et al., 2000; Papagno et al., 2017) whereas an increasing working memory load modulates the region of the brain that plays the role of the visuospatial sketchpad (Salmon et al., 1996; Ragland et al., 2002). For instance, working memory tasks with low demand result in activity indicative of the visuospatial sketchpad within the occipital lobe and distinct prefrontal cortex activation whereas working memory tasks with high demand lead to elevated activity in the parietal lobe and different prefrontal cortex regions. Responses indicative of episodic buffer-like activity were observed bilaterally in the frontal and temporal lobes, particularly in the left hippocampus (Rudner et al., 2007).

1.5.3 Long-term memory

Working memory sits at the intersection between short-term memory and long-term memory, where the latter is a stable form of memory that may persist for a few min to a lifetime (Fig. 1.6). Long-term memory may be divided into two broad categories: declarative memory and nondeclarative memory (Andersen et al., 2007). Declarative memory, also known as explicit or conscious memory, is dependent upon the medial temporal lobe and includes semantic memory and episodic memory. Semantic memory relates to the recollection of facts and concepts whereas episodic memory is associated with recalling information linked to experiences and particular events. Nondeclarative memory is also referred to as implicit or subconscious memory and is primarily hippocampal-independent. Forms of nondeclarative memory include procedural memory, priming, simple conditioning, and non-associative learning. Procedural memory is dependent upon basal ganglia or putamen function and is related to learning skills and habits. Priming, a neocortical-dependent form of nondeclarative memory, suggests that previous exposure to a particular stimulus shapes a future response to the same stimulus. Simple conditioning relates to emotional responses or musculoskeletal coordination where the former is dependent upon amygdalar activity whereas the latter relies on cerebellar function. Non-associative learning is a form of learning that does not pair a stimulus and behavior, resulting in this particular form of implicit memory depending on circuits such as the reflex pathway. For example, habituation and sensitization are two forms of non-associative learning. While short-term memory, working memory, and long-term memory depend upon and function via distinct circuitry, each form of memory shapes one another. For instance, reports suggest that a low demand working memory task may require the activity of short-term memory systems such as the prefrontal and parietal cortices (Squire and Wixted, 2011) whereas high demand working memory tasks may increase prefrontal cortex activity while also recruiting circuitry related to long-term memory (Lewis-Peacock and Postle, 2008; Jeneson and Squire, 2012). Interestingly, the episodic buffer may function as a point of cross-talk between short-term

and long-term memory systems with working memory. Additionally, the conversion of short-term memory, working memory, and long-term memory involve complex communication between the medial temporal lobe and prefrontal cortex (Stern et al., 2001; Yoon et al., 2008; Schon et al., 2009; Preston and Eichenbaum, 2013). For instance, elevated working memory load leads to enhanced medial temporal lobe and prefrontal cortex activity, suggesting each region plays a unique role in the transition of one form of memory to another (Yoon et al., 2008).

1.5.3 Overview of the stages of memory formation

The generation of short-term, working, and long-term memories in the context of a neural timeline includes three fundamental stages: encoding, consolidation, and retrieval (McGaugh, 2000). Current evidence suggests the hippocampus plays a role in each of these stages in declarative memory (Tulving, 1983; Squire, 1992), serving as a transient relay station for long-term storage in the prefrontal cortex.

1.5.3.1 Memory encoding

The processing of somatosensory stimuli into a distinct memory trace within a neural network is known as encoding or acquisition (McGaugh, 2000). An abundance of evidence suggests the hippocampus performs an imperative function in encoding contextual-based memories, particularly the dH CA3 subfield (Rolls et al., 1998; Nakazawa et al., 2003; Lee and Kesner, 2004; Daumas et al., 2005). Specifically, Daumas et al. (2005) observed that the dH CA3 subfield was required for the encoding phase of contextual fear conditioning, which is in line with a study by Nakazawa et al. (2003) that indicated dH CA3 NMDA receptors were required for the acquisition of contextual-based learning and memory. The dH CA1 subfield was also reported to play a role in the acquisition of contextual-based learning and memory tasks (Daumas et al., 2005). Other studies demonstrated the CA1 subfield interprets temporal components whereas the CA3 subfield deciphers spatial elements during associative learning tasks (Lee et al., 2005; Hunsaker et al., 2006). An increased delay between trials in a sequential nonspatial learning and memory task also distinctly recruited dH CA1 circuitry, suggesting that

this hippocampal subfield is progressively imperative as temporal demand increases (Farovik et al., 2010). Overall, these studies and others indicate substantial crosstalk between the CA1 and CA3 subfields facilitates the encoding of contextual-based experiences (Daumas et al., 2005; Lee et al., 2005; Hunsaker et al., 2006; Hunsaker et al., 2008; Farovik et al., 2010; Bahar et al., 2011).

1.5.3.2 Memory consolidation

Consolidation may be defined as the transformation of a short-term memory trace to a long-term memory trace (Dudai, 2012). A long-term, enduring memory trace is known as an engram. Consolidation generally occurs at the synaptic-level and the brain system-level, where the former is related to stimuli-mediated activation of signaling cascades that lead to gene synthesis a few h after the novel experience whereas the latter denotes the days to years-long circuit-level process of post-encoding long-term memory restructuring. Notably, intensity of a learning and memory experience impacts synaptic alterations such as synaptic efficacy and membrane excitability, consequently modulating the effectiveness of memory consolidation at the system-level. Therefore, salient learning and memory traces may be consolidated more effectively than those with less trace strength, facilitating recollection of the former more readily than the latter. Memory consolidation is recognized through evaluating the temporal-dependent impact of a lesion or an amnesic agent on the capacity to learn and remember a task (Shema et al., 2007). Many classic experiments using lesions or amnesic agents revealed the crucial, time-dependent role the hippocampus plays in memory consolidation. For instance, Zola-Morgan and Squire (1990) observed that monkeys with hippocampal and parahippocampal lesions exhibited comparable performance to nonlesioned monkeys in an object discrimination task learned 12-16 weeks prior to surgery but displayed impaired performance in a new object discrimination task that was first experienced shortly before surgery. Nonprimate investigations utilizing a retrograde amnesia approach also discovered a temporal-dependent role of the hippocampus in memory consolidation (Winocur, 1990; Kim and Fanselow, 1992; Bolhuis et al., 1994;

Anagnostaras et al., 1999; Squire et al., 2001). Further, hippocampal subfields serve a unique function in the synaptic-level and system-level consolidation process. Specifically, studies found that infusion of a CaMKII inhibitor into the dH CA1 subfield prevented LTP induction and memory consolidation immediately after training in hippocampal-dependent task, indicating that the dH is the central hub for initial phases in memory consolidation at the synaptic level (Barros et al., 1999; Malenka and Nicoll, 1999). Further, Daumas et al. (2005) demonstrated the dH CA1 subfield is essential for temporal-dependent contextualization during memory consolidation whereas other researchers observed the dH CA3 subfield facilitates the consolidation of memory from short-term to long-term storage (Stupien et al., 2003; Florian and Roullet, 2004; Gilbert and Kesner, 2006; Gilbert and Brushfield, 2009). Collectively, these experiments have led to the hypothesis that the hippocampal formation is a temporary site for labile memories, undergoing processing prior to stable, long-term storage in the neocortex. This proposition is supported by the robustness of particularly long-term memories even after bilateral medial temporal lobe lobectomy (Scoville and Milner, 1957). Notably, studies described unidirectional, monosynaptic projections from the CA1 subfield as well as the subiculum to the prefrontal cortex (Jay and Witter, 1991; Jay et al., 1992; Parent et al., 2010). During a spatial working memory task, a pathway from the hippocampus to the medial prefrontal cortex displayed activity indicative of coordinated phase-lock theta rhythms (Jones and Wilson, 2005), activity that was progressively enhanced as working memory demand increased (Tamura et al., 2017). These data are in line with an interpretation that suggests long-term memory storage occurs in the prefrontal cortex and crosstalk between this structure and the hippocampus facilitates long-term memory processes such as retrieval.

1.5.3.3 Memory retrieval

The recollection of an engram is defined as retrieval. Evidence indicates that retrieval of particular forms of memory is not dependent upon hippocampal function alone. For instance, animals with dH CA1 and CA3 subfield lesions exhibited no impairments in contextual fear

conditioning retrieval when a recognition memory procedure was employed 24 h after learning (Daumas et al., 2005). A study by Fortin et al. (2002) observed that complete hippocampal lesions impaired the ability to learn sequential events but had no effect on the ability to recognize items occurring in a distinct series. On the other hand, Kim and Fanselow (1992) detected substantial contextual memory deficits when hippocampal lesions were conducted one day after conditioning while Nakazawa et al. (2002) demonstrated that the dH CA3 subfield enabled recollection of pattern completion. The dH CA3 subfield was also shown to be crucial in the retrieval precision of long-term memories (Chadwick et al., 2014). These reports illustrate a complex role of the hippocampus in memory retrieval, an illustration that is supported by highly-specified activities of neocortical structures in memory retrieval (Squire and Wixted, 2011). For instance, studies revealed that particular neocortical structures were activated during encoding for experiences related to distinct behavioral domains, resulting in each particular neocortical structure storing a unique characteristic of the learning and memory experience (Wheeler et al., 2000; Polyn et al., 2005; Woodruff et al., 2005). Accordingly, lesioning of each particular neocortical structure resulted in impaired recollection of the particular feature of the experience that corresponded to the damaged structure (Squire and Wixted, 2011). Overall, these investigations suggest that while the hippocampus may be an essential structure in memory encoding and consolidation, engrams are primarily stored in the neocortex, which the hippocampus and extrahippocampal structures shape and modulate during memory retrieval. Further, patient H.M. and other clinical studies indicate that extrahippocampal projections provide alternative routes for memory recollection upon removal or severe damage of the hippocampus.

1.5.4 Preclinical paradigms used in investigating learning and memory

Numerous behavioral tasks were developed throughout decades of researching different forms and phases of learning and memory in rodents; however, the present review will highlight two particular paradigms: the Morris water maze (MWM) and radial arm maze (RAM; Fig. 1.7).

1.5.4.1 The Morris water maze

The MWM is a highly robust and reproducible hippocampal-dependent spatial learning and memory task that commonly assesses reference memory (Morris, 1984). Reference memory is a form of long-term memory that employs previously learned information to complete a task. In the case of the MWM, the animal learns and remembers a set of extramaze visual cues to repeatedly navigate the maze (Fig. 1.7.1). Specifically, the animal utilizes the extramaze visual cues to orient themselves and subsequently find a hidden escape platform submerged beneath the surface of an opaque water pool. The animals are particularly motivated to find the escape platform to escape any adverse consequences such as excessive energy expenditure or death. Several common paradigms employed in the MWM include assessment of animal escape latency, performance on a probe trial, and efficiency in reversal training. For instance, escape latency is a measurement of reference memory and is determined by evaluating the time required for animals to find the hidden platform in the pool over a series of training days (Fig. 1.7.1A). After sequential days of the hidden platform being located at the same position in the pool, the platform is removed in a probe trial, and performance is assessed via the amount of time spent in the quadrant where the hidden platform was previously found (Fig. 1.7.1B). The researcher may then measure reversal training by moving the hidden platform to a different location in the pool and determining the time required for animals to relearn the task (Fig. 1.7.1C). Common response variables for the MWM include escape latency, time in the target quadrant, path length to the target quadrant, and platform crossings; however, these responses may be impacted by reduced swim velocity or thigmotaxis (Wolfer et al., 1998), particularly in older animals where a comparable reference memory task that is less stressful like the Barnes maze may be more effective (Barnes, 1979).

1.5.4.2 The radial arm maze

While the MWM is an effective paradigm for assessing hippocampal-dependent spatial reference memory, the RAM developed by Olton and Samuelson (1976) is a more versatile task

for evaluating hippocampal-related learning and memory deficits (Fig. 1.7.2). Similar to the MWM, animals utilize extramaze cues to learn tasks in the RAM, permitting the detection of impairments in different forms of spatial working memory and spatial reference memory. The general protocol of this task begins with an animal being placed in a circular central hub surrounded by inaccessible arms protruding from the hub. After a short habituation period, doors to each arm open and allow the animal to access and explore the maze. At the end of each arm is a trough where the animal may receive a food reward. The animals commonly undergo food restriction and are therefore highly motivated to complete the task in a manner that maximizes food reward yet minimizes energy expenditure. Over a series of days, the animal will use extramaze cues to learn and remember the behavioral tests required of them to obtain their reward. Common eight arm RAM paradigms include RAM foraging tasks and Win-shift tasks. The RAM foraging task may be designed as a short-term spatial working memory task or as a reference memory task. In the case of the former, an animal is required to visit all RAM arms (i.e., eight of eight arms) one time and a revisit is defined as an error in the short-term spatial working memory foraging task (Fig. 1.7.2A). Impaired performance in this short-term spatial working memory foraging task was observed in pretrained animals that received dH infusion of D-APV, suggesting that acquisition of this task is hippocampal-dependent (Kawabe et al., 1998; Yoshihara and Ichitani, 2004). Interestingly, Potvin et al. (2006) demonstrated that dH lesions, but not vH lesions, resulted in impaired performance in this eight-arm RAM foraging task, suggesting that the RAM may be used to disassociate the role of hippocampal sectors in spatial learning and memory tasks as well as to detect sector-specific impairments. A reference memory version of the foraging task may require an animal to visit the same subset of RAM arms to obtain food reward every trial (i.e., four of eight arms are baited with food reward), and a visit to an unbaited arm is defined as a reference memory error whereas a revisit to a baited or unbaited arm may be defined as an error in short-term working memory (Fig. 1.7.2B). Yamada et al. (2015) demonstrated that acquisition and consolidation of experiences from this task are

hippocampal-dependent as infusion of D-APV into the dH of animals pretrained in this task resulted in impaired subsequent performance compared to animals that did not receive D-APV. The Win-shift task is a test that further highlights the versatility and precision of the RAM paradigm in detecting hippocampal-dependent learning and memory deficits (Fig. 1.7.2C). Specifically, this task consists of two phases where the first study phase allows animal access to only four of eight arms, requiring the animal to visit each arm one time to acquire a food reward. A revisit to an arm in this task is defined as an error in short-term spatial working memory. After completing the study phase in this Win-shift task, the animal is removed from the maze for an interposed delay. Following the interposed delay, the animal completes a test phase where it must visit the four arms that were inaccessible in the study phase, and a visit to an arm from the study phase or a revisit is defined as a long-term spatial working memory error. This Win-shift task necessitates the use of a different search strategy in phase two and requires a greater working memory demand than the foraging task, revealing subtle or profound hippocampal-dependent learning and memory deficits. Intracerebroventricular or dH infusion of D-APV prior to phase one or phase two of the Win-shift task resulted in impaired short-term and long-term spatial working memory, suggesting that acquisition and consolidation of experiences from this task are hippocampal-dependent (Bolhuis and Reid, 1992; Yoshihara and Ichitani, 2004). Interestingly, Floresco et al. (1997) demonstrated that lidocaine injections into the ventral subiculum impaired the performance of animals in the Win-shift task, suggesting that the vH is essential in this particular RAM task. These data are in line with an interpretation suggesting that the increased working memory demand of the Win-shift task requires memory consolidation achieved by a monosynaptic vH-medial prefrontal cortex projection. Indeed, a different study utilizing a hippocampal-dependent task that contained an interposed delay revealed hippocampal-medial prefrontal cortex theta-gamma coupling that was elevated as the duration of the interposed delay was increased (Tamura et al., 2017). O'Neill et al. (2013) also observed medial prefrontal cortex theta oscillations were synchronized with vH, but not dH, activity during

a spatial working memory task that contained an interposed delay, further supporting the necessity of the monosynaptic projection of the vH to the medial prefrontal cortex in tasks like the RAM Win-shift.

Collectively, these studies underscore the elegant crosstalk between hippocampal and neocortical structures in different types and phases of short-term, working, and long-term learning and memory tasks. Additionally, decades of research have led to the development of highly flexible, versatile, and sensitive behavioral paradigms to identify impairments in learning and memory. Furthermore, particular tasks like the RAM exhibit the unique capacity to disassociate the complex role of the dH and the vH in different types and phases of learning and memory. Sensitive tasks such as the RAM may be particularly useful to detect subtle deficits in hippocampal-dependent learning and memory in disease states that present in a heterogenous manner like Gulf War Illness.

1.6 Gulf War Illness Introduction

In response to the Iraqi invasion of Kuwait in August 1990, the United States (U.S.) and a coalition of 38 countries initiated the buildup of military forces in the Persian Gulf, an undertaking known as Operation Desert Shield. Following the buildup of forces, the combat phase of the conflict, Operation Desert Storm, began in January 1990 and ended before March 1991, although some U.S. personnel remained in the Persian Gulf until June. While aerial assaults only lasted for six weeks, ground warfare persisted for four days, and coalition casualties were remarkably low, within six months of the conclusion of Operation Desert Storm a consistent constellation of symptoms known as chronic multisymptom illness began to present in many coalition troops that returned home (Gavaghan, 1994; Robinson, 1995; Pennisi, 1996). This constellation of symptoms is presently known as Gulf War Illness (GWI) and is a persisting condition affecting approximately a third of the 700,000 U.S. military forces deployed in the 1990-1991 Gulf War (RACGWI, 2008; RACGWI, 2014; White et al., 2016). While many symptoms present in GWI, the cardinal symptoms of the disease include cognitive deficits,

fatigue, and chronic musculoskeletal pain (Haley et al., 1997; Fukuda et al., 1998; Steele, 2000). Notably, GWI symptom onset is latent (Li et al., 2011; Fappiano and Baraniuk, 2020) and symptom severity becomes progressively worse over time (Zundel et al., 2019; Porter et al., 2020; Zundel et al., 2020). Although the precise etiology of GWI is unknown, overexposures to neurotoxic pesticides, nerve agent prophylactics, organophosphate (OP) nerve agents, and prolonged wartime stress have been associated with the induction and persistence of GWI (Haley and Kurt, 1997; Cherry et al., 2001; Wolfe et al., 2002; Winkenwerder, 2003; Steele et al., 2012). Gulf War veterans (GWV) experienced substantial chemical exposure. Accordingly, GWI symptom severity is correlated to the degree, duration, and type of GWI-relevant (GWIR) chemical exposures (Wolfe et al., 2002; Steele et al., 2012; Maule et al., 2018). Further, an increasing number of studies report that GWI presents in a sex- (Coughlin, 2016; Coughlin et al., 2017; Brown et al., 2019; Heboyan et al., 2019; Porter et al., 2020; Sullivan et al., 2020) and race-specific (Steele, 2000; Steele et al., 2012; Coughlin and Heboyan, 2018; Porter et al., 2020; Sullivan et al., 2020) manner. There is currently no single case definition of GWI due to the heterogeneity of GWI subpopulations, although particular criteria are preferably used to define GWI in clinical (i.e., Centers for Disease Control criteria) and preclinical (i.e., Kansas criteria) settings. No objective biomarker is presently accepted and employed in clinical GWI diagnosis. Additionally, current treatments for GWI are targeted and symptomatic, underscoring the demand for investigations into safe and efficacious treatments for this progressive condition.

The present review surveys preclinical, GWIR genetic, molecular, synaptic, structural, and behavioral abnormalities related to nervous system function and clinical correlates (Fig. 1.8). A particular emphasis is placed upon hippocampal function and hippocampal-dependent learning and memory within the context of GWI. Preclinical drug candidates for the treatment of GWI neuropathology will also be described. The majority of reports that will be discussed were published after 2010.

1.7 Gulf War Illness in a Preclinical Setting

Recapitulating the dose, duration, and frequency of exposure to GWI causal factors presents a significant challenge in developing a GWI animal model. Therefore, a variety of GWI models have been developed in the mouse (Abdullah et al., 2011; O'Callaghan et al., 2015; Zakirova et al., 2015; Carreras et al., 2018) and the rat (Abdel-Rahman et al., 2002; Parihar et al., 2013; Macht et al., 2018). GWIR chemicals commonly included in these models are pyridostigmine bromide (PB), permethrin (PM), N, N-Diethyl-meta-toluamide (DEET), and a nerve agent surrogate, diisopropylfluorophosphate (DFP; Abdel-Rahman et al., 2002; O'Callaghan et al., 2015; Zakirova et al., 2015). Extensive characterization of genetic, epigenetic, and molecular alterations in the nervous system of these GWI animal models has been conducted. Emerging evidence suggests synaptic and network irregularities in GWI animal models. Further, cellular and structural abnormalities are presented in GWI animal models. Aberrant behavior exhibited in GWI animal models is also consistent with neuropsychological dysfunction described in a clinical setting. The present section will review some of the genetic, epigenetic, molecular, synaptic, cellular, structural, and behavioral anomalies presented in GWI animal models.

1.7.1 Genetic, epigenetic, and molecular alterations

Preclinical GWI investigations have observed wide-ranging alterations in genetic, epigenetic, and molecular signaling in the nervous system. Studies have evaluated genetic and epigenetic abnormalities presented in GWI animal models. Characterization of GWI-related alterations in monoaminergic and cholinergic activity has been described. Neurotransmitter-independent effects of GWIR chemical and stress on molecular activity in the nervous system have also been explored. While many GWI animal models have been developed, we will summarize the genetic and molecular aberrations reported in the central and autonomic nervous systems from five consistently used GWI animal models here.

The acute and persisting impact of genetic and epigenetic alterations have been characterized in GWI animal models (Pierce et al., 2016; Ashbrook et al., 2018). Short-term genetic and epigenetic changes were documented in an animal model by Ashbrook et al. (2018). In this study, the authors observed modulated expression of genes related to oligodendrocyte functionality in the frontal cortex as well as distinct activity of immunological and synaptic-related genes in the frontal cortex and hippocampus. Additionally, histone modification and DNA methylation aberrations in genes related to neuroinflammation were demonstrated. In particular, reduced gene activity and cellular proportions of frontal cortex myelinating oligodendrocytes were found, a result that is in line with a recent preclinical study demonstrating substantial oligodendrocyte pathology in GWI (Belgrad et al., 2019) as well as clinical reports describing impaired white matter myelination in GWI veterans (Heaton et al., 2007; Rayhan et al., 2013; Chao et al., 2015). Altered gene expression related to immune signaling, including a notable modulation of *Tlr2*, likely underlies modulated microglial and astrocyte activity, glial activity that was consistently reported across other GWI animal models. Along with augmented expression of inflammatory genes, the authors also found elevated expression of oxidative stress-related genes, which may provide insight into sickness behavior described in GWI. Further, significant alterations in genes related to ACh were identified, suggesting impaired cholinergic neurotransmission. A recent preprint by a similar corticosterone-DFP exposure paradigm observed a distinct impact of GWIR chemical and stress exposure across 30 different mouse strains at an acute timepoint, suggesting that GWI differentially manifests based on genetic composition (Jones et al., 2020). The authors also demonstrated that GWIR chemical exposure impacted male mice genetic activity significantly more than in females, which is in line with clinical reports of GWI resulting in sex-specific effects (Sullivan et al., 2020). Another short-term study exposed 30 BXD mice strains as well as parental C57BL/6J and DBA/2J strains to corticosterone-DFP, detecting a litany of differentially expressed genes in the prefrontal cortex which were indicative of dysfunction immune and inflammatory signaling (Xu et al., 2020). For

instance, animals exposed to corticosterone and DFP exhibited significant enrichments in Kyoto Encyclopedia of Genes and Genomes (KEGG) terms such as *TNF signaling pathway*, *Th17 cell differentiation*, *inflammatory bowel disease*, and *nuclear factor-kappa B (NF-κB) signaling pathway* compared to animals exposed to DFP alone, responses which were consistent with previous preclinical and clinical GWI studies (Broderick et al., 2011; Seth et al., 2019; Vashishtha et al., 2020). As GWI presents in a mixed manner across genetically heterogeneous clinical populations, the use of a myriad of mouse strains in the aforementioned studies allowed for the assessment of preclinical GWI while considering genetic diversity, revealing substantial transcriptomic variations indicative of genetically distinct alterations in cytokine activity patterns, oligodendrocyte responses, and mitochondrial-related gene expression following corticosterone and DFP exposure. While these studies only evaluated genetic and epigenetic responses at a short-term timepoint (i.e., not translatable to the current age of GWV), the results were consistent with clinical investigations describing changes in peripheral NF-κB-related genes (Broderick et al., 2013), alterations in GWIR biomarkers (Abou-Donia et al., 2017; Abou-Donia et al., 2020; Abou-Donia et al., 2021), shifts in CRP expression (Johnson et al., 2016), aberrant mitochondrial-related function (Chen et al., 2017), and impaired white matter myelination (Heaton et al., 2007; Rayhan et al., 2013; Chao et al., 2015). Persisting genetic and epigenetic changes in a GWI animal model were described in Pierce et al. (2016). Abnormalities in DNA methylation were reported in GWIR chemical and stress-exposed animals where hippocampal 5-methylcytosine levels were upregulated whereas cortical and cerebellar 5-hydroxymethylcytosine expression was downregulated. Elevated hippocampal expression of two miRNA, miR-29b-3p and miR-124-3p, were found in GWIR chemical and stress-exposed animals. Human progranulin, a glycoprotein associated with frontotemporal dementia in patients with decreased levels of the protein, is a target of miR-29b (Jiao et al., 2010), suggesting that prolonged enhancement of miR-29b-3p levels may lead to impaired cognition. Collectively, these studies suggest genetic and epigenetic expression are acutely and persistently impacted

following GWIR exposures, a result that is consistent with clinical findings (Baraniuk and Shivapurkar, 2017; Trivedi et al., 2019; Latimer et al., 2020).

Emerging evidence suggests that GWIR exposures alter the short-term and long-term activity of neurotransmitters and proteins that facilitate neurotransmitter synthesis, release, and recycling (Terry Jr et al., 2011; Ojo et al., 2014; Zakirova et al., 2015; Locker et al., 2017; Carpenter et al., 2020). For instance, acute and persisting enhancement of brain ACh levels in GWI animal models were described (Ojo et al., 2014; Zakirova et al., 2015; Locker et al., 2017). Interestingly, synaptophysin, a protein indicative of functional vesicular release from the presynaptic terminal, was reduced in hippocampal pyramidal cells of C57BL/6J mice exposed to chlorpyrifos (CPF), PB, and PER days following exposure (Ojo et al., 2014). Hippocampal synaptophysin levels were also reduced 18 days and five months post-GWIR chemical exposure in a different GWI animal model, suggesting acute and persisting impairment of presynaptic functionality (Zakirova et al., 2015). Additionally, reduced α_7-nicotinic acetylcholine receptor density in the basal forebrain and decreased choline acetyltransferase activity in the basal forebrain were reported two weeks after repeated, low-level DFP exposure, indicating acute deficits in cholinergic transmission (Terry Jr et al., 2011). A report using a paradigm consisting of individual or combinatorial exposure to CPF, DFP, PB, and DEET identified aberrant signaling of the D1 receptor/cyclic AMP/protein kinase A pathway and dysregulation of Cdk5 activity, responses that are associated with neurodegeneration and dysfunctional neurotransmission (Torres-Altoro et al., 2011). Further, a recent analysis of acute monoaminergic alterations in two different GWI animal models suggests a broad impact of GWIR chemical and stress exposure on neurochemistry (Carpenter et al., 2020). This study observed increased serotonin usage and dopamine dyshomeostasis in an array of limbic structures such as the striatum, amygdala, vH, and dH, brain regions which are implicated in behavior that is persistently abnormal in GWI animal models and veterans (White et al., 2016). Collectively, these studies suggest that GWIR chemical and stress exposure induces acute and

persistent aberrations in neurotransmitter levels and expression of machinery associated with neurotransmitter functionality, effects which may underlie preclinical behavioral aberrations in GWI animal models.

GWIR chemicals have been shown to induce a variety of neurotransmitter-independent effects that are implicated in GWI pathophysiology such as adversely impacting structural proteins related to axoplasmic transport, hindering lipid homeostasis, modulating mitochondria functionality, impeding trophic factor production, and impairing Ca^{2+} homeostasis (Grigoryan et al., 2008; Grigoryan et al., 2009a; Grigoryan et al., 2009b; Jiang et al., 2010; Middlemore-Risher et al., 2010; Terry Jr et al., 2011; Torres-Altoro et al., 2011; Naughton et al., 2018; Phillips and Deshpande, 2018; Phillips et al., 2019; Bose et al., 2020; Naughton et al., 2020; Shi et al., 2020). For instance, OPs like soman, sarin, chlorpyrifos oxon (CPO, the active metabolite of CPF), and DFP covalently bind to tubulin (Grigoryan et al., 2008). DFP and CPO also covalently interact with lysine residues of albumin, keratin, actin, tubulin, and transferrin while other OPs were shown to bind to tyrosine residues of axonal transport-related proteins, suggesting that GWIR chemicals may hinder the function of axoplasmic transport via off-target (i.e., non-serine) interactions with structural proteins (Grigoryan et al., 2009a; Grigoryan et al., 2009b). Notably, a recent study demonstrated that two drugs approved by the FDA for non-OP-related disorders mitigated DFP-induced axoplasmic transport deficits in vitro (Naughton et al., 2020). The same group also found that OPs impact structural protein function in vivo (Jiang et al., 2010; Naughton et al., 2018). Specifically, mice exposed to CPF and CPO displayed significantly smaller and disrupted microtubules in brain tissue compared to unexposed mice (Jiang et al., 2010). A recent report indicated that repeated, low-level DFP exposure impairs the myelination of axons and inhibits axoplasmic transport in rats (Naughton et al., 2018). A different group observed acute hyperphosphorylation of tau protein in the brain of animals that underwent individual or combinatorial exposure to CPF, DFP, PB, and DEET (Torres-Altoro et al., 2011). An investigation by Bose et al. (2020) also detected persisting accumulation of

hyperphosphorylated tau protein in the frontal cortex five months after PB-PM exposure. Impairments in lipid metabolism following GWIR chemical exposure were recently detailed in Shi et al. (2020). Specifically, this report detected acute inhibition of hippocampal AChE and neuropathy target esterase in guinea pigs exposed to low-level sarin for 14 days. Neuropathy target esterase plays a fundamental role in enabling membrane lipid metabolism, and metabolomic data revealed that low-level, repeated sarin exposure led to acute dyshomeostasis of phospholipid and sphingolipid metabolism, responses that are consistent with studies of lipid aberrations in GWI veterans (Abdullah et al., 2012). GWIR chemical-induced mitochondrial dysfunction was also described. For instance, rats in a separate study displayed decreased quantity but increased length of mitochondria as well as reduced axonal movement following exposure to CPF or CPO (Middlemore-Risher et al., 2010).

Further, impaired trophic factor expression may play a role in underlying GWI pathophysiology. Interestingly, following repeated, low-level DFP exposure in vivo, nerve growth factor expression was enhanced whereas tropomyosin receptor kinase A density was reduced in the hippocampus and the basal forebrain 14 days post-DFP exposure, suggesting impaired trophic factor production and function (Terry Jr et al., 2011). Emerging evidence also indicates that brain-derived neurotrophic factor (BDNF) secretion was persistently reduced by PB-PM or DFP exposure in the frontal cortex and hippocampus, respectively (Bose et al., 2020; Kimono et al., 2020; Ribeiro et al., 2020). Biomarkers indicative of persistent disruption of Ca^{2+} homeostasis suggest that prolonged dysregulation of Ca^{2+} may play a role in GWI pathophysiology (Abou-Donia et al., 2017; Abou-Donia et al., 2020; Abou-Donia et al., 2021). Aberrant Ca^{2+} levels was observed within hippocampal pyramidal cells found in the CA1 subfield up to six months after DFP exposure in a GWI animal model (Phillips and Deshpande, 2018), an effect that may be attributed to reduced expression of a regulator of ryanodine receptor permeability, calstabin 2 (Phillips et al., 2019). Overall, these studies suggest that

neurotransmitter-independent targets of GWIR chemicals may play a role in persistence of GWI-specific pathology.

1.7.1.1 Findings from a DEET, PB, PM, and restraint stress-based GWI model

One of the first and most studied GWI rat models consisted of exposure to DEET, PB, PM, and restraint stress daily for 28 days (Abdel-Rahman et al., 2002). Early research assessing molecular changes in this GWI animal model observed significant reductions in peripheral BuChE and brain-wide AChE activity as well as alterations in mAChR2 density days after GWIR exposures (Abdel-Rahman et al., 2002; Abdel-Rahman et al., 2004a). These effects were not found in animals that received only GWIR chemicals or underwent restraint stress, suggesting a detrimental synergistic effect between chemical and stress exposure. Moreover, alterations identified in the animals that received both GWIR chemicals and stress were consistent with the capacity for PB and other GWIR chemicals to cross the blood-brain barrier as a result of the stressful conditions within the animal model. Additionally, lipidomic analysis revealed impaired expression of brain lipids related to the transport, uptake, storage, and synthesis of ACh, and activity of brain ACh functional pathways were increased in C57BL/6J mice exposed to DEET, PB, PM, and restraint stress 42 days post-exposure, underscoring the wide-ranging effect of GWIR chemicals and stress on nervous system functionality (Abdullah et al., 2012). A caveat of these reports is that they evaluated molecular changes shortly after exposure to GWIR chemicals and stress. Studies that assessed persisting molecular alterations as a result of GWIR exposures using this model such as Carreras et al. (2018) observed decreased hippocampal GABA, GAD67, and n-acetyl-aspartate levels in male and female rats three months post-exposure. Findings from this study also suggested impaired trophic factor production and function as a result of modulated TrkB expression in the hippocampus of males and females. Additionally, the authors described sex-specific effects of GWIR chemical and stress exposure, effects which are consistent with clinical reports indicating GWI symptomology presents in a sex-specific manner (Coughlin et al., 2017). Another study investigating prolonged

molecular alterations presented in this animal model discovered persistent oxidative stress, mitochondrial dysfunction, Nrf2 activation, and neuroinflammation in the hippocampus six months after exposure to GWIR chemicals and stress (Shetty et al., 2017). The authors state that while the observed oxidative stress, mitochondrial abnormalities, and pro-inflammatory cytokines were previously reported, the novel observation of enhanced Nrf2 activation may reflect an innate response aimed at reducing persistent activation of brain microglia, highlighting the neuroinflammatory phenotype of GWI. Using an identical exposure paradigm, the same group revealed elevated pro-inflammatory cytokines (such as IL1-β and TNF-α) and inflammatory mediators (like high mobility group box-1) in the cerebral cortex of rats 10 months (~29 human years) post-GWIR chemical and stress exposure, a time point that is translationally-relevant to the current age of GWI veterans (Sengupta, 2013; Madhu et al., 2019).

1.7.1.2 Findings from a DFP and corticosterone-based GWI models

Numerous studies have evaluated the acute and persisting inflammatory molecular alterations observed in a GWI model recently developed by O'Callaghan et al. (2015) (O'Callaghan et al., 2015; Locker et al., 2017; Craddock et al., 2018; Koo et al., 2018; Miller et al., 2018; Michalovicz et al., 2019; Michalovicz et al., 2020). In this model, C57BL/6J mice are exposed to PB, DEET, DFP, and corticosterone at varying timepoints over two weeks. Animals preexposed to corticosterone exhibited a significantly increased neuroinflammatory response to DFP, a response that contradicts the classical anti-inflammatory effect of glucocorticoids. A variation of this model detected elevated expression of cortical pro-inflammatory cytokines and unique inflammatory-induced alterations in neuroanatomical structures in Sprague Dawley rats shortly after exposure to corticosterone and DFP when compared to animals that were treated with only corticosterone or DFP (Koo et al., 2018). In particular, high-order diffusion MRI revealed distinct hippocampal and hypothalamic diffusivity changes in animals exposed to both corticosterone and DFP, changes that were associated with alterations in pro-inflammatory

cytokine signaling. These data suggest that low-level exposure to a nerve agent during prolonged stress may augment pro-inflammatory cytokine expression in the brain and lead to microstructural alterations in regions like the hippocampus, results which were observed in a clinical study (Chao and Zhang, 2018). Interestingly, the neuroinflammatory phenotype presented in this model was mediated by a noncholinergic mechanism (Locker et al., 2017; Miller et al., 2018; Michalovicz et al., 2020). A caveat of these preclinical studies is that the endpoint for the investigated molecular markers is days to a few weeks post-GWIR chemical and stress exposure, a time point that is not relevant to the current age of GWI veterans.

1.7.1.3 Findings from a PB-PM-based GWI model

A PB-PM exposure paradigm that recapitulates clinical GWI pathology has been validated across multiple mouse species over the past decade, resulting in the discovery of a plethora of acute and persisting GWI-specific proteomic, lipidomic, neuroinflammatory, trophic factor, and mitochondrial molecular alterations (Abdullah et al., 2011; Abdullah et al., 2013; Abdullah et al., 2016; Emmerich et al., 2017; Zakirova et al., 2017; Joshi et al., 2019; Bose et al., 2020; Kimono et al., 2020). For instance, proteomic analysis of CD1 mice exposed to PB-PM revealed a constellation of altered proteins related to lipid metabolism, endocrine functionality, and immune system regulation five months post-exposure (Abdullah et al., 2011). Using the same model, a follow-up by Abdullah et al. (2013) discovered enhanced phosphatidylcholine and sphingomyelin-related lipid species levels in the brain. Later work by this group observed distinct lipid expression in GWV that was comparable to those found in a rodent PB-PM exposure paradigm (Emmerich et al., 2017), underscoring the translational relevance of the preclinical lipid abnormalities and suggesting that continued investigation of these lipid disruptions is warranted. Additionally, a proteomic study detected persistent immune system dysregulation and mitochondrial dyshomeostasis five months after PB-PM exposure (Zakirova et al., 2017). Expression of autoantibodies indicative of long-term innate and adaptive immune system crosstalk mediated by metabolites of PM was also found in GWI veterans and

PB-PM-exposed mice, a response that may contribute to the persisting neuroinflammatory phenotype presented in GWI (Joshi et al., 2019). A separate group employed an adapted form of the PB-PM exposure paradigm to reveal a widespread impact of GWI-related inflammation (Bose et al., 2020; Kimono et al., 2020). Specifically, this group detected elevations in peripheral, frontal cortex, and hippocampal proinflammatory cytokines such as IL-6 and IL-1β, cytokines that were correlated with other pathological immune markers in the gut microbiome and the enteric nervous system (Bose et al., 2020). A different report found prolonged PB-PM-induced enhancement of frontal cortex IL-1β, IL-18, and IL-6 protein levels as well as peripheral IL-1β, TNF-α, and IL-6 expression were associated with NLRP3 inflammasome activation, detrimental responses that were reversed in NLRP3 knockout mice (Kimono et al., 2020). This investigation also revealed long-term neuroinflammatory-induced radical oxygen species production in the frontal cortex that was correlated with NLRP3 inflammasome activity. Emerging evidence also indicates PB-PM-induced persisting impairments in the production of frontal cortex and hippocampal trophic factors such as BDNF (Bose et al., 2020; Kimono et al., 2020). Further, expression of proteins comprising mitochondrial complex IV and V were reduced in PB-PM exposed animals, results that were indicative of compromised ATP synthase activity and subsequent bioenergetic perturbations. Importantly, a separate proteomic, lipidomic, and metabolomic reported revealed disrupted mitochondrial energetics and aberrant metabolites indicative mitochondrial dysfunctionality 16 months after PB-PM exposure, results that were consistent with clinical findings (Abdullah et al., 2016).

1.7.1.4 Findings from a PB and restraint stress-based GWI model

A recently developed GWI rat model consisting of PB exposure and repeated restraint stress produced molecular evidence of acute and prolonged impairments in glucocorticoid signaling, augmented neuroinflammation, and deficits in cholinergic as well as glutamatergic neurotransmission (Macht et al., 2018; Macht et al., 2019; Macht et al., 2020). Specifically, a persisting enhancement of plasma corticosterone levels was revealed three months after PB

and stress exposure, suggesting that concurrent exposures resulted in prolonged alterations of glucocorticoid activity (Macht et al., 2018). Neuroimmune homeostatic feedback loops were shown to be persistently disrupted by the exposure paradigm as evidenced by reduced plasma IL-4 and TNF-α levels. The authors also observed an initial decrease of plasma AChE activity 10 days after PB and stress exposure compared to vehicle-treated animals; however, AChE activity in PB and stress-exposed animals was enhanced three months post-exposure, indicating a prolonged impact on the cholinergic system. Additionally, this group employed their animal model to evaluate the short-term effects of innate immune and immobilization challenges on contextual and cue-based fear conditioning responses as well as extracellular ACh levels via microdialysis (Macht et al., 2019). PB exposure modulated the physiological response to immunological and stress-based challenges, consequently shaping extracellular ACh expression across multiple brain regions. The simultaneous functional effect of modulated ACh levels was found in impaired contextual and cue-based fear learning. A follow-up study using this animal model demonstrated that brain region-specific extracellular glutamate levels following lipopolysaccharide exposure or immobilization challenge were differentially impacted based on previous exposure to stress (Macht et al., 2020). While the two most recent studies evaluated animal responses at an acute timepoint, these studies suggest that concomitant exposure to PB and stress modulates neurotransmission as a result of complex neuroimmune crosstalk, which may result in persistent aberrations in cognition and behavior. Future studies investigating the prolonged effect of repeated PB and stress exposure on neurotransmission and neuroimmune signaling is warranted.

Collectively, these studies suggest an array of genetic, epigenetic, and molecular abnormalities are presented in the nervous system of GWI animal models. Importantly, many of these reported abnormalities in animal models are consistent with findings from the clinical GWI literature. Continued investigation into genetic, epigenetic, and molecular aberrations described in preclinical GWI models may facilitate the development of objective, GWI-specific biomarkers.

Additionally, genetic, epigenetic, and molecular alterations shown in animal models may be related to GWI-related impairments in synaptic, cellular, structural, and behavioral processes.

1.7.2 Synaptic aberrations

Essential cognitive functions such as hippocampal-dependent learning and memory are, in part, regulated by synaptic plasticity and transmission, making synaptic activity critical for behavioral functionality (Whitlock et al., 2006). Preclinical GWI studies have demonstrated acute and persisting impairments in synaptic plasticity and transmission. Short-term abnormalities in synaptic plasticity and transmission were observed via bath application of GWIR chemicals or in GWI animal models. We will summarize recent preclinical studies evaluating acute and persisting impairments in synaptic plasticity and transmission here.

Acute exposure to GWIR chemicals such as DEET, PB, and OPs have been shown to adversely impact synaptic plasticity and transmission (Friedman et al., 1996; Pavlovsky et al., 2003; Corbel et al., 2009; Nutter et al., 2013; Nutter et al., 2015; Cooper et al., 2016; Flunker et al., 2017; Cooper et al., 2018). For instance, acute DEET exposure increased hippocampal pyramidal cell excitability, an effect attributed to enhanced cholinergic transmission (Corbel et al., 2009). Specifically, acute DEET application to a cockroach neuronal preparation resulted in a biphasic response where the initial amplitude of field excitatory postsynaptic potentials (fEPSPs) was elevated followed by decreased fEPSP amplitude, an effect that was blocked by pretreatment with a nonselective muscarinic receptor antagonist (Corbel et al., 2009). Acute DEET exposure also altered the kinetics of mouse endplate potentials and directly inhibited human and insect AChE/BuChE. Collectively, these data suggest that acute DEET exposure modulates cholinergic signaling, an effect that may have contributed to the initiation of GWI. Additionally, studies found acute PB exposure modulates mammalian glutamatergic transmission (Friedman et al., 1996; Friedman et al., 1998; Pavlovsky et al., 2003). Notably, a dose-dependent enhancement of the hippocampal fEPSP amplitude was observed throughout acute PB exposure, an effect that was reported at GWIR concentrations (Friedman et al., 1996;

Friedman et al., 1998). In a separate study, acute PB exposure increased hippocampal spontaneous excitatory postsynaptic current (sEPSC) frequency, suggesting PB-induced enhancement of glutamatergic transmission is mediated via augmented presynaptic vesicular release (Pavlovsky et al., 2003). A different investigation demonstrated that acute application of CPO, the active metabolite of CPF, to a striatal slice preparation resulted in elevated sEPSC frequency, which is indicative of increased presynaptic glutamate release (Torres-Altoro et al., 2011). Multiple reports indicate that acute bath application of organophosphates like paraoxon, soman, and DFP to hippocampal slices results in reduced glutamatergic transmission (Eterović et al., 2011; Ferchmin et al., 2015; Hoffman et al., 2019). A recent study by Brown et al. (2020) suggests cholinergic and noncholinergic mechanisms underlie modulatory effects of acute DFP exposure on glutamatergic transmission along the dorsoventral axis of the hippocampus slice. Additionally, voltage-gated cation channel kinetics of rat vascular pain receptors were modulated weeks after CPF, PB, PM, and DEET exposure (Nutter et al., 2013; Nutter et al., 2015; Flunker et al., 2017; Cooper et al., 2018). In particular, an animal model consisting of a 60 day exposure period to CPF, PB, and PM revealed impaired dorsal root ganglion nociceptor kinetics of voltage-gated Na^+ and K^+ channels expressed in muscle and vascular nociceptors up to 12 weeks post-exposure (Nutter et al., 2013; Nutter et al., 2015). Further, 60-day exposure to CPF, PB, and PM detrimentally impacted muscarinic receptor signaling of muscle nociceptors 12 weeks post-exposure (Cooper et al., 2016). The inclusion of DEET to a four-week CPF, PB, and PM exposure paradigm marginally enhanced vascular nociceptor voltage-gated K^+ channel conductance impairments 12 weeks post-exposure (Flunker et al., 2017). Nociceptor voltage-gated Na^+ channel and TRPA1 kinetics were also modulated up to 16 weeks after four weeks of exposure to CPF, PB, PM, and DEET (Cooper et al., 2018). Importantly, modulations of channel kinetics described by these studies occurred concomitantly with pain-like behavior that was consistent with clinically relevant GWI-specific pain.

While there are a number of reports characterizing the acute effects of GWIR exposures on synaptic plasticity and transmission, there is a paucity of available information characterizing the persisting impact of GWIR chemical and stress exposure on synaptic plasticity and transmission. Specifically, to the best of our knowledge, only two published reports employed electrophysiology to assess persisting deficits in synaptic plasticity and transmission following GWIR exposures. For instance, Speed et al. (2011) investigated the acute and persisting effects of CPF exposure on mouse synaptic plasticity and transmission and observed a biphasic effect of the OP. Notably, hippocampal basal synaptic transmission was enhanced days after exposure but was reduced three months later, an effect that was a result of a delayed decrease in spontaneous excitatory postsynaptic current frequency that occurred concurrently with reduced dendritic branching. A recent report by Wang et al. (2020) also characterized persisting aberrations in synaptic plasticity and transmission, findings that will be covered in detail in subsection 1.10.1.

Collectively, these studies suggest that GWIR exposures induce acute and persisting impairments in synaptic plasticity and transmission in GWI animal models. It is presently unclear if these impairments are predominately mediated by a presynaptic or postsynaptic mechanism as the aforementioned studies described aberrations on both sides of the synapse. Detriments in cognition reported by GWI veterans are in line with preclinical synaptic plasticity and transmission deficits induced by GWIR chemical and stress exposure in structures related to neuropsychological function such as the hippocampus. Future preclinical GWI investigations evaluating persisting modulations in synaptic plasticity and transmission (i.e., defining a GWI-related presynaptic or postsynaptic pathological mechanism that is conserved across models) as well as network activity (i.e., theta rhythm, gamma rhythm, sharp-wave ripples, etc.) may address a substantial knowledge gap in GWI research that would provide additional context for behavioral deficits presented by GWI animal models as well as GWI veterans.

1.7.3 Cellular and structural pathology

Short-term and prolonged neural pathology has been demonstrated in GWI preclinical studies. Wide-spread neuronal cell death has been described for decades in GWI animal models; however, emerging evidence suggests glial pathology plays a critical role in the underlying pathophysiology of GWI. Persisting neuroanatomical structural degradation and long-term impairments in neurogenesis have also been reported in GWI animal models. GWI-induced pathology in neural cells and anatomical structures likely contributes to synaptic deficits and behavioral aberrations. We will summarize recent preclinical studies evaluating GWI-related acute and persisting cellular as well as structural neuropathology here.

Early reports indicated wide-ranging acute neuropathology induced by GWIR chemicals and stress in rats (Abdel-Rahman et al., 2002; Abdel-Rahman et al., 2004a; Abdel-Rahman et al., 2004b; Ojo et al., 2014). Specifically, a study showed that blood-brain barrier integrity was substantially compromised shortly after combined exposure to DEET, PB, PM, and restraint stress (Abdel-Rahman et al., 2002). Neuronal cell death was found in the cingulate cortex, thalamus, hypothalamus, and dentate gyrus, regions in which blood-brain barrier integrity was also diminished. Abdel-Rahman et al. (2002) also detected dentate gyrus neuron death immediately after GWIR chemical and stress exposure. A later report using a different GWI exposure paradigm also demonstrated reduced blood-brain barrier integrity at a short-term timepoint (Ojo et al., 2014). A separate investigation observed acute hippocampal subfield-specific neuronal cell death as well as neuron death in the motor cortex and the Purkinje cell layer of the cerebellum shortly after GWIR chemical and stress exposure (Abdel-Rahman et al., 2004a). Individual or combined exposures to GWIR pesticides malathion, DEET, and PM also induced short-term neuron death in the Purkinje cell layer of the cerebellum, brainstem, midbrain, dentate gyrus, CA1, and CA3 hippocampal subfields (Abdel-Rahman et al., 2004b).

A drawback of the aforementioned studies was the use of acute neuropathological endpoints, limiting their relevance to the current clinical condition of GWI veterans. Recent

reports have shown persisting disruptions in blood-brain barrier integrity (Kimono et al., 2020) and neuropathology in regions involved in cognition and motor functions. For instance, Parihar et al. (2013) discovered neuron loss in the hippocampal CA1 subfield, the CA3 subfield, and the dentate gyrus in mice four months after GWIR chemical and stress exposure. Five days of repeated, low-dose DFP exposure in a GWI animal model led to hippocampal damage as characterized by the presence of FJC+ staining in the polymorphic layer and along the hilus/granule cell border of the dentate gyrus three months post-exposure (Phillips and Deshpande, 2016). Additionally, interneuron pathology was observed in a GWI animal model where reduced levels of parvalbumin-expressing interneurons in the dentate gyrus and neuropeptide Y-expressing interneurons in the CA1/CA3 hippocampal subfields were detected three months following GWIR chemical and stress exposure (Megahed et al., 2015). The same study found that the quantity of parvalbumin and neuropeptide Y-expressing interneurons were decreased in the hippocampus overall, an effect that may contribute to hyperexcitability of glutamatergic circuitry and subsequent aberrant behavior (i.e., anxiety). Evidence of disrupted glial functionality in GWI animal models continues to emerge. Specifically, a recent investigation employing in vitro and ex vivo hippocampal slices found disrupted oligodendrocyte development and cholinergic signaling in a DFP and corticosterone exposure paradigm up to three weeks after exposure, indicating a novel role for this glial cell in the pathophysiology of GWI (Belgrad et al., 2019). Persisting activation of glia involved in regulating neuroimmune signaling has also been demonstrated. For instance, while some reports found no changes in GFAP+ immunostaining (O'Callaghan et al., 2015; Carreras et al., 2018), most studies observed astrogliosis up to 22.5 months following GWIR chemical and stress exposure, particularly in the hippocampus (Abdel-Rahman et al., 2002; Abdel-Rahman et al., 2004a; Abdullah et al., 2011; Abdullah et al., 2012; Parihar et al., 2013; Ojo et al., 2014; Zakirova et al., 2015; Abdullah et al., 2016; Zakirova et al., 2016; Zakirova et al., 2017; Madhu et al., 2019). Moreover, studies that evaluated long-term (Parihar et al., 2013; Abdullah et al., 2016; Carreras et al., 2018; Joshi et

al., 2018), but not short-term (Ojo et al., 2014; O'Callaghan et al., 2015), hippocampal Iba1+ levels reported persisting increases in this stain, suggesting prolonged activation of microglia. Elevated activation of microglia was also detected in the cerebral cortex five and 10 months after GWIR chemical and stress exposure (Madhu et al., 2019; Bose et al., 2020; Kimono et al., 2020). Interestingly, one investigation did not find enhanced Iba1+ staining 22.5 months after repeated exposure to PB and PM, a response the authors suggest may be due to confounding effects of age with GWI-related neuropathology (Zakirova et al., 2016). These results are in line with clinical data suggesting that GWI is chronic neuroimmune illness.

Structural changes observed in the brain of GWI animal models are consistent with anomalies presented in GWI veterans. For instance, decreased total hippocampal volume, CA3 subfield volume, and dentate gyrus volume was identified months after GWIR chemical exposure in an animal model (Parihar et al., 2013). Maladaptive structural changes may also be indicative of aberrant hippocampal neurogenesis. Reduced hippocampal neuronal cell proliferation and neurogenesis were detected days to months following GWIR chemical and stress exposure (Abdel-Rahman et al., 2002; Joosen et al., 2009; Parihar et al., 2013; Ojo et al., 2014; Megahed et al., 2015; Kott et al., 2016; Kodali et al., 2018; Liu et al., 2020). Namely, reduced hippocampal neurogenesis was found three days after exposure to CPF alone or combined with PM and PB (Ojo et al., 2014). A single, acute soman exposure decreased hippocampal neurogenesis four weeks after exposure (Joosen et al., 2009). Recent studies using the same exposure paradigm as Abdel-Rahman et al. (2002) observed reduced hippocampal neurogenesis immediately after exposure (Parihar et al., 2013; Liu et al., 2020), three months after exposure (Megahed et al., 2015), and four months after exposure (Parihar et al., 2013; Kodali et al., 2018). Interestingly, a preprint by Liu et al. (2020) demonstrated concomitant minocycline treatment ameliorated reduced hippocampal neurogenesis days after the termination of the GWI exposure paradigm. Furthermore, the introduction of GWI-relevant concentrations of corticosterone in the drinking water of mice over a prolonged period

decreased neurogenesis in the ventral hippocampus but not in the dorsal hippocampus (Kott et al., 2016). Nevertheless, a report described no changes in hippocampal neurogenesis 22.5 months post-PB-PM exposure (Zakirova et al., 2016). The authors suggest that the lack of differences in neurogenesis in these animals may be due to the confounding effects of old age. Collectively, these preclinical studies suggest that acute and persisting neuronal, glial, and neuroanatomical pathology may be a component of GWI. Further, prolonged impairments in neurogenesis may contribute to neuropsychological deficits and neuropathology in GWI veterans.

1.7.4 Behavioral impact

Wide-ranging abnormalities in neuropsychological function among GWI veterans have been described (White et al., 2016). Studies have also found acute and prolonged behavioral anomalies in GWI animal models. Specifically, behavioral deficits in the cognitive domain like learning and memory as well as impairments in the mood domain such as anxiety-like behavior have been consistently reported in preclinical investigations. Additionally, motor dysfunction has been demonstrated in GWI animal models. We will summarize recent preclinical studies evaluating acute and persisting aberrations in neuropsychological function here.

Preclinical studies employing different GWI animal models have consistently demonstrated acute and persisting aberrant behavior in the cognitive domain, particularly in learning and memory function (Prendergast et al., 1997; Abdullah et al., 2011; Terry Jr et al., 2011; Abdullah et al., 2012; Terry Jr et al., 2012; Parihar et al., 2013; Hattiangady et al., 2014; Zakirova et al., 2015; Abdullah et al., 2016; Phillips and Deshpande, 2016; Zakirova et al., 2016; Carreras et al., 2018; Macht et al., 2018; Phillips and Deshpande, 2018; Macht et al., 2019; Madhu et al., 2019; Phillips et al., 2019; Liu et al., 2020). Low-level exposure to DFP resulted in no change in performance in a spatial working memory-dependent Win-shift radial arm maze (RAM) task 50 days post-exposure but induced clear impairments in the reference memory-dependent Morris water maze (MWM) 140 days post-exposure (Prendergast et al., 1997; Terry

Jr et al., 2012). These data suggest a delayed, but not immediate, impairment in hippocampal-dependent learning and memory after exposure to GWIR chemicals. This is supported by a publication using a PB-PM exposure paradigm that observed persisting, but not acute, impairments in the reference memory-dependent Barnes maze task (Zakirova et al., 2015). Further, using an exposure paradigm of DEET, PB, PM, and restraint stress in rats, Parihar et al. (2013) found reference memory deficits in the MWM three months after exposure. CD1 male mice also exhibited diminished performance in the MWM task nearly four months after exposure to PB-PM (Abdullah et al., 2011). Impairments in performance in the Barnes maze task were detected 16 months after PB-PM exposure in a different study (Abdullah et al., 2016), suggesting that exposure to GWIR chemicals and stress results in delayed impairments in reference memory. Interestingly, a different group using the DEET, PB, PM, and restraint stress GWI paradigm did not detect altered performance in the MWM three months post-GWIR chemical exposure (Carreras et al., 2018). In a PB-PM exposure paradigm in C57BL/6J mouse model, no impairments in MWM performance were identified, a result that may have been confounded by sensorimotor deficits (Abdullah et al., 2012). Additionally, another report found no changes in performance in the water RAM or Barnes maze tasks 13 months and 22.5 months post-GWIR chemical exposure, respectively; however, the authors argue that no differences in performance may be a result of a confounding age effect (Zakirova et al., 2016). Short-term recognition memory impairments in the novel object recognition (NOR) task were observed after repeated exposure to DFP (Terry Jr et al., 2011). A recent preprint described diminished NOR performance in animals days after exposure to DEET, PB, PM, and restraint stress, an effect reversed by concomitant minocycline treatment (Liu et al., 2020). Persisting deficits in the NOR and object location tasks were reported three months after exposure in a rat GWI model that consisted of repeated, low-level DFP (Phillips and Deshpande, 2016; Phillips et al., 2019). A different study by these authors reproduced their findings in the NOR six months post-DFP exposure (Phillips and Deshpande, 2018). Additionally, prolonged recognition

memory impairments in the NOR task and the object location task were also demonstrated three months after DEET, PB, PM, and restraint stress exposure, suggesting GWIR chemical and stress exposure induces persisting deficits in hippocampal-dependent as well as perirhinal cortex-dependent learning and memory (Hattiangady et al., 2014). A recent report evaluated the effects of DEET, PB, PM, and restraint stress exposure on cognition and observed impairments in the NOR and object-in-place associative recognition memory task 10 months post-exposure, suggesting deficits in spatial memory and recognition memory that persist to a transitionally relevant timepoint (i.e., 10 months in rat corresponds to approximately 29 human years) to GWI veterans (Sengupta, 2013; Madhu et al., 2019). Other prolonged learning and memory deficits such as impaired contextual and cue-based fear conditioning following exposure to PB and stress were reported in separate studies (Macht et al., 2018; Macht et al., 2019). Collectively, these preclinical studies are in line with clinical investigations suggesting that persisting deficits in hippocampal-dependent learning and memory is a component of GWI.

Abnormal anxiety levels are another consistently described mood anomaly in GWI veterans. Short-term and long-term aberrant anxiety-like responses have also been found in GWI animal models (Abdullah et al., 2011; Abdullah et al., 2012; Parihar et al., 2013; Hattiangady et al., 2014; Phillips and Deshpande, 2016; Zakirova et al., 2016; Carreras et al., 2018; Phillips et al., 2019; Liu et al., 2020). For instance, enhanced anxiety-like behavior in the open field test (OFT) was observed immediately after using a 28-day GWIR chemical exposure paradigm in C57BL/6J mice, particularly in females (Abdullah et al., 2012). A preprint by Liu et al. (2020) also reported enhanced anxiety-like activity in the OFT days after exposure to a DEET, PB, PM, and restraint stress GWI paradigm, a response that was prevented by concomitant treatment with minocycline. Abdullah et al. (2011) identified increased anxiety-like behavior in the OFT 30 days post-PB-PM exposure in CD1 mice. Using an exposure paradigm consisting of DEET, PB, PM, and restraint stress, a preprint by Liu et al. (2020) observed elevated anxiety-like behavior in the elevated plus-maze (EPM) at a short-term timepoint,

aberrant behavior that was ameliorated via concomitant minocycline treatment. Employing an identical exposure protocol, Parihar et al. (2013) found enhanced anxiety-like behavior in the EPM three months after GWIR chemical exposure. The same group demonstrated no changes in anxiety-related behaviors in the OFT three months post-GWIR chemical exposure in a different report, a response that may be a result of the OFT being less challenging than the EPM (Hattiangady et al., 2014). A different group using the DEET, PB, PM, and stress GWI paradigm also demonstrated enhanced anxiety-related behavior in the EPM three months post-exposure (Carreras et al., 2018). Additionally, increased anxiety in the EPM was identified three months after exposure in a rat GWI model that consisted of repeated, low-level DFP (Phillips and Deshpande, 2016; Phillips et al., 2019). Interestingly, a separate report showed disinhibition of anxiety-like behavior 13 months post-PB-PM exposure but no changes in anxiety or social behavior 22.5 months after exposure, a response the authors argue may be due to confounding effects of age (Zakirova et al., 2016). Collectively, these preclinical studies are in line with clinical investigations of elevated anxiety in GWI veterans.

Motor deficits reported in GWI veterans have been recapitulated in rodents exposed to GWIR chemicals and stress (Prendergast et al., 1997; Abou-Donia et al., 2001; Abdel-Rahman et al., 2004b; Terry Jr et al., 2011; Abdullah et al., 2012; Hattiangady et al., 2014; Ramirez-Sanchez et al., 2020). Sensorimotor deficits were observed in CD1 mice seven days after exposure to DEET, PB, PM, and restraint stress as indicated by impaired performance on the rotarod task (Abdullah et al., 2012). Additionally, animals exposed to DEET, PB, PM, and stress individually or in combination exhibited diminished performance in the inclined plane, forepaw grip, and beam-walk tasks 30 days post-exposure (Abou-Donia et al., 2001; Abdel-Rahman et al., 2004b). Significantly decreased limb strength, as well as time and distance traveled on a treadmill, were reduced three weeks post-DEET, PB, PM, and stress exposure (Ramirez-Sanchez et al., 2020). Low-level exposure to DFP induced impairments in the OFT and the grip strength task immediately and up to 30 days post-exposure in rats (Prendergast et al., 1997;

Terry Jr et al., 2011). On the contrary, another group demonstrated no changes motor-related behaviors in the OFT three months post-GWIR chemical and stress exposure (Hattiangady et al., 2014). These data suggest that short-term, but not long-term, motor deficits are presented in animals following exposure to GWIR chemicals and stress. Future studies investigating persisting motor deficits in GWI animal models via the use of clinically relevant and consistent behavioral tasks such as those outlined by Janulewicz et al. (2017) are warranted.

1.8 Treating Gulf War Illness: Preclinical Studies

1.8.1 Overview of Gulf War Illness treatment

Many clinical and preclinical studies investigating the safety and efficacy of candidates for the treatment of GWI have been completed; however, 30 years after the 1990-1991 Persian Gulf War, GWI treatments are targeted and symptomatic. Additionally, emerging evidence suggests there are inconsistencies and inadequacies in the treatment of GWI veterans (Baldwin et al., 2019). As veterans are aging, their symptom severity is becoming progressively worse (Zundel et al., 2019; Porter et al., 2020; Zundel et al., 2020). Therefore, there is substantial demand for the rapid development of safe and efficacious therapies for extended remission of GWI. This section will focus on recent preclinical investigations of potential therapies for treating the long-term health challenges presented in GWI. Recent reviews have also surveyed the current state of studies evaluating preclinical and clinical therapeutic candidates for the treatment of GWI (Dickey et al., 2020; Nugent et al., 2020).

1.8.2 Gulf War Illness treatment candidates: preclinical studies

Numerous recent studies have investigated the safety and efficacy of novel therapeutics in treating GWI-related symptomology presented in animal models (Joshi et al., 2018; Kodali et al., 2018; Laferriere et al., 2019; Phillips et al., 2019; Joshi et al., 2020; Keledjian et al., 2020; Ribeiro et al., 2020; Shetty et al., 2020; Wang et al., 2020; Zhu et al., 2020). These preclinical reports consistently targeted fundamental components of GWI, namely lipid abnormalities, oxidative stress, aberrant neuroimmune signaling, and mitochondrial dysfunctionality.

Therapeutics impacting emerging mechanisms underlying GWI pathophysiology such as epigenetic irregularities, glutamatergic signaling, disrupted Ca^{2+} homeostasis were also evaluated. This subsection will focus on a few recent studies assessing possible therapeutics for treating persistent neurological abnormalities presented in GWI animal models.

1.8.2.1 Preclinical Gulf War Illness treatment candidates: oleoylethanolamide

The current review has previously established that prolonged lipid dyshomeostasis is accompanied by oxidative stress and a neuroinflammatory phenotype in a 10-day PB-PM exposure paradigm in C57Bl/6J mice (Zakirova et al., 2015; Abdullah et al., 2016; Emmerich et al., 2017). Importantly, abnormalities identified in the lipidomic profile of this GWI animal model were consistent with some of the aberrations observed in a clinical setting, particularly omega-3 docosahexaenoic acid, ether-containing phospholipids, and very-long-chain fatty acid (VLCFA) containing sphingomyelin species. Plasma VLCFA and thiobarbituric acid reactive substances were also shown to be significantly increased in GWI veterans compared to healthy GWV, suggesting abnormalities in lipid processing as well as elevated lipid peroxidation, respectively (Joshi et al., 2018). The authors of Joshi et al. (2018) argue GWI may be a result of prolonged impairments in peroxisomal activity as peroxisomes are involved in the β-oxidation of VLCFA as well as the synthesis of omega-3 docosahexaenoic acid and ether-containing phospholipids, processes that enhance oxidative stress and neuroinflammation when dysfunctional. Therefore, oleoylethanolamide (OEA), a peroxisome proliferator-activated receptor (PPAR) α agonist, was investigated as a potential treatment for mitigating prolonged lipid dyshomeostasis, reducing inflammatory activity, and ameliorating cognition. Indeed, OEA treatment, beginning five months post-PB-PM exposure, resulted in a reduction of brain VLCFA levels that were comparable to the levels of age-matched vehicle-treated animals. Moreover, expression of thiobarbituric acid reactive substances in OEA-treated animals exposed to PB-PM were not significantly different compared to controls. Abnormal NF-κB and signal transducer and activator of transcription 3 (STAT3) phosphorylation, indicators of inflammation in a litany of CNS disorders, were observed

in PB-PM-exposed animals months after exposure, an effect reversed by OEA treatment. Additionally, persistent activation of astrocytes, as well as activation and proliferation of microglia, were reduced in PB-PM-exposed animals that received OEA treatment. These lipid, neuroinflammatory, and neuropathological changes were all detected 11 months post-PB-PM exposure (i.e., six months of OEA treatment), corresponding to alterations that are clinically relevant to the current age of GWI veterans (e.g., 11 months in mice approximately corresponds to more than 30 human years (Sengupta, 2013)). Six months after PB-PM exposure, reference memory deficits in the Barnes maze were reversed in PB-PM exposed animals that received OEA treatment. The authors also argued that OEA treatment reduced fatigue in PB-PM exposed animals as indicated by PB-PM-vehicle animals exhibiting significantly enhanced immobility time in the FST compared to all other groups 10 months after PB-PM exposure. Abnormal anxiety-like behavior was observed in PB-PM-vehicle animals in the EPM 11 months post-exposure, an effect reversed by OEA treatment. Overall, these data suggest that OEA may be an efficacious therapy for treating persisting lipid dysfunction, attenuating pro-inflammatory activity, and improving cognition in GWI. There are a number of advantages and limitations of this report. For instance, an advantage of this study includes the significant delay prior to animals receiving OEA treatment. This recapitulates the brief exposure period and prolonged delay before GWI veterans receiving GWI-specific therapy. Further, the 11-month endpoint of the study facilitates the construct validity of the model and the predictive validity of the OEA treatment as the age of the animals is comparable to the current age of GWI veterans. Limitations of this study include unexpected anxiolytic activity in the EPM and enhanced immobility time in the FST in vehicle animals that received OEA, responses that warrant further evaluation of the toxicological profile of OEA. Although a biorepository of plasma samples from GWV was consulted in analyzing clinical data, the low number of GWV employed in the study (control veterans: n=10; GWI veterans: n=12) limits the impact of the report. Additionally, the lack of female subjects and animals in this study restricts the scope of the results.

1.8.2.2 Preclinical Gulf War Illness treatment candidates: curcumin and monosodium luminol-GVT

The Shetty group recently published reports evaluating the efficacy of two candidates for treating GWI, treatments that are fundamentally dependent upon restoration of redox homeostasis and mitigating the production of pro-inflammatory cytokines in a GWI animal model (Kodali et al., 2018; Shetty et al., 2020). One candidate that was evaluated, curcumin, has a well-established safety profile (Leibowitz and Ormerod, 2018) and was shown to induce prolonged anti-inflammatory, antioxidant, and neuroprotective effects in a GWI animal model (Kodali et al., 2018). The same group also evaluated the efficacy of a different agent, monosodium luminol-GVT (MSL), in the treatment of GWI (Shetty et al., 2020). The authors note that MSL was shown to restore redox homeostasis in animal models of disease states involving prolonged neuroinflammation and oxidative stress, symptoms which are consistent with GWI. Four months after a 28 day exposure to DEET, PB, PM, and restraint stress, rats began MSL treatment for two months to assess the safety and efficacy of the therapeutic in treating GWI-specific neuropsychological impairments, increased oxidative stress, elevated inflammation, augmented neuropathology, and diminished neurogenesis. Three doses (40, 80, or 160 mg/kg) were used to investigate dose-response effects. After two months of MSL treatment (six months post-GWIR chemical and stress exposure), animals that underwent GWIR chemical and stress exposure exhibited impaired performance in the NOR task, pattern separation task, sucrose preference test, and novelty suppressed feeding test, effects that were reversed in animals treated with 160 mg/kg MSL. These findings suggest that MSL ameliorated hippocampal-dependent learning and memory, improved motivation, enhanced sensitivity to rewarding stimuli, and reduced anxiety-like behavior, respectively. Additionally, MSL normalized genes related to combating oxidative stress to levels that were comparable to naïve age-matched control animals. Brain and peripheral markers of oxidative stress in GWIR chemical and stress-exposed animals were also reduced to levels comparable to naïve animals following

MSL treatment, suggesting that the therapeutic rebalanced redox homeostasis in a long-term GWI animal model. Peripheral pro-inflammatory cytokines and chemokines (i.e., IL-1α, IL-6, TNF-α) levels were also decreased in GWIR chemical and stress-exposed animals that received MSL treatment, suggesting MSL mitigated pro-inflammatory cytokine expression. Additionally, glial functionality was recovered in MSL-treated rats six months after GWIR chemical and stress exposure. Specifically, a reduction in hippocampal astrocyte hypertrophy and a bias of activated microglia back to a resting state was observed in animals that received MSL treatment. While three weeks of treatment with MSL in GWIR chemical and stress exposed animals did not result in restored hippocampal neurogenesis, eight weeks of MSL treatment recovered the proliferation of putative neural stem cells and rescued hippocampal neurogenesis to levels comparable to naïve control animals. This study suggests that MSL may be an efficacious agent for the treatment of underlying pathophysiology leading to the persistence of GWI. Notably, the authors employed a four-month delay before initiating MSL treatment. Accordingly, this timeline more effectively recapitulates clinical GWI than studies that begin treating animals immediately after GWIR chemical and stress exposure. Moreover, the variety of endpoints (i.e., neuroinflammatory, oxidative stress, histopathological, and behavioral) investigated in this report provides a comprehensive picture of the effects of GWIR chemical and stress exposure as well as MSL treatment in the GWI animal model. A noticeable drawback of this study includes the lack of a dose-response effect mediated by MSL in some experiments. For instance, a biphasic, U-shaped response was observed in MSL-treated rats in the object location test and pattern separation test. The highest dose utilized in this study, 160 mg/kg, most consistently restored measurements to levels comparable to naïve control animals, suggesting this high dose may hold the most promise for the treatment of GWI. Accordingly, toxicological analysis is warranted as some of these responses may be a result of compensatory mechanisms elicited in response to the large dose of MSL.

1.8.2.3 Preclinical Gulf War Illness treatment candidates: LDN/OSU-215111

Clinical (Brandley et al., 2020; Holton et al., 2020a; Holton et al., 2020b; Joyce and Holton, 2020; Kirkland et al., 2020) and preclinical studies (Torres-Altoro et al., 2011; Macht et al., 2019; Macht et al., 2020) suggest that aberrant glutamatergic signaling may be an underlying component of GWI pathophysiology. Positioned at the intersection of neuronal and glial synaptic communication is the tripartite synapse, a term defined as a functional combination of the presynaptic bouton, postsynaptic density, and proximal glial cells that facilitate and regulate the activity of a synapse. The aforementioned reports of atypical glutamatergic transmission and dysfunctional glial signaling in GWI may be indicative of tripartite synapse impairments. Accordingly, a recent report by Wang et al. (2020) targeted preclinical GWI-specific abnormalities in tripartite synapse function via a novel pyridazine derivative, LDN/OSU-215111. LDN/OSU-215111 was previously shown to enhance the function and structure of glutamatergic synapses as well as astrocytic cells at tripartite synapses, a response which was related to elevating the production of proteins in perisynaptic astrocytic processes (PAP) and subsequent enhancement of forms of plasticity related to PAP activity such as LTP (Foster et al., 2018). Wang et al. (2020) assessed the efficacy of LDN/OSU-215111 in treating GWI symptomology following exposure to a modified paradigm developed by Abdel-Rahman et al. (2002). Following six weeks of GWIR chemical and stress exposure, persisting GWI-related pathology was observed in animals including aberrant glutamatergic molecular markers, mood abnormalities, cognitive impairment, elevated fatigue, deficits in synaptic plasticity and transmission, hippocampal neural atrophy, reduced hippocampal neurogenesis, and enhanced hippocampal glial dysfunction, responses that were generally reversed in animals following 10 mg/kg LDN/OSU-215111 (p.o.) treatment. Specifically, hippocampal glutamate levels were elevated in vivo, an effect that was not observed in GWIR chemical and stress-exposed mice that received a LDN/OSU-215111 treatment. Daily treatment

with the pyridazine derivative during and for three months following GWIR chemical and stress exposure prevented the development of anxiety- and depressive-like behavior in males and females as well. Additionally, deficits in the NOR task and Barnes maze were generally prevented by LDN/OSU-215111 treatment three months post-GWIR exposure in males and females. Male, but not female, animals exposed to GWIR chemical and stress exhibited reduced LTP magnitude three months after exposure, a response that was ameliorated by LDN/OSU-215111 treatment. A different subset of animals underwent testing in the aforementioned tasks that assessed anxiety- and depressive-like behavior five months after GWIR chemical and stress exposure followed by four weeks of daily LDN/OSU-215111 treatment. Subsequent retesting in anxiety- and depressive-like behavioral tasks revealed normalization of performance in mood-related tests following four weeks of daily treatment with the pyridazine derivative (six months post-GWIR exposure), suggesting that delayed treatment with the novel therapeutic may ameliorate GWI-related mood dysfunction. A different subset of animals exhibited more profound impairments in recognition and reference memory six months post-GWIR exposure compared to three months post-exposure. These hippocampal-dependent learning and memory impairments were ameliorated in animals that began receiving daily LDN/OSU-215111 treatment for four weeks following a five-month delay after GWIR exposure. Additionally, LTP magnitude was reduced in both male and female animals six months after exposure to GWIR chemicals and stress, a response that was not detected in animals that received four weeks of daily LDN/OSU-215111 treatment. These persisting changes in LTP magnitude were accompanied by prolonged alterations in excitatory and inhibitory neurotransmission. Notably, reduced frequency and amplitude of miniature EPSCs and sEPSCs were observed in animals six months post-GWIR exposure, responses that were not observed following LDN/OSU-215111 treatment. Further, miniature IPSC frequency, as well as spontaneous IPSC frequency and amplitude, were reduced in GWIR chemical and stress-exposed animals; however, none of these inhibitory responses were ameliorated by treatment

with the pyridazine derivative. These data are in line with GWIR chemical and stress-induced elevations in hippocampal excitatory transmission as a result of augmented extracellular glutamate levels, leading to persistent aberrations in synaptic efficacy. The authors also detected a prolonged reduction in markers of glutamatergic presynaptic function such as synaptophysin and vesicular glutamate transporter 1 as well as decreases in indicators of postsynaptic integrity like PSD-95, responses which are consistent with electrophysiological data suggesting impairments in glutamatergic neurotransmission. Importantly, LDN/OSU-215111 treatment reversed the detrimental impact of GWIR exposure on these glutamatergic presynaptic and postsynaptic molecular markers. Additionally, these data are consistent with impaired interneuron function. Indeed, while the authors did not observe persistently reduced density of hippocampal pyramidal cells, they did detect diminished density of parvalbumin-positive interneurons in the DG as well as CA1 and CA3 subfields six months post-GWIR exposure, decreases that were ameliorated by LDN/OSU-215111 treatment. Moreover, decreased expression of functional proteins found in the presynaptic terminal of GABAergic synapses like vesicular GABA transporter (vGAT) but no detected alterations in functional postsynaptic GABAergic cell indicators such as gephyrin and $GABA_A$ receptor density suggests a complex yet significant impact of GWIR exposure on inhibitory neurotransmission in the hippocampus. Importantly, LDN/OSU-215111 treatment ameliorated the observed reduction in hippocampal vGAT expression. A persistent reduction in hippocampal neurogenesis induced by GWIR exposure was also rebalanced by LDN/OSU-215111 treatment. Overall, these data suggest that GWIR chemical and stress exposure persistently elevate intracellular glutamate levels, leading to impaired neural structure and function that manifests as deficits in neurogenesis, behavior, and synaptic plasticity and transmission. Importantly, targeting the tripartite synapse by employing concomitant or delayed LDN/OSU-215111 treatment augmented the translation of transcripts in the PAP, generally ameliorating GWI-specific aberrations in glutamate homeostasis, neural integrity, neurogenesis, neurotransmission,

synaptic plasticity, mood, and cognition. This study presents several strengths. An advantage of this investigation was the wide-ranging assays employed, including experiments that assessed a myriad of molecular, cellular, and behavioral responses. Notably, this is one of the first published reports to evaluate persisting impairments in synaptic plasticity and transmission in a GWI animal model, which aids in addressing a substantial knowledge gap in GWI research. The authors included male and female animals in this study, bolstering the potential scope and impact of the report as increasing evidence suggests GWI differentially manifests across sexes (Dursa et al., 2019; Sullivan et al., 2020). The authors also evaluated the efficacy of LDN/OSU-215111 at immediate and delayed timepoints, allowing researchers to assess how the therapeutic may ameliorate GWI-specific pathology over time. Nevertheless, there are a number of drawbacks to this report. While the authors employed a well-established GWI exposure paradigm, the present study utilized a novel, more intense, and longer lasting stress protocol, potentially limiting the capacity to compare the present report to other preclinical and clinical literature. Moreover, the authors do not indicate if electrophysiology recordings are conducted in the dH or the vH, and the protocol employed in LTP experiments limits the report. Specifically, a relatively short period (five min) was utilized to establish basal synaptic transmission, contributing to the observed variability in the time course. The authors only recorded the potentiated fEPSP response for 30 min post-TBS, monitoring a response that may be more appropriately defined as STP rather LTP. Accordingly, this substantially hinders the ability of the authors to assess the persisting impact of GWIR chemical and stress exposure on hippocampal synaptic plasticity.

1.8.2.4 Preclinical Gulf War Illness treatment candidates: Intracellular Ca^{2+} modulating agents

Persisting pathology and aberrant behavior presented in a GWI model consisting of repeated, low-level DFP exposure was associated with abnormal intracellular Ca^{2+} signaling (Phillips and Deshpande, 2016; Phillips and Deshpande, 2018; Phillips and Deshpande, 2020). A recent study evaluated if levetiracetam, an FDA-approved antiepileptic drug that induces

pharmacological activity via inhibition of Ca^{2+}-induced Ca^{2+} release, can restore Ca^{2+} signaling and associated behavioral function in this GWI animal model (Phillips et al., 2019). After five consecutive days of DFP exposure, rats underwent a three-month waiting period prior to receiving four consecutive days of levetiracetam treatment. The next day, DFP-exposed animals exhibited significantly elevated intracellular Ca^{2+} levels, an effect that was partially prevented by levetiracetam treatment. Additionally, DFP-exposed animals that received levetiracetam treatment did not display deficits in sensitivity to reward in the sucrose preference test like DFP-exposed animals did. Anxiety-like behavior and enhanced immobility time in the EPM and FST, respectively, were reversed in DFP-levetiracetam treated animals. Moreover, DFP-exposed animals exhibited impaired recognition memory in the NOR, an impairment that was not observed in DFP-levetiracetam treated rats. Analysis of protein levels involved in the regulation and release of intracellular Ca^{2+} revealed a reduction in calstabin 2 levels in DFP exposed rats, a critical regulatory subunit of the ryanodine receptor 2 (RyR2) complex that selectively binds and stabilizes RyR2 to a closed state. Importantly, RyR2 is implicated in modulating cognitive behavior (Abu-Omar et al., 2018; Kushnir et al., 2018). Accordingly, the authors propose that DFP-induced aberrations in glutamatergic neurotransmission may contribute to excitotoxic activation of NMDA receptors, leading to sustained leakage of Ca^{2+} from intracellular stores such as RyR2 as a result of reduced expression of regulatory proteins like calstabin 2. Therefore, targeting aberrant intracellular Ca^{2+} signaling may hold promise in treating neurological abnormalities and behavioral dysfunction in GWI veterans. Using the same model and timeline, two recent studies by this group followed-up this potential mechanism by evaluating the efficacy of ketamine and its enantiomers (i.p. ROA) as anti-depressants in treating GWI-related molecular and behavioral aberrations three months post-DFP exposure (Ribeiro et al., 2020; Zhu et al., 2020). As doses of ketamine above 10 mg/kg impacted locomotor activity in the OFT, the authors selected a concentration of 10 mg/kg to evaluate the therapeutic effects of this competitive NMDA receptor antagonist. A dose-dependent reduction

in depressive/fatigue-like behavior in the FST was observed up to 24 h post-ketamine treatment in rats that were exposed to DFP three months prior. Additional indicators of depressive/fatigue-like behavior were observed in DFP exposed animals such as decreased sucrose preference consumption as well as anxiety-like behavior in the EPM, responses that were reversed up to 24 h and one h, respectively, after 10 mg/kg ketamine treatment. Interestingly, one enantiomer of ketamine, (*R*-)-ketamine, appeared to be more efficacious in persistently ameliorating GWIR behavioral abnormalities in the FST and EPM than the other. A significant elevation in intracellular Ca^{2+} levels was observed in CA1 hippocampal cells, an effect that was reversed one h but not 24 h post-ketamine treatment. Ketamine treatment also recovered hippocampal BDNF levels 24 h, but not one h, post-ketamine treatment in DFP-exposed animals. Interestingly, a different competitive NMDA receptor antagonist, memantine, reversed persistent elevations in hippocampal CA1 intracellular Ca^{2+} levels one h after treatment but did not impact immobility time in the FST 24 h post-treatment or hippocampal BDNF levels. Administration of a TrkB inhibitor, ANA-12, five min prior to ketamine treatment had no effect on ameliorated immobility time in the FST one h post-ketamine treatment but blocked the previously described reduction in immobility time 24 h post-ketamine treatment. These data suggest that the therapeutic impact of 10 mg/kg ketamine treatment on aberrant GWIR molecular and behavioral responses is initially driven by normalization of intracellular Ca^{2+} levels followed by a persistent TrkB-mediated recovery of BDNF levels. While the authors note that the half-life of ketamine is approximately one h, other studies demonstrated that ketamine metabolites induce sustained therapeutic effects that are independent of the NMDA receptor such as persistent activation of AMPA receptors and modulatory effects on BDNF signaling (i.e., hydroxynorketamine). Further, the (*R*-)-ketamine enantiomer induces unique physiological effects independent of the traditional impact on NMDA receptor conductance, which may underlie the observed elevations in antidepressant-like effects compared to its (*S*+)-ketamine counterpart in the present reports as well as reduced psychotomimetic effects described in other studies. Nevertheless, these studies

present several shortcomings. For instance, each report is particularly limited by the duration of the investigations. Specifically, the three-month waiting period corresponds to approximately 10 human years, a period that is not translationally relevant to the 30 year period since the 1990-1991 Persian Gulf War (Sengupta, 2013). This limits the construct validity of the model and the predictive validity of levetiracetam and ketamine. The i.p. ROA for levetiracetam and ketamine also hinders the translational relevance of these studies as this is not a practical route for clinical administration of these potential therapeutics. Additionally, the authors of Phillips et al. (2019) propose that the etiological basis of the sustained dysfunction presented in their GWI model is due to intracellular Ca^{2+} dyshomeostasis, resulting from reduced expression of regulatory proteins like calstabin 2. Nevertheless, their study lacks an experimental group assessing the effects of levetiracetam treatment on calstabin 2 expression. Therefore, future studies assessing the efficacy of levetiracetam in restoring calstabin 2 levels may aid in interpreting improvements in persisting GWI-specific behavioral anomalies. Furthermore, the authors of the latter two reports speculate that a substantial component of the persisting therapeutic effects of ketamine is mediated via metabolites of the NMDA receptor antagonist. Accordingly, it may be fruitful for future reports assessing the sustained effects of ketamine in improving GWIR symptomology and underlying molecular aberrations to include a treatment group of a previously demonstrated therapeutic ketamine metabolite such as hydroxynorketamine. Moreover, the authors did not indicate if the dH or the vH were evaluated, hippocampal sectors that are increasingly recognized as functionally distinct structures (Papatheodoropoulos, 2018). Additionally, studies suggest ketamine induces sex-specific therapeutic effects (Freeman et al., 2019). As increasing evidence suggests that GWI presents in a sex-specific manner (Coughlin et al., 2017; Heboyan et al., 2019), subsequent studies evaluating the safety and efficacy of ketamine in treating GWI should include female animals or subjects to maximize the impact and reproducibility of the investigation.

1.8.3 Preclinical Gulf War Illness treatment candidates: summary

Collectively, these studies illustrate an optimistic outlook for the development of a potentially safe and efficacious GWI treatment. For instance, therapeutics investigated by the field of preclinical GWI researchers are converging on translationally relevant mechanisms of clinical GWI, namely lipid abnormalities, oxidative stress, aberrant neuroimmune signaling, mitochondrial dysfunctionality, epigenetic irregularities, glutamatergic signaling, and disrupted Ca^{2+} homeostasis. Nevertheless, the litany of mechanistic options to target a drug candidate around presents an obvious question for researchers developing a safe and efficacious treatment for GWI: Which individual, or group of, therapeutic mechanism(s) should a drug candidate be targeted towards? And how efficacious would this drug candidate be in treating a disease state that presents as heterogeneously as GWI? Challenges related to these questions and others will be addressed in the 6.2 subsection of this document.

1.9 Conclusion

The hippocampus is a bilateral structure located in the medial temporal lobe of mammalian organisms and exhibits a distinct subregion, subfield, and laminar organization that facilitates relatively simple investigation of circuit-level mechanisms compared to other brain regions. Complex cellular characteristics of the hippocampus drive unique forms of synaptic plasticity as well as diverse manifestations of excitatory, inhibitory, and modulatory neurotransmission. These intricate forms of synaptic plasticity and transmission play multifaceted functions in behavioral responses, particularly learning and memory. Emerging evidence indicates that the dorsal and ventral sectors of the hippocampus are functionally distinct structures, resulting in novel research evaluating intrinsic and extrinsic differences between these sectors. The establishment of differential synaptic, cellular, and structural properties within the dH compared to the vH has provided insight into subtle and profound roles these sectors play in behavioral responses.

GWI is a chronic multisymptom illness affecting approximately one-third of 700,000 GWV. In the present review, we have described preclinical GWI studies detailing neurological

aberrations at the genetic, epigenetic, molecular, synaptic, cellular, anatomical, and behavioral levels. Investigations of preclinical drug candidates for the treatment of GWI were also reviewed. Collectively, extensive clinical and preclinical GWI research has been conducted, yet there is still clear demand for enhanced progress in GWI investigations as treatments for the disease state remain targeted and symptomatic.

The degree, duration, and type of GWIR exposures are correlated to the symptom severity of GWI, resulting in a disease state that presents heterogeneously among veterans. Accordingly, the varied severity of GWI symptomology as a result of unique exposure conditions indicates that assessing multiple forms of GWIR exposures (i.e., in vitro vs. in vivo) and presentation of their adverse effects within distinct physiological systems (i.e., ex vivo vs. in vivo) may address knowledge gaps in GWI research. Additionally, emerging evidence suggests that GWI is a progressive disease as indicated by the gradual increase in reported GWI symptom severity and frequency over time. Therefore, assessing experimental endpoints at multiple time points (i.e., acute vs. persisting) in preclinical GWI studies may provide insight into the apparent worsening of the disease. Further, neurological (i.e., cognitive) and immunological (i.e., inflammation) dysfunction has been consistently featured in GWI studies highlighted throughout this review, underscoring the necessity to address aberrations in these physiological domains via a safe and efficacious treatment. The following preclinical studies detailed in this document specifically aimed to address knowledge gaps related to the progressive neuropathological mechanisms initiated by in vitro and in vivo GWIR exposures, particularly along the hippocampal dorsoventral axis. These investigations also aimed to determine the efficacy of an immunotherapeutic, lacto-n-fucopenatose-III, in ameliorating neurological abnormalities in two animal models of GWI when the therapeutic was coadministered with GWIR exposures as well as months after exposures.

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Figures

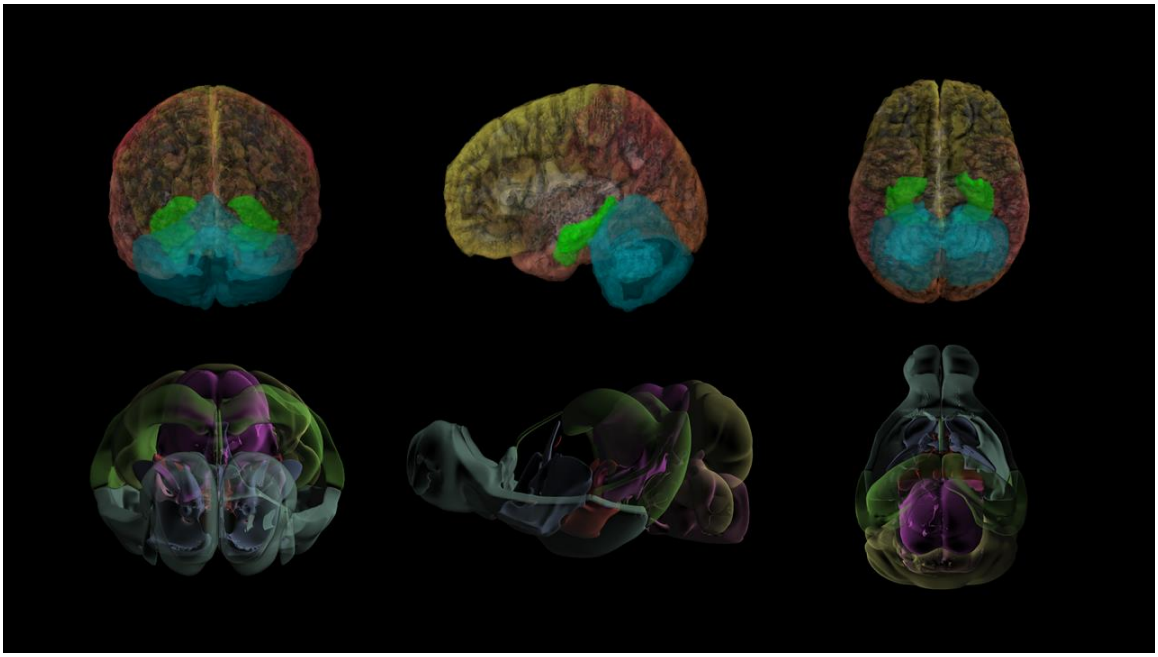

Figure 1.1. Neuroanatomical location of the hippocampal formation (green) in human (upper) and rodent (lower) brain.

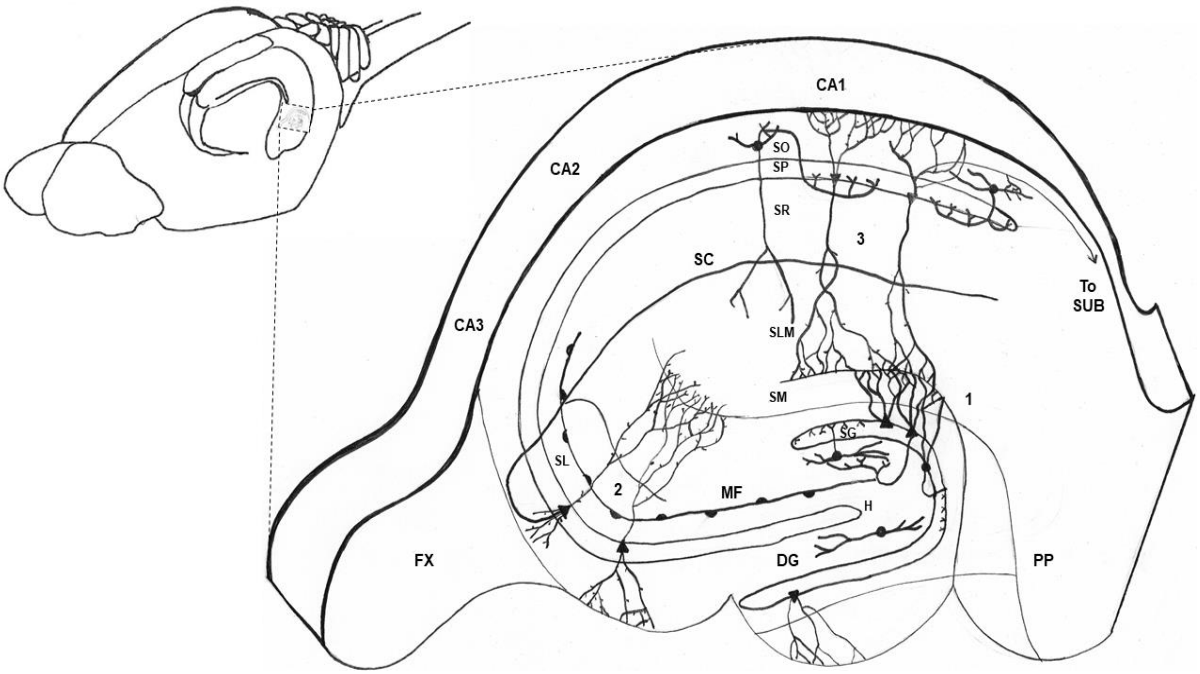

Figure 1.2. Hippocampal laminar cytoarchitecture with perforant and alvear pathways. 1, 2, and 3 denote the three major synapses along the perforant (trisynaptic) pathway. Abbreviations: CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; DG, dentate gyrus; FX, fornix; H, hilus; MF, mossy fiber; PP, perforant pathway; SC, Schaffer collateral; SG, stratum granulosum; SL, stratum lucidum; SLM, stratum lacunosum-moleculare; SM, stratum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SUB, subiculum.

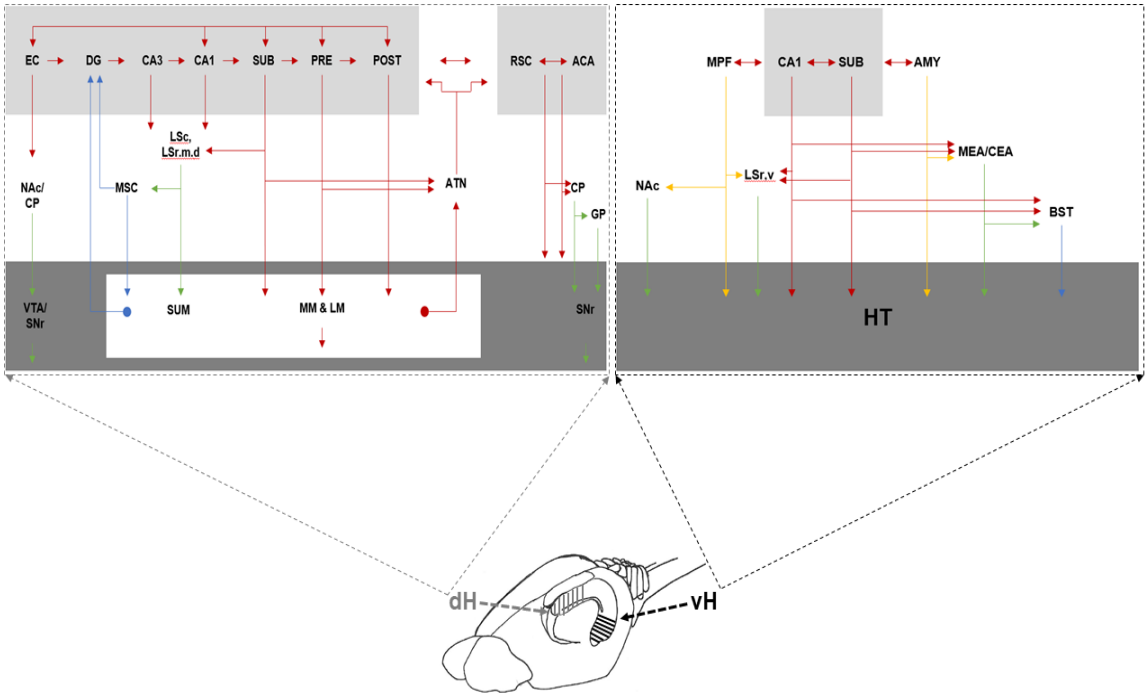

Figure 1.3. Afferent and efferent projections in the dorsal hippocampus (dH) and the ventral hippocampus (vH). Modified from Fanselow and Dong. *Neuron* 2010. Abbreviations: *dH, dorsal hippocampus panel*: ACA, anterior cingulated area; ATN, anterior thalamic complex; CA1, dorsal domain of cornu ammonis 1; CA3, dorsal domain of cornu ammonis 3; CP, caudoputamen; DG, dorsal domain of the dentate gyrus; EC, the caudolateral band of the entorhinal cortex; GP, globus pallidus; LM, lateral mammilary nucleus; LSc, the caudal part of the lateral septal nucleus; LSr.m.d, rostromediodorsal part of the lateral septal nucleus; MM, medial mammilary nucleus; MSC, medial septal complex; NAc, nucleus accumbens; PRE, presubiculum; POST, postsubiculum; RSC, retrosplenial cortex; SNr, reticular part of the substantial nigra; SUB, dorsal subiculum; SUM, supramammillary nucleus; VTA, ventral tegmental area. *vH, ventral hippocampus panel*: AMY, cortical-like amygdalar areas (nuclei); BST, bed nuclei of the stria terminalis; CA1, ventral domain of cornu ammonis 1; CEA, central amygdalar nucleus; LSr,v, the rostral and ventral parts of the lateral septal nucleus; MEA,

medial amygdalar nucleus; MPF, medial prefrontal cortex; NAc, nucleus accumbens; SUB, the ventral subiculum.

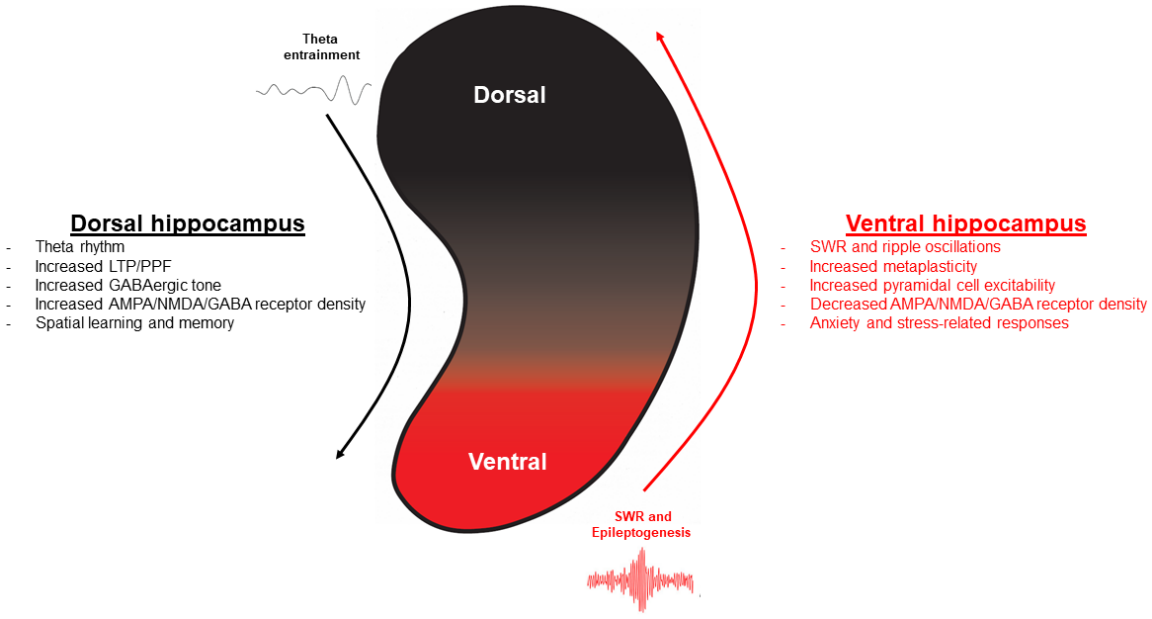

Figure 1.4. Differential functionality along the hippocampal dorsoventral axis. Modified from Papatheodoropoulos. *Front Biosci* 2018. Abbreviations: AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; PPF, paired-pulse facilitation; SWR, sharp-wave ripple.

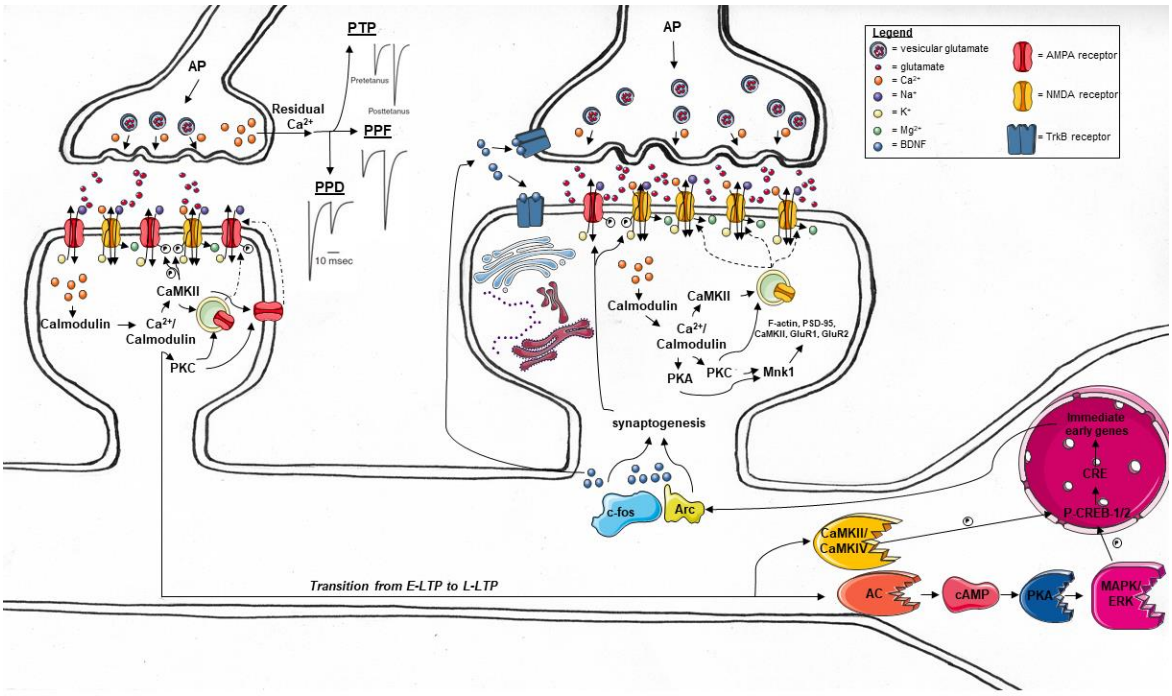

Figure 1.5. Cellular mechanisms of short-term and long-term plasticity in the hippocampus. Abbreviations: AC, adenylate cyclase; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, action potential; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium ion; CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIV, calcium/calmodulin-dependent protein kinase II; cAMP, cyclic adenosine monophosphate; CRE, cyclic adenosine monophosphate response element; E LTP, early-long-term potentiation; ERK, extracellular signal-regulated kinase; GluR1, ionotropic glutamate receptor 1 subunit; GluR2, ionotropic glutamate receptor 2 subunit; L-LTP, late long-term potentiation; MAPK, mitogen-activated protein kinase; Mnk1, mitogen-activated protein kinase–interacting kinase 1; NMDA, N-methyl-D-aspartate; P-CREB-1/2, phospho-cyclic adenosine monophosphate-response element binding protein; PKA, protein kinase A; PKC, protein kinase C; PPD, paired-pulse depression; PPF, paired-pulse facilitation; PTP, post-tetanic potentiation; PSD-95, postsynaptic density protein 95; TrkB, tropomyosin receptor kinase B.

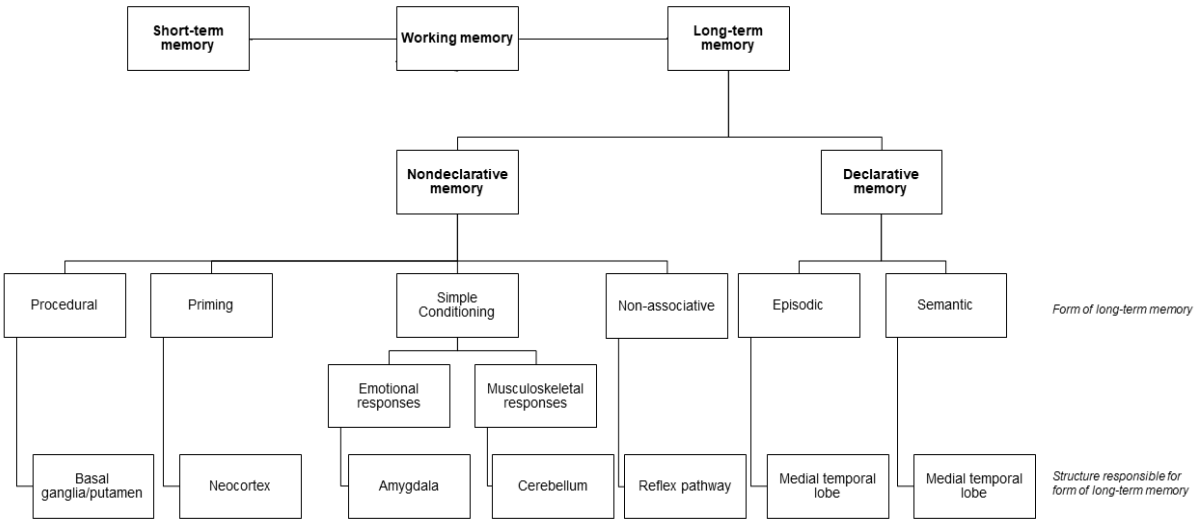

Figure 1.6. General forms of learning and memory. Modified from García-Lázaro et al. *Neurol India* 2012.

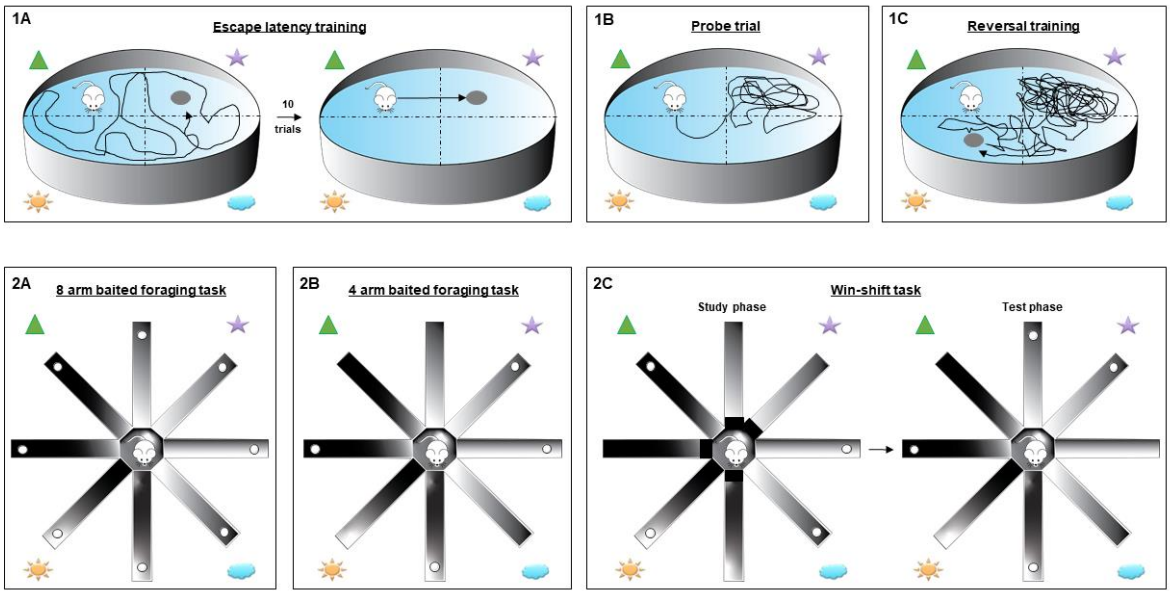

Figure 1.7. Common tasks employed in the Morris water maze (panel 1) and the eight-arm radial arm maze (panel 2).

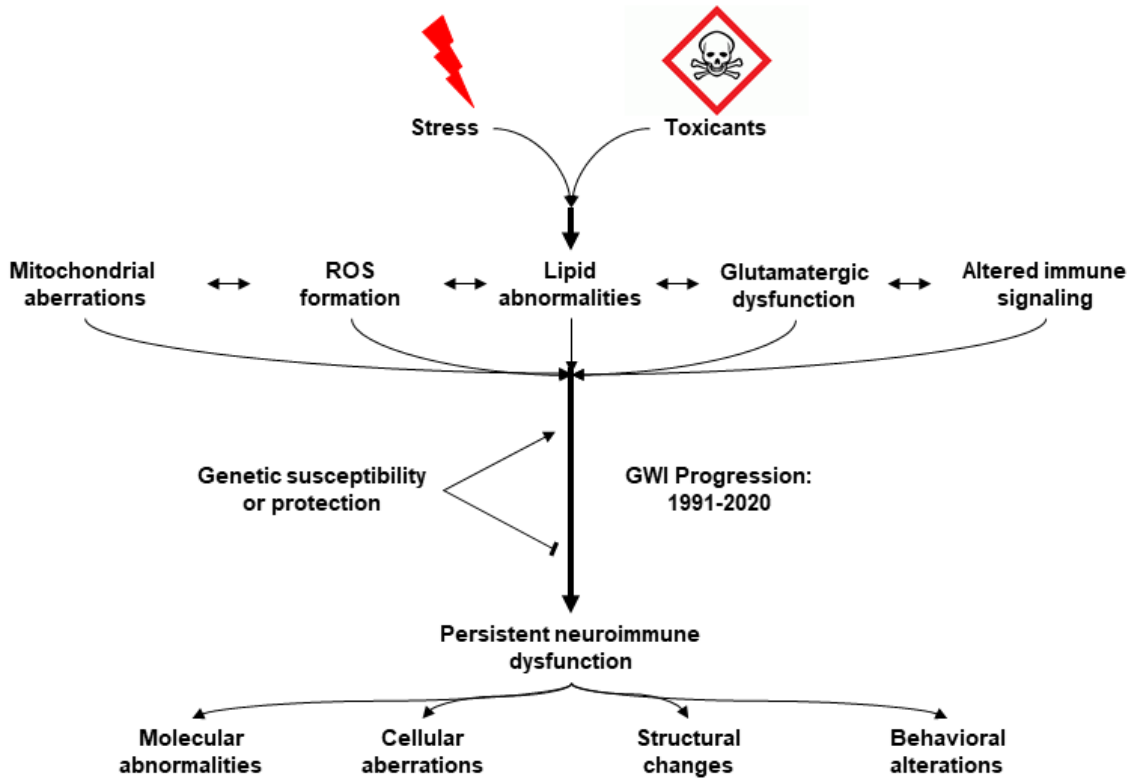

Figure 1.8. Current hypothesis of the initiation and progression of GWI pathophysiology.

Abbreviations: ROS, radical oxygen species.

Table 1.1. Differential expression of receptors along the hippocampal dorsoventral axis.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-Methyl-D-aspartate; mGluR, metabotropic glutamate receptor; GABA, γ-aminobutyric acid; CB, cannabinoid; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; RyR, ryanodine receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor. See subsection 1.2.6 for references.

Receptor	**Expression in the dH**	**Expression in the vH**
AMPA	Greater than in the vH	
NMDA	Greater than in the vH	
Class I mGluR	Greater than in the vH	
α_1 $GABA_A$	Greater than in the vH	
$\alpha_{2/5}$ $GABA_A$		Greater than in the dH
$GABA_B$	Greater than in the vH	
CB_1	Greater than in the vH	
mAChR	Slightly greater than in the vH	
nAChR		Slightly greater than in the dH
RyR1		Greater than in the dH
GR	Greater than in the vH	
MR		Greater than in the dH

CHAPTER 2

DORSOVENTRAL-SPECIFIC EFFECTS OF NERVE AGENT SURROGATE DIISOPROPYLFLUOROPHOSPHATE ON SYNAPTIC TRANSMISSION IN THE MOUSE HIPPOCAMPUS[1]

[1] Brown KA, Filipov NM, Wagner JJ. *Dorsoventral-Specific Effects of Nerve Agent Surrogate Diisopropylfluorophosphate on Synaptic Transmission in the Mouse Hippocampus.* Journal of Pharmacology and Experimental Therapeutics. 2020 Apr; 373 (1) 10-23.
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Abstract

Although there has been an increasing appreciation for functional differences between the dorsal (dH) and ventral (vH) hippocampal sectors, there is a lack of information characterizing the cholinergic and noncholinergic mechanisms of acetylcholinesterase inhibitors on synaptic transmission along the hippocampal dorsoventral axis. Diisopropylfluorophosphate (DFP) is an organophosphate (OP) that is commonly employed as a nerve agent surrogate in vitro as well as in rodent models of disease states, such as Gulf War Illness. The present study investigated the cholinergic and noncholinergic mechanisms responsible for the effects of acute DFP exposure on dH and vH synaptic transmission in a hippocampal slice preparation. A paired-pulse extracellular recording protocol was used to monitor the population spike (PS) amplitude as well as the PS paired-pulse ratio (PS-PPR) in the CA1 subfield of the dH and the vH. We observed that DFP-induced PS1 inhibition was produced by a cholinergic mechanism in the dH, whereas a noncholinergic mechanism was indispensable in mediating the inhibitory effect of DFP on the PS1 in the vH. PS-PPR in both dH and vH sectors was increased by acute DFP exposure, an effect that was blocked by an N-methyl-D-aspartate receptor antagonist but not by cholinergic antagonists. Clinical reports have indicated dorsoventral-specific hippocampal abnormalities in cases of OP intoxications. Therefore, the observed dorsoventral-specific noncholinergic mechanisms underlying the effects of DFP on hippocampal synaptic transmission may have important implications for the treatment of OP overexposures.

2.1 Introduction

The hippocampus is a bilateral structure located in the medial temporal lobe of mammalian organisms and plays a fundamental role in learning and memory. Over the past decade, there has been increasing appreciation for differential functionality between the posterior (dorsal,[dH]) and anterior (ventral,[vH]) hippocampal sectors (Bannerman et al., 2004; Fanselow and Dong, 2010). For instance, the dH is associated with spatial memory whereas the vH is linked to emotional and stress-related responses (Bannerman et al., 2004). In both sectors, hippocampal-dependent behavior is regulated by synaptic plasticity and transmission (Whitlock et al., 2006). Hippocampal synaptic transmission is driven by excitatory neurotransmitters like glutamate and shaped by inhibitory neurotransmitters such as γ-aminobutyric acid (GABA, Watkins and Evans, 1981). Another neurotransmitter, acetylcholine (ACh), modulates hippocampal pyramidal cell activity (Benardo and Prince, 1982a; Benardo and Prince, 1982b; Madison et al., 1987), consequently influencing hippocampal memory functions (Hasselmo, 2006). For instance, cholinergic projections from the medial septum and diagonal band to the hippocampal formation are involved in controlling hippocampal theta rhythms (Stewart and Fox, 1990; Vertes and Kocsis, 1997; Hasselmo, 2006). While there is some evidence for differential cholinergic projection (Milner et al., 1983; Amaral and Kurz, 1985) and receptor density (Ruiz et al., 1993; Jung et al., 1994; Huang and Winzer-Serhan, 2006; Kenney et al., 2012) in the dH compared to the vH, there is a paucity of reports investigating the distinct role of ACh in synaptic transmission along the hippocampal dorsoventral axis.

Organophosphates (OPs) are potent neurotoxicants that are frequently employed in agricultural and military settings (Naughton and Terry Jr, 2018). OPs phosphorylate the serine hydroxyl group in the catalytic triad of acetylcholinesterase (AChE), irreversibly inhibiting the enzyme (Abou-Donia, 2003). Subsequent enhancement of synaptic ACh levels leads to cholinergic modulation of hippocampal glutamatergic transmission via activation of metabotropic muscarinic (mAChR) and ionotropic nicotinic (nAChR) ACh receptors. If not aggressively

treated, acute OP-induced cholinergic hyperactivity precipitates the presentation of cholinergic crisis followed by glutamatergic-mediated persistence of seizures and ultimately death (Abou-Donia et al., 2016).

Exposure to OPs also induces persisting toxicity via multiple noncholinergic mechanisms, i.e., by affecting oxidant formation, mitochondrial energetics, axoplasmic transport, immune system functionality, and brain endocannabinoid homeostasis (Casida and Quistad, 2004; Pope et al., 2005; Mense et al., 2006; Nallapaneni et al., 2006; Kaur et al., 2007; Grigoryan et al., 2008; Nallapaneni et al., 2008; Grigoryan et al., 2009; Jiang et al., 2010; Middlemore-Risher et al., 2010; Wright et al., 2010; Rohlman et al., 2011; Banks and Lein, 2012; O'Callaghan et al., 2015; Locker et al., 2017; Miller et al., 2018; Naughton et al., 2018; Naughton and Terry Jr, 2018; Michalovicz et al., 2019). Nevertheless, it is unknown if such noncholinergic effects differentially impact synaptic transmission along the hippocampal dorsoventral axis.

Low-level exposure to OPs in a variety of settings has been consistently associated with learning and memory impairments in humans. Repeated subacute OP insecticide exposure in agricultural settings has been linked with impairments in short-term memory (Kaplan et al., 1993; Eaton et al., 2008; Rohlman et al., 2011; Ross et al., 2013). Aerotoxic syndrome, an occupational disease linked to exposure to OPs circulating in unfiltered aircraft cabin air, is associated with cognitive deficits (Michaelis et al., 2017). Additionally, civilians exposed to OP nerve agents in the Tokyo metro terror attacks (Hood, 2001; Nishiwaki et al., 2001; Miyaki et al., 2005) and in Kurdistan during the Iran-Iraq war (Talabani et al., 2018) present persisting neurological impairments, including cognitive deficits. Moreover, U.S. veterans of the 1990-1991 Persian Gulf War exhibit dose-dependent associations between OP exposure and impaired performance on neurobehavioral tasks (Proctor et al., 2006), as well as decreased white matter volume (Heaton et al., 2007). Notably, the performance of veterans with Gulf War Illness (GWI)

and predicted sarin exposure in a learning and memory task is negatively correlated with estimated exposure levels (Chao et al., 2017).

The prototypical alkylphosphate OP diisopropylfluorophosphate (DFP) was developed for the study of chemical weapons (Saunders, 1957). DFP is structurally homologous, but significantly less volatile, than nerve agents like sarin, making DFP useful for investigating the mechanisms of sarin and other highly toxic OPs. Accordingly, DFP is one of the most widely studied OP alkylphosphates in the laboratory (Prendergast et al., 1997; Prendergast et al., 1998; Pope et al., 2005; Grigoryan et al., 2008; Nallapaneni et al., 2008; Wright et al., 2010; Terry Jr et al., 2011; Terry Jr et al., 2012; Ferchmin et al., 2015; Naughton et al., 2018; Naughton and Terry Jr, 2018). Moreover, DFP is currently employed as an initiator of status epilepticus in seizure models (Deshpande et al., 2010; Todorovic et al., 2012; Pouliot et al., 2016) and a nerve agent surrogate in models of Gulf War Illness (O'Callaghan et al., 2015; Phillips et al., 2019). Nonetheless, there is a lack of information characterizing the cholinergic and noncholinergic mechanisms of OPs like DFP on hippocampal synaptic transmission. While there is evidence of OPs inducing dorsoventral-specific pathology in the hippocampus (Apland et al., 2010), there are no reports in the literature investigating dorsoventral-specific effects of AChE inhibitors on hippocampal synaptic transmission. It is also unknown if dorsoventral-specific effects of AChE inhibitors are dependent upon cholinergic and noncholinergic mechanisms resulting from the functional division of the two hippocampal sectors. Therefore, using a hippocampal slice preparation, the aim of the present work was to investigate the cholinergic and noncholinergic mechanisms that mediate the effects of DFP exposure on synaptic transmission in the dH and the vH.

2.2 Methods

2.2.1 Animal Maintenance

Male C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice, aged 7-13 weeks, were housed in groups of 5. Animals were acclimated to their home cages for at least 1 week after

arrival and were maintained on a 12 h light/dark cycle (lights on from 0700-1900 h). Food and water were provided *ad libitum*. All experiments were performed in compliance with the University of Georgia Animal Care and Use Committee guidelines.

2.2.2 Slice Preparation and Electrophysiology

Hippocampal slice preparation and electrophysiology experiments were conducted as previously described (Keralapurath et al., 2017; Preston et al., 2019). Briefly, mice were sacrificed following deep anesthetization with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane). Brains were removed and quickly submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120mM NaCl, 3mM KCl, 4mM $MgCl_2$, 1mM NaH_2PO_4, 26mM $NaHCO_3$, and 10mM glucose). A mid-sagittal cut was made, and half of the brain was mounted on its caudal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm slices containing the dH. The other half of the brain was mounted on its rostral pole and sectioned along the horizontal plane with a vibratome to acquire 400 µm slices containing the vH. The dH and vH were subdissected free from the rest of the slice and the CA3 subfield was removed. These slices were then quickly placed in a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120mM NaCl, 3mM KCl,1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, and 10mM glucose). Slices were continuously perfused with ACSF (1 mL/min) during a 45 min recovery period at room temperature followed by a 45 min recovery period at the recording temperature of 30 °C. Schaffer collateral fibers were stimulated by placing a bipolar electrode (Kopf Instruments, Tujunga, CA) in the stratum radiatum of the CA1 subfield whereas a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments, Sarasota, FL) was inserted in the stratum pyramidale of CA1. During recording experiments, a stimulus pulse was applied once every minute to elicit responses from Schaffer collateral-CA1 synapses.

A graphical representation of the protocol is shown in Fig.2.1A. A stimulus- response curve (I/O curve) was acquired at the beginning of each experiment by incrementally delivering

single 20 µA stimulus pulses ranging from 40-200 µA in intensity and measuring the population spike (PS1) response (mV) from PS trough to PS peak. The stimulus intensity was adjusted to elicit stable baseline responses of 75% of the maximal PS1 amplitude. Baseline paired-pulse ratio (PS-PPR) was then monitored each minute for 30 min via paired-pulse stimulation with an interpulse interval of 10 ms. Baseline responses were recorded for 40 min during antagonist wash-in experiments. Antagonists were present for the entire duration of the experiment following 20 min of antagonist-free baseline recording. After establishing the baseline response, DFP was washed-in to the bath for 30 min and an I/O curve was immediately recorded at the end of the wash-in period. Slice PS-PPR was monitored for the following 1 h. A final I/O curve was measured to determine the persisting effects of DFP exposure on basal synaptic transmission.

2.2.3 Determination of AChE Activity

Briefly, each assay was completed using 400 µm individual dH or vH slices superfused with 1 mL/min ACSF ± DFP for 4.75 h ± 15 min at 37 °C. Slices were immediately stored at -80°C following completion of electrophysiology experiments. On the day of the assay, slices were kept frozen on dry ice until homogenized with Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT) in ice-cold 0.05 M Tris-HCl (pH 7.4) buffer. Sixty slices (30 from the dH and 30 from the vH) were randomly selected for homogenization. Six slices from each sector (dH or vH) and treatment level (0, 3, 10, 30, and 100 µM DFP) were prepared for the activity assay. AChE activity of each slice homogenate was measured in triplicate wells (3 replicates/slice homogenate).

AChE activity was measured spectrophotometrically using a modification (Chambers et al., 1988; Chambers and Chambers, 1989) of Ellman et al. (1961) with acetylthiocholine as the substrate and 5,5'-dithio-bis(nitrobenzoic acid) as the chromogen. The method from Chambers et al. (1988) was scaled to a microplate format. Following 20 min incubation of each slice homogenate with the reaction mixture described in Chambers et al. (1988), absorbance was

measured via BioTek Epoch™ Microplate spectrophotometer at 412 nM. An AChE standard curve was included alongside the samples at the following concentrations: 0, 1, 3, 10, 30, 100, 300, 1000 mU/mL (diluted in 0.05 M Tris-HCl [pH 7.4] buffer; AChE source: *Electrophorus electricus* [Sigma-Aldrich, St. Louis, MO]). Physostigmine hemisulfate (10 µM) was incubated with a subset of control samples to serve as the positive control. AChE enzyme activity was normalized on a per mg of tissue protein basis. Protein content in the homogenized samples was quantified via the Bradford protein assay method (Bradford, 1976) in a 96-well plate using Bradford reagent from Bio-Rad (Hercules, CA). Bovine serum albumin (Fisher Scientific, Pittsburgh, PA) was used as the standard for the protein quantification assay.

2.2.4 Drugs

AFDX-116, atropine sulfate, acetylthiocholine iodide, acetylcholinesterase, 5,5'-dithio-bis(nitrobenzoic acid), and diisopropylfluorophosphate (Purity: 99%, Lot: MKCD1107) were purchased from Sigma-Aldrich (St. Louis, MO). Dantrolene-sodium, D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV), mecamylamine HCl, physostigmine hemisulfate were purchased from Tocris Bioscience (Minneapolis, MN). Pirenzepine was purchased from Alfa Aesar (Tewksbury, MA). 2.5 mM AFDX-116 and 20 mM dantrolene stock solutions were prepared in DMSO. 0.1% DMSO vehicle control experiments were conducted to confirm that DMSO did not induce a significant effect on population spike responses.

2.2.5 Data Analysis

Electrophysiology data was digitized at 10kHz, low-pass filtered at 1kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunnyvale, CA). Synaptic responses of the first PS (PS1) were normalized to 1 using the average PS1 amplitude of the last 5 min of baseline. Thirty min DFP wash-in and 60 min DFP wash-out PS1 values were calculated using the average PS1 amplitude values 26-30 min after beginning DFP wash in and 56-60 min into DFP wash-out, respectively. The effect of an antagonist on the PS1 was evaluated by comparing the average PS1 amplitude of the last 5 min prior to antagonist wash-in (16-20 min) compared to

the average PS1 amplitude of the last 5 min prior to DFP wash-in (36-40 min). PS-PPR values were obtained by pairing a conditioning stimulus, which evoked PS1, with a second stimulus, evoking PS2, and calculating the percent change of PS2 with respect to PS1 (PS-PPR = PS2/PS1). Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. All statistical analysis and graphic production were completed using R version 3.3.2 (The R Foundation for Statistical Computing, Vienna, Austria). Statistical analysis was completed by using the aov() function for analysis of variance (ANOVA) and the t.test function for the Student's *t*-test. Graphic production was completed via R package "ggplot2". DFP dose-response curve (DRC) statistical analysis was conducted via one-way repeated measures ANOVA followed by Dunnett's multiple comparison test. The Student's *t*-test (one-tailed) was utilized for all other pairwise comparisons of data. An α level of 0.05 was used as the criterion for statistical significance. All data are presented as mean ± SEM.

2.3 Results

2.3.1 Population spike inhibition and concurrent population spike paired-pulse facilitation were observed in dorsal and ventral hippocampal slices following DFP wash-in.

A significant main effect of DFP exposure on the PS_1 amplitude was observed in the dH ($F_{4,39}=38.2$, $p < 0.001$) and the vH ($F_{4,38}=46.6$, $p < 0.001$; Fig. 2.1B,2.1C, respectively). A Dunnett's multiple comparison test revealed significant inhibition of the PS_1 amplitude in dH ($p < 0.05$) and vH ($p < 0.001$) slices exposed to 30 μM DFP compared to unexposed slices. Additionally, Dunnett's test revealed that 100 μM DFP wash-in induced significant PS1 inhibition in dH ($p < 0.001$) and vH slices ($p < 0.001$) compared to unexposed slices. Interestingly, marginal, nonsignificant recovery of the PS1 amplitude was observed in both dH and vH slices treated with 100 μM DFP. DH and vH slices that were not exposed to DFP displayed no significant change in PS1 amplitude approximately 2 h after establishing baseline. A one-way ANOVA (DFP treatment as a factor) revealed a significant main effect of DFP on AChE activity, indicating that dH and vH slices exposed to DFP exhibited a significant reduction in AChE

activity ($F_{4,55}=15.2$, $p<0.001$; IC50 = 2.70 µM; Fig. 2.1D,2.1E). DH and vH slices were combined in AChE activity assay analyses as a two-way ANOVA (sector and DFP treatment as factors) revealed no significant main effect of hippocampal sector on AChE activity ($F_{1,50}=0.0220$, $p=0.88$). Based on these results, 30 µM DFP was utilized as the DFP concentration for all remaining experiments throughout this study.

2.3.2 Nonselective nAChR antagonism enhances DFP-mediated population spike inhibition in the dorsal and the ventral hippocampus.

The nonselective, noncompetitive nAChR antagonist mecamylamine (MEC) was employed to investigate the role of the nAChR in DFP-mediated PS1 inhibition. Pretreatment with 30 µM MEC significantly enhanced DFP-mediated PS1 inhibition in the dH ($t(17)=2.00$, $p=0.031$) and the vH ($t(15)=1.86$, $p=0.042$) compared to 30 µM DFP alone (Fig. 2.2A,2.2B, respectively). Furthermore, significantly enhanced PS1 inhibition in the dH ($t(17)=2.77$, $p=0.0066$) and the vH ($t(15)=4.26$, $p<0.001$) was observed following 60 min DFP wash-out. The magnitude of PS1 inhibition in dH slices treated with DFP and MEC was significantly less than the inhibition observed in the vH after 30 min DFP wash-in ($t(18)=2.74$, $p=0.0068$) and 60 min DFP wash-out ($t(18)=2.82$, $p=0.0057$). MEC alone did not induce a significant effect on PS1 amplitude in the dH or the vH.

2.3.3 Nonselective mAChR blockade prevents DFP-mediated population spike inhibition in the dorsal hippocampus but not in the ventral hippocampus.

The nonselective mAChR antagonist atropine (ATR) was selected to investigate the role of the mAChR in DFP-mediated PS1 inhibition. Preexposure to 3 µM ATR prevented DFP-mediated PS1 inhibition the dH ($t(13)=3.32$, $p=0.0027$; Fig. 2.2C). Interestingly, PS1 in the dH was enhanced following 60 min DFP wash-out ($t(13)=4.11$, $p<0.001$). Conversely, ATR pretreatment prevented DFP-induced PS1 inhibition in the vH after 30 min application of DFP ($t(15)=2.20$, $p=0.022$), but this effect did not persist following 60 min DFP wash-out ($t(15)=0.0941$, $p=0.46$; Fig. 2.2D). The magnitude of PS1 inhibition between dH and vH slices

treated with DFP and ATR was significantly different after 30 min DFP wash-in (t(14)=3.41, p=0.0021) and 60 min DFP wash-out (t(14)=2.64, p=0.0097). ATR alone did not induce a significant effect on PS1 amplitude in the dH or the vH.

2.3.4 Selective M2R, but not M1R, mAChR blockade prevents DFP-mediated population spike inhibition in the dorsal hippocampus but not in the ventral hippocampus.

Pretreatment of slices with 100 nM of the M1 mAChR (M1R) selective antagonist pirenzepine (PZP) did not prevent DFP-mediated PS1 inhibition in either the dH (t(12)=0.645, p=0.27) or the vH (t(13)=0.126, p=0.45; Fig. 2.2E,2.2F, respectively). Pretreatment with 300 nM AFDX-116, a selective M2 mAChR (M2R) antagonist, prevented DFP-mediated PS1 inhibition in the dH (t(17)=2.01, p=0.030; Fig. 2.2G). Strikingly, PS1 in the dH quantitively resembled the enhancement observed in ATR experiments following 60 min DFP wash-out (t(17)=3.08, p=0.0034). Conversely, AFDX-116 pretreatment had no significant effect on DFP-induced PS1 inhibition in the vH after 30 min application of DFP (t(14)=1.02, p=0.16) nor after 60 min DFP wash-out (t(14)=0.277, p=0.39; Fig. 2.2H). The magnitude of PS1 inhibition in dH and vH slices treated with DFP and AFDX-116 was significantly different following 30 min DFP wash-in (t(17)=2.27, p=0.018) and 60 min DFP wash-out (t(17)=3.02, p=0.0039). Neither PZP nor AFDX-116 alone induced a significant effect on PS1 amplitude in the dH or the vH.

2.3.5 Concurrent nonselective mAChR and nAChR blockade prevents DFP-mediated population spike inhibition in the dorsal hippocampus but not in the ventral hippocampus.

Coapplication of 3 μM ATR and 30 μM MEC to dH slices prior to wash-in of DFP prevented the DFP-mediated PS1 inhibition after 30 min of DFP application (t(12)=2.18, p=0.025) and 60 min of DFP wash-out (t(12)=2.16, p=0.026; Fig. 2.3A). The DFP-mediated PS1 inhibition was significantly prevented in the vH following 30 min DFP application (t(13))=2.09, p=0.028), but the effect of cholinergic antagonist pretreatment did not persist following a 60 min DFP wash-out period (t(13)=0.649, p=0.26; Fig. 2.3B). The dorsoventral-specific effect of DFP-mediated PS1 inhibition observed in previous cholinergic antagonist experiments was also

observed in ATR and MEC co-application experiments, where dH PS1 was significantly greater than vH PS1 following 30 min DFP wash-in ($t(11)=1.80$, $p=0.049$) and 60 min DFP wash-out ($t(11)=2.96$, $p=0.0065$) of DFP. Coapplication of ATR and MEC did not induce a significant effect on PS1 amplitude in the dH or the vH.

2.3.6 M2R, NMDAR, and nonselective nAChR blockade prevents DFP-mediated population spike inhibition in the dorsal and the ventral hippocampus.

Coapplication of 300 nM AFDX-116, 25 µM D-APV (an N-methyl-D-aspartate receptor [NMDAR] competitive antagonist), and 30 µM MEC prior to wash-in of DFP resulted in no observable effect of DFP on the PS1 in the dH ($t(14)=1.89$, $p=0.040$) or the vH ($t(15))=2.86$, $p=0.0059$; Fig. 2.3C,2.3D, respectively). Prevention of DFP-mediated PS1 inhibition persisted in the dH ($t(14)=4.42$, $p<0.001$) and the vH ($t(15)=2.12$, $p=0.026$) following 60 min of DFP wash-out. The dorsoventral-specific effect of DFP-mediated PS1 inhibition that was present in cholinergic antagonist pretreatment experiments was not observed in this triple antagonist wash-in experiment following 30 min DFP wash-in ($t(15)=0.808$, $p=0.22$) and 60 min DFP wash-out ($t(15)=0.124$, $p=0.45$). Coapplication of AFDX-116, D-APV, and MEC did not induce a significant effect on PS1 amplitude in the dH or the vH. These data and those previously shown suggest that DFP-induced PS1 inhibition is mediated by the M2R in the dH whereas the effect of DFP is mediated by both cholinergic receptors and the NMDAR in the vH.

2.3.7 NMDAR antagonism prevents DFP-mediated population spike inhibition in the dorsal and the ventral hippocampus.

Pretreatment of dH slices with 25 µM D-APV did not significantly prevent DFP-mediated PS1 inhibition following 30 min DFP wash-in ($t(15)=1.33$, $p=0.10$); however, a significant reduction in PS1 inhibition was observed following 60 min DFP wash-out ($t(15)=3.12$, $p=0.0035$; Fig. 2.3E). D-APV pretreatment of vH slices did not significantly prevent DFP-mediated PS1 inhibition compared to slices that received DFP alone following 30 min DFP wash-in ($t(12)=1.24$, $p=0.12$) or 60 min DFP wash-out ($t(12)=1.05$, $p=0.15$; Fig. 2.3F); however,

vH slices pretreated with D-APV did not display significantly different PS1 amplitude compared to control vH slices at the 30 min DFP wash-in ($t(15)=1.69$, $p=0.56$) or the 60 min DFP wash-out timepoint ($t(15)=0.421$, $p=0.34$). Interestingly, the magnitude of DFP-induced PS1 inhibition was not significantly different in the dH compared to the vH following 30 min DFP wash-in ($t(13)=1.56$, $p=0.072$) and 60 min DFP wash-out ($t(13)=0.692$, $p=0.25$) when D-APV was present in the bath. Prior to DFP application D-APV alone did not induce a significant effect on PS1 amplitude in the dH or the vH (Supplemental Figure 2.2A, Supplemental Figure 2.2B, respectively).

2.3.8 RyR antagonism enhances DFP-mediated population spike inhibition in the dorsal and the ventral hippocampus.

As the NMDAR is involved in hippocampal Ca2+-induced Ca2+ release, we investigated the role of intracellular Ca2+ ([Ca2+]i) stores in DFP-mediated PS1 inhibition by blocking ryanodine receptors (RyRs). Pretreatment with 20 µM dantrolene (DAN) significantly enhanced DFP-mediated PS1 inhibition in the dH ($t(14)=4.88$, $p<0.001$) and the vH ($t(12)=2.44$, $p=0.016$) compared to 30 µM DFP alone (Fig. 2.3G,2.3H, respectively). Furthermore, inhibition of the PS_1 in the dH ($t(14)=8.11$, $p<0.001$) and the vH ($t(12)=4.33$, $p<0.001$) was significantly enhanced following 60 min DFP wash-out. There was no significant difference in PS_1 inhibition in dH and vH slices pretreated with DAN after 30 min DFP wash-in ($t(12)=1.07$, $p=0.15$) and 60 min DFP wash-out ($t(12)=0.117$, $p=0.45$). DAN alone did not induce a significant effect on PS1 amplitude in the dH or the vH.

2.3.9 Population spike paired-pulse facilitation observed in dorsal and ventral hippocampus slices exposed to DFP is mediated by a noncholinergic mechanism.

The PS-PPR recorded from control dH and vH slices initially exhibit PS-paired-pulse inhibition (PS-PPI, ratio <1.0), an indicator of functional recurrent inhibition in the CA1 subfield (Supplemental Figure 2.1A, Supplemental Figure 2.1B). Following 30 µM DFP treatment, PS-paired-pulse facilitation (PS-PPF, ratio >1.0) in the dH ($t(7)=3.00$, $p=0.010$) and the vH

(t(7)=3.29, p=0.0067) was observed (Fig.2.4A,2.4B). Persisting PS-PPF was observed in the dH (t(7)=4.26, p=0.0019) and the vH (t(7)=3.20, p=0.0075) following DFP wash-out. DH (t(6)=0.380, p=0.36) and vH (t(10)=1.34, p=0.11) slices that were not exposed to DFP displayed no significant change in PS-PPR approximately 2 h after establishing baseline (data not shown). Pretreatment of dH slices with the nAChR antagonist MEC resulted in significantly enhanced PS-PPR compared to slices that received DFP alone (t(17)=1.84 p=0.040; Fig. 2.4A). Likewise, pretreatment of vH slices with MEC resulted in significantly enhanced PS-PPR compared to slices that received DFP alone (t(15)=2.60 p=0.010; Fig. 2.4B). DH slices pretreated with the mAChR antagonist ATR did not exhibit significantly different PS-PPR (t(13)=0.958, p=0.18) from slices that received only DFP (Fig. 2.4C). Moreover, we did not observe a significant difference in PS-PPR in vH slices pretreated with ATR compared to those that received DFP alone (t(15)=1.38, p=0.09; Fig. 2.4D). Preexposure of dH slices to the M1R selective antagonist PZP did not prevent the significant increase of PS-PPR observed following DFP wash-in (t(12)=0.0937, p=0.46; Fig. 2.4E). Likewise, preexposure of vH slices to PZP did not prevent the significant increase of PS-PPR after DFP wash-in (t(13)=0.0622, p=0.48; Fig. 2.4F). However, dH slices that received preexposure to the M2R selective antagonist AFDX-116 exhibited significantly reduced PS-PPR compared to slices that received DFP alone (t(17)=1.89, p=0.038; Fig. 2.4G) whereas pretreatment of vH slices with AFDX-116 had no effect on PS-PPR following DFP wash-in (t(14)=0.372, p=0.36; Fig. 2.4H). Coexposure of dH (t(12)=0.488, p=0.32) and vH (t(13)=1.12, p=0.14) slices to ATR and MEC did not result in a significant reduction in the PS-PPR that was observed following DFP wash-in (Fig. 2.5A,2.5B, respectively). Together, these data suggest that cholinergic receptors do not mediate the PS-PPF that is observed following exposure of dH and vH slices to 30 μM DFP.

2.3.10 NMDAR blockade prevents population spike paired-pulse facilitation that is observed in dorsal and ventral hippocampus slices exposed to DFP.

Interestingly, PS-PPI was preserved in both dH ($t(14)=1.82$, $p=0.045$) and vH ($t(15)=2.24$, $p=0.020$) slices treated with AFDX-116, MEC, and the NMDAR antagonist D-APV prior to DFP exposure (Fig. 2.5C,2.5D, respectively). PS-PPI persisted in dH ($t(14)=2.98$, $p=0.0049$) and vH ($t(15)=2.21$, $p=0.021$) slices preexposed to the three antagonists after 60 min DFP wash-out. No significant difference in PS-PPR was observed between dH and vH slices that received triple antagonist pretreatment. Additionally, 20 min exposure to only D-APV resulted in a significant reduction in PS-PPR in dH ($t(8)=2.17$, $p=0.031$) and vH ($t(5)=2.19$, $p=0.040$) slices (Supplemental Figure 2C, Supplemental Figure 2D, respectively). D-APV pretreatment alone preserved PS-PPI in the dH ($t(15)=3.24$, $p=0.0028$) and the vH ($t(12)=2.28$, $p=0.021$) following DFP wash-in (Fig. 2.5E,2.5F, respectively). When D-APV was the only antagonist present in the bath, PS-PPI persisted in the dH and vH following 60 min DFP wash-out ($t(15)=3.59$, $p=0.0013$; $t(12)=2.68$, $p=0.010$; respectively). No significant difference in PS-PPR was observed between dH and vH slices pretreated with D-APV following DFP wash-in ($t(13)=0.436$, $p=0.33$) and 60 min DFP wash-out ($t(13)=0.720$, $p=0.24$). Additionally, pretreatment with the RyR antagonist DAN resulted in a significant enhancement of PS-PPF observed in the dH ($t(14)=3.22$, $p=0.0031$) and the vH ($t(12)=2.42$, $p=0.016$) following DFP exposure (Fig. 2.5G,2.5H, respectively). Collectively, these data suggest that the NMDAR plays an indispensable role in mediating the PS-PPF that is observed in dH and vH slices exposed to 30 μM DFP.

2.4 Discussion

The data in the present report demonstrate that DFP-induced PS1 inhibition in the dH and vH CA1 subfield is mediated by cholinergic receptors and NMDARs. Increased PS-PPR observed in dH and vH slices exposed to DFP is prevented by an NMDAR antagonist. To the best of our knowledge, this is the first report characterizing both cholinergic and noncholinergic

effects of OP exposure on dH and vH synaptic transmission in a hippocampal slice preparation. Additionally, our findings of a DFP-NMDAR interaction affecting synaptic transmission are novel. Our results provide insight into the circuit-level role of the NMDAR in maintaining functional recurrent inhibition (RI) in the dH and the vH following exposure to an OP. The observed dorsoventral-specific effects may provide insight into OP-induced deficits in cognition and mood. A circuitry schematic containing the minimal components needed to explain the proposed dorsoventral-specific effects is presented in Fig. 2.6.

DFP has previously been employed to investigate the acute effects of an OP on the PS1, a response variable which can be directly correlated to the number of functional pyramidal cells in the stratum pyramidale of CA1 (Williamson and Sarvey, 1985; Jones et al., 1990; Ferchmin et al., 2015). Our results are consistent with these prior reports indicating acute DFP exposure inhibits CA1 glutamatergic transmission and that a substantial component of this inhibition is mediated via cholinergic receptors. More specifically, following irreversible inhibition of AChE, enhanced synaptic ACh leads to hyperactivation of mAChRs, a well-established mechanism of OP-induced neurotoxicity (Abou-Donia et al., 2016). Previous studies have shown that mAChR blockade via ATR pretreatment partially prevents DFP-induced PS1 inhibition (Ferchmin et al., 2015). The present study showed that ATR pretreatment not only prevented DFP-mediated inhibition of the dH PS1 amplitude, but the amplitude was in fact significantly enhanced by DFP application. Similarly, dH slices pretreated with AFDX-116 quantitatively resembled ATR experiments, suggesting that DFP-mediated PS1 inhibition is due to activation of M2Rs located in dH CA1. These receptors may be located at the Schaffer Collateral-CA1 synapse and somata of pyramidal cells (Vilaró et al., 1992; Auerbach and Segal, 1996; Seeger and Alzheimer, 2001; Seeger et al., 2004), where activation of these M2Rs enhances K+ conductance, hyperpolarizing principal cells and decreasing excitatory glutamatergic transmission (Drever et al., 2011). In support of these findings, prior evidence indicates OPs preferentially act as indirect agonists via M2Rs (Liu et al., 2002).

ACh-mediated activation of nAChRs on pyramidal cells enhances cation conduction, facilitating principal cell depolarization (Albuquerque et al., 2009). Interestingly, nAChR blockade enhanced DFP-mediated PS1 inhibition in both the dH and the vH. Prior evidence indicates that acute OP exposure accelerates the rate of nAChR desensitization (Tattersall, 1990), an effect that may contribute to the enhanced PS1 inhibition observed in the present study. Specifically, this enhanced inhibition may be a result of reducing the excitatory tone of α7-like nAChRs located on stratum pyramidale principal cells (Castro and Albuquerque, 1993; Mike et al., 2000). Additionally, reducing the activity of CA1 interneuron-selective interneurons following the blockade of α4β2-like nAChRs would also decrease pyramidal cell excitability, reducing the PS1 amplitude (Bell et al., 2015).

Direct interactions of OPs with cholinergic receptors are well-established (Pope, 1999; Pope et al., 2005). A number of reports have indicated that OPs and other AChE inhibitors directly compete at the agonist site of M2Rs (Bakry et al., 1988; Silveira et al., 1990; Fitzgerald and Costa, 1992; Ward et al., 1993; Ward and Mundy, 1996; Rocha et al., 1999; Santos et al., 2003) or directly phosphorylate (Bomser and Casida, 2001) M2Rs. Moreover, OPs have been shown to preferentially interact with M2/M4 mAChRs and activate their Gα/i-coupled pathways (Ward et al., 1993; Ward and Mundy, 1996). We postulate that AChE-independent mechanisms may underlie some of the results observed in the present study. In particular, no increase in AChE inhibition was observed at DFP concentrations greater than 10 μM yet progressively greater inhibition of the PS1 was observed at higher concentrations. Furthermore, we observed a noteworthy recovery of the PS1 amplitude in dose-response experiments utilizing 100 μM DFP. We attribute this recovery to wash-out of DFP following direct interaction with nAChRs on pyramidal cells. This interpretation is supported by previous reports of DFP directly inducing nAChR desensitization at concentrations near 100 μM (Albuquerque et al., 1984; Bakry et al., 1988; Eldefrawi et al., 1988).

A number of noncholinergic mechanisms mediating OP effects have been characterized. DFP and other OPs covalently interact with lysine and tyrosine residues of structural proteins such as tubulin, actin, and kinesin, resulting in axonal transport deficits (Grigoryan et al., 2008; Grigoryan et al., 2009; Jiang et al., 2010). A recent report demonstrated that subacute DFP exposure leads to structural disruptions of myelinated axons, a putative result of axonal transport deficits (Naughton et al., 2018). AChE-independent decreases in mitochondrial number and energetics following OP exposure have been described (Kaur et al., 2007; Middlemore-Risher et al., 2010). Moreover, a series of reports have emphasized the role of the NMDAR in initiating Ca2+-induced Ca2+ release mechanisms after OP exposure (Deshpande et al., 2010; Phillips et al., 2019). Interestingly, DFP has been shown to directly interact with the glutamate binding pocket of the NMDAR, irreversibly inhibiting the receptor (Johnson and Michaelis, 1992). In the present report, we observed a partial reduction of DFP-mediated pyramidal cell inhibition in dH and vH slices pretreated with an NMDAR competitive antagonist. Experiments in which slices were preexposed to AFDX-116, D-APV, and MEC completely prevented DFP-mediated pyramidal cell inhibition. There is evidence that bath application of an NMDAR antagonist disinhibits glutamatergic transmission (Čapek and Esplin, 1991; Grunze et al., 1996), which may contribute to preventing the DFP-mediated PS1 inhibition. Blockade of RyRs, receptors involved in Ca2+-induced Ca2+ release, enhanced DFP-mediated PS1 inhibition in the dH and the vH, an effect that may involve decreased pyramidal cell excitability due to disruption of [Ca2+]i homeostasis. There is also evidence suggesting OPs directly interact with receptors involved in Ca2+-induced Ca2+ release (Sun et al., 2000), an interaction that may further contribute to the disruption of [Ca2+]i homeostasis. Noncholinergic mechanisms unexplored in the present study, such as endocannabinoid signaling, promote OP-induced modulations in the hippocampus (Nallapaneni et al., 2006; Nallapaneni et al., 2008; Wright et al., 2010; Hoffman et al., 2019). Future studies investigating such noncholinergic

mechanisms may be fruitful, particularly because there is a paucity of reports characterizing the effects of OPs on cannabinoid signaling along the hippocampal dorsoventral axis.

Recurrent inhibition modulates principal cell excitability and network functionality by ensuring a sufficient GABAergic inhibitory tone is present in the CA1 subfield (Andersen et al., 1963; Papatheodoropoulos and Kostopoulos, 1998). When using the double orthodromic stimulation protocol to monitor RI in the CA1 subfield of healthy hippocampal tissue (Michelson and Lothman, 1989; Sloviter, 1991), a paired-pulse interpulse interval of 10 ms results in fast-acting, ionotropic GABAAR-mediated PS paired-pulse-inhibition (PS-PPI, PS2/PS1 < 1) (Papatheodoropoulos et al., 2002; Petrides et al., 2007). It has been noted that a linear increase in PPR is observed as GABAA-R-mediated inhibition decreases (da Silva et al., 1995). Therefore, we monitored the PS-PPR as a measurement of functional synaptic inhibition in CA1, where it would be expected that a decrease in RI would result in an increase in PS-PPR. This was observed in dH and vH slices treated with 30 µM DFP, where an initial PS-PPI response was shifted to PS-PPF. Interestingly, individual or coapplication of mAChR and nAChR antagonists had no significant effect on the PS-PPF that was observed following DFP application. Nonetheless, we observed a marginal preservation of PS-PPI when slices were pretreated with AFDX-116. A previous report demonstrated that activation of mAChRs reduced the frequency of tetrodotoxin-insensitive miniature inhibitory postsynaptic currents (mIPSCs) as well as monosynaptically evoked IPSCs, resulting in weakened RI (Behrends and Ten Bruggencate, 1993). M2Rs are frequently expressed on parvalbumin-positive (PV(+)) interneurons and blocking these receptors increases GABA release from perisomatic PV(+) cells (Hajos et al., 1997; Fukudome et al., 2004; Szabó et al., 2010) as illustrated in Fig. 2.6, a result consistent with our AFDX-116 experiments.

Strikingly, NMDAR antagonist pretreatment alone or combined with cholinergic antagonists preserved PS-PPI following DFP treatment. Previous reports have shown that GABAergic input onto CA1 principal neurons is modulated by NMDAR-dependent transmission,

where bath application of D-APV reduces CA1 pyramidal cell RI postsynaptic potential by more than 30% (Grunze et al., 1996). The unique subunit composition and receptor density of NMDAR-expressing interneurons facilitate enhanced sensitivity to D-APV compared to NMDAR-expressing principal cells (Monyer et al., 1994; Martina et al., 2003; Nyiri et al., 2003; Avignone et al., 2005; Martina et al., 2013). Therefore, we postulate that the preservation of PS-PPI in D-APV pretreatment experiments is a result of the modulatory role of NMDAR-expressing interneurons. Specifically, blockade of the NMDAR results in disinhibition of pyramidal cells, thereby promoting excitation of perisomatic interneurons mediating RI and preserving PS-PPI. We also observed a significant reduction of dH and vH PS-PPR during D-APV application, which is consistent with our NMDAR-mediated disinhibition hypothesis. As this is the first report of the NMDAR modulating the PS-PPR in the presence of an AChE inhibitor, further investigation into this mechanism is warranted.

The long-range *in vivo* projections comprising the septohippocampal system are complex (Teles-Grilo Ruivo and Mellor, 2013), making the hippocampal slice preparation a sufficient but limited model for investigating cholinergic and noncholinergic mechanisms that mediate the effects of OPs on synaptic transmission along the hippocampal dorsoventral axis. At the behavioral level, the dH is associated with spatial memory whereas the vH facilitates emotional-related responses (Floresco et al., 1997; Moser and Moser, 1998; Bannerman et al., 2004; Pothuizen et al., 2004; Tamura et al., 2017). Receptor densities differ between the dH and vH sectors, resulting in intrinsic synaptic distinctions (Pandis et al., 2006; Petrides et al., 2007; Segal et al., 2010; Papatheodoropoulos, 2018). In the context of hippocampal cholinergic transmission, differential cholinergic projection (Milner et al., 1983; Amaral and Kurz, 1985) and receptor densities (Ruiz et al., 1993; Jung et al., 1994; Huang and Winzer-Serhan, 2006; Kenney et al., 2012) are found along the hippocampal dorsoventral axis. In light of these previous reports, accounting for dorsoventral-specific effects of OP exposure may be critical in interpreting the impact of exposure to these agents. For instance, we observed a reduction of

DFP-mediated pyramidal cell inhibition in dH and vH slices pretreated with an NMDAR antagonist, an effect that was statistically significant in the dH but not in the vH. These statistical differences may be attributed to significantly greater expression of GluN2A and GluN2B subunits in the dH CA1 compared to the vH CA1 (Pandis et al., 2006). This may be particularly relevant to OP exposure in a low-level exposure setting like the 1990-1991 Gulf War, where OP nerve agent exposure has been associated with a dose-dependent impairment of cognition and structural hippocampal abnormalities (Heaton et al., 2007; Chao et al., 2017). Additionally, cognitive impairments following low-level exposure to OP pesticides in agricultural settings have been reported (Rohlman et al., 2011). Given the present evidence for NMDAR involvement of DFP-mediated effects on synaptic transmission, our findings underscore the value of future investigations of therapeutic agents that target noncholinergic mechanisms in cases of low-level OP exposures that lead to persisting cognitive impairments.

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Figures

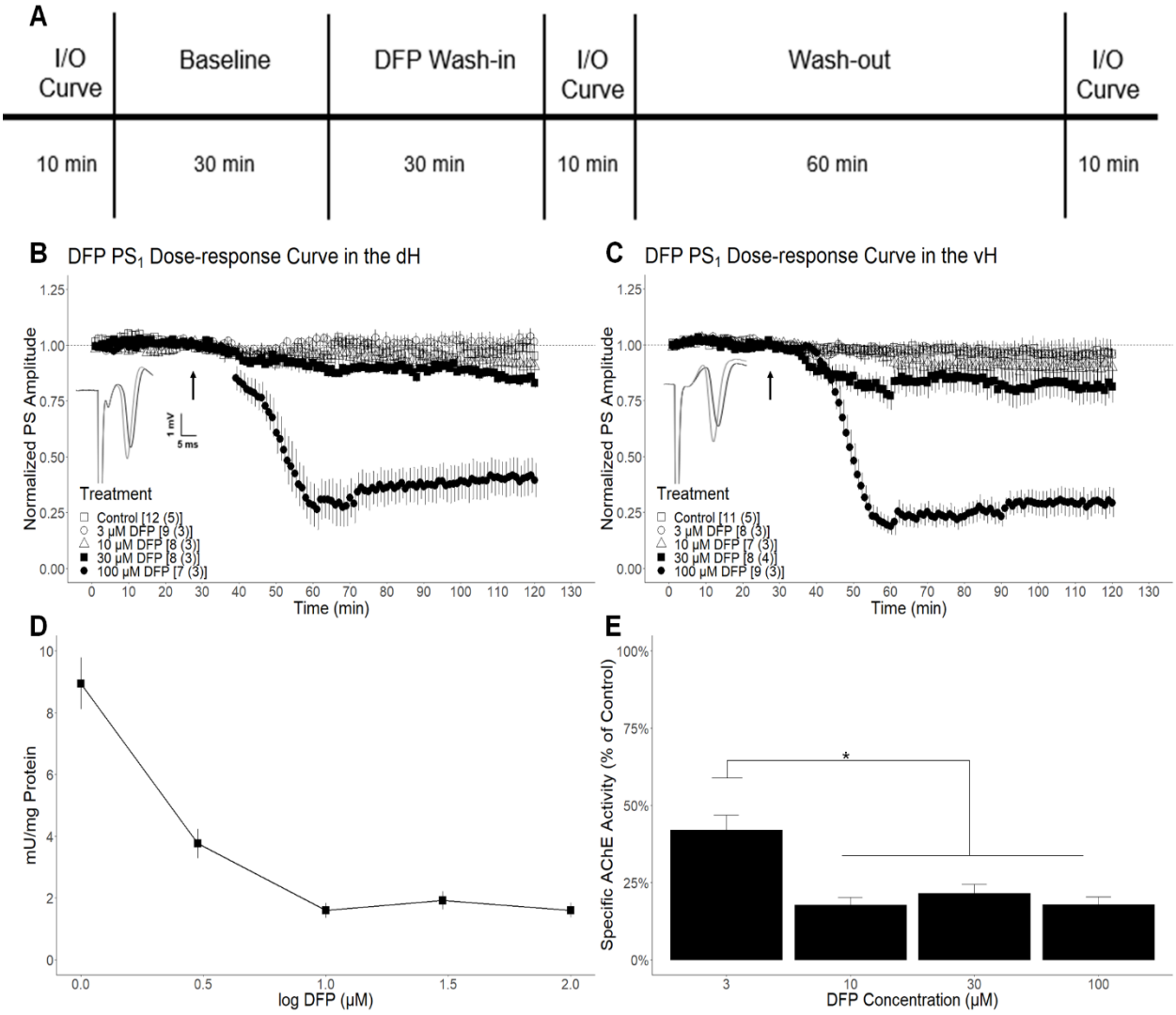


Figure 2.1. The effect of diisopropylfluorophosphate (DFP) wash-in on the population spike (PS1) and AChE activity in the dorsal (dH) and the ventral (vH) hippocampus. A. Timeline of DFP wash-in experiments. **B and C.** Results of dose-response curve experiments investigating the effect of DFP on the PS1 in the dH (**B**) and the vH (**C**). Slices were exposed to DFP as indicated by the arrow at 30 min. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. Representative traces of the baseline PS1 (gray) and the PS1 following 60 min 30 µM DFP wash-out (black) are shown. **D and E.** Acetylcholinesterase (AChE) activity in dH and vH slices exposed to DFP for 30 min and

washed with ACSF for 80 min (IC50 = 2.70 μM). Each data point represents the average AChE activity from 12 slices (collected from the dH and the vH). Data are expressed as mean ± S.E.M. I/O curve = input- output curve.

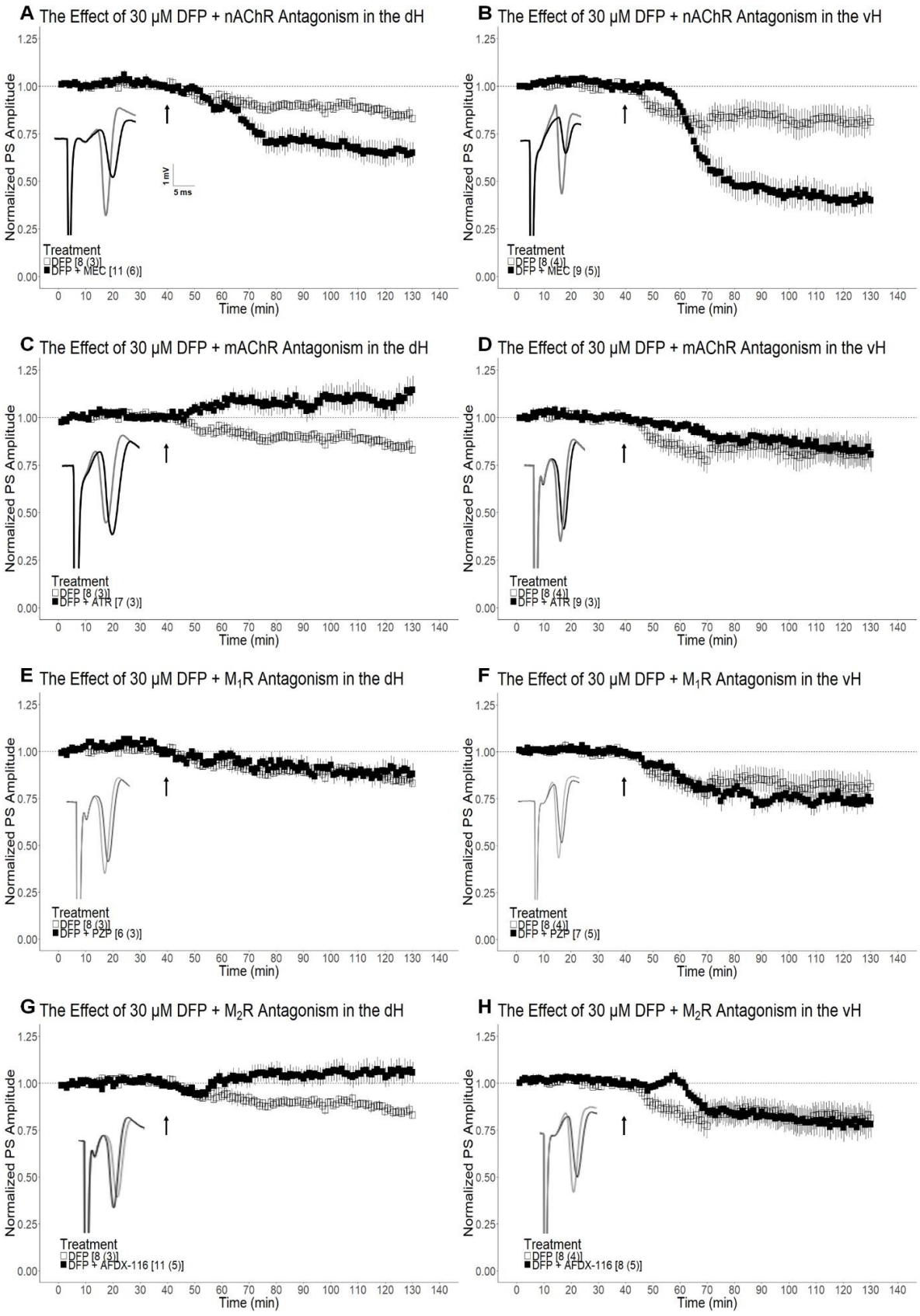

A The Effect of 30 μM DFP + nAChR Antagonism in the dH
B The Effect of 30 μM DFP + nAChR Antagonism in the vH
C The Effect of 30 μM DFP + mAChR Antagonism in the dH
D The Effect of 30 μM DFP + mAChR Antagonism in the vH
E The Effect of 30 μM DFP + M1R Antagonism in the dH
F The Effect of 30 μM DFP + M1R Antagonism in the vH
G The Effect of 30 μM DFP + M2R Antagonism in the dH
H The Effect of 30 μM DFP + M2R Antagonism in the vH
Normalized PS Amplitude
Time (min)
1 mV
5 ms
Treatment
DFP [8 (3)]
DFP + MEC [11 (6)]
DFP [8 (4)]
DFP + MEC [9 (5)]
DFP + ATR [7 (3)]
DFP + ATR [9 (3)]
DFP + PZP [6 (3)]
DFP + PZP [7 (5)]
DFP + AFDX-116 [11 (5)]
DFP + AFDX-116 [8 (5)]

Figure 2.2. Cholinergic antagonist pretreatment prevents DFP-mediated PS1 inhibition in the dH, but not in the vH. A and B. The effect of 30 μM mecamylamine (MEC) pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**A**) and the vH (**B**). **C and D.** The effect of 3 μM atropine (ATR) pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**C**) and the vH (**D**). **E and F.** The effect of 100 nM pirenzepine (PZP) pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**E**) and the vH (**F**). **G and H.** The effect of 300 nM AFDX-116 pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**G**) and the vH (**H**). In antagonist experiments, slices were pretreated with antagonist(s) following 20 min of baseline recording. Baseline was then monitored for an additional 20 min (40 min total baseline). Slices that did not receive antagonist pretreatment (□) and slices that received antagonist pretreatment (■) were exposed to DFP as indicated by the arrow at 40 min. Representative traces are shown for antagonist experiments in which the baseline PS1 (gray) and the PS1 following 60 min DFP wash-out (black) are shown. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. Data are expressed as mean ± S.E.M.

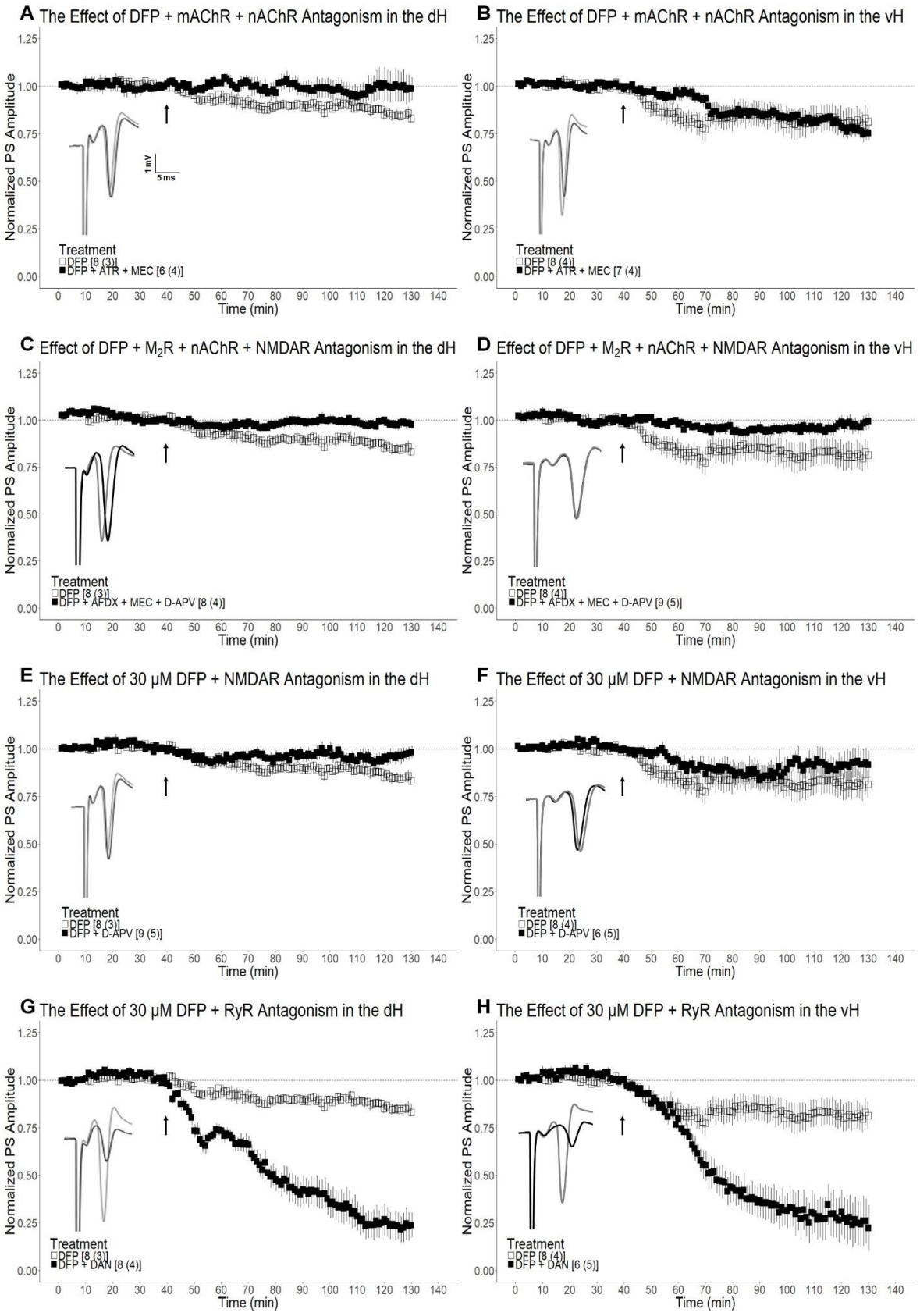

A The Effect of DFP + mAChR + nAChR Antagonism in the dH
B The Effect of DFP + mAChR + nAChR Antagonism in the vH
C Effect of DFP + M2R + nAChR + NMDAR Antagonism in the dH
D Effect of DFP + M2R + nAChR + NMDAR Antagonism in the vH
E The Effect of 30 µM DFP + NMDAR Antagonism in the dH
F The Effect of 30 µM DFP + NMDAR Antagonism in the vH
G The Effect of 30 µM DFP + RyR Antagonism in the dH
H The Effect of 30 µM DFP + RyR Antagonism in the vH
Normalized PS Amplitude
Time (min)
1 mV
5 ms
Treatment
DFP [8 (3)]
DFP + ATR + MEC [6 (4)]
DFP [8 (4)]
DFP + ATR + MEC [7 (4)]
DFP [8 (3)]
DFP + AFDX + MEC + D-APV [8 (4)]
DFP [8 (4)]
DFP + AFDX + MEC + D-APV [9 (5)]
DFP [8 (3)]
DFP + D-APV [9 (5)]
DFP [8 (4)]
DFP + D-APV [6 (5)]
DFP [8 (3)]
DFP + DAN [8 (4)]
DFP [8 (4)]
DFP + DAN [6 (5)]

Figure 2.3. Either cholinergic or NMDAR blockade is sufficient in preventing DFP-mediated PS1 inhibition in the dH but vH NMDAR blockade is indispensable. A and B. The effect of 3 μM atropine (ATR) and 30 μM mecamylamine (MEC) pretreatment on 30 μM DFP- mediated PS1 inhibition in the dH (**A**) and the vH (**B**). **C and D.** The effect of 300 nM AFDX-116, 30 μM MEC, and 25 μM D-APV pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**C**) and the vH (**D**). **E and F.** The effect of 25 μM D-APV pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**E**) and the vH (**F**). **G and H.** The effect of 20 μM dantrolene (DAN) pretreatment on 30 μM DFP-mediated PS1 inhibition in the dH (**G**) and the vH (**H**). Slices that did not receive antagonist pretreatment (□) and slices that received antagonist pretreatment (■) were exposed to DFP as indicated by the arrow at 40 min. Representative traces are shown for antagonist experiments in which the baseline PS1 (gray) and the PS1 following 60 min DFP wash-out (black) are shown. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. Data are expressed as mean ± S.E.M.

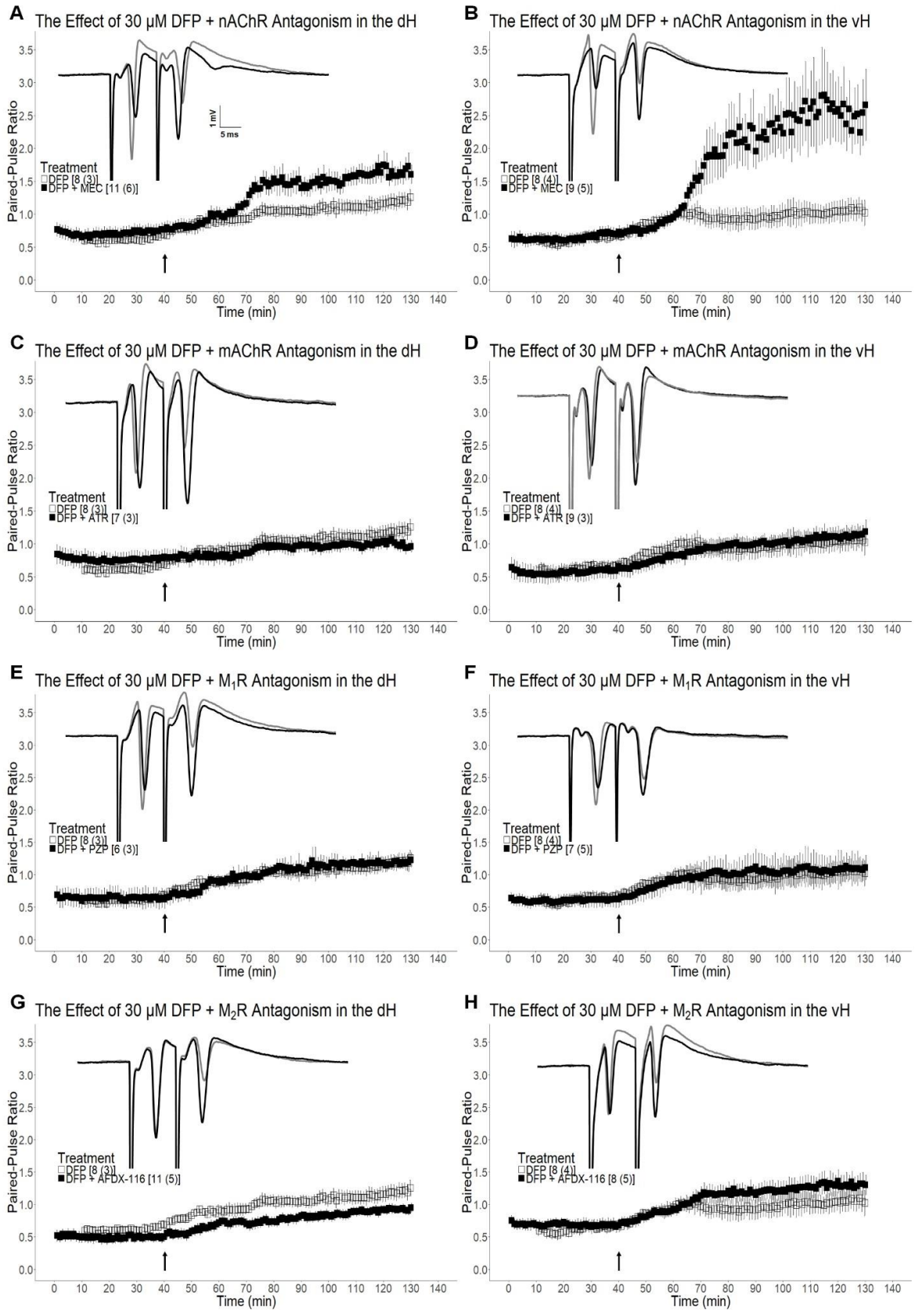

A
The Effect of 30 μM DFP + nAChR Antagonism in the dH
Paired-Pulse Ratio
Time (min)
1 mV
5 ms
Treatment
DFP [8 (3)]
DFP + MEC [11 (6)]
B
The Effect of 30 μM DFP + nAChR Antagonism in the vH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (4)]
DFP + MEC [9 (5)]
C
The Effect of 30 μM DFP + mAChR Antagonism in the dH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (3)]
DFP + ATR [7 (3)]
D
The Effect of 30 μM DFP + mAChR Antagonism in the vH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (4)]
DFP + ATR [9 (3)]
E
The Effect of 30 μM DFP + M1R Antagonism in the dH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (3)]
DFP + PZP [6 (3)]
F
The Effect of 30 μM DFP + M1R Antagonism in the vH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (4)]
DFP + PZP [7 (5)]
G
The Effect of 30 μM DFP + M2R Antagonism in the dH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (3)]
DFP + AFDX-116 [11 (5)]
H
The Effect of 30 μM DFP + M2R Antagonism in the vH
Paired-Pulse Ratio
Time (min)
Treatment
DFP [8 (4)]
DFP + AFDX-116 [8 (5)]

Figure 2.4. Cholinergic antagonist pretreatment does not preserve population spike paired- pulse inhibition (PS-PPI) in neither dH nor vH slices exposed to DFP. A and B. The effect of 30 μM mecamylamine (MEC) pretreatment on 30 μM DFP-mediated population spike paired- pulse ratio (PS-PPR) modulation in the dH (**A**) and the vH (**B**). **C and D.** The effect of 3 μM atropine (ATR) pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**C**) and the vH (**D**). **E and F.** The effect of 100 nM pirenzepine (PZP) pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**E**) and the vH (**F**). **G and H.** The effect of 300 nM AFDX-116 pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**G**) and the vH (**H**). Slices that did not receive antagonist pretreatment (□) and slices that received antagonist pretreatment (■) were exposed to DFP as indicated by the arrow at 40 min. Representative traces are shown for antagonist experiments in which the baseline PS-PPR (gray) and the PS-PPR following 60 min DFP wash-out (black) are shown. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. PS-PPR < 1.0 = paired-pulse inhibition (PS-PPI); PS-PPR > 1.0 = paired-pulse facilitation (PS- PPF). Data are expressed as mean ± S.E.M.

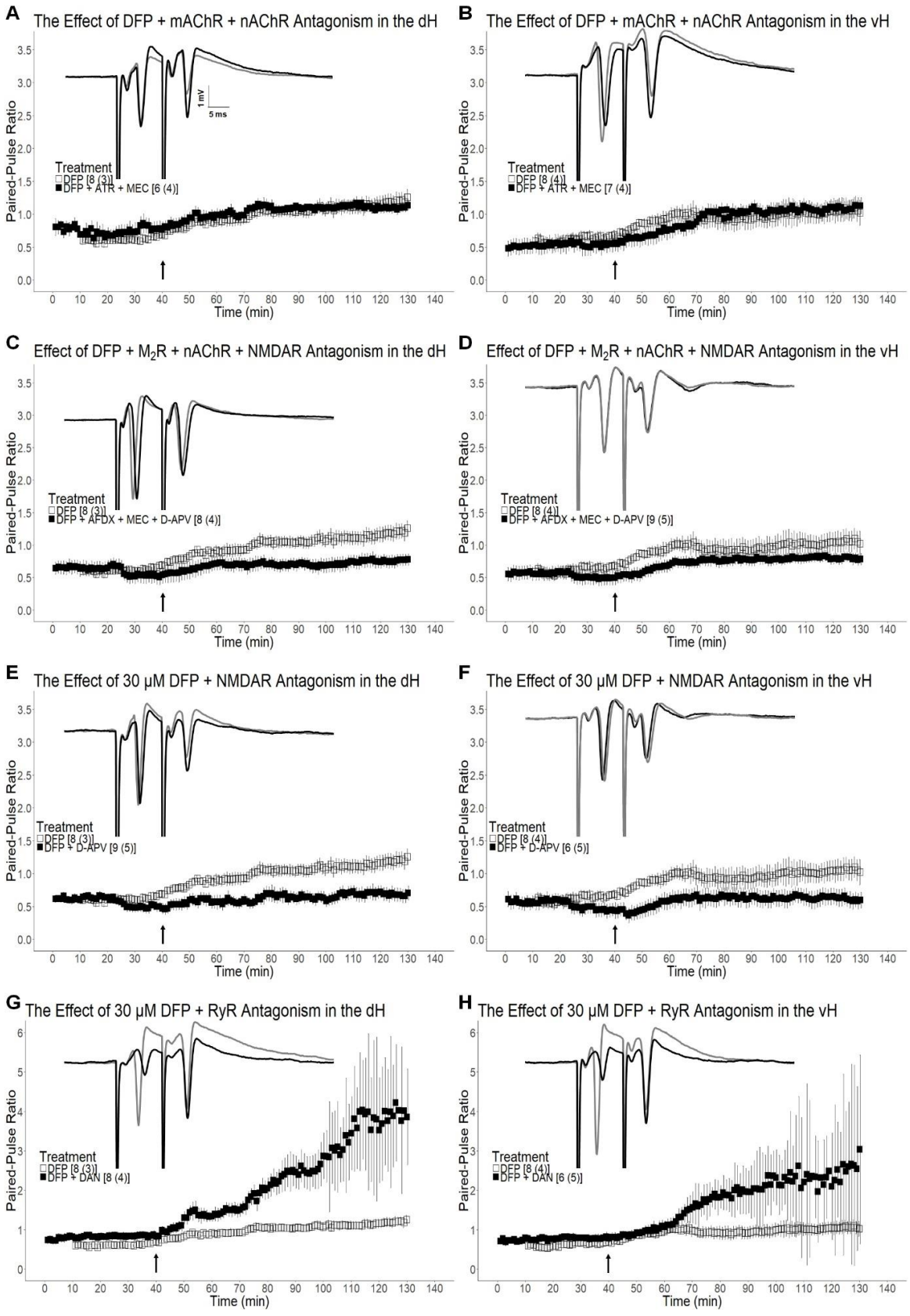

A
The Effect of DFP + mAChR + nAChR Antagonism in the dH
1 mV
5 ms
Treatment
DFP [8 (3)]
DFP + ATR + MEC [6 (4)]
Paired-Pulse Ratio
Time (min)
B
The Effect of DFP + mAChR + nAChR Antagonism in the vH
Treatment
DFP [8 (4)]
DFP + ATR + MEC [7 (4)]
Paired-Pulse Ratio
Time (min)
C
Effect of DFP + M2R + nAChR + NMDAR Antagonism in the dH
Treatment
DFP [8 (3)]
DFP + AFDX + MEC + D-APV [8 (4)]
Paired-Pulse Ratio
Time (min)
D
Effect of DFP + M2R + nAChR + NMDAR Antagonism in the vH
Treatment
DFP [8 (4)]
DFP + AFDX + MEC + D-APV [9 (5)]
Paired-Pulse Ratio
Time (min)
E
The Effect of 30 μM DFP + NMDAR Antagonism in the dH
Treatment
DFP [8 (3)]
DFP + D-APV [9 (5)]
Paired-Pulse Ratio
Time (min)
F
The Effect of 30 μM DFP + NMDAR Antagonism in the vH
Treatment
DFP [8 (4)]
DFP + D-APV [6 (5)]
Paired-Pulse Ratio
Time (min)
G
The Effect of 30 μM DFP + RyR Antagonism in the dH
Treatment
DFP [8 (3)]
DFP + DAN [8 (4)]
Paired-Pulse Ratio
Time (min)
H
The Effect of 30 μM DFP + RyR Antagonism in the vH
Treatment
DFP [8 (4)]
DFP + DAN [6 (5)]
Paired-Pulse Ratio
Time (min)

Figure 2.5. NMDAR antagonist pretreatment preserves PS-PPI in dH and vH slices exposed to DFP. A and B. The effect of 3 μM atropine (ATR) and 30 μM mecamylamine (MEC) pretreatment on 30 μM DFP-mediated population spike paired-pulse ratio (PS-PPR) modulation in the dH (**A**) and the vH (**B**). **C and D.** The effect of 300 nM AFDX-116, 30 μM MEC, and 25 μM D-APV pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**C**) and the vH (**D**). **E and F.** The effect of 25 μM D-APV pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**E**) and the vH (**F**). **G and H.** The effect of 20 μM dantrolene (DAN) pretreatment on 30 μM DFP-mediated PS-PPR modulation in the dH (**G**) and the vH (**H**). Slices that did not receive antagonist pretreatment (□) and slices that received antagonist pretreatment (■) were exposed to DFP as indicated by the arrow at 40 min. Representative traces are shown for antagonist experiments in which the baseline PS-PPR (gray) and the PS-PPR following 60 min DFP wash-out (black) are shown. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. PS-PPR < 1.0 = paired-pulse inhibition (PS-PPI); PS-PPR > 1.0 = paired-pulse facilitation (PS- PPF). Data are expressed as mean ± S.E.M.

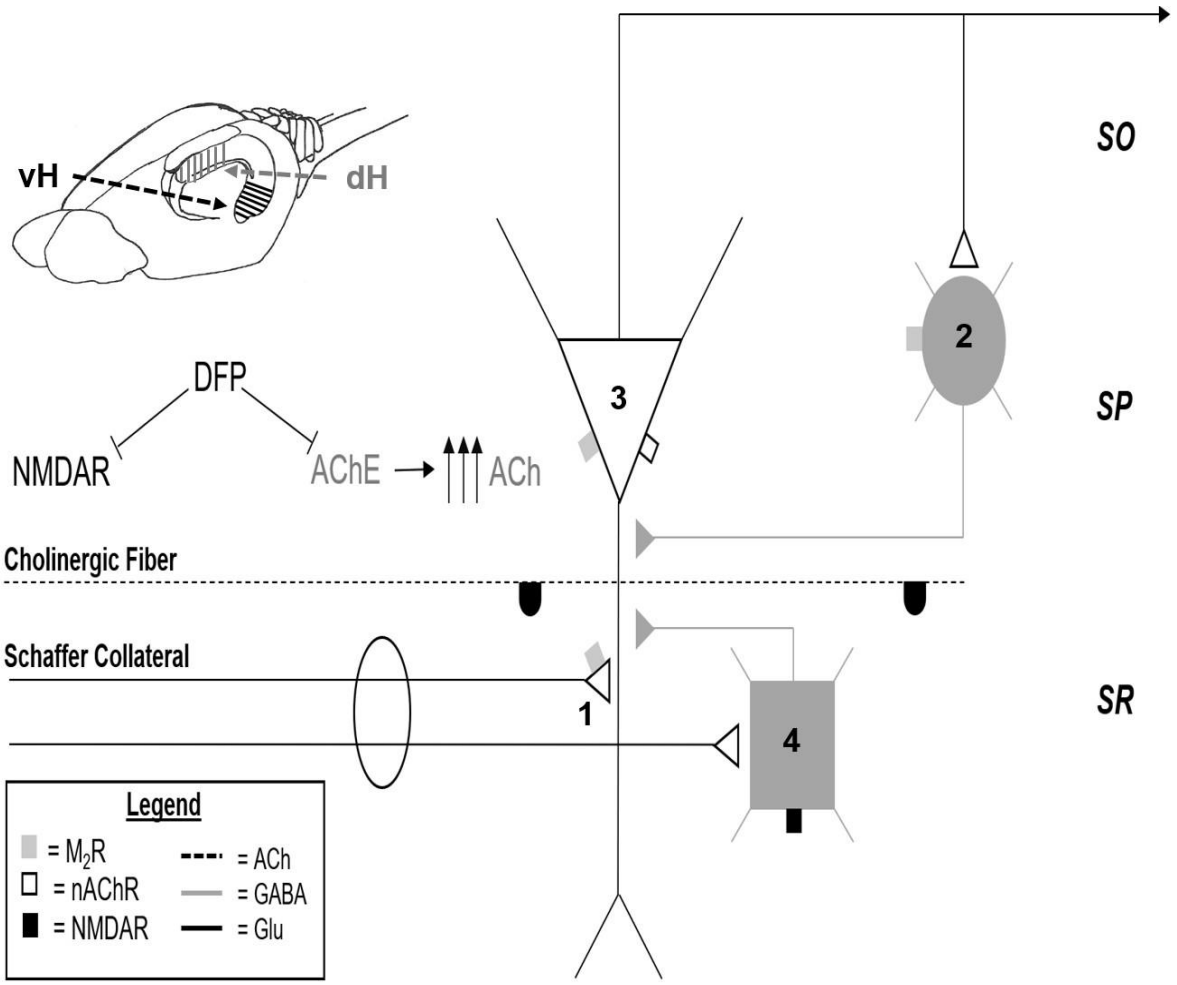
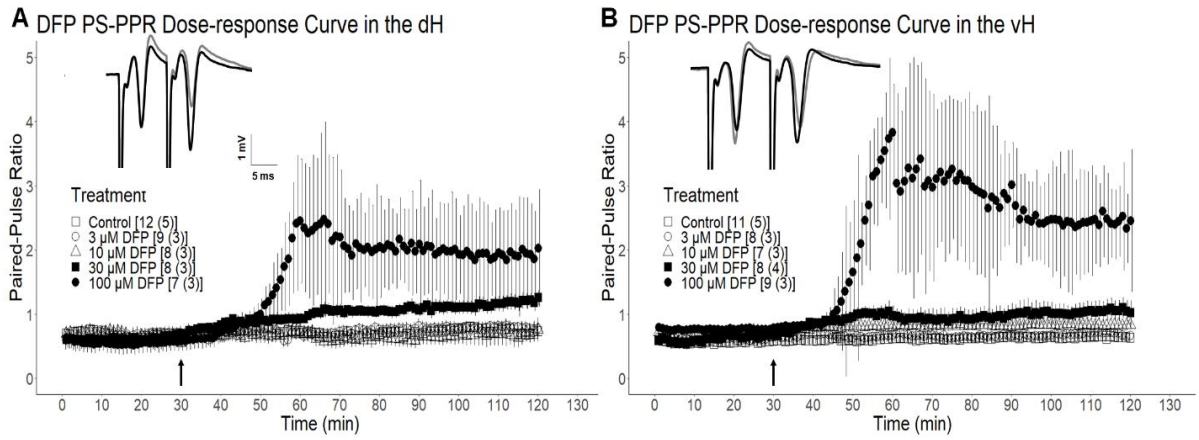
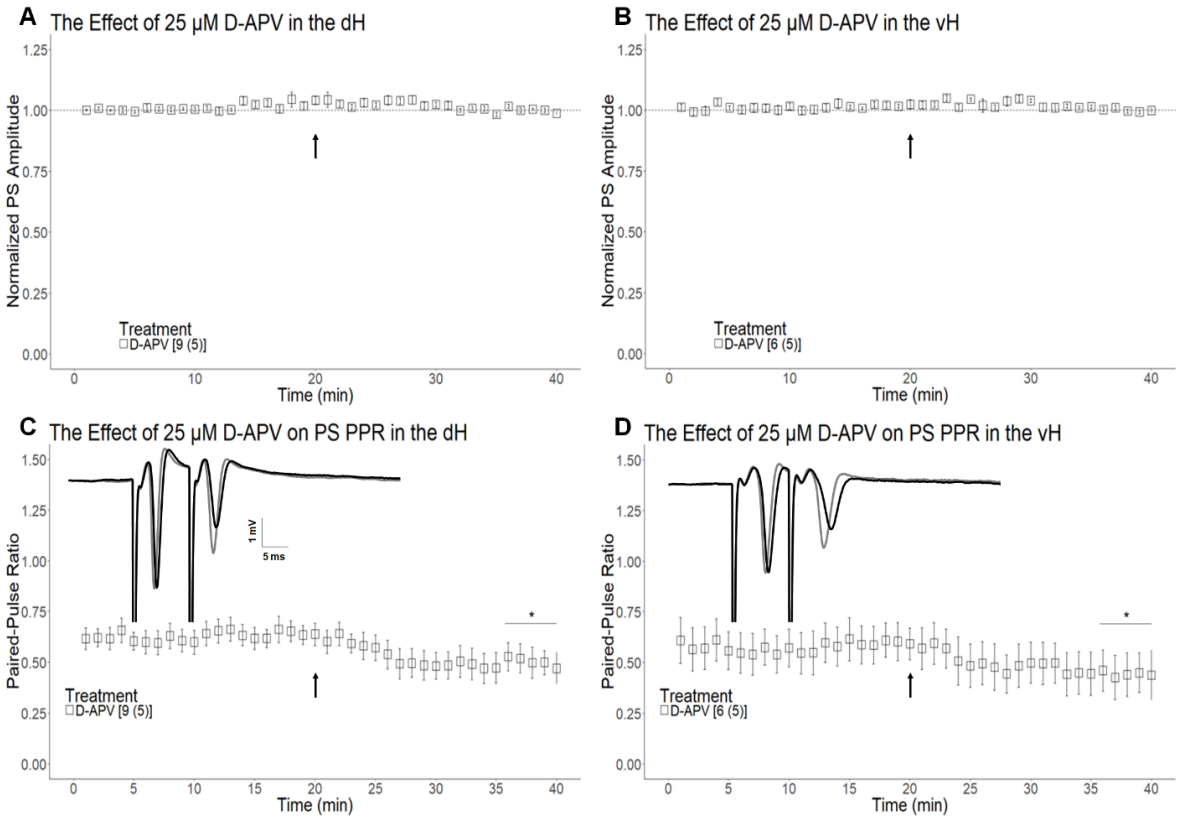


Figure 2.6. Proposed schematic illustrating the effects of DFP on dorsal (dH) and ventral (vH) hippocampal neurocircuitry in relation to the mAChR, the nAChR, and the NMDAR. DFP induces irreversible AChE inhibition, leading to M2R-mediated PS1 inhibition in the dH (1,3; gray). DFP induces PS1 inhibition through an M2R, nAChR, and NMDAR-dependent mechanism in the vH (1, 3, 4; black). Following DFP exposure, PS PPI shifts to PS PPF in the dH and the vH. PS-PPI can be partially preserved by M_2R blockade in the dH (2) or completely preserved by NMDAR blockade in the dH or the vH (4). SO = stratum oriens, SP = stratum pyramidale, SR = stratum radiatum.



Supplemental Figure 2.1. The effect of DFP wash-in on the population spike paired-pulse ratio (PS-PPR) in the dH and the vH. A and B. Results of dose-response curve experiments investigating the effect of DFP on the PS-PPR in the dH (**A**) and the vH (**B**). Slices were exposed to DFP as indicated by the arrow at 30 min. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. Representative traces of the baseline PS-PPR (gray) and the PS-PPR following 60 min 30 µM DFP wash-out (black) are shown. Data are expressed as mean ± S.E.M.



Supplemental Figure 2.2. The effect of D-APV on the population spike (PS_1) and the population spike paired-pulse ratio (PS-PPR) in the dorsal hippocampus (dH) and the ventral hippocampus (vH). A and B. The effect of 25 µM D-APV on PS_1 amplitude in the dH (**A**) and the vH (**B**). **C and D.** The effect of 25 µM D-APV on PS PPR in the dH (**C**) and the vH (**D**). Slices were exposed to D-APV as indicated by the arrow at 20 min. Representative traces of the baseline PS-PPR (gray) and the PS-PPR following 20 min D-APV wash-in (black) are shown. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. PS-PPR < 1.0 = paired-pulse inhibition (PS-PPI); PS-PPR > 1.0 = paired-pulse facilitation (PS-PPF). * denotes $p < 0.05$. Data are expressed as mean ± S.E.M.

CHAPTER 3

LACTO-N-FUCOPENTAOSE-III (LNFPIII) AMELIORATES ACUTE ABERRATIONS IN HIPPOCAMPAL SYNAPTIC PLASTICITY AND TRANSMISSION IN A GULF WAR ILLNESS ANIMAL MODEL[2]

[2] Brown KA, Preston CJ, Carpenter JM, Ludwig HD, Norberg T, Harn DA, Filipov NM, Wagner JJ. *Lacto-N-fucopentaose-III (LNFPIII) ameliorates acute aberrations in hippocampal synaptic plasticity and transmission in a Gulf War Illness animal model.*
Submitted to Brain Research.

Abstract

Approximately one-third of Persian Gulf War veterans are afflicted by Gulf War Illness (GWI), a chronic multisymptom condition that fundamentally presents with cognitive deficits (i.e., learning and memory impairments) and neuroimmune dysfunction (i.e., inflammation). Factors associated with GWI include overexposures to neurotoxic pesticides and nerve agent prophylactics such as permethrin (PM) and pyridostigmine bromide (PB), respectively. GWI-related neurological impairments associated with PB-PM overexposures have been recapitulated in animal models; however, there is a paucity of studies assessing PB-PM-related aberrations in hippocampal synaptic plasticity and transmission that may underlie behavioral impairments. Importantly, FDA-approved neuroactive treatments are currently unavailable for GWI. In the present study, we assessed the efficacy of an immunomodulatory therapeutic, lacto-N-fucopentaose-III (LNFPIII), on ameliorating acute effects of in vivo PB-PM exposure on synaptic plasticity and transmission as well as trophic factor/cytokine expression along the hippocampal dorsoventral axis. PB-PM exposure resulted in hippocampal synaptic transmission deficits 48 h post-exposure, a response that was ameliorated by LNFPIII coadministration, particularly in the dorsal hippocampus (dH). LNFPIII coadministration also enhanced synaptic transmission in the dH and the ventral hippocampus (vH). Notably, LNFPIII coadministration elevated long-term potentiation in the dH. Further, PB-PM exposure did not alter key inflammatory cytokine expression in the vH whereas LNFPIII coadministration enhanced brain-derived neurotrophic factor expression in PB-PM-exposed animals. Collectively, these findings demonstrate that PB-PM exposure impaired hippocampal synaptic responses 48 h post-exposure, impairments that differentially manifested along the dorsoventral axis. Importantly, LNFPIII ameliorated GWI-related electrophysiological deficits, a beneficial effect indicating the potential efficacy of LNFPIII for treating GWI.

3.1 Introduction

Gulf War Illness (GWI) is a chronic multisymptom condition that affects approximately a third of the 700,000 United States military forces that served in the 1990-1991 Persian Gulf War (White et al., 2016). Clinical symptoms of GWI include fatigue, cognitive deficits, and musculoskeletal pain, symptoms that progressively worsen over time and are likely related to neuroimmune dysfunction (Coughlin, 2017; Zundel et al., 2020). While the exact etiology of GWI is unknown, persistent wartime stress and excessive exposures to neurotoxic pesticides like permethrin (PM) and nerve agent prophylactics such as pyridostigmine bromide (PB) have been implicated in the pathogenesis of GWI (White et al., 2016). Currently, only symptomatic and targeted treatments of GWI are available with no approved neuroactive therapeutics.

Preclinical studies have characterized acute and persisting pyridostigmine bromide-permethrin (PB-PM)-induced neurological aberrations at the molecular, cellular, and behavioral levels in an established GWI model (Zakirova et al., 2015; Joshi et al., 2018; Carpenter et al., 2020). These neurological aberrations specifically include dysregulation of cytokine expression and signaling, undesirable activation of glial cells (i.e., astrocytes and microglia), and cognitive deficits (Zakirova et al., 2015; White et al., 2016; Joshi et al., 2018; Carpenter et al., 2020). Notably, learning and memory impairments in GWI veterans (Jeffrey et al., 2019) have been recapitulated in animals exposed to PB-PM (White et al., 2016), impairments that may be related to neuroimmune dysfunction (Trageser et al., 2020). Specifically, dorsal hippocampal (dH)-dependent learning and memory deficits (Zakirova et al., 2015; Joshi et al., 2018), as well as ventral hippocampal (vH)-related behavioral aberrations (i.e. abnormal anxiety-like behavior; Joshi et al., 2018) after PB-PM exposure were reported. As increasing evidence suggests the dH and the vH are functionally distinct structures (Papatheodoropoulos, 2018), monitoring dorsoventral-specific effects of PB-PM exposure on hippocampal-dependent behavioral abnormalities may be crucial for interpreting abnormalities in synaptic plasticity and transmission along the hippocampal dorsoventral axis. However, reports evaluating the impact

of PB-PM exposure on synaptic plasticity and transmission are unavailable. There is an additional paucity of investigations assessing the benefits of any therapeutic on GWI-related electrophysiological impairments. Given the emerging evidence of the substantial role neuroimmune dysfunction plays in GWI symptomology (O'Callaghan et al., 2016; Coughlin, 2017; Georgopoulos et al., 2017; Michalovicz et al., 2020), testing an immunotherapeutic for ameliorating PB-PM-induced abnormalities in hippocampal synaptic plasticity and transmission would address a substantial knowledge gap in the field of GWI research.

Recent reviews highlight the potential efficacy of a number of GWI therapeutics (Reviewed in: Dickey et al., 2020; Nugent et al., 2020), yet targeted and symptomatic treatments are available. Neuroimmune dysfunction is increasingly associated with the underlying pathobiology of GWI (Coughlin, 2017; Georgopoulos et al., 2017; Trageser et al., 2020). A particular approach that holds potential for extended remission of GWI is rebalancing inflammatory cytokines (Craddock et al., 2015); however, investigations utilizing immunotherapeutics to target GWI-induced immune system dysregulation are limited. The neoglycoconjugate lacto-N-fucopentaose-III (LNFPIII) was previously shown by our group to be a safe and efficacious agent for restoring central and peripheral immune function in disease states (Bhargava et al., 2012; Zhu et al., 2012; Tundup et al., 2015). LNFPIII induces therapeutic effects by rebalancing the anti-inflammatory:inflammatory cytokine population as well as direct stimulation of kinases involved in trophic factor production such as the mitogen-activated protein kinase ERK-1/2 (Tundup et al., 2015). Importantly, recent work by our group demonstrated that LNFPIII coadministration ameliorated short-term neuroinflammatory and neurochemical-related aberrations in multiple GWI models (Carpenter et al., 2020) and it rebalanced persisting pathology such as gastrointestinal dysbiosis and inflammation when treatment was initiated months after GWI-related exposures (Mote et al., 2020). However, LNFPIII's efficacy in ameliorating hippocampal synaptic plasticity and transmission in a model of GWI is unknown. Accordingly, the present study investigated the efficacy of LNFPIII in

ameliorating short-term PB-PM-induced deficits in dH and vH synaptic plasticity and transmission. We hypothesized that synaptic responses in the dH and the vH would be impaired 48 h post-PB-PM exposure and that LNFPIII coadministration would ameliorate synaptic activity in these sectors while also enhancing trophic factor production.

3.2 Methods

3.2.1 Materials

Animals were exposed to pyridostigmine bromide (PB; ≥ 98% purity; Sigma Aldrich, St. Louis, MO) and permethrin (PM; 29.5% cis/69.5% trans isomer; 99% purity; Chem Service Inc., West Chester, PA). Production of the LNFPIII dextran conjugate was described in Tundup et al. (2015). Unless specifically indicated, all other chemicals and reagents used in this study were acquired from Sigma Aldrich or Fisher Scientific (Hampton, NH).

3.2.2 Animals

Eight to nine-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in groups of four in an environmentally controlled room (22-24°C), were maintained on a 12 h light/dark cycle (0700-1900 lights on) and received food and water access *ad libitum*. Animals underwent one week of acclimation and were then handled daily for one week before the start of the study. All procedures were in accordance with the latest National Institutes of Health guidelines and were approved in advance by the University of Georgia Institutional Animal Care and Use Committee.

3.2.3 GWI Model

Mice (n=23; weight: 24.4±0.19 g [mean±SEM]) were randomly divided via random number generator into GWI (PB-PM) and vehicle (DMSO) treatment groups as follows: Vehicle-Vehicle (DMSO-Dextran), Vehicle-LNFPIII (DMSO-LNFPIII), PB-PM-Vehicle (PB-PM-Dextran), and PB-PM-LNFPIII. The body weight of randomized treatment groups did not significantly differ from one another. Mice then received 10 days of concurrent PB-PM exposure and LNFPIII treatment as described in Fig. 3.1A and Carpenter et al. (2020). Briefly, mice received daily

treatment for 10 days with a combination of PB and PM (0.7 and 200 mg/kg, respectively, intraperitoneal [i.p.]) or DMSO vehicle (i.p.), immediately followed by an injection of LNFPIII or dextran vehicle (both 35 μg/mouse, subcutaneous) diluted in sterile saline. Body weights were monitored daily during PB-PM exposure until study completion. Forty-eight h after the last exposure, vH tissue was collected for qPCR analysis, and electrophysiology experiments were conducted on dH and vH tissues. Researchers were blinded to treatment groups throughout qPCR analysis, electrophysiology experiments, and subsequent data analysis.

3.2.4 Quantitative PCR (qPCR) Analysis

qPCR was completed on vH tissue for key inflammatory cytokines (IL-6, IL-1β, and TNFα) and growth factors (brain-derived neurotrophic factor, nerve growth factor, and ciliary neurotrophic factor) as described in Carpenter et al. (2020) with additional details provided in Table 3.1. Briefly, total RNA from a single vH brain punch (1.5 mm diameter, 500 μm thick section) was isolated by an E.Z.N.A total RNA isolation kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's directions. RNA was quantified with a Take3 micro-volume plate and Epoch microplate spectrophotometer (BioTek, Winooski, VT). Seventy-five ng RNA/sample was used to synthesize cDNA with a Maxima first-strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, Waltham, MA) and a Peltier thermal cycler (Bio-Rad, Hercules, CA; 10 min at 25 °C, 15 min at 50 °C, 5 min at 85 °C). Using 1 or 2 ng of cDNA per sample, expression of various inflammatory genes and the growth factors (Table 3.1) were determined by a qPCR with mouse-specific primers (RealTimePrimers, Elkins Park, PA) and Maxima SYBR Green/lowRox qPCR Master Mix (2x) (Thermo Scientific). Amplifications were performed on Mx3005 P qPCR machine (Stratagene, San Diego, CA) and treatment differences were calculated as a fold change by the $^{\Delta\Delta}$ Ct method with 18S as the house keeping gene, as described previously (Lin et al., 2013; Krishna et al., 2016).

3.2.5 Slice Preparation and Electrophysiology

Brain dissection and hippocampal slice recovery were completed as described in Brown et al. (2020). Briefly, mice were sacrificed following deep anesthetization with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane). Brains were removed and quickly submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120mM NaCl, 3mM KCl, 4mM $MgCl_2$, 1mM NaH_2PO_4, 26mM $NaHCO_3$, and 10mM glucose). A mid-sagittal cut was made, and half of the brain was mounted on its caudal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm slices containing the dorsal hippocampus. The other half of the brain was mounted on its rostral pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm slices containing the ventral hippocampus. The dorsal hippocampus and ventral hippocampus were subdissected free from the rest of the slice and the CA3 subfield was removed. These slices were then quickly placed in a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120mM NaCl, 3mM KCl, 1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, and 10mM glucose). Slices were continuously perfused with ACSF (one mL/min) during a 45 min recovery period at room temperature followed by a 45 min recovery period at the recording temperature of 30 °C. Schaffer collateral fibers were stimulated in the stratum radiatum of the CA1 subfield via a bipolar stimulating electrode (Kopf Instruments, Tujunga, CA) whereas recording was conducted via a 1.0 MΩ tungsten microelectrode (World Precision Instruments, Sarasota, FL). A stimulus pulse was applied once every min to elicit field excitatory postsynaptic potentials (fEPSPs) at Schaffer collateral-CA1 synapses.

An input-output curve (I/O curve) was generated at the beginning of each experiment by incrementally introducing individual 15 µA stimulus pulses from 45-180 µA in intensity. The fEPSP slope (mV/ms) was monitored in a one ms interval immediately following the fiber volley. The stimulus intensity was modified to elicit 35% of the maximal fEPSP slope and paired-pulse fEPSPs (50 ms interpulse interval) were recorded each minute for five min after recording the

I/O curve. A stimulus pulse was then applied every min for 30 min to assess the baseline fEPSP response. After establishing the baseline fEPSP response, a high-frequency stimulation (HFS) protocol (three x 100 Hz/one-sec train at 20-sec intervals) was utilized to induce long-term potentiation (LTP) and fEPSP responses were monitored for the subsequent 60 min. fEPSP responses were normalized to the average fEPSP slope value recorded during 26-30 min of baseline recording. LTP magnitude was calculated by averaging the normalized fEPSP slope values from 56-60 min post-HFS.

3.2.6 Statistical Analysis

Two-way analysis of variance (ANOVA) was utilized to assess the effects of factor A (PB-PM) and factor B (LNFPIII) on electrophysiology response variables. Pairwise comparisons were conducted by Student-Newman-Keuls *post hoc* test or planned pairwise comparisons (Student's t-test, as appropriate) in the case of a significant main or interaction effect. qPCR statistical analysis was conducted as described in section 3.2.4. Statistical analysis was completed using R version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) and figures were generated using GraphPad Prism 5 (San Diego, CA).

3.3 Results

3.3.1 LNFPIII coadministration ameliorated PB-PM-induced impairments in hippocampal basal synaptic transmission, particularly in the dorsal hippocampus

A significant reduction by PB-PM was detected in dH basal synaptic transmission at stimulus intensities of 120,135,150,165, and 180 μA when compared to vehicle-vehicle mice (p's ≤ 0.05; Fig. 3.1B).). LNFPIII administration resulted in enhanced dH basal synaptic transmission compared to non-LNFPIII-treated groups at stimulation intensities of 150,165, and 180 μA (main effect: p's ≤ 0.05) as well as 135 μA (main effect: $p \leq 0.1$). Notably, LNFPIII treatment ameliorated the PB-PM reduction of dH basal synaptic transmission by elevating activity in PB-PM-LNFPIII mice at stimulus intensities of 45,135,150,165, and 180 μA (p's $\leq$ 0.05). In the vH, a two-way ANOVA revealed an effect of PB-PM exposure on basal synaptic

transmission at stimulation intensities of 105,150,165, and 180 μA (main effect: $p \leq 0.05$) as well as 45,90,120, and 135 μA (main effect: p's ≤ 0.1; Fig. 3.1C). This effect was driven mostly by a large, stimulus intensity-independent increase in basal synaptic activity in vehicle mice treated with LNFPIII, decreasing vH basal synaptic activity. LNFPIII treatment elevated vH basal synaptic transmission at stimulus intensities of 90 μA ($p < 0.05$) as well as 105 and 120 μA (p's ≤ 0.1) in vehicle-LNFPIII mice compared to vehicle-vehicle mice.

3.3.2 LNFPIII coadministered to PB-PM-exposed animals enhanced dorsal hippocampal long-term potentiation

A two-way ANOVA revealed a PB-PM x LNFPIII interaction effect ($p = 0.05$) on dH long-term potentiation (LTP) magnitude after PB-PM exposure; concurrent PB-PM exposure and LNFPIII treatment led to an increase in dH LTP magnitude compared to the PB-PM-vehicle group ($p \leq 0.05$, preplanned Student's *t*-test; Fig. 3.2A-2C). No significant main effects of PB-PM exposure or LNFPIII treatment were observed on dH paired-pulse facilitation (PPF) magnitude (Fig. 3.2D). There were no significant main effects of PB-PM or LNFPIII on LTP magnitude in the ventral hippocampus (vH) post-PB-PM exposure (Fig. 3.3A-3C). Additionally, there were no significant main effects of PB-PM exposure or LNFPIII treatment on vH PPF magnitude (Fig. 3.3D).

3.3.3 The effect of PB-PM exposure ± LNFPIII coadministration on the expression of key inflammatory cytokines and trophic factors in the ventral hippocampus

There were no significant effects of PB-PM exposure or LNFPIII treatment on the expression of vH inflammatory markers IL-6, IL-1β, or TNF-α 48 h post-exposure (Table 3.1). Further, no significant effects of PB-PM exposure or LNFPIII treatment on vH trophic factors were detected; however, LNFPIII treatment numerically enhanced brain-derived neurotrophic factor (BDNF). The trend was most pronounced in the PB-PM-LNFPIII group ($p < 0.08$).

3.4 Discussion

Presentation of pathology related to neuroimmune dysfunction has been consistently reported in the PB-PM GWI animal model (Zakirova et al., 2015; Joshi et al., 2018), which is in line with clinical reports indicating GWI is a neuroimmune disease (Coughlin, 2017). Elimination of PB and PM occurs rapidly (Aquilonius et al., 1980; Anadon et al., 1991) and results in direct PB-PM-induced enhancement of hippocampal pro-inflammatory expression six h (Carpenter et al., 2020) but not 48 h post-exposure as shown here, a response that yields indirect, persisting, GWI-specific neuroinflammation (O'Callaghan et al., 2016). To the best of our knowledge, this transient enhancement in hippocampal inflammatory cytokine expression following GWI-related exposures has been described in a different GWI model (O'Callaghan et al., 2015; Michalovicz et al., 2019), but not after PB-PM exposure, a finding that addresses a knowledge gap related to the temporal progression of GWI-related neuroinflammation in this model. Additionally, acute enhancement of inflammatory cytokine secretion leads to aberrant activation of microglia and astrocytes, which, while transient, has lasting effects on glial homeostasis, bringing forth impaired hippocampal synaptic plasticity and transmission in a temporal-specific manner along the dorsoventral axis (Prieto and Cotman, 2017; Onufriev et al., 2018). Irregular microglia stimulation facilitates an immune system response that produces undesirable activation of astrocytes (Ramesh et al., 2013), enabling secretion of inflammatory cytokines that impair hippocampal synaptic transmission (Prieto and Cotman, 2017). Furthermore, enhanced expression of pro-inflammatory cytokines reduces the secretion of critical trophic factors involved in hippocampal synaptic transmission such as BDNF (Kang and Schuman, 1995; Barrientos et al., 2004). Increased activation of hippocampal microglia following PB-PM exposure has been described (Abdullah et al., 2016; Joshi et al., 2018). Reports using the PB-PM GWI model also observed astrocyte activation, particularly in the hippocampus (Abdullah et al., 2011; Zakirova et al., 2015; Joshi et al., 2018). Additionally, elevated central and peripheral

pro-inflammatory cytokine expression were found both 6 h and months after PB-PM exposure (Joshi et al., 2018; Carpenter et al., 2020; Mote et al., 2020).

In the present study, we observed reduced dH and vH basal synaptic transmission 48 h post-PB-PM exposure, an effect that was quantitatively greater in the dH compared to the vH. This observation is consistent with the aforementioned investigations describing a PB-PM-induced neuroinflammatory phenotype that consequently leads to detrimental microglia and astrocyte activation, driving impairments in hippocampal synaptic transmission. Moreover, we assessed hippocampal LTP, a well-established synaptic substrate for learning and memory (Whitlock et al., 2006), to determine if PB-PM exposure adversely impacted hippocampal synaptic plasticity. Interestingly, we did not observe PB-PM-induced impairments in dH or vH LTP magnitude. An investigation by Onufriev et al. (2018) detected rapid production of pro-inflammatory cytokines as well as time-matched impairments in dH synaptic plasticity but a slower response in the vH following acute proinflammatory stress, results that are in-line with the current findings of a more substantial PB-PM-induced effect on dH synaptic measurements shortly after exposure yet not on vH synaptic responses. These sector-specific effects may be of particular interest as a GWI-relevant chemical was recently shown to induce differential acute effects on synaptic transmission along the hippocampal dorsoventral axis (Brown et al., 2020). Further, while there is a lack of information regarding PB-PM-induced modulations in hippocampal synaptic plasticity and transmission, studies assessing the PB-PM GWI animal model have found modulations in hippocampal presynaptic protein levels. For instance, a reduction in hippocampal synaptophysin (SYP) levels, a marker for presynaptic vesicles, was reported from two-three weeks and up to five months following PB-PM exposure (Zakirova et al., 2015); however, SYP knockout mice do not exhibit changes in PPF magnitude, a measurement indicative of presynaptic-mediated alterations in synaptic plasticity (Janz et al., 1999). Moreover, SYP knockout did not impact hippocampal LTP magnitude in this study. These results are consistent with our findings in the dH and the vH, where we did not observe

PB-PM-induced variations of PPF or LTP magnitude. Coupled with the acute PB-PM-mediated impairment of dH and vH synaptic transmission, the lack of abnormalities in PPF magnitude between treatment groups is consistent with an interpretation indicating a decrease in the density of synapses in the CA1 subfield. This interpretation is in line with preclinical studies that observed reduced hippocampal CA1 apical dendritic spine density (Speed et al., 2011) and hippocampal neurogenesis (Parihar et al., 2013; Shetty et al., 2020; Wang et al., 2020) in GWI animal models that included PB and PM in the exposure protocol as well as exposure to another GWI-relevant pesticide.

We previously demonstrated that LNFPIII rebalanced central and peripheral immune function in immunological disease states such as experimental autoimmune encephalomyelitis (Zhu et al., 2012) as well as GWI (Carpenter et al., 2020; Mote et al., 2020). LNFPIII induces anti-inflammatory chemokine and cytokine production via the CD14/TLR-MAPK (ERK) axis (Tundup et al., 2015). ERK is a kinase critical for the production of trophic factors, such as BDNF (Alonso et al., 2004). ERK-1/2 phosphorylation is also essential for the expression of late-LTP (English and Sweatt, 1997), a form of LTP that is dependent upon *de novo* protein synthesis and trophic factor production (Bekinschtein et al., 2007). Notably, studies investigating GWI animal models found decreased cortical (Kimono et al., 2020) and hippocampal BDNF levels (Ribeiro et al., 2020) as well as reduced tropomyosin receptor kinase B density in the hippocampus (Carreras et al., 2018), a receptor fundamentally involved in facilitating the actions of BDNF (Minichiello, 2009), indicating GWI-related chemical exposures may impact secretion of the trophic factor in addition to its signaling activity. In the present study, LNFPIII treatment recovered dH basal synaptic transmission in PB-PM treated animals, an effect that may be attributed to either LNFPIII-mediated rebalancing of anti-inflammatory cytokine production (Carpenter et al., 2020; Mote et al., 2020) or LNFPIII-mediated effects on ERK (Tundup et al., 2015). Further, the trend in the vH of an LNFPIII-induced enhancement of BDNF secretion may be particularly pertinent to the observed recovery of dH basal synaptic transmission as

downstream effectors like BDNF have been shown to increase hippocampal basal synaptic activity (Kang and Schuman, 1995). LNFPIII only enhanced vH basal synaptic transmission in vehicle animals, a response that may be attributed to the observed trending LNFPIII effect on BDNF secretion or differential baseline activity of immune mediators such as microglia and astrocytes along the hippocampal dorsoventral axis (Ogata and Kosaka, 2002; Jinno et al., 2007). Notably, we did not observe an enhancement of dH LTP magnitude in vehicle-LNFPIII-treated animals, indicating that underlying non-measurable effects of PB-PM are needed for LNFPIII to exhibit this result.

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Figures and tables

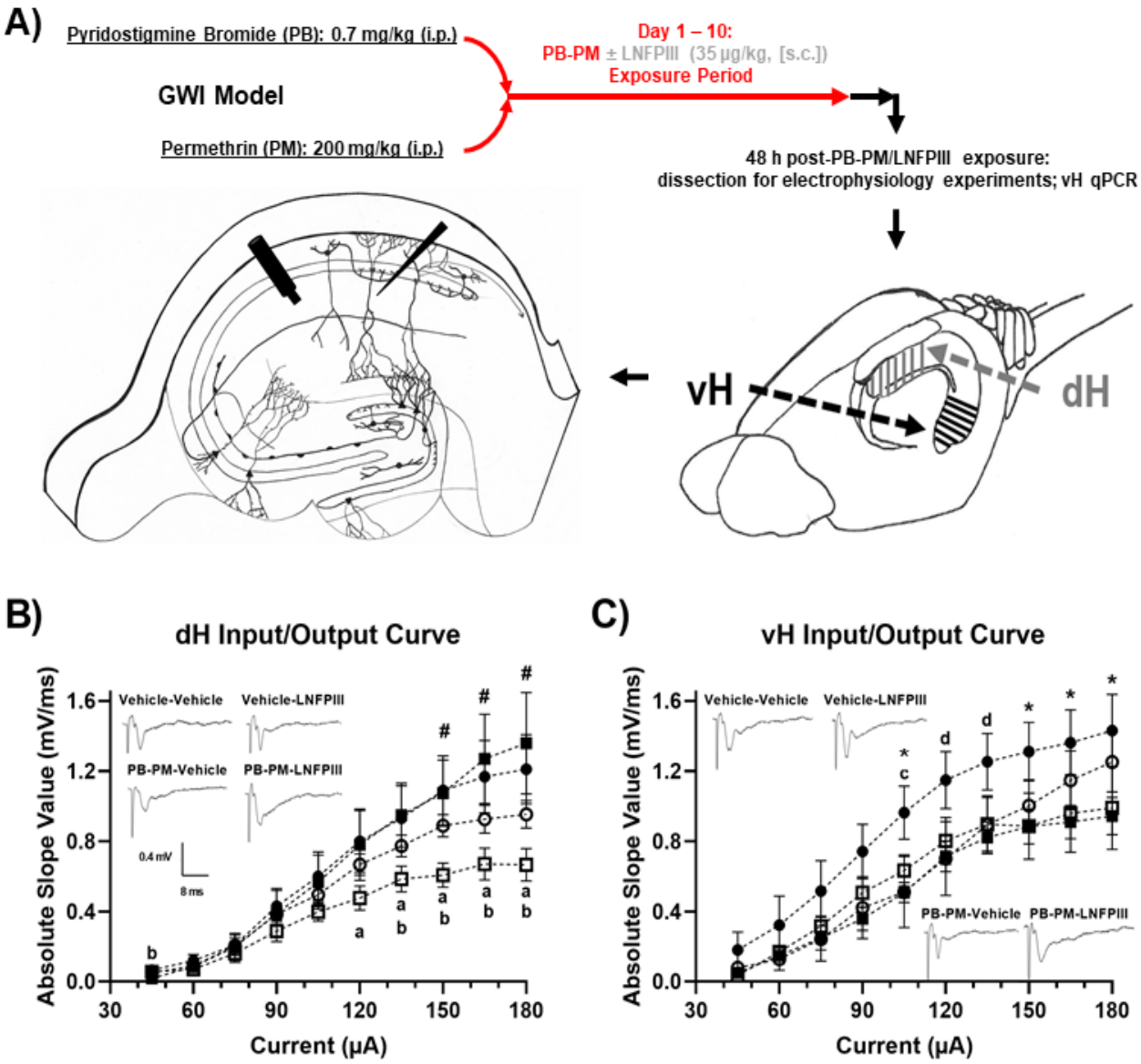

Figure 3.1. Experimental design of pyridostigmine bromide-permethrin (PB-PM) exposure ± LNFPIII coadministration and their effect on dorsal (dH) and ventral (vH) hippocampal synaptic transmission 48 h post-exposure. A. Animals were randomly divided into GWI (pyridostigmine bromide-permethrin; PB-PM) and vehicle (DMSO) treatment groups. Mice then received 10 days of daily exposure with a combination of PB and PM (0.7 and 200 mg/kg, respectively; i.p.) or DMSO (i.p.). Animals were also randomly selected to receive 10 days of concurrent daily treatment with either lacto-N-fucopentaose-III (LNFPIII) or dextran vehicle (both

35 µg/mouse; S.C., diluted in sterile saline). Electrophysiology experiments were conducted 48 h following the last exposure. The cylinder found in the hippocampal slice represents a stimulating electrode along the Schaffer collateral-CA1 pathway found in the stratum radiatum whereas the triangle represents a recording electrode in the CA1 stratum radiatum. **B.** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities from vehicle-vehicle (○; n=9(3)), vehicle-LNFPIII (●; n=9(4)), PB-PM-vehicle (□; n=7(4)), and PB-PM-LNFPIII (■; n=6(3)) treatment groups recorded from the dH 48 h after PB-PM exposure. **C.** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities from vehicle-vehicle (○; n=5(4)), vehicle-LNFPIII (●; n=7(4)), PB-PM-vehicle (□; n=8(4)), and PB-PM-LNFPIII (■; n=12(3)) treatment groups recorded from the vH 48 h after PB-PM exposure. Insets show representative traces composed of average sweeps from 135 µA stimulation intensity. * denotes main effect of PB-PM ($p < 0.05$) whereas # denotes main effect of LNFPIII ($p < 0.05$). [a] compares vehicle-vehicle and PB-PM-vehicle; [b] compares PB-PM-vehicle and PB-PM-LNFPIII; [c] and [d] compare vehicle-vehicle and vehicle-LNFPIII groups. [a], [b], [c] indicates $p < 0.05$. [d] indicates $p < 0.1$. Data are expressed as mean ± SEM.

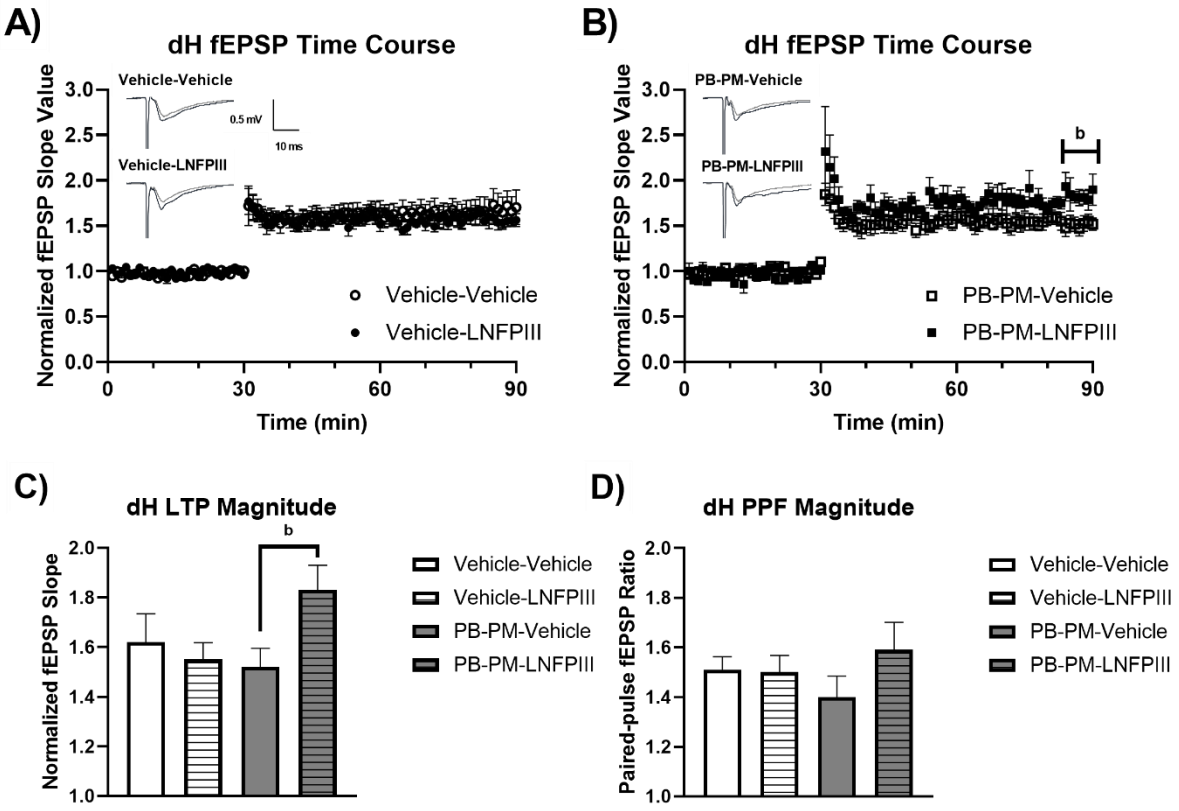

Figure 3.2. The effect of pyridostigmine bromide-permethrin (PB-PM) exposure ± LNFPIII coadministration on measurements of dorsal hippocampal (dH) synaptic plasticity 48 h post-PB-PM exposure. A and B. Summary plots of normalized fEPSP values recorded from the dH of vehicle-treated (**A**) or PB-PM exposed (**B**) animals 48 h post-PB-PM exposure. (**A**) compares vehicle-vehicle (○) and vehicle-LNFPIII (●) treated mice whereas (**B**) compares PB-PM-vehicle (□) and PB-PM-LNFPIII (■) treated mice. Insets show summary traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post-tetanus (black). **C.** Summary quantification of dH LTP magnitude recorded 48 h after PB-PM exposure from vehicle-vehicle (1.62 ± 0.11, n=9(3)), vehicle-LNFPIII (1.55 ± 0.07, n=9(4)), PB-PM-vehicle (1.52 ± 0.08, n=7(4)), and PB-PM-LNFPIII (1.83 ± 0.10, n=6(3)) treated animals. **D.** The paired-pulse ratio for vehicle-vehicle (1.51 ± 0.05), vehicle-LNFPIII (1.50 ± 0.07), PB-PM-vehicle (1.40 ± 0.08), and PB-PM-LNFPIII (1.59 ± 0.11) treatment groups recorded from the dH 48 h after PB-

PM exposure (interpulse interval = 50 ms). [b] compares PB-PM-vehicle and PB-PM-LNFPIII ($p < 0.05$ via preplanned Student's *t*-test). Data are expressed as mean ± SEM.

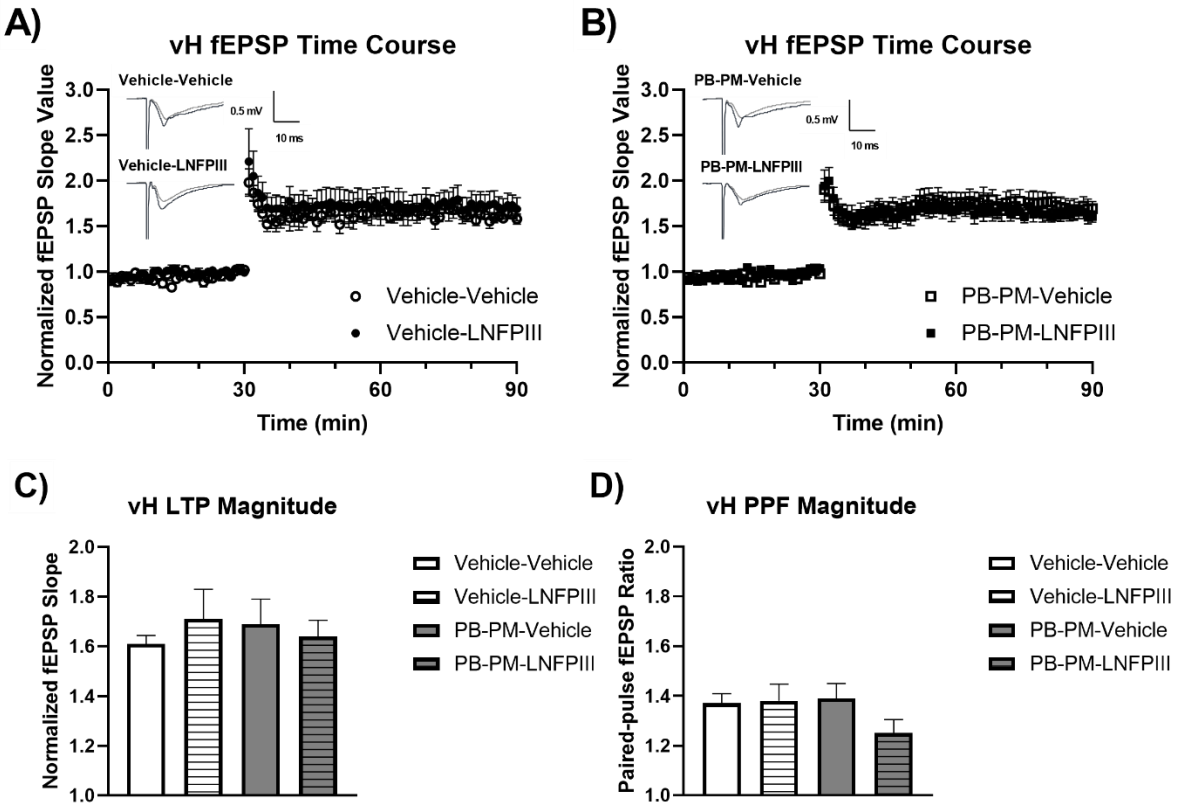

Figure 3.3. The effect of PB-PM exposure ± LNFPIII coadministration on measurements of ventral hippocampal (vH) synaptic plasticity 48 h post-PB-PM exposure. A and B. Summary plots of normalized fEPSP values recorded from the vH of vehicle-treated (**A**) or PB-PM exposed (**B**) animals 48 h post-PB-PM exposure. (**A**) compares vehicle-vehicle (○) and vehicle-LNFPIII (●) treated mice whereas (**B**) compares PB-PM-vehicle (□) and PB-PM-LNFPIII (■) treated mice. Insets show traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post-tetanus (black). **C.** Summary quantification of vH LTP magnitude recorded 48 h after PB-PM exposure from vehicle-vehicle (1.61 ± 0.04, n=5(4)), vehicle-LNFPIII (1.71 ± 0.12, n=7(4)), PB-PM-vehicle (1.70 ± 0.10, n=8(4)), and PB-PM-LNFPIII (1.64 ± 0.06, n=12(3)) treated animals. **D.** The paired-pulse ratio for vehicle-vehicle (1.37 ± 0.04), vehicle-LNFPIII (1.38 ± 0.07), PB-PM-vehicle (1.39 ± 0.06), and PB-PM-LNFPIII (1.25 ± 0.06) treatment

groups recorded from the vH 48 h after PB-PM exposure (interpulse interval = 50 ms). Data are expressed as mean ± SEM.

Table 3.1. The effect of PB-PM exposure ± LNFPIII coadministration (10 d) on the expression of key inflammatory cytokines and growth factors in the ventral hippocampus (vH) measured by qPCR 48 h post-PB-PM exposure. vH samples from a separate cohort of mice (n=5, 6, 6, 4, for the, respectively, Vehicle-Vehicle, Vehicle-LNFPIII, PB-PM-Vehicle, PB-PM-LNFPIII groups) treated identically to the cohort used for electrophysiology were used for RNA isolation and subsequent qPCR analyses. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with 18S as the housekeeping gene and are expressed as a mean fold change from the Vehicle-Vehicle control ± SEM. P-values reflect the comparison for each respective group compared to the Vehicle-Vehicle control. Gene abbreviations: IL-6, Interleukin 6; IL-1β, Interleukin 1 beta; TNFα, Tumor necrosis factor alpha; BDNF, Brain-derived neurotrophic factor; NGF, Nerve growth factor; CNTF, Ciliary neurotrophic factor.

Gene	**Treatment**	**p-value**	**Treatment**	**p-value**	**Treatment**	**p-value**
	Vehicle-LNFPIII		**PB-PM-Vehicle**		**PB-PM-LNFPIII**	
IL-6	0.93 ± 0.089	0.886	0.87 ± 0.083	0.624	0.89 ± 0.085	0.779
IL-1β	1.05 ± 0.402	0.941	0.89 ± 0.343	0.874	0.94 ± 0.362	0.94
TNFα	0.62 ± 0.209	0.249	0.72 ± 0.241	0.421	0.77 ± 0.259	0.494
BDNF	1.66 ± 0.415	0.227	1.37 ± 0.344	0.416	**2.5 ± 0.625**	**0.076**
NGF	0.95 ± 0.126	0.855	0.97 ± 0.129	0.902	1.14 ± 0.152	0.687
CNTF	0.80 ± 0.092	0.379	1.01 ± 0.117	0.963	1.16 ± 0.134	0.716

CHAPTER 4

DELAYED TREATMENT WITH THE IMMUNOTHERAPEUTIC LNFPIII AMELIORATES MULTIPLE NEUROLOGICAL DEFICITS IN A MOUSE MODEL OF GULF WAR ILLNESS[3]

[3] Brown KA*, Carpenter JM*, Diaz AN, Dockman RL, Preston CJ, Benbow RA, Harn DA, Norberg T, Wagner JJ, Filipov NM. *Delayed treatment with the immunotherapeutic LNFPIII ameliorates multiple neurological deficits in a mouse model of Gulf War Illness.*
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* Authors contributed equally.

Abstract

Thought to stem mostly from deployment-related chemical overexposures, residual effects of the 1990-1991 Gulf War (GW) still plague veterans nearly 30 years later as Gulf War Illness (GWI), a disease with multiple neurological symptoms with likely immunological underpinnings. Currently, GWI remains untreatable, and the long-term neurological disease manifestation is not fully characterized. The present study sought to expand and evaluate the long-term implications of prior GW chemicals exposure on neurological function 6-8 months post-GWI-like symptomatology induction. Additionally, the beneficial effects of delayed treatment with the glycan immunotherapeutic lacto-N-fucopentaose III (LNFPIII) were evaluated. Male C57BL/6J mice underwent a 10-day combinational exposure to the nerve agent prophylactic pyridostigmine bromide (PB) and the insecticide permethrin (PM) (0.7 and 200 mg/kg, respectively). Beginning four months after the GW chemicals exposure, a subset of the mice were treated twice a week until study completion with LNFPIII. Evaluation of cognition/memory, motor function, and mood was performed beginning 1 month after LNFPIII treatment initiation. Prior exposure to PB/PM produced multiple locomotor, neuromuscular, and sensorimotor deficits across several motor tests. Subtle anxiety-like behavior was also present in PB/PM mice in mood tests. Further, PB/PM exposed mice learned at a slower rate, mostly during early phases of the learning and memory tests employed. LNFPIII treatment restored or improved many of these maladaptive behaviors particularly in motor and cognition/memory domains. Electrophysiology data collected 8 months post PB/PM exposure revealed modest aberrations in basal synaptic transmission and long-term potentiation in the dorsal or ventral hippocampus that were improved by LNFPIII treatment. Immunohistochemical analysis (tyrosine hydroxylase; TH) did not detect major PB/PM effects along the nigrostriatal pathway, but LNFPIII increased striatal TH. Additionally, GFAP+ astrocytes and IBA-1+ microglia were increased in PB/PM mice, an effect reduced by LNFPIII. Collectively, long-term neurobehavioral and neurobiological dysfunction associated with prior PB/PM exposure was characterized;

delayed LNFPIII treatment provided many behavioral and biological protective effects in the context of GWI, highlighting its potential as a GWI therapeutic.

4.1 Introduction

Documented associations of wartime related chemical exposures with delayed adverse health outcomes in veterans exist for several conflicts. One notable example is the Vietnam War where increased incidence of certain cancers, reproductive abnormalities, and neurological problems in the years following the war have been attributed, in part, to extensive exposures to pesticides, such as components and contaminants of the herbicidal mixture Agent Orange (NASEM et al., 2018; Stellman and Stellman, 2018). More recent war-related exposures that have impacted veterans' health negatively occurred during the 1990-1991 Gulf War (GW). Nearly 30 years have passed since this war and approximately 30% of GW veterans suffer from Gulf War Illness (GWI), a complex, multi-symptomatic chronic disease (White et al., 2016; DDGWIRP, 2018). The symptoms, including neurological, immunological, and gastrointestinal ailments, appeared shortly after veterans returned from deployment and progressively worsened over time (White et al., 2016; DDGWIRP, 2018; Zundel et al., 2019; Porter et al., 2020). Precise etiology and pathophysiology of this disease are still being investigated, but combinational and frequent exposures to pesticides (eg. Permethrin: PM, chlorpyrifos), insect repellants (N-Diethyl-3-methylbenzamide [DEET]), a nerve agent prophylactic (pyridostigmine bromide: PB), and, in some cases, chemical nerve agents (sarin and cyclosarin) are implicated and have been investigated in laboratory models of GWI (Sullivan et al., 2003; White et al., 2016).

The symptomatology experienced by GWI veterans encapsulates a number of neurological complaints including cognitive and memory dysfunction, motor impairments, and affect alterations such as anxiety and depression (White et al., 2016). Multiple reports highlight impairments in cognition, memory, and reaction speed, with the latter suggesting sensorimotor deficits (Anger et al., 1999; Proctor et al., 2006; Toomey et al., 2009; Hubbard et al., 2014; White et al., 2016; Sullivan et al., 2018). Many of these deficits have been recapitulated in GWI-centered laboratory studies. For instance, varying GW chemical exposure combinations lead to

impairments in spatial working and reference memory, decreases in motor function, and increases in affect measures, i.e., anxiety, in rodents (Abou-Donia et al., 2002; Abou-Donia et al., 2004; Abdullah et al., 2011; Torres-Altoro et al., 2011; Abdullah et al., 2013; Parihar et al., 2013; Hattiangady et al., 2014; Ojo et al., 2014; Joshi et al., 2018; Macht et al., 2019; Joshi et al., 2020).

Several pathogenic factors within GWI, including central nervous system structural changes, neurotransmitter dyshomeostasis, and immune system disruption, are associated with and/or could be responsible for these behavioral deficits. Specifically, brain structural abnormalities, such as reduced hippocampal volume and increased diffusivity in white matter connections, are featured prominently in GWI (Heaton et al., 2007; Chao et al., 2010; Chao et al., 2011; Rayhan et al., 2013; Chao et al., 2015; White et al., 2016). Neurotransmitter alterations such as changes in GABAergic activity in areas maintaining cognition (Megahed et al., 2014; Carreras et al., 2018) and monoaminergic disbalance in areas that regulate motor function, memory, and affect (Megahed et al., 2014; Carreras et al., 2018; Carpenter et al., 2020) have also been demonstrated in GWI models. Recently, a consensus for immune disruption emerged as a common denominator for GWI pathogenesis (O'Callaghan et al., 2015; Michalovicz et al., 2020). This persistent neuroimmune dysfunction manifests as increased systemic inflammation and notable glial activation in the brain of GWI veterans compared to matched controls (Broderick et al., 2013; Parkitny et al., 2015; Broderick et al., 2018; Alshelh et al., 2020). Further, inflammation, especially neuroinflammation, is consistent among several GWI laboratory studies; widespread brain increases in gliosis and inflammatory markers, particularly in the hippocampus, are common findings (Parihar et al., 2013; Megahed et al., 2014; O'Callaghan et al., 2015; Zakirova et al., 2015; Zakirova et al., 2016; Carreras et al., 2018; Miller et al., 2018; Macht et al., 2019; Madhu et al., 2019; Carpenter et al., 2020). In some GWI studies where neuroinflammation was demonstrated, the authors also reported cognitive

and affect deficits (Parihar et al., 2013; Zakirova et al., 2015; Zakirova et al., 2016; Carreras et al., 2018; Joshi et al., 2018).

Because the precise etiology of GWI is still unknown, laboratory models have utilized several combinations of GW-relevant chemicals. It has been reported that veterans with GWI were exposed to higher rates of the nerve agent prophylactic, PB, and pesticides; thus, it is critical to use models incorporating such components (Steele et al., 2012; White et al., 2016). One established GWI model that includes short-term exposure to PB and the insecticide PM (Zakirova et al., 2015) has been used by our group to study the acute and chronic effects of these GWI chemicals (Carpenter et al., 2020; Mote et al., 2020). This model produced behavioral changes consistent with GWI clinical symptomology including anxiety, psychomotor, and cognitive problems, months after exposure termination (Abdullah et al., 2011; Zakirova et al., 2015; Zakirova et al., 2016; Joshi et al., 2018; Joshi et al., 2020). Further, this GWI chemical exposure paradigm leads to increases in hippocampal neuroinflammation, manifested with increased inflammatory cytokines as well as glial activation in the hippocampus and the cortex (Zakirova et al., 2015; Zakirova et al., 2016; Carpenter et al., 2020). However, the magnitude of short-term neuroinflammation following PB/PM exposure is reduced when compared to the acute effects of a model that incorporates a nerve agent surrogate and corticosterone-induced stress (O'Callaghan et al., 2015), suggesting the PB/PM model may exhibit effective construct and etiological validity for milder versions of GWI (Carpenter et al., 2020). Utilizing distinct animal models to recapitulate more or less severe forms of GWI may be particularly relevant to clinical GWI as this disease state has been shown to present in a heterogeneous manner depending on the duration, frequency, and intensity of chemical exposure (Steele et al., 2012). Moreover, the multiple neurobehavioral and neuropathological alterations observed in the PB/PM model overlap with clinical presentation of GWI (Zakirova et al., 2015; Zakirova et al., 2016; Joshi et al., 2018; Joshi et al., 2020), which makes the model suitable for an in-depth

investigation of GWI pathobiology and for evaluating the safety and efficacy of novel GWI treatments.

Efficacious treatment options for GWI are limited, in part due to the heterogeneous presentation of disease symptomology (DDGWIRP, 2018). Nevertheless, therapeutic interventions have been explored and have provided some symptom benefits (Donta et al., 2004; Baraniuk et al., 2013; Golomb et al., 2014; Golier et al., 2016; DDGWIRP, 2018; Helmer et al., 2020; Holton et al., 2020). Because of the underlying immune dysfunction in GWI, immunomodulatory therapies may be an advantageous treatment option. Lacto-N-fucopentaose III (LNFPIII) is an immunomodulatory glycan found in human milk that, to date, has had no documented adverse effects (Atochina et al., 2008; Bhargava et al., 2012; Zhu et al., 2012; Srivastava et al., 2014; Tundup et al., 2015; Carpenter et al., 2020). When conjugated to a dextran carrier, LNFPIII activates CD14/TLR-4 signaling for extracellular signal-regulated kinase (ERK)-dependent production of anti-inflammatory mediators to skew the inflammatory balance of the innate immune system in an anti-inflammatory direction, including in pathological disease states such as experimental autoimmune encephalomyelitis and diet-induced obesity (Atochina et al., 2008; Bhargava et al., 2012; Zhu et al., 2012; Srivastava et al., 2014; Tundup et al., 2015). In fact, in an earlier study where we evaluated LNFPIII treatment effects during GWI chemical exposure, LNFPIII was beneficial in preventing acute monoaminergic disbalance in multiple brain regions and reducing inflammation within the hippocampus (Carpenter et al., 2020). Further, laboratory GWI studies found decreases in cortical (Kimono et al., 2020) and hippocampal (Ribeiro et al., 2020) brain-derived neurotrophic factor (BDNF) levels and density of the tropomyosin receptor kinase B (TrkB) in the hippocampus (Carreras et al., 2018), a receptor fundamentally involved in facilitating the actions of BDNF (Minichiello, 2009). Given that LNFPIII enhances phosphorylation of ERK-1/2 (Tundup et al., 2015), and ERK-1/2 is critical for the production of trophic factors such as BDNF (Alonso et al., 2004), suggesting that LNFPIII may be a promising GWI neurotherapeutic.

Our earlier study utilizing this established PB/PM exposure paradigm indicated acute neuroinflammation and monoaminergic disbalance in multiple cognition- and motor-regulating brain regions were ameliorated by LNFPIII treatment (Carpenter et al., 2020). However, while this therapeutic induced beneficial effects when given in conjunction with GWI chemical exposure, it is unknown whether it will be beneficial when given long after the termination of GWI exposure, i.e., restorative effects. Additionally, the long-term impact of PB/PM exposure on motor and sensorimotor function, as well as electrophysiological aberrations that may shape cognitive deficits associated with this model are yet to be characterized. Thus, the present study sought to expand and evaluate the long-term implications of prior PB/PM exposure on neurobehavioral and neurobiological function 6-8 months post-GWI symptomatology induction. Further, we characterized the neuroinflammatory profile of this model by evaluating glial markers. Finally, we examined whether delayed treatment with the immunotherapeutic LNFPIII was beneficial.

4.2 Methods

4.2.1 Materials

Pyridostigmine bromide (PB; ≥ 98% purity; Sigma Aldrich, St. Louis, MO) and permethrin (PM; 29.5% cis/69.5% trans isomer; 99% purity; Chem Service Inc., West Chester, PA) were used for animal treatments in this study. Lacto-N-fucopentaose III (LNFPIII) dextran conjugate was produced as previously described (Tundup et al., 2015). All additional chemicals and reagents, unless otherwise noted, were of analytical or higher grade and were obtained from Sigma Aldrich or Fisher Scientific (Hampton, NH).

4.2.2 Animals

Male C57BL/6J mice (8-9 weeks old; Jackson Laboratories, Bar Harbor, ME) were housed 4 per cage in an environmentally controlled room (22-24° C) and maintained on a 12 h light/dark cycle (0700-1900 lights on) for one week of acclimation and throughout the study. Mice were handled daily for one week prior to the start of the study to minimize experimenter-

induced stress. Food and water were available *ad libitum*. All procedures were approved in advance by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with the latest National Institutes of Health guidelines.

4.2.3 GWI Model

Following (Zakirova et al., 2015), mice (N=61) weighing 24.4 ± 0.19 g (mean ± SEM) were randomly divided into GWI (PB/PM) and vehicle (DMSO) treatment groups and treated daily for 10 days with a combination of PB and PM (0.7 and 200 mg/kg, respectively) or DMSO vehicle IP. Four months after the initial GWI-chemicals exposure, mice within each of the two original treatments were randomly subdivided into an LNFPIII or a vehicle group and were treated twice a week until study completion with LNFPIII or dextran vehicle (both 35 µg/mouse; SC) diluted in sterile saline as in (Carpenter et al., 2020). Thus, from this point onward there were 4 treatment groups: Vehicle-Vehicle (DMSO-Dextran, n=14), Vehicle-LNFPIII (DMSO-LNFPIII, n=15), PB/PM-Vehicle (PB/PM-Dextran, n=16), and PB/PM-LNFPIII (n=16). Body weights were measured daily during GWI chemicals exposure and biweekly after the last GWI exposure until study completion. During the radial arm maze (RAM), in which mice underwent food restriction, weight measurements were taken daily for monitoring as described under the RAM testing protocol. Refer to Figure 4.1A for a detailed experimental timeline.

4.2.4 Behavioral Tests

One month after LNFPIII treatment inception, a battery of behavioral tests for assessment of cognition/memory, mood, and motor function were performed as described below. All behavioral testing, scoring, and analysis were done in a treatment-blind manner. Refer to Figure 4.1B for a detailed behavioral timeline.

4.2.4.1 Nest Building

Nest building was assessed prior to the Sucrose Preference test to measure cognitive and motor functions; it has been shown that severe hippocampal damage leads to deficits in nest building (Deacon, 2012). Marked striatal damage also leads to these deficits, likely through

a substantial impairment in forelimb and orofacial dexterity (Hofele et al., 2001). Mice were individually housed with a fresh paper nestlet (Bed-r'Nest, The Anderson's Inc., Maumee, OH); pictures of the nests were taken from above at different time points (30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 300 min, 360 min, and 24 h) after nestlet placement and scored based on a 4-point scale (1 = untouched, 4 = nest completed) as in (Deacon, 2012).

4.2.4.2 Sucrose Preference (SP)

The SP test, used to measure anhedonia in mice (two-bottle choice paradigm) (Eagle et al., 2016), was performed during behavioral days 1-8. Briefly, individually housed mice were given access to two bottles filled with tap water for 4 days to establish a baseline and then given access to one bottle of tap water and one bottle of 1.5% sucrose water for 4 additional days. Solution intake was measured daily by weighing bottles, and bottle position was switched after each measurement to prevent side preference. Sucrose preference (%) was determined using the following equation [(total sucrose intake / (sucrose + water intake)) x 100].

4.2.4.3 Elevated Zero Maze (EZM)

The EZM is a test frequently used to assess anxiety-like behaviors in mice but can also be expanded to assess certain motor function-related parameters (Braun et al., 2011; Conrad et al., 2011). On day 12, an EZM apparatus (Stoelting Co., Wood Dale, IL) was used under dim illumination (8W red bulb). Lighting conditions, measured with an URCERI light meter, were 30-40 lux and 10-15 lux in the open and closed quadrants, respectively. Starting at a central, open quadrant position facing a closed quadrant, mice were allowed to freely explore the maze for 5 min. All behaviors were tracked and scored using ANY-Maze software (Stoelting). Mice were considered to be in a quadrant when 70% of their body was in the area. Scored parameters of interest included time spent in the open and closed areas, number of entries into the open and closed areas, and the latency to enter the closed area at the start of the test.

4.2.4.4 Marble Burying (MB)

Following a 3 h resting period post EZM, anxiety-like behaviors were assessed using the MB test as previously described (Krishna et al., 2016) with slight modifications. Briefly, clean testing cages were filled with 5 cm of corn cob bedding (Bed-o'Cobs ¼ inch, The Anderson's Inc., Maumee, OH), and mice were individually placed in these cages for 10 min (habituation phase). After a 1 h resting period, mice were returned to the leveled testing cages with 20 marbles (diameter 10 mm, Panacea Products Corp., Columbus, OH) aligned 4 x 5 in the cage for 20 min (testing phase). A baseline and post-test picture were captured, and the number of marbles buried (>70%) was counted for statistical analysis as in (Krishna et al., 2016).

4.2.4.5 Open field (OF)

Locomotor activity was assessed in an OF test as previously described (Krishna et al., 2016) on behavior day 14. Briefly, mice were individually placed into an open field arena (25 cm x 25 cm x 40 cm; Coulbourn Instruments, Whitehall, PA) and allowed to freely explore for 30 min. The distance traveled, time spent in the center and periphery, and the number of rearings was tracked and scored with the Limelight software (Actimetrics, Wilmette, IL) for both the total 30 min and 5 min intervals.

4.2.4.6 Pole Test (PT)

Following a 5 min resting period after the OF, a PT for motor coordination was used as described (Krishna et al., 2016). Mice were placed upright on a vertical pole (1 x 55 cm; d x h) and the times to turn and complete the test by reaching the bottom (total time) of the pole were recorded. Each mouse underwent 4 trials (5 min inter-trial interval) with the last 3 used for statistical analysis.

4.2.4.7 Grip Strength (GS)

After a resting period following the PT, forelimb GS (in newtons, N) was measured using a gauge attached to a mouse-specific wire grid (Bioseb, France) as previously described (Krishna et al., 2016). Mice were gently placed on the grid and the grip strength was assessed

over 4 trials (1-min inter-trial interval). Body-weight-normalized average and max grip strengths were used for statistical analysis.

4.2.4.8 Novel Object Recognition (NOR)

The NOR test was performed on day 15 as previously described (Krishna et al., 2016). Briefly, the OF on the previous day was used as a habituation phase for the NOR. During the identical phase of the NOR, mice were placed in the open field with two identical objects and allowed to freely explore them for 5 min. Mice were then returned to their home cage for 1 h. During the novel phase, one identical object was replaced with a novel object. Mice were placed in the open field and allowed to explore the novel and identical objects. The number of approaches and time spent at the novel object were scored for the first 20 s of the novel phase with Limelight software (Actimetrics). The novel preference index (NPI) was calculated as described in (Lin et al., 2013).

4.2.4.9 Gait Test

Following a 3 h resting period after the NOR, motor function was evaluated using the gait test as described in (Mulherkar et al., 2013) and (Wang et al., 2017) with slight modifications. Briefly, a runway (82 cm long, 5.5 cm wide with 8 cm high walls) lined with paper was used; an empty cage with home cage bedding was placed at the end of the runway during testing for escape. Prior to testing, the front and hind paws of the mice were painted with non-toxic red and black ink, respectively (Office Depot, item: #839-994 and #839-967). Two trials were conducted (5 min apart), a pre-trial and the actual trial that was used for subsequent statistical analyses. Between each trial, the runway was cleaned with 70% EtOH and replaced with a new paper strip. Gait stride length, base width, inter/intra-step distance, stride variability, total steps, and velocity were determined from (Mulherkar et al., 2013) and (Wang et al., 2017).

4.2.4.10 Sticker Removal (SR)

The SR test was used to determine sensorimotor function (Fleming et al., 2013) on behavioral day 17. Food was removed from the rodents' home cage and ¾ of the bedding was

transferred to a clean holding cage where the mice acclimated for 1 h. During the testing phase, a small circular sticker (Avery ¼" round label 5793, Office Depot item: # 113019) was applied using forceps to the nose of the mouse, and the mouse was placed into the home cage. This test was performed over three 90 s trials with a 5 min inter-trial rest. Parameters used for statistical analysis included the average and fastest times for contact and removal of the sticker (Fleming et al., 2013).

4.2.4.11 Swim Test (ST)

Following a 3 h rest after the SR, depressive-like behaviors were assessed using the ST as previously described (Krishna et al., 2016). Briefly, mice were placed in a large beaker filled with 3 L of water (29 ± 2 ºC) for 15 min. After each test, the beaker was sanitized thoroughly. Total climbing counts and time spent climbing, mobile, and immobile were scored using Limelight software (Actimetrics). Additionally, the time to the first immobile bout was measured using ANY-Maze (Stoelting).

4.2.4.12 Barnes Maze (BM)

The BM test was used to assess spatial learning and memory on behavioral testing days 19-24 (Sunyer et al., 2007). Using a 20-hole circular maze (Stoelting) in which one is equipped with an escape box (target hole; TH). The maze is brightly illuminated to promote anxiogenic escape motivation (~1000 lux measured by URCERI light meter). During the acquisition phase (days 1-4), mice were trained to learn and escape into the TH over four 3-min trials, and after trial completion, mice remained or were manually placed into the TH for 1 min. For the probe trial, the escape box was removed, and mice explored the maze for 90 s. Between mice, the maze was rotated and sanitized to remove any residual olfactory cues. The latency, distance, and number of errors to reach the TH were tracked and scored automatically using ANY-Maze software (Stoelting).

4.2.4.1 Radial Arm Maze (RAM)

An 8-arm RAM (Med Associates, St. Albans, VT) was used to assess learning and memory function (Babb and Crystal, 2006; Preston et al., 2019) in animals 7 months post-PB/PM exposure (refer to Figure 4.1). The maze has a food trough at the end of each arm with a photosensor activated dispenser (20 mg sucrose pellet). External visual cues were located around the room to facilitate spatial navigation. MED-PC 4.0 (Med Associates) was used to control and record photosensor, food, and door signals from an adjacent room. Prior to RAM testing, mice were single housed and underwent a 14-day food restriction protocol until they reached 85% of their free-fed body weight; this was used to motivate learning of the RAM apparatus. During the last 3 days of the food restriction period, mice were behaviorally acclimated to the RAM, and the apparatus was cleaned as in (Preston et al., 2019).

Spatial short-term working memory was assessed by an 8-arm RAM foraging task over 10 consecutive days. Following a 20 min room acclimation period, each mouse was placed in the central RAM hub for 1 min before all 8 doors to each arm opened and allowed free access to all arms. In the foraging task, each arm is “baited” so that one food reward was dispensed upon the first head poke into the food trough of each arm. An error in spatial short-term working memory was defined as an animal revisiting a trough after the initial visit, and the total number of errors for each animal was recorded. Animal performance was assessed by the average total number of errors of the first three days of testing, the last three days of testing, and comparing the two.

Spatial long-term working memory was evaluated over 10 consecutive days using a modified delayed spatial Win-Shift task after the last day of the foraging task (Furgerson et al., 2014; Clark et al., 2015). This test incorporates a 2-phase (the study phase and the test phase) procedure with an interposed delay. During the study phase, 4 arms were randomly opened, allowing the animal to receive a reward from 4 baited arms. Following the completion of the study phase, the animal was returned to the home cage for a 4 min retention interval, and the

maze was cleaned. Then, the animal was returned to the central hub for 1 min (total retention interval of 5 min) before the initiation of the test phase in which all 8 doors were opened, allowing free access to all arms. However, only the 4 arms that were unopened in the study phase dispensed food reward. An error in spatial long-term working memory was defined as a visit to an arm that was baited in the study phase and the total number of errors was recorded. Animal performance was determined by assessing the mean errors from the first three days of testing, the last three days of testing, and comparing the two as in (Clark et al., 2015).

4.2.5 Slice Preparation and Electrophysiology

Two weeks after completion of the RAM, brains from the mice slated for electrophysiological assessment were removed following deep anesthetization with halothane (Sigma) and were submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120mM NaCl, 3mM KCl, 4mM $MgCl_2$, 1mM NaH_2PO_4, 26mM $NaHCO_3$, and 10mM glucose). A mid-sagittal cut was made, and half of the brain was mounted on its dorsal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm sections containing the vH. The other half was mounted on its caudal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm sections containing the dH. The vH and dH were subdissected from the rest of the slice and the CA3 region was removed. Slices were then transferred to a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120mM NaCl, 3mM KCl, 1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, and 10mM glucose) and allowed to recover for 45 min at room temperature followed by 45 min at the recording temperature of 30 °C. Next, a bipolar stimulating electrode (Kopf Instruments, Tujunga, CA) and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments, Sarasota, FL) were placed in the stratum radiatum of CA1. A stimulus pulse was applied once every min consisting of a single square wave of 270 µs duration to elicit field excitatory postsynaptic potentials (fEPSPs) at Schaffer collateral-CA1 synapses.

A stimulus-response curve was acquired at the beginning of each experiment by incrementally delivering single 15 µA stimulus pulses ranging from 45-180 µA in intensity and measuring fEPSP slope (mV/ms) in a 1 ms interval immediately following the fiber volley. The stimulus intensity was adjusted to elicit stable baseline responses of approximately 35% of the maximal fEPSP slope. Paired-pulse responses were monitored each min for 5 min via paired-pulse stimulation with an interpulse interval of 50 ms. Baseline responses were recorded each min for 30 min prior to a high-frequency stimulation (HFS) protocol (3 x 100 Hz/1 s train at 20 s intervals) to induce long-term potentiation. fEPSP responses were monitored for the subsequent 60 min following HFS. fEPSP responses were normalized to baseline using the average value of the five responses immediately prior to HFS. LTP magnitude was determined via averaging the normalized fEPSP slope values at 56–60 min post-HFS. Electrophysiology data was digitized at 10kHz, low-pass filtered at 1kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunnyvale, CA).

4.2.6 Tissue Collection

Following euthanasia, blood was collected in Na citrate (0.109M) containing tubes (BD, San Jose, CA) for plasma harvesting. Immediately afterward, organs (brain, inguinal lymph nodes, spleen, thymus, liver, and kidney) were weighed and frozen on dry ice. All tissues were stored at -80° C until analysis. For the brain only, a sagittal cut was made, and one half was quickly frozen on dry ice while the other half was immersion fixed as in (Krishna et al., 2016).

4.2.7 Immunohistochemistry

Fixed brains were coronally sectioned into 40 µm thick sections that were placed in phosphate buffer at 4°C until staining. Free-floating sections containing the striatum (STR) were labeled with anti-tyrosine hydroxylase (TH) for assessment of dopaminergic neurons/ terminal health as done previously (Filipov et al., 2009; Krishna et al., 2014). Briefly, for both bright field and immunofluorescence, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide (30%) and 0.3% normal goat serum in 1x TBS for 5 min and blocked in 3% normal

goat serum for 30 min at room temperature (RT). Following blocking, sections for bright field were immunostained with anti-TH primary antibody (1:2000; Millipore AB152) for 48 h at 4°C. Following primary antibody incubation, sections were thoroughly washed and incubated with the appropriate biotinylated secondary antibody (1:200; Vector Laboratories) for 30 min at RT, followed by a 30 min incubation with a Vector ABC reagent standard detection kit (Vector Labs, Burlingame, CA). TH was visualized by development in 3,3′ diaminobenzidine (DAB) for approximately 3 min, followed by incubation in cresyl violet (CV) for 3 min to stain all neurons. For immunofluorescent sections, sections were incubated with a primary antibody cocktail (1:1000 rabbit anti-IBA-1 microglia marker, Wako Chemicals, Richmond, VA; and 1:3000 chicken anti-GFAP astrocyte marker, Aves Labs, Davis, CA) diluted in 0.1% TritonX-100 in PBS for 48 h at 4°C. Following primary antibody incubation, sections were thoroughly washed and incubated with the appropriate secondary fluorescent antibodies (GFAP: 1:1000 goat anti-rabbit 594, Abcam; and IBA-1: 1:1000 goat anti-chicken 488, Abcam) for 2 h at RT in the dark, followed by incubation with a nuclear stain for 5 min (Hoechst 33258, Invitrogen). Following the final washes, sections were mounted to slides, fixed with an ethanol gradient followed by xylene clearing, and coverslipped with VectaMount (Vector Labs).

Images were taken on a Zeiss Axioscope A1 and analysis of TH intensity, GFAP+ or IBA-1+ cells and signal intensity was done using ImageJ software. Regions of interest (ROIs) in the STR included the dorsolateral and ventromedial STR and were captured at 5x (n = 5 animals per group/3 sections per animal). The hilus region of the hippocampus was captured at 40x (11-19 images per treatment group). At the higher magnification (40x) GFAP+/Hoescht+ and IBA-1+/Hoescht+ cells were counted in the hilus region of the hippocampus using the Cell counter plugin of ImageJ.

4.2.8 Statistical Analysis

A two-way analysis of variance (ANOVA) was used to determine main effects of treatments or treatment interactions. If an ANOVA was significant ($p \leq 0.05$), treatment means

were separated by Student-Newman-Keuls (SNK) *post hoc* test or planned pairwise comparisons (Student's t-test, as appropriate). All data, except electrophysiology and the RAM, were analyzed using SigmaPlot 12.5 (San Jose, CA), and all graphs were generated using GraphPad Prism 5 (San Diego, CA). Statistical analyses for electrophysiology and RAM data were analyzed using R version 3.3.2 (R Development Core Team, 2016, https://www.r-project.org/) and was completed via aov() function for analysis of variance (ANOVA) and the t.test function for the Student's *t*-test and paired *t*-test.

4.3 Results

4.4.1 Body Weights

Body weights of the vehicle and PB/PM groups were not different at the start of the GWI treatment (24.45 ± 0.29 g vs. 24.33 ± 0.26 g, $p > 0.75$) or subsequent bi-weekly weigh-ins (p's ≥ 0.29) up to 4.5 months after the initial PB/PM exposure (Fig. 4.1C). At 5 months, the vehicle and PB/PM groups were randomly subdivided into an LNFPIII or a vehicle group for a total of 4 groups. There were no significant treatment differences for months 5-9, but at sacrifice (9 months), there was a trend ($p \leq 0.08$) for increased body weight in the PB/PM-treated mice (Fig. 4.1C).

4.3.2 Motor effects after prior PB/PM exposure and delayed LNFPIII treatment

4.3.2.1 Pole Test (PT)

PT performance was unaffected by either PB/PM or LNFPIII. The means and fastest times to turn ($p \geq 0.18$) and complete the test ($p \geq 0.18$) were not different among treatments (data not shown).

4.3.2.2 Grip Strength (GS)

Weight-normalized GS, an indicator of neuromuscular function, tended to be decreased by prior PB/PM exposure (average GS $p = 0.08$; max GS $p = 0.09$; Fig. 4.2A). LNFPIII treatment did not affect the performance of mice in this test significantly; that is, the immunotherapeutic only numerically increased GS, regardless of PB/PM treatment (Fig. 4.2A).

4.3.2.3 Sticker Removal (SR)

Both fastest contact (numerically) and fastest removal ($p \leq 0.05$; Fig. 4.2B) times were increased in PB/PM-treated mice. LNFPIII treatment eliminated these increased latencies for removal or contact with the sticker. In the case of the fastest removal, where the PB/PM effect was significant, the beneficial effect of LNFPIII was also significant ($p \leq 0.01$; Fig. 4.2B).

4.3.2.4 Open Field (OF)

Locomotor activity was significantly affected by prior PB/PM exposure regardless of LNFPIII as evident by decreases in the distance traveled ($p \leq 0.01$; Fig. 4.2C), line crossings ($p \leq 0.01$; data not shown), and vertical activity ($p \leq 0.05$; Fig. 4.2C) during the first 5 min in the exploratory phase of the test. These locomotor effects did not persist, although there was a similar non-significant decrease in the distance traveled for the overall 30 min (data not shown). As expected, all mice habituated to the arena as all groups traveled less distance (Fig. 4.2D) and made fewer line crossings over time (data not shown) (p's ≤ 0.001).

4.3.2.5 Gait Test

Prior PB/PM exposure caused few specific alterations in gait. PB/PM mice took significantly more steps ($p \leq 0.05$; Fig. 4.2E) and exhibited a numerical increase in hindlimb base width (data not shown). The step deficit caused by prior PB/PM treatment was not apparent in mice treated with LNFPIII (Fig. 4.2E). Additionally, LNFPIII treatment had beneficial effects on gait irrespective of PB/PM; it increased the stride length ($p \leq 0.01$; Fig. 4.2F) and forepaw interstep distance ($p = 0.08$; data not shown) while decreasing stride length variability ($p \leq 0.05$; Fig. 4.2G) and paw overlap (right paw, $p \leq 0.05$; data not shown). Average speed, cadence, or time to finish were unaffected (data not shown).

4.3.2.6 Elevated Zero Maze (EZM)

Motor function with the EZM was evaluated by timing the closed-arm entry. There were no significant differences, but PB/PM treated mice were numerically slower to enter the closed

arm after initial placement on the maze (p = 0.12; Fig 4.2H). LNFPIII shortened the latencies to enter the closed arm (p > 0.35).

4.3.3 Long term mood effects of prior PB/PM exposure and delayed LNFPIII treatment

4.3.3.1 Marble Burying (MB) Test

The average number of buried marbles (≥ 70%) during the test, irrespective of treatment, was 9 (45%). Mice's performance in this test was unaffected statistically by PB/PM exposure, LNFPIII, or their interaction. There was, however, a trend (p = 0.09; Fig. 4.3A) for prior PB/PM treatment to decrease the number of buried marbles, likely due to a neuromuscular deficit rather than an anxiolytic effect.

4.3.3.2 Elevated Zero Maze (EZM)

A trending treatment interaction (PB/PM x LNFPIII, p = 0.055; Fig. 4.3B) was present for open entries, in which PB/PM mice made fewer entries into the open arm than control (p = 0.07), a trend that was abrogated with delayed LNFPIII treatment. A similar trend was observed when times in open vs closed-arm were analyzed, i.e., PB/PM mice spent less and more time on the open and closed arms, respectively, and LNFPIII prevented this anxiety-like trend (p = 0.10).

4.3.3.3 Open Field (OF)

PB/PM treated mice exhibited more anxiety-like behavior in the OF; they spent less and more time in the center and periphery of the OF arena, respectively (p's ≤ 0.05; Fig. 4.3C). These anxiety-like deficits caused by PB/PM exposure were not seen in mice treated with LNFPIII (Fig. 4.3C).

4.3.3.4 Swim Test (ST)

Climbing attempts and times spent mobile vs. immobile were not affected by PB/PM and/or LNFPIII during the 1st 5 min of the test or for the total 15 min test duration (immobile time: p ≥ 0.12; Fig. 4.3D), albeit the climbing was numerically decreased by PB/PM treatment (data not shown). When the latency to the first bout of immobility was analyzed, there was a trend (p

= 0.06) for a potentially anxiety-driven increase in the latency in the PB/PM treated mice, a trend that was mitigated in the mice that were also treated with LNFPIII (Fig. 4.3E).

4.3.3.5 Sucrose Preference (SP)

All groups exhibited strong sucrose preference ($p \leq 0.001$; data not shown) and there were no significant main treatment differences (%: $p \geq 0.16$; Fig 4.3F). Interestingly, there was a significant treatment interaction (PB/PM x LNFPIII, $p \leq 0.05$) for total sucrose consumed in which LNFPIII increased sucrose consumption within PB/PM groups, suggesting LNFPIII may be beneficial in increasing sensitivity to rewarding stimuli in animals previously exposed to GWI chemicals.

4.3.4 Cognition and memory effects of prior PB/PM exposure and delayed LNFPIII treatment

4.3.4.1 Nest Building

Nest building was not affected by either treatment as all groups built full nests over the course of 24 h (time effect, $p \leq 0.001$; Fig. 4.4A). Moreover, at each time point, there were no differences between PB/PM or LNFPIII treatments, suggesting prior PB/PM exposure did not cause severe hippocampal and/or striatal deficits that are associated with decreased performance in this test.

4.3.4.2 Barnes Maze (BM) Test

Significant main effects of day for responses variables such as path length, latency to the target hole, and errors demonstrated that all animals learned the target hole (TH) location as they took less time, made fewer errors, and traveled less distance over the 4 days of maze training (p's ≤ 0.001; errors, Fig. 4.4B). However, prior PB/PM treatment led to acquisition phase deficits. Specifically, PB/PM mice were slower to learn and remember the TH location as evident on the last day of training (day 4), where PB/PM treated groups, irrespective of LNFPIII treatment, exhibited significant increases in path length ($p \leq 0.01$; data not shown), latency to TH ($p \leq 0.01$; data not shown), and errors ($p \leq 0.001$; Fig. 4.4B). Mice's performance in the probe trial was unaffected (e.g., probe trial errors; $p \geq 0.85$; data not shown).

4.3.4.3 Novel Object Recognition (NOR)

Short-term object recognition memory assessed with the NOR was impaired by PB/PM. Prior PB/PM exposure led to significant decreases in the novelty preference index (NPI)($p \leq 0.05$; Fig. 4.4C), as well as novel object approaches and time spent at the novel object (p's ≤ 0.05; data not shown). LNFPIII treatment did not modulate these effects in a significant manner.

4.3.4.4 Radial Arm Maze (RAM)

Following 10 consecutive days of training in a dH-dependent 8-arm radial arm maze (RAM) uninterrupted foraging task (Potvin et al., 2006), all mice significantly improved their performance over time ($p \leq 0.001$; Fig. 4.4D). These data suggest that PB/PM exposure did not significantly impair dH-dependent short-term spatial working memory. Interestingly, LNFPIII treated mice initially (days 1-3) made significantly fewer errors (LNFPIII main effect $p \leq 0.01$; Fig. 4.4D), suggesting that LNFPIII improves early performance in this task irrespective of PB/PM treatment.

Similar to the foraging task, following 10 consecutive days of training in a vH-dependent modified Win-shift task to assess long-term spatial working memory (Floresco et al., 1997; O'Neill et al., 2013; Clark et al., 2015; Tamura et al., 2017), a significant main effect of training (Days 1-3 vs Days 8-10; $p \leq 0.05$), but not treatment, on performance over time was present (Fig. 4.4E). This main training effect was not due to a group-specific error reduction. Rather, it was due to a significantly worse (more errors) performance of PB/PM mice compared to control mice on days 1-3 ($p \leq 0.05$; Fig. 4.4E), an effect not seen in PB/PM mice that were also treated with LNFPIII.

4.3.5 Dorsal and ventral hippocampal electrophysiology 8 months post PB/PM exposure and 4 months post-LNFPIII treatment initiation

At 8 months post-PB/PM exposure and roughly 4 months after LNFPIII treatment initiation, no significant main effect of PB/PM exposure on dH LTP magnitude was observed. However, a significant main effect of LNFPIII treatment resulted in increased dH LTP magnitude

($p \leq 0.01$), leading to enhanced dH LTP magnitude in LNFPIII treated mice compared to their vehicle counterparts (p's ≤ 0.05; Fig. 4.5A). A significant treatment interaction effect (PB/PM x LNFPIII; $p \leq 0.05$) on basal synaptic transmission resulted in reduced dH basal synaptic activity at 90, 105, 135, 150, 165, and 180 μA stimulus intensities in PB/PM exposed animals (p's $\leq$ 0.05); this was not seen in animals also treated with LNFPIII (Fig. 4.5B). Further, dH paired-pulse facilitation (PPF) was not impacted by either treatment (Supplemental Fig. 4.1A).

In the vH, LTP magnitude was unaffected by PB/PM; however, LNFPIII enhanced vH LTP magnitude (PB/PM-LNFPIII vs PB/PM-vehicle mice; $p < 0.05$; Fig. 4.5C). A significant PB/PM treatment x stimulus intensity interaction effect resulted in a reduction of vH basal synaptic transmission in PB/PM exposed animals ($p \leq 0.001$). Specifically, there was a significant reduction in vH basal synaptic transmission at stimulus intensities of 90, 120, 135, and 150 μA (p's ≤ 0.05) and a trending reduction at 105 and 165 μA ($p = 0.053$ and $p = 0.054$, respectively) in PB/PM mice compared to control mice (Fig. 4.5D). Further, LNFPIII treatment significantly recovered vH basal synaptic transmission at 75, 90, and 105 μA (p's ≤ 0.05) and numerically at 120, 150, and 165 μA stimulus intensities (p's ≤ 0.10) in PB/PM exposed mice (Fig. 4.5D). No significant treatment effects were observed on vH PPF 8 months after PB/PM exposure (Supplemental Fig. 4.1B).

4.3.6 Tyrosine hydroxylase (TH) immunoreactivity in the dorsolateral and ventromedial striatum 9 months post PB/PM exposure and delayed LNFPIII treatment

In the dorsolateral striatum (DL STR), there were no significant main treatment effects for TH immunoreactivity. However, LNFPIII increased TH optical density in the vehicle-LNFPIII mice ($p \leq 0.05$; Fig. 4.6A). A similar effect was also apparent in the ventromedial (VM) STR, in which there were no significant main effects of either treatment on TH levels, but there was a trend for TH increase in the vehicle-LNFPIII group ($p = 0.06$; Fig. 4.6B). See Figure 4.6C for representative images.

4.3.7 GFAP and IBA-1 immunoreactivity in the hilus region of the hippocampus 9 months post PB/PM exposure and delayed LNFPIII treatment

Analysis of astrocytes (GFAP+/Hoescht+) in the hilus region of the hippocampus revealed a trending interaction (PB/PM x LNFPIII; $p = 0.06$); pairwise-comparisons between the vehicle-vehicle and PB/PM-vehicle groups revealed increased GFAP by PB/PM ($p \leq 0.05$; Fig. 4.7A). This PB/PM increase in GFAP was eliminated by LNFPIII ($p \leq 0.05$; Fig. 4.7A). Similar analysis of microglia (IBA-1+/Hoescht+) in the hilus revealed a significant interaction (PB/PM x LNFPIII; $p \leq 0.05$) in which LNFPIII reduced the abundance of IBA-1 immunoreactivity within PB/PM-treated mice ($p \leq 0.05$; Fig. 4.7B). See Figure 4.7C for representative GFAP/IBA-1 staining.

4.4 Discussion

Multiple preclinical studies have characterized acute and persisting neurological aberrations in PB/PM-based GWI models (Abdullah et al., 2011; Abdullah et al., 2013; Zakirova et al., 2015; Zakirova et al., 2016; Zakirova et al., 2017; Joshi et al., 2018; Joshi et al., 2019; Joshi et al., 2020), but some knowledge gaps remain. The present study sought to extensively evaluate the long-term implications of prior GWI chemical (PB/PM) exposure on neurobehavioral and neurobiological function 6-8 months post GWI-related symptomology induction. Additionally, treatment with an immunomodulatory agent, LNFPIII, was initiated several months post PB/PM exposure to assess its beneficial effects in the context of GWI. As expected, prior PB/PM-treatment led to multiple behavioral deficits in motor, mood, and cognitive/memory functions that, at this stage, were mild to moderate in nature. LNFPIII treatment, initiated months after PB/PM exposure, improved multiple outcomes. The PB/PM-related deficits and benefits of LNFPIII were further examined through ex-vivo electrophysiology recordings for hippocampal function and post-mortem immunohistochemical analysis of dopamine homeostasis and glial status in the brain. Overall, delayed LNFPIII treatment ameliorated numerous PB/PM-elicited

maladaptive changes in neurobehavioral and neurobiological function months after the initial exposure period (refer to radar plot summary, Fig. 4.8).

To identify potential mechanisms influencing the observed motor and mood behavioral deficits, striatal tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine (DA) synthesis, was evaluated immunohistochemically. In an earlier study, we examined the effects of PB/PM on DA 6 h post-exposure found that PB/PM increased DA turnover (utilization) in the striatum (STR) (Carpenter et al., 2020); however, whether these effects persist long term remained undetermined. Historically, neuropathological alterations along the nigrostriatal pathway, comprised of DA projections from the substantia nigra pars compacta (SNpc) to the STR (medial, dorsolateral), are associated with motor impairments (dyskinesia, rigidity, and posture instability) seen in neurodegenerative disorders, such as Parkinson's Disease (PD) (Kalia and Lang, 2015). Additionally, disruption in the mesolimbic pathway, consisting of DA projections from the ventral tegmental area (VTA) to the ventromedial (VM) STR, can lead to affect disorders such as anhedonia, anxiety, and depression, also observed in some patients with PD (Cooney and Stacy, 2016). Current data indicate that 9 months post PB/PM exposure, nigral VTA DA neuron terminals are not affected significantly. Given that DA neurons, especially nigrostriatal ones, decline with neuronal aging and GWI veterans experience accelerated aging (Zundel et al., 2019), it will be prudent to evaluate this axis 10-14 months post-exposure. Interestingly, LNFPIII treatment, independent of PB/PM exposure, increased the levels of DA terminal field (TH) intensity in both the dorsolateral (DL) and VM STR, suggesting that LNFPIII may be beneficial in the absence of GWI exposures. LNFPIII treatment may also be beneficial later in the progression of the disease, e.g. in the aged veterans with GWI.

In this study, several parameters of motor function were impacted by PB/PM GWI treatment. Neuromuscular function (grip strength) was moderately decreased by prior PB/PM exposure. These results are consistent with an earlier study demonstrating a short-term decrease in grip strength post-PB/PM exposure, where PM was applied topically and the PB/PM

ratio/duration of exposure differed from the present study (Abou-Donia et al., 2004). Of note, sensorimotor function was impaired as PB/PM mice performed worse than controls in the sticker removal (SR) task, and this deficit was eliminated by LNFPIII. This new information is in line with reports of GWI veterans exhibiting sensorimotor problems (Axelrod and Milner, 1997; Proctor et al., 2006; Toomey et al., 2009), and suggests that LNFPIII might ameliorate this aberration. Additionally, PB/PM exposure decreased marble-burying activity, an effect normally interpreted as an inhibition of anxiety-like behavior. However, considering the neuromuscular and sensorimotor deficits observed, the decreased burying might be indicative of a motor impairment. Impaired locomotor performance was present in the EZM, as PB/PM mice entered the closed arms more slowly than controls post placement on the maze; this abnormal effect was ameliorated numerically by LNFPIII. Open Field locomotor deficits presented as decreases in distance traveled and rearing during the exploratory phase of the test. These results align with a similar study in which 1 month post-GW chemicals exposure, there was a decrease in total distance traveled in PB/PM mice (Abdullah et al., 2011). Together, the present study and (Abdullah et al., 2011), suggest that motor deficits, especially in exploratory behavior, manifest early and likely persist post-PB/PM exposure. Further, these deficits in locomotor function may be propagated by PB/PM gait deficiencies as PB/PM mice took significantly more steps and exhibited increased hindlimb base width in the gait test. No fat distribution differences existed among groups (data not shown), suggesting that PB/PM mice exhibited a wider stance for postural stability. This interpretation is in line with a previous report demonstrating that PB/PM treatment-induced subtle, ataxic-like effects (Abdullah et al., 2011). Taken together with previous motor data, gait deficiencies observed in this study suggest that PB/PM mice have a compromised ability and/or compensate more to achieve normal locomotor function from controls. LNFPIII prevented the increased step number, stride length, and it also reduced stride length variability, suggesting this immunotherapeutic has gait and postural effects that may not only be beneficial in the context of GWI, but overall as well.

Altered mood has been consistently reported in veterans with GWI (Sullivan et al., 2003; Janulewicz et al., 2017) and experimental GWI models (Abdullah et al., 2013; Parihar et al., 2013; Zakirova et al., 2016; Carreras et al., 2018; Joshi et al., 2018; Phillips et al., 2019). Here, months after PB/PM exposure, mood impairments were predominantly anxiety-like. In the EZM, subtle, anxiety-like behaviors were identified by numerical decreases in open arm entries and time spent in open arms for PB/PM mice with the former response variable approaching significance. PB/PM-associated anxiety-like behavior also manifested as significant decreases and increases in time spent in the center and periphery in the OF, respectively; LNFPIII prevented these anxiety-like effects. Differences in depressive-like behaviors were not observed among groups for behavioral despair (ST) or anhedonia (SP). Although climbing attempts, an escape behavior in the ST, were numerically decreased in PB/PM mice, this aligned more so with the observed motor impairments than mood dysfunction. Interestingly, PB/PM mice were mobile for a longer duration prior to the first bout of immobility, suggesting this effect may also be anxiety-related. It has been suggested that certain forms of anxiety (i.e., hyperactive and panic states) may precede depression; thus, it is possible that this increased latency to behavioral despair is due to the initial anxiogenic effect of the ST (Paul, 1988; Boyer, 2000; Merikangas et al., 2003; Polani, 2004). Of note, this effect was not observed with LNFPIII, further suggesting that this novel treatment ameliorates GWI-related affect dysfunction. Mood alterations in experimental GWI models vary and depend on the animal's age and GW chemicals used to recapitulate the disease (Abdullah et al., 2011; Zakirova et al., 2016; Carreras et al., 2018; Joshi et al., 2018). For example, studies investigating behaviors at early time points post-GW chemicals/manipulations (e.g. DEET, diisopropylfluorophosphate, and stress) indicated increases in both anxiety- and depressive-like behaviors (Parihar et al., 2013; Carreras et al., 2018; Phillips et al., 2019). However, studies utilizing the PB/PM paradigm only detected anxiety-like behaviors shortly after exposure (Abdullah et al., 2012). Interestingly, long-term studies did not observe persistent anxiety-like behavior in PB/PM treated mice on the

Elevated Plus Maze and OF, but did observe depressive-like behavior in the ST 10 and 13 months post-exposure (Zakirova et al., 2016; Joshi et al., 2018). The present findings observed anxiety-like, but not depressive-like, behavior 6-7 months post PB/PM exposure, providing insight into the progression of mood alterations of animals tested 6-7 months post-PB/PM exposure. Our findings shed additional light into a possible shift from anxiety to depression in the context of GWI and other conditions over time (Paul, 1988; Merikangas et al., 2003).

Consistent with clinical complaints of GWI veterans (White et al., 2016), previous animal studies have observed several learning and memory deficits months after PB/PM exposure that are generally attributed to pathological changes in the hippocampus (Abdullah et al., 2011; Zakirova et al., 2015; Zakirova et al., 2016; Joshi et al., 2018). The dorsal (dH) and the ventral (vH) hippocampus are increasingly appreciated as functionally distinct structures where the dH primarily serves as a center for learning and memory whereas the vH is fundamentally involved in regulating stress/emotional responses (Fanselow and Dong, 2010; Papatheodoropoulos, 2018). In the present study, profound PB/PM-induced impairments were not observed in dH- and vH- specific learning and memory tasks such as the Radial Arm Maze (RAM), Barnes Maze (BM), and nesting behavior tests. However, short-term, dH- and perirhinal cortex-specific recognition memory was impaired in the Novel Object Recognition (NOR) task by prior PB/PM exposure. Further, although PB/PM exposed mice improved their performance in the BM, they did so at a significantly slower rate than controls in the training phase; LNFPIII did not improve these measures. This finding is consistent with a previous study examining performance in the BM 15 months post PB/PM exposure (Zakirova et al., 2016). Similar studies assessing the impact of PB/PM exposure on BM performance did not observe this effect at 5- or 22-months post-exposure, highlighting a potential transient, adverse neurological effect on reference memory at 8-15 months after PB/PM exposure (Zakirova et al., 2015; Zakirova et al., 2016), but this needs further investigation. Likewise, all groups improved their performance in the dH-dependent short-term spatial working memory RAM foraging task. Of note, LNFPIII groups

performed significantly better early in this test. No observable deficits in the vH-dependent long-term spatial working memory RAM Win-shift task were noted by the end of training in the paradigm. However, similar to the training phase of the BM, PB/PM mice performed significantly worse early-on, an effect that was not observed in PB/PM mice that received LNFPIII treatment. Overall, these data suggest modest aberrant and ameliorative effects of PB/PM exposure and LNFPIII treatment, respectively, on dH and vH-dependent behavioral function. Identifying hippocampal dorsoventral-specific effects of prior PB/PM exposure and delayed LNFPIII treatment on synaptic plasticity and transmission may be critical for interpreting the subtle hippocampal-dependent behavioral abnormalities/ameliorations presented here.

Previous studies indicate PB/PM exposure can lead to a neuroinflammatory phenotype that manifests with prolonged aberrations in glial function and sustained inflammation (Abdullah et al., 2011; Zakirova et al., 2016; Joshi et al., 2018; Joshi et al., 2019; Carpenter et al., 2020). Under conditions of neuroinflammation, enhanced inflammatory cytokine production leads to aberrant activation of glial cells and subsequent impairment of hippocampal synaptic plasticity and transmission, resulting in deficits in learning and memory (Maher et al., 2006; Kelly et al., 2013; Habbas et al., 2015; Prieto and Cotman, 2017). Furthermore, enhanced expression of pro-inflammatory cytokines reduces the secretion of hippocampal brain-derived neurotrophic factor (BDNF) (Barrientos et al., 2004; Yirmiya and Goshen, 2011; Littlefield et al., 2015), a critical trophic factor involved in synaptic transmission (Kang and Schuman, 1995). Studies utilizing the PB/PM GWI model have found varying results surrounding glial activation in the hippocampus during the progression of GWI. In one study, at 5 months post PB/PM exposure, astrogliosis but not microgliosis was apparent (Abdullah et al., 2011), whereas both were observed at 15 months (Abdullah et al., 2016; Joshi et al., 2018), but not different from controls at 22 months (Zakirova et al., 2016). These results are in line with the current study. Here, at 9 months post-prior PB/PM exposure, immunohistochemical analysis of GFAP (astrocytes) and IBA-1 (microglia) revealed a PB/PM-dependent GFAP increase and a less pronounced one of

IBA-1 in the hippocampus. Importantly, LNFPIII treatment reduced both GFAP and IBA-1 within the GWI context, indicative of its anti-inflammatory properties. This sustained neuroinflammation post-GWI exposures may underlie the persisting impairments in hippocampal synaptic transmission as well as the beneficial effects of LNFPIII discussed herein.

Hippocampal electrophysiology data revealed reduced dH and vH basal synaptic transmission by PB/PM, an effect that is consistent with a GWI study employing a different exposure paradigm that observed prolonged deficits in excitatory and inhibitory hippocampal neurotransmission (Wang et al., 2020). These preclinical findings of persisting disruptions in glutamatergic neurotransmission are in line with a recent clinical trial that observed amelioration of some GWI-specific symptoms in veterans that received a low glutamate diet (Holton et al., 2020). Interestingly, LNFPIII recovered vH, but not dH, basal synaptic transmission, which may be attributed to LNFPIII-mediated normalization of glial function, namely of pro-inflammatory cytokine secretion and enhancement of anti-inflammatory cytokine production in this inflammation-sensitive hippocampal sector (Onufriev et al., 2017; Pearson-Leary et al., 2017; Zheng et al., 2017; Kyran et al., 2018; Pearson-Leary et al., 2019; Pearson-Leary et al., 2020).

Finally, hippocampal LTP, a well-established synaptic substrate for learning and memory (Bliss and Collingridge, 1993; Whitlock et al., 2006), was assessed to determine if PB/PM exposure adversely impacted hippocampal synaptic plasticity and if LNFPIII would be beneficial. While PB/PM exposure did not affect LTP, an enhancement of dH and vH LTP magnitude was detected in LNFPIII-treated mice, suggesting prolonged treatment likely increased ERK-1/2 phosphorylation and potentiated hippocampal synaptic efficacy (Tundup et al., 2015). Additionally, a lack of a PB/PM-induced effect on a presynaptic-related mechanism of synaptic plasticity (i.e paired-pulse facilitation) in addition to PB/PM-mediated impairments of dH and vH synaptic transmission is consistent with a decrease in the density of synapses in the CA1 subfield. This interpretation is in line with preclinical GWI studies that observed reductions in hippocampal CA1 apical dendritic spine density (Speed et al., 2011), CA1 principal cell

postsynaptic density protein 95 expression (Wang et al., 2020), as well as decreased hippocampal neuronal cellular proliferation and neurogenesis months after GWI-related exposures (Parihar et al., 2013; Megahed et al., 2014; Kodali et al., 2018; Shetty et al., 2020; Wang et al., 2020). Although previous studies indicated synaptic density and hippocampal neurogenesis may be persistently impaired in animals exposed to GW chemicals, normalization of the fEPSP slope value in the LTP response provides the opportunity to detect the LNFPIII induced enhancement of LTP magnitude. Accordingly, while delayed LNFPIII treatment enhanced dH and vH LTP magnitude irrespective of prior PB/PM exposure, given the progressive nature of the disease, LNFPIII may be an even more efficacious treatment for ameliorating synaptic anomalies that underlie GWI-related cognitive impairments later in the course of the disease.

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Figures

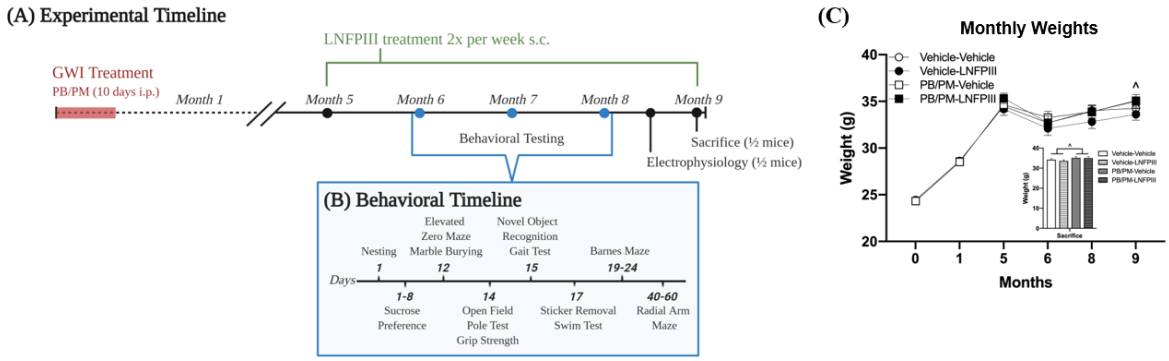


Figure 4.1. Experimental and Behavioral Timelines. (A) depicts the experimental timeline for both the short- and long-term studies in which mice received pyridostigmine bromide (PB) and permethrin (PM) daily for 10 days. Lacto-N-fucopentaose III (LNFPIII) treatment began 4 months after PB/PM exposure. Electrophysiology was performed during month 8. **(B)** is a detailed timeline of behavioral tests that were performed during months 6-8. **(C)** Monthly weights were monitored and are presented for the start of study (0), post PB/PM treatment (1), start of LNFPIII treatment (5), prior to RAM FR (6), post RAM FR (8), and sacrifice (9). ^ indicates trend for PB/PM effect $p \leq 0.10$. *Abbreviations:* FR: food restriction; GWI: Gulf War Illness; i.p.: intraperitoneally; LNFPIII: Lacto-N-fucopentaose III; PB: pyridostigmine bromide; PM: permethrin; RAM: radial arm maze; s.c.: subcutaneously.

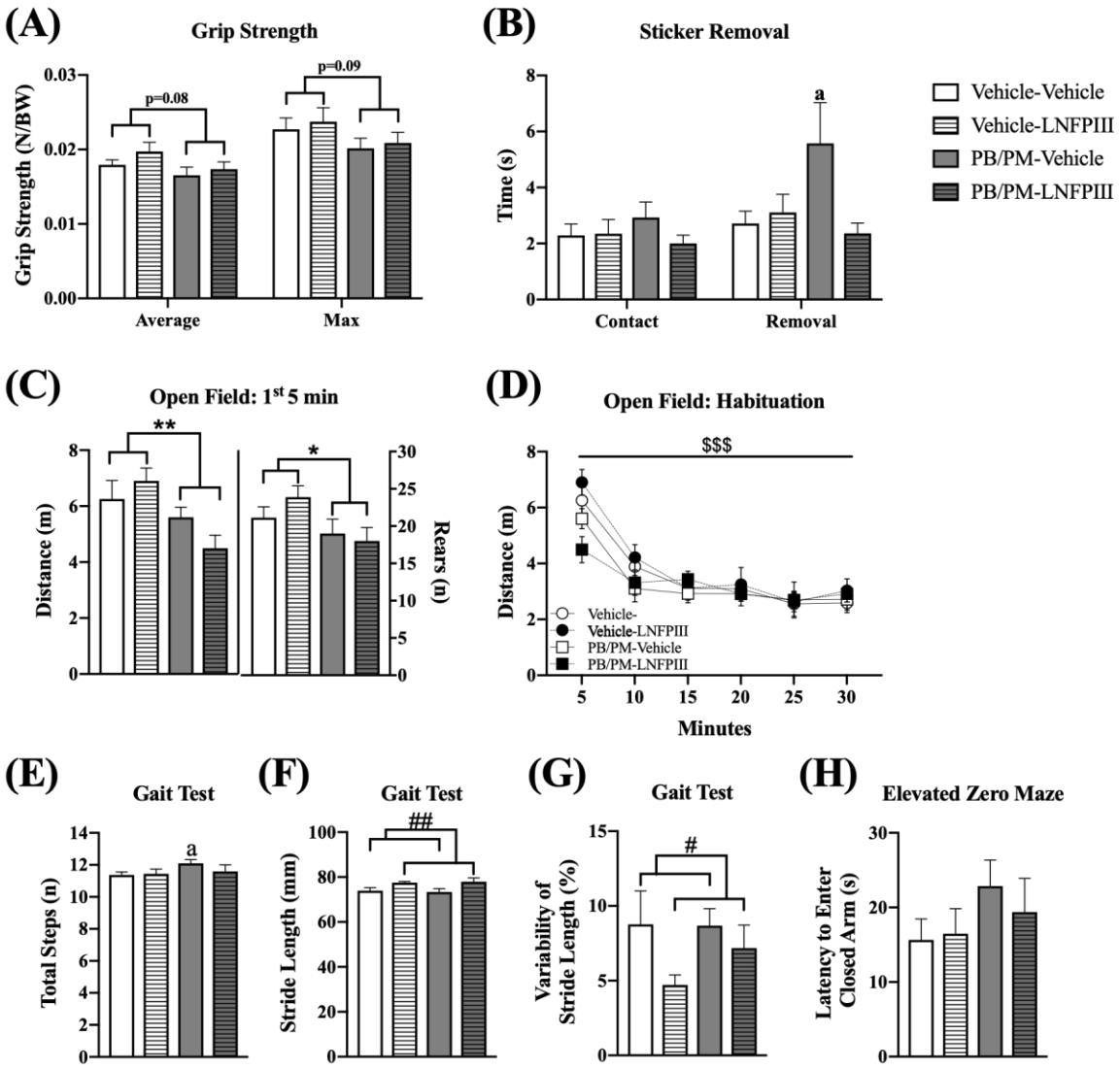


Figure 4.2. Effects of prior PB/PM exposure and delayed LNFPIII treatment on motor function. Several motor effects of prior PB/PM exposure and LNFPIII treatment were evaluated by grip strength (N/body weight) **(A)**; sticker removal time **(B)**; 5 min distance and rears **(C)** and habituation over time **(D)** in the open field; total steps **(E),** stride length **(F),** and stride length variability **(G)** in the gait test; entry time into the closed arm (**H)** of the elevated zero maze. Data are present as mean ± SEM (n=8-10 for grip strength, OF, and gait test; n=14-16 for sticker removal and elevated zero maze). * and ** indicate $p < 0.05$ and 0.01, respectively for PB/PM main effects. # and ## indicates $p < 0.05$ and 0.01, respectively for main effect of LNFPIII

treatment. $^{\$\$\$}$ indicates $p < 0.001$ of time in the OF. ^{a} indicates $p < 0.05$ for Vehicle-Vehicle vs. PB/PM-Vehicle.

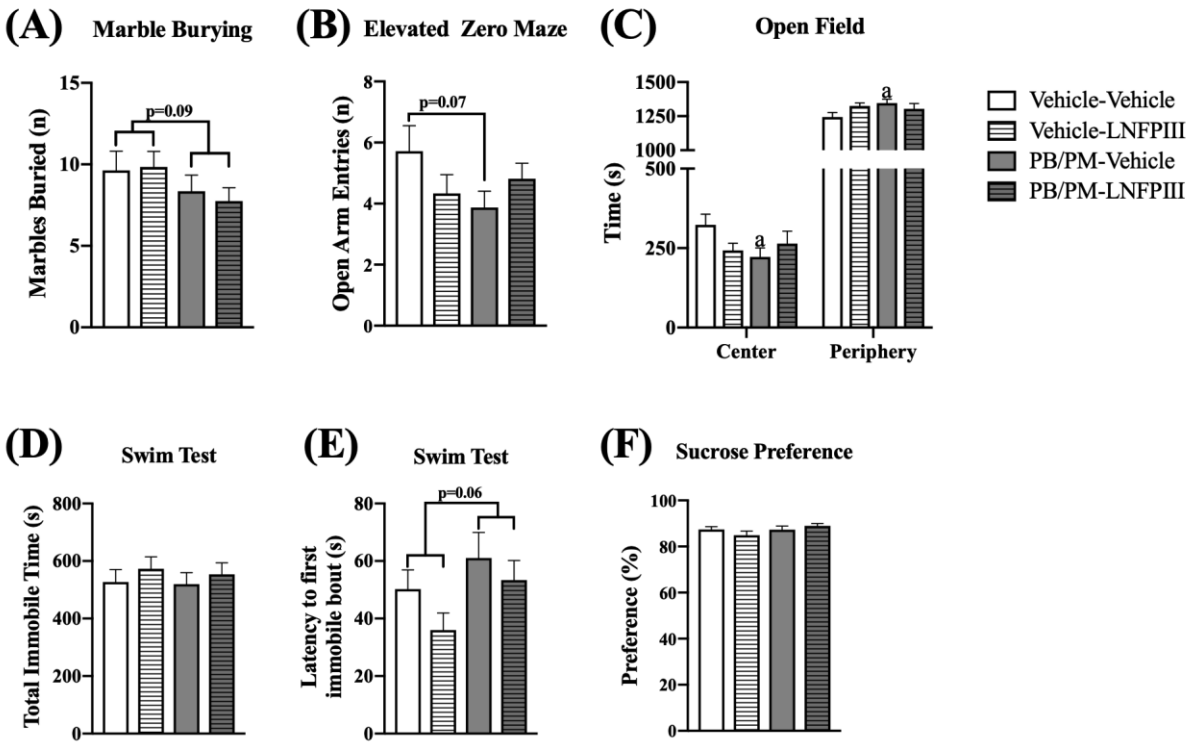


Figure 4.3. Long term mood effects of prior PB/PM exposure and delayed LNFPIII treatment. Mood disturbances of prior PB/PM exposure and LNFPIII treatment were evaluated by several tests for anxiety-like and depressive-like behaviors: number of marbles buried **(A)** in the marble burying test; open arm entries **(B)** in the elevated zero maze; center and periphery time **(C)** in the open field; immobile time **(D)** and latency to first immobile bout **(E)** in the swim test; sucrose preference **(F)** in the sucrose preference test. Data are presented as mean ± SEM (n = 8-10 for open field; n = 14-16 for marble burying, elevated zero maze, swim, and sucrose preference test). [a] indicates $p < 0.05$ for Vehicle-Vehicle vs PB/PM-Vehicle.

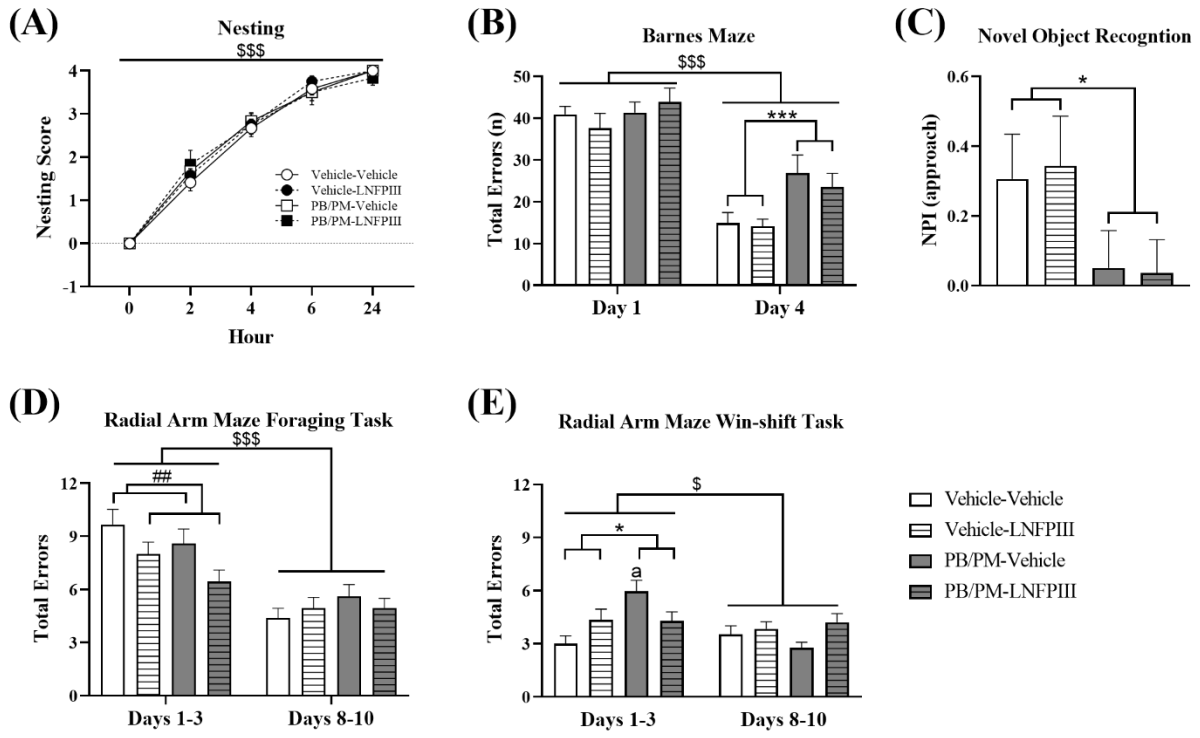


Figure 4.4. Cognition and memory effects of prior PB/PM exposure and delayed LNFPIII treatment. Cognitive/memory effects of prior PB/PM exposure and LNFPIII treatment were evaluated by several tests: nest-building ability **(A)** in the nesting test; total errors **(B)** during the training phase of the Barnes maze; approach novelty preference index (NPI) **(C)** in the novel object recognition test; total errors in the radial arm maze foraging **(D)** and Win-shift tasks **(E)**. Data are presented as mean ± SEM (n = 14-16 for nesting, Barnes maze, and novel object recognition; n = 13-15 for radial arm maze). $ and $$$ indicate $p < 0.05$ and 0.001 for time. * and *** indicate $p < 0.05$ and 0.001, respectively, for PB/PM main effects. ## indicates $p < 0.01$ for main effect of LNFPIII treatment. a indicates $p < 0.05$ for Vehicle-Vehicle vs. PB/PM-Vehicle.

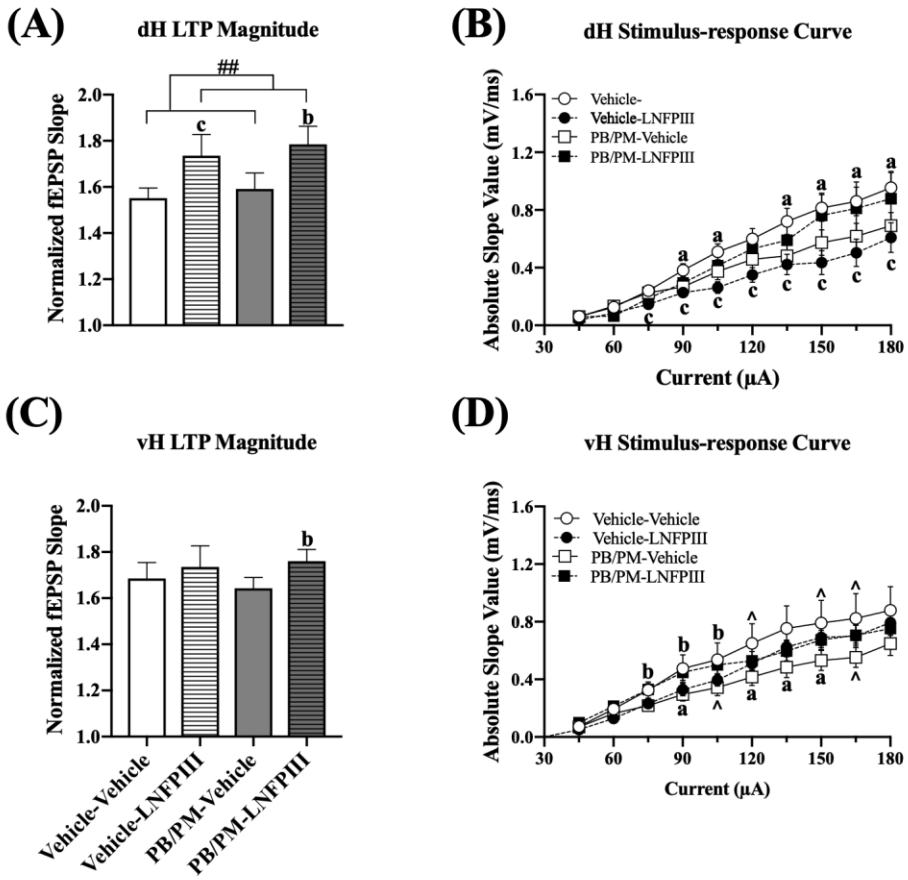


Figure 4.5. Hippocampal measurements of synaptic plasticity and transmission after prior PB/PM exposure and delayed LNFPIII treatment. A) and **C)** show summary quantification of dorsal hippocampus (dH) and ventral hippocampus (vH), respectively, LTP magnitude recorded 8 months after PB/PM exposure. **B)** and **D)** show the stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities from the dH and vH, respectively. Data are presented as mean ± SEM. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed: Vehicle-Vehicle (dH, n=14(6); vH, n=14(6)), Vehicle-LNFPIII (dH, n=9(6); vH, n=20(6)), PB/PM-Vehicle (dH, n=12(6); vH, n=21(6)), and PB/PM-LNFPIII (dH, n=13(6); vH, n=17(6)). $^{\#\#}$ indicates main effect of LNFPIII treatment. $^{a, b,}$ and ^{c} indicate $p < 0.05$ for Vehicle-Vehicle vs PB/PM-Vehicle, PB/PM-Vehicle vs PB/PM-LNFPIII, and Vehicle-Vehicle vs Vehicle-LNFPIII groups, respectively. ^ indicates trend $p < 0.10$ for either Vehicle-Vehicle vs PB/PM-Vehicle or PB/PM-Vehicle vs PB/PM-LNFPIII.

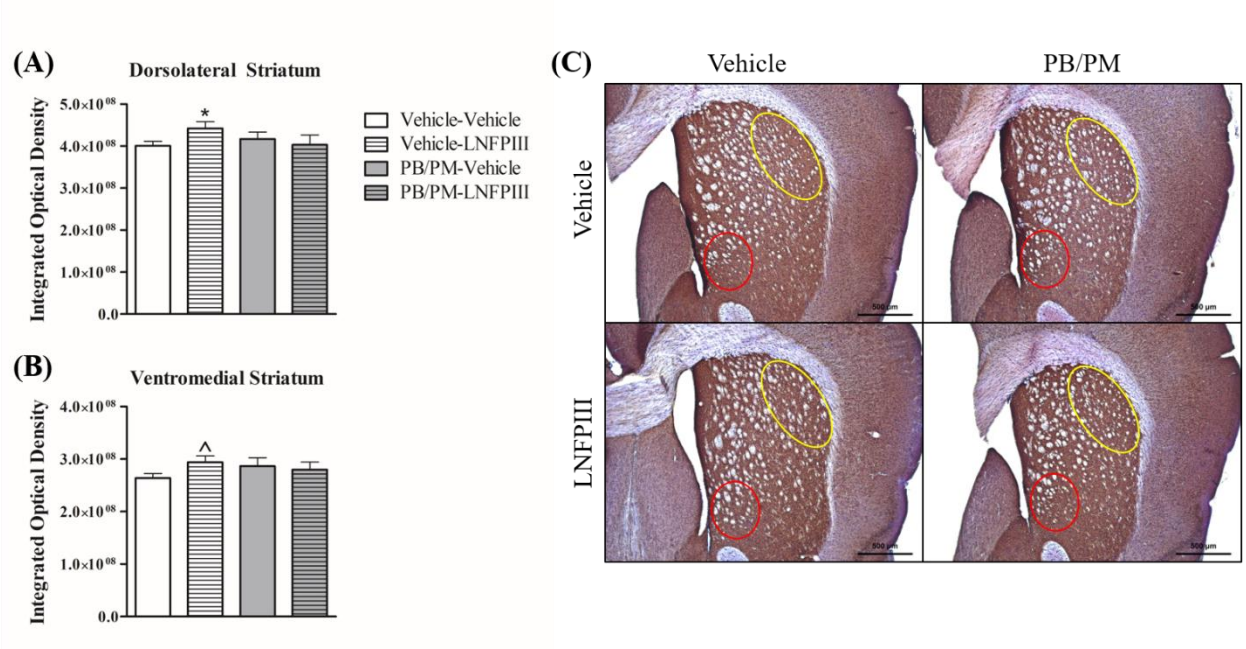


Figure 4.6. Tyrosine hydroxylase (TH) immunoreactivity in the dorsolateral and ventromedial striatum 9 months post PB/PM exposure and delayed LNFPIII treatment. Semi-quantitative analysis of TH+ CV+ cells in the dorsolateral (yellow oval) and ventromedial (red oval) striatum. Data are presented as mean ± SEM. n = 5 animals per group/3 sections per animal. ***** and ^ indicate $p \leq 0.05$ or $p \leq 0.10$ for Vehicle-Vehicle vs. Vehicle-LNFPIII, respectively.

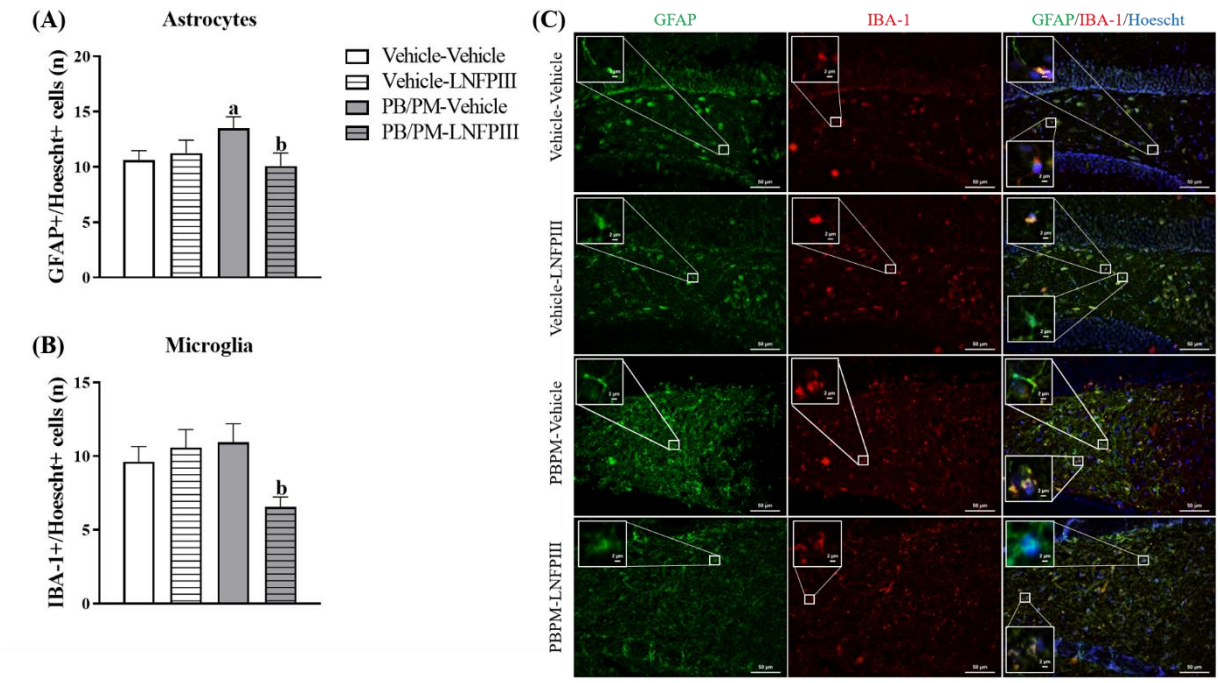


Figure 4.7. GFAP and IBA-1 immunoreactivity in the hilus region of the hippocampus 9 months post-PB/PM exposure and delayed LNFPIII treatment. Double-stained astrocytes (GFAP/Hoescht)**(A)** or microglia (IBA-1/Hoescht) **(B)** were counted using ImageJ. **(C)** shows representative staining at 40x in the hilus region of the hippocampus for astrocytes (GFAP), microglia (IBA-1), and nuclear stain (Hoescht). Data are presented as mean ± SEM. n = 11-19 images per group. [a] and [b] indicate $p \leq 0.05$ for Vehicle-Vehicle vs. PB/PM-Vehicle and PB/PM-Vehicle vs. PB/PM-LNFPIII comparisons, respectively.

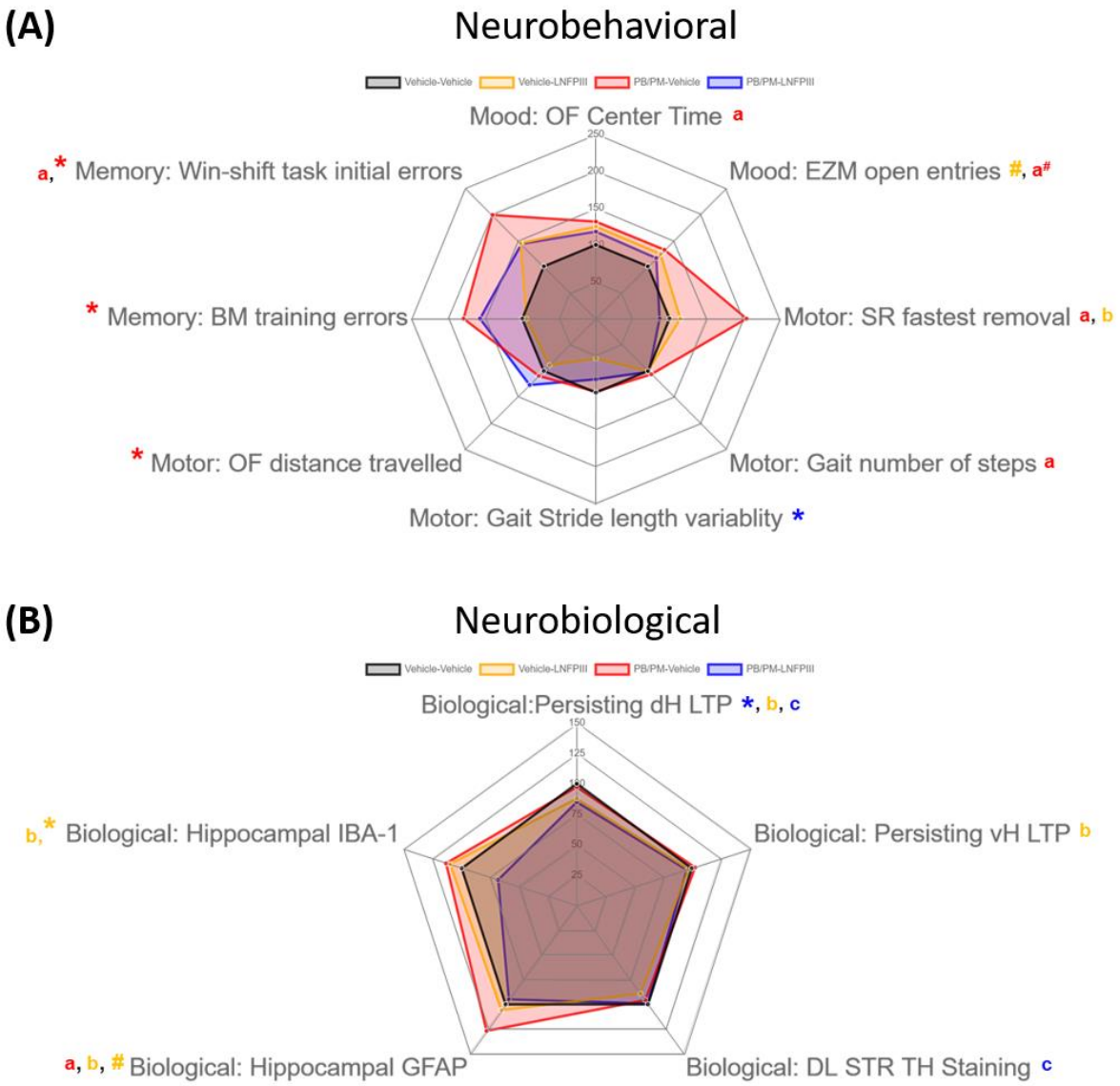
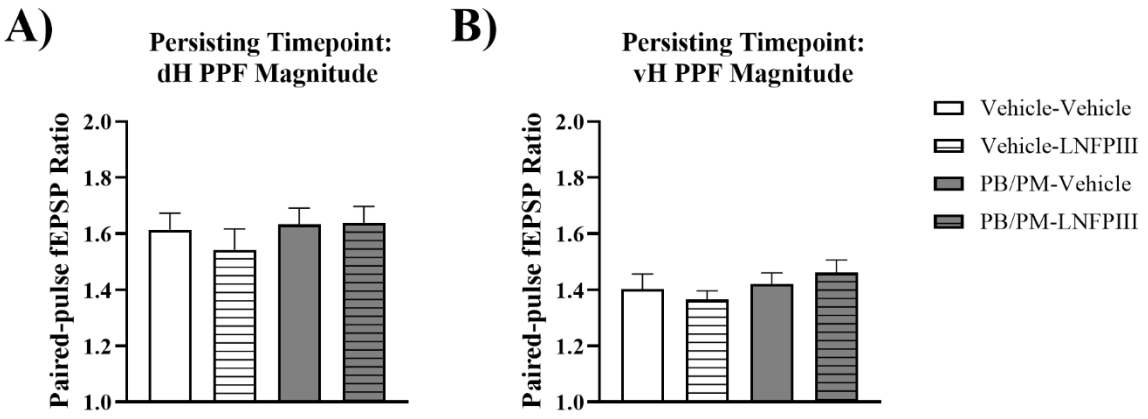


Figure 4.8. Radar Plots Summary. The radar plots highlight the neurobehavioral **(A)** and neurobiological **(B)** effects of prior PB/PM exposure and delayed LNFPIII treatment at study completion. * denotes main/interaction effect ($p<0.05$) whereas # denotes trending main/interaction effect ($p<0.1$). Blue indicates an LNFPIII effect, red denotes a PB/PM effect, and orange denotes a beneficial effect of LNFPIII in the presence of PB/PM. [a, b,] and [c] denote a significant ($p<0.05$) pairwise effect for Vehicle-Vehicle vs PB/PM-Vehicle (red), PB/PM-Vehicle

vs PB/PM-LNFPIII (orange), and Vehicle-Vehicle vs Vehicle-LNFPIII (blue), respectively. [a#] indicates a trending ($p<0.1$) pairwise effect for Vehicle-Vehicle vs PB/PM-Vehicle (red), PB/PM-Vehicle vs PB/PM-LNFPIII (orange), and Vehicle-Vehicle vs Vehicle-LNFPIII (blue), respectively.



Supplemental Figure 4.1. Paired-pulse facilitation magnitude post PB/PM exposure and delayed LNFPIII treatment. The PPF magnitude for dH **(A)** and vH **(B)** recorded after PB/PM chemicals exposure (interpulse interval = 50 ms). Data are expressed as mean ± SEM. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed: Vehicle-Vehicle (dH, n=14(6); vH, n=14(6)), Vehicle-LNFPIII (dH, n=9(6); vH, n=20(6)), PB/PM-Vehicle (dH, n=12(6); vH, n=21(6)), and PB/PM-LNFPIII (dH, n=13(6); vH, n=17(6)).

CHAPTER 5

LACTO-N-FUCOPENATOSE-III AMELIORATES ACUTE AND PERSISTING HIPPOCAMPAL SYNAPTIC PLASTICITY AND TRANSMISSION DEFICITS IN A GULF WAR ILLNESS MOUSE MODEL[4]

[4] Brown KA, Carpenter JM, Preston CJ, Ludwig HD, Clay KB, Harn DA, Norberg T, Wagner JJ, Filipov NM. *Lacto-N-fucopentaose-III ameliorates acute and persisting hippocampal synaptic plasticity and transmission deficits in a Gulf War Illness mouse model.*
Submitted to Life Sciences.

Abstract

Aims: The present study investigated if treatment with the immunotherapeutic, lacto-N-fucopentaose-III (LNFPIII), resulted in amelioration of acute and persisting deficits in synaptic plasticity and transmission as well as trophic factor expression along the hippocampal dorsoventral axis in a mouse model of Gulf War Illness (GWI).

Main methods: Mice received either coadministered or delayed LNFPIII treatment throughout or following, respectively, exposure to a 15-day GWI induction paradigm. Subsets of animals were subsequently sacrificed 48 h, seven months, or 11 months post GWI-related (GWIR) exposure for hippocampal qPCR or *in vitro* electrophysiology experiments.

Key findings: Progressively worsened impairments in hippocampal synaptic plasticity, as well as a biphasic effect on hippocampal synaptic transmission, were detected in GWIR-exposed animals. Dorsoventral-specific impairments in hippocampal synaptic responses became more pronounced over time, particularly in the dorsal hippocampus. Notably, delayed LNFPIII treatment ameliorated GWI-related aberrations in hippocampal synaptic plasticity and transmission seven and 11 months post-exposure, an effect that was consistent with enhanced hippocampal trophic factor expression in animals treated with LNFPIII.

Significance: Approximately a third of Gulf War Veterans have GWI; however, GWI therapeutics are presently limited to targeted and symptomatic treatments. As increasing evidence underscores the substantial role of persisting neuroimmune dysfunction in GWI, efficacious neuroactive immunotherapeutics may hold substantial promise in yielding GWI remission. The findings in the present report indicate that LNFPIII may be an efficacious candidate for ameliorating persisting neurological abnormalities presented in GWI.

5.1 Introduction

Gulf War Illness (GWI) is a chronic multisymptom condition that affects approximately one-third of the 700,000 U.S. military personnel deployed in the 1990-1991 Gulf War (White et al., 2016). Neurological (i.e., cognitive) and immunological (i.e., inflammation) deficits feature in GWI prominently (White et al., 2016). Although the etiology of GWI is unknown, overexposures to neurotoxic pesticides, organophosphate (OP) nerve agents, nerve agent prophylactics, and wartime stress have been associated with GWI symptoms (Cherry et al., 2001; Winkenwerder, 2003; Steele et al., 2012). GWI symptom severity is correlated to the degree, duration, and type of GWI-related (GWIR) exposures (Steele et al., 2012; Sullivan et al., 2018). Notably, the reported incidence (Zundel et al., 2019) and symptom severity (Porter et al., 2020; Zundel et al., 2020) of GWI has increased over time, suggesting disease progression. This underscores the critical need to employ GWI animal models reflecting the progressive nature of the disease and use such models for identifying efficacious GWI therapeutics.

GWI models have been established in the mouse (Abdullah et al., 2011; O'Callaghan et al., 2015). These models recapitulate GWI by utilizing GWIR chemical exposures that, in some models, are interfaced with modeling of stress-like conditions, i.e., by administering glucocorticoids such as corticosterone (Cort) (O'Callaghan et al., 2015). GWIR chemicals used to recapitulate GWI include pesticides such as N,N-diethyl-meta-toluamide (DEET), nerve agent prophylactics such as pyridostigmine bromide (PB), and a nerve agent surrogate, diisopropylfluorophosphate (DFP) (O'Callaghan et al., 2015). DEET preferentially targets insect olfactory receptors; however, reports have indicated that overexposures may produce mammalian toxicity (Corbel et al., 2009). The carbamate PB, a reversible acetylcholinesterase (AChE) inhibitor, functions as a nerve agent prophylactic in the absence of stress; however, during chronic stress, PB might cross the blood-brain barrier and be neurotoxic (Blick et al., 1991; Friedman et al., 1996). Military operations exposed thousands of U.S. personnel to low levels of OP nerve agent, making the incorporation of a relatively less volatile OP like DFP an

appropriate component of recapitulating GWIR exposures (Winkenwerder, 2002; Haley and Tuite, 2013).

While a number of preclinical and clinical studies have evaluated potential GWI therapeutics (Reviewed in: Dickey et al., 2020; Nugent et al., 2020), only symptomatic treatments are available. Increasing evidence indicates that prolonged symptoms of GWI are a result of neuroimmune dysfunction (Coughlin, 2017; Georgopoulos et al., 2017; Trageser et al., 2020). Inhibition of pro-inflammatory cytokines is a strategy that holds potential for extended remission of GWI (Craddock et al., 2015); however, studies employing immunotherapeutics to target persisting GWI-induced immune system dysregulation are limited. We previously demonstrated that the neoglycoconjugate lacto-N-fucopentaose-III (LNFPIII) is a safe and efficacious agent for restoring central and peripheral immune function in disease states (Bhargava et al., 2012; Zhu et al., 2012; Tundup et al., 2015). This neoglycoconjugate induces therapeutic effects, in part, by rebalancing the anti-inflammatory:inflammatory cytokine milieu, but it also directly stimulates the mitogen-activated protein kinase ERK-1/2 (Tundup et al., 2015). Importantly, recent work by our group demonstrated that LNFPIII rebalanced short-term neurochemical and inflammatory-related aberrations in two GWI models (Carpenter et al., 2020) and it ameliorated gut dysbiosis and inflammation when treatment was initiated months after GWIR exposures (Mote et al., 2020). However, LNFPIII's efficacy in a progressive model of GWI is unknown.

Reports of impaired hippocampal-dependent behavior in GWI animal models (Joshi et al., 2020; Shetty et al., 2020; Wang et al., 2020) and GW veterans (Odegard et al., 2013; Chao et al., 2017; Sullivan et al., 2018) are abundant. Persisting hippocampal structural abnormalities have been documented in clinical GWI studies (Li et al., 2011; Chao et al., 2017; Cheng et al., 2020). Loss of hippocampal principal cells and interneurons (Parihar et al., 2013; Megahed et al., 2015; Wang et al., 2020) and aberrant glial activity (Parihar et al., 2013; Joshi et al., 2018; Belgrad et al., 2019) in GWI models have also been characterized. Electrophysiological studies

describing alterations in hippocampal synaptic plasticity or transmission in GWI models are sparse. The available preclinical reports indicate that glutamatergic, GABAergic, and dopaminergic synaptic plasticity and transmission are impaired in GWI (Torres-Altoro et al., 2011; Wang et al., 2020). Yet, studies accounting for the progressive nature of GWI that are focused on hippocampal neurophysiology are critically needed but unavailable. Accordingly, we evaluated the efficacy of LNFPIII in ameliorating acute and persisting electrophysiological, trophic factor, and cytokine aberrations presented in a well-established PB/DEET/CORT/DFP GWI model (O'Callaghan et al., 2015).

5.2 Methods

5.2.1 Animal Maintenance

Male C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice, aged eight-nine weeks, were housed in groups of five. Animals were acclimated to their home cages for at least one week after arrival and were maintained on a 12 h light/dark cycle (lights on from 0700-1900 h). Food and water were provided *ad libitum*. Following acclimation, animals were randomly assigned to one of four treatment groups: vehicle-vehicle, vehicle-LNFPIII, GWI-vehicle, and GWI-LNFPIII groups. All experiments were approved in advance by the University of Georgia Animal Care and Use Committee and were in accordance with the latest NIH and ARRIVE guidelines.

5.2.2 Materials

The following chemicals were used for animal treatments: corticosterone (Cort; Steraloids, Newport, RI), N,N-diethyl-meta-toluamide (DEET; Sigma Aldrich, St. Louis, MO), diisopropylfluorophosphate (DFP; Sigma Aldrich), and pyridostigmine bromide (PB; Sigma Aldrich). Lacto-N-fucopentaose-III (LNFPIII) dextran conjugate was produced as previously described (Tundup et al., 2015). All additional chemicals and reagents used in this study, unless otherwise noted, were of analytical or higher grade and were obtained from Sigma Aldrich or Fisher Scientific (Hampton, NH).

5.2.3 GWI Exposure Protocol

The exposure protocol has been previously described (O'Callaghan et al., 2015; Carpenter et al., 2020) and is presented in Fig. 5.1A. Briefly, mice from the GWI-vehicle and GWI-LNFPIII groups were treated with a combination of PB (2 mg/kg, subcutaneous [s.c.]) and DEET (30 mg/kg, s.c.) daily for 14 days. PB and DEET were both delivered in 0.9% saline. Home cage drinking water of GWI-vehicle and GWI-LNFPIII groups were treated with Cort (200 mg/L in 1.2% ethanol [EtOH]) on days 8-14 to emulate combat-related stress. Following the same timeline as described for GWI-vehicle and GWI-LNFPIII treatment groups, vehicle-vehicle and vehicle-LNFPIII treated animals received saline vehicle injections and 1.2% EtOH/water. On day 15, animals from the GWI-vehicle and GWI-LNFPIII groups received a single injection of DFP (3.75 mg/kg, intraperitoneal [i.p.] in sterile saline). Vehicle-vehicle and vehicle-LNFPIII treated animals were treated with the saline vehicle (i.p.). The LNFPIII dosage used in the present report was selected based on our previous studies (Atochina et al., 2008; Bhargava et al., 2012; Tundup et al., 2012; Zhu et al., 2012; Tundup et al., 2015; Carpenter et al., 2020; Mote et al., 2020). Animals received vehicle carrier or LNFPIII treatment based on the following timelines:

Acute effects time point: The exposure protocol and electrophysiology schedule are presented in Fig. 5.1A. A subset of animals was selected to evaluate the acute effects of GWIR exposure, as well as the efficacy of LNFPIII coadministration in ameliorating GWIR exposure effects. On days 1-14, mice from the vehicle-LNFPIII and GWI-LNFPIII groups received daily treatments of LNFPIII (35 µg/mouse, s.c.) whereas mice from the vehicle-vehicle and GWI-vehicle groups received equivalent amounts of the dextran vehicle carrier (35 µg/mouse, s.c.) diluted in sterile saline. Animals were sacrificed for experiments 48 h post GWIR exposure ± LNFPIII treatment. The bodyweight of animals sacrificed at the acute time point was monitored daily for the duration of the study as well as immediately prior to sacrifice for electrophysiology experiments.

Early persisting time point: The exposure protocol and electrophysiology schedule are presented in Fig. 5.2A. A subset of animals was utilized to evaluate the early persisting effects of GWIR exposure, as well as the efficacy of delayed LNFPIII treatment in ameliorating GWIR exposure-induced abnormalities seven months after exposure termination. Notably, these animals did not receive vehicle or LNFPIII during the GWIR exposure duration. Vehicle or LNFPIII treatment began four months after GWIR exposure termination and continued twice per week until the animals were sacrificed for electrophysiology experiments three months later (seven months post GWIR exposure).

Late persisting time point: The exposure protocol and electrophysiology schedule are presented in Fig. 5.3A. A subset of animals was used to evaluate the late persisting effects of GWIR exposure and the efficacy of delayed LNFPIII treatment in ameliorating GWIR exposure-induced abnormalities 11 months after exposure. Mice began receiving vehicle or LNFPIII treatment eight months post GWIR exposure, which continued twice per week until the animals were sacrificed for electrophysiology experiments three months later (11 months post GWIR exposure termination).

The bodyweight of animals used for the early and late persisting time points was monitored every other week for the duration of the study, as well as immediately before sacrifice for electrophysiology experiments.

5.2.4 Slice Preparation and Electrophysiology

Hippocampal slice preparation and electrophysiology experiments were conducted as previously described (Keralapurath et al., 2017; Preston et al., 2019). Briefly, mice were sacrificed following deep anesthetization with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane). Brains were removed and quickly submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120mM NaCl, 3mM KCl, 4mM $MgCl_2$, 1mM NaH_2PO_4, 26mM $NaHCO_3$, and 10mM glucose). A mid-sagittal cut was made, and half of the brain was mounted on its caudal pole and sectioned along the horizontal plane with a

vibratome to obtain 400 µm slices containing the dorsal hippocampus. The other half of the brain was mounted on its rostral pole and sectioned along the horizontal plane with a vibratome to acquire 400 µm slices containing the ventral hippocampus. The dorsal hippocampus and ventral hippocampus were subdissected free from the rest of the slice and the CA3 subfield was removed. These slices were then quickly placed in a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120mM NaCl, 3mM KCl, 1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, and 10mM glucose). Slices were continuously perfused with ACSF (one mL/min) during a 45 min recovery period at room temperature followed by a 45 min recovery period at the recording temperature of 30 °C. Schaffer collateral fibers were stimulated by placing a bipolar electrode (Kopf Instruments, Tujunga, CA) in the stratum radiatum of the CA1 subfield; a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments, Sarasota, FL) was inserted in the same layer of CA1. During recording experiments, a stimulus pulse was applied once every minute to elicit responses from Schaffer collateral-CA1 synapses.

An input-output curve (I/O curve) was acquired at the beginning of each experiment by incrementally applying individual 15 µA stimulus pulses from 45-180 µA in intensity. The fEPSP slope (mV/ms) was measured in a one ms interval immediately following the fiber volley. After recording the I/O curve, the stimulus intensity was modified to elicit 35% of the maximal fEPSP slope and paired-pulse fEPSPs (50 ms interpulse interval) were recorded each minute for five min. Individual stimulus pulses were then applied each min for 30 min and baseline fEPSP responses were monitored. After recording baseline responses for 30 min, a high-frequency stimulation (HFS) protocol (three x 100 Hz/one-sec train at 20-sec intervals) was utilized to induce long-term potentiation (LTP) and fEPSP responses were monitored for the subsequent 60 min. fEPSP responses were normalized to the average fEPSP slope value recorded during 26-30 min of baseline recording. LTP magnitude was calculated by averaging the normalized fEPSP slope values from 56-60 min post-HFS.

5.2.5 Quantitative PCR (qPCR) Analysis

Analyses were completed on brain tissue (48 h: ventral hippocampus; 11 months: dorsal hippocampus and ventral hippocampus) as described in (Carpenter et al., 2020). Briefly, total RNA from a single hippocampal brain punch (1.5 mm diameter, 500 µm thick section) was isolated by an E.Z.N.A total RNA isolation kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's directions. RNA was quantified with a Take3 micro-volume plate and Epoch microplate spectrophotometer (BioTek, Winooski, VT). Seventy-five ng RNA/sample was used to synthesize cDNA with a Maxima first-strand cDNA synthesis kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA) and a PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA; 10 min at 25 °C, 15 min at 50 °C, 5 min at 85 °C). Using one or two ng of cDNA per sample, expression of various inflammatory genes and growth factors (48 h), or growth factors-only (11 months) were determined by a qPCR with mouse-specific primers (RealTimePrimers, Elkins Park, PA) and Maxima SYBR Green/lowRox qPCR Master Mix (2x) (Thermo Fisher Scientific, Waltham, MA). Amplifications were performed on Mx3005 P qPCR machine (Stratagene, San Diego, CA) programed for initial warming cycle (95°C, 10 min) followed by 45 cycles (95°C, 15 s, 60°C, 1 min) with each sample run in duplicate. Treatment differences detected in qPCR data were calculated using the $^{\Delta\Delta}$Ct method and presented as a fold change normalized to 18S as the housekeeping gene, as described previously (Lin et al., 2013; Krishna et al., 2016; Carpenter et al., 2020). Primer sequences are in Table 1.

5.2.6 Data Analysis

Electrophysiology data was digitized at 10kHz, low-pass filtered at 1kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunnyvale, CA). Electrophysiology statistical analysis was completed using R version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). For electrophysiology data, statistical analysis was completed by using the aov() function for analysis of variance (ANOVA), the PostHocTest() function under the DescTools package (version 0.99.38) for Student-Newman-Keuls *post hoc* test, and the t.test

function for preplanned Student's *t*-tests. Graphic production was completed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Electrophysiology data were analyzed via two-way ANOVA with factor A being GWI treatment (vehicle or GWIR treatment) and factor B being LNFPIII treatment (vehicle or LNFPIII treatment). If a two-way ANOVA was significant ($p \leq 0.05$), treatment means were separated via Student-Newman-Keuls *post hoc* test or pairwise comparisons (as appropriate). An α level of 0.05 was used as the criterion for statistical significance. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed. All data are presented as mean ± SEM.

5.3 Results

5.3.1 Coadministration of LNFPIII enhanced LTP magnitude whereas both GWIR exposure and LNFPIII treatment increased hippocampal basal synaptic transmission 48 h post-exposure

The GWIR exposure ± LNFPIII coadministration timeline is represented in Fig. 5.1A. No significant main effect of GWIR exposure on LTP was observed 48 h post-exposure ($p=0.80$; Fig. 5.1B, 5.1C, 5.1D). Coadministration of LNFPIII resulted in a significant main effect of LNFPIII on LTP magnitude 48 h post GWIR/LNFPIII treatments ($p=0.018$; Fig. 5.1B, 5.1C, 5.1D). An interaction effect between GWIR X LNFPIII on LTP magnitude also approached significance ($p=0.077$). A *post hoc* comparison revealed a significant LTP enhancement in vehicle-LNFPIII animals compared to vehicle-vehicle animals ($p=0.0059$; Fig. 5.1D).

Significant main GWIR (p's<0.05; stimulus intensities: 45-180 µA) and LNFPIII (p's$=0.05$; stimulus intensities: 105-180 µA) effects on basal synaptic transmission were detected, resulting in increased basal synaptic activity 48 h post-exposure (Fig. 5.1E). A main effect of LNFPIII treatment on basal synaptic transmission approached significance at stimulus intensities of 75 and 90 µA ($p<0.1$). A *post hoc* analysis showed significant increases in basal synaptic transmission in GWI-vehicle mice compared to vehicle-vehicle mice at stimulus intensities of 60, 75, 90, 105, 120, 135, 150, 165, and 180 µA ($p<0.05$). Additionally, significant increases in basal synaptic transmission in GWI-LNFPIII mice compared to GWI-vehicle mice at stimulus

intensities of 45, 120, 135, 150, 165, and 180 μA were observed ($p<0.05$). Finally, basal synaptic transmission in vehicle-LNFPIII mice compared to vehicle-vehicle mice was increased at stimulus intensities of 105, 120, 135, 150, 165, and 180 μA ($p<0.05$). Neither GWIR exposure ($p=0.67$) nor LNFPIII treatment ($p=0.27$) had a significant main effect on paired-pulse facilitation (PPF; Fig. 5.1F).

5.3.2 LTP magnitude is reduced seven months after GWIR exposure, a reduction that is ameliorated by LNFPIII treatment initiated four months after GWIR exposure termination

Four months after GWIR exposure termination, a subset of animals began receiving vehicle or LNFPIII treatment twice per week until the animals were sacrificed for electrophysiology experiments (Fig. 5.2A). Following three months of vehicle or LNFPIII treatment (seven months post GWIR exposure termination), we found a significant effect of LNFPIII treatment on LTP magnitude in GWIR exposed animals ($p=0.027$), resulting in a trending main effect of LNFPIII on LTP ($p=0.09$; Fig. 5.2B, 5.2C, 5.2D). Specifically, a trend of a significant difference in LTP magnitude between vehicle-vehicle and GWI-vehicle animals was observed ($p=0.063$), whereas a significant decrease in LTP magnitude in GWI-vehicle animals compared to GWI-LNFPIII animals was detected ($p=0.013$; Fig. 5.2D). No significant differences in LTP magnitude between vehicle-vehicle, vehicle-LNFPIII, and GWI-LNFPIII animals were revealed ($p's>0.05$).

Interestingly, no significant main effects of GWIR exposure or LNFPIII treatment on basal synaptic transmission were detected at any assessed stimulation intensities ($p>0.05$; Fig. 5.2E). Similar to the 48 h time point, no significant main effects of GWIR exposure ($p=0.44$) or LNFPIII treatment ($p=0.80$) on PPF were found (Fig. 5.2F).

5.3.3 Dorsal hippocampal LTP magnitude and basal synaptic transmission is reduced 11 months after GWIR exposure, an effect that is ameliorated by delayed LNFPIII treatment

Eight months after GWIR exposure termination, a subset of animals began receiving LNFPIII or vehicle treatment twice per week until the end of the study (Fig. 5.3A). Following

three months of LNFPIII or vehicle treatment (11 months post-GWIR exposure), a significant main effect of GWIR exposure, driven by the decrease in the GWI-vehicle group, on dorsal hippocampal (dH) LTP magnitude was revealed ($p=0.029$; Fig. 5.3B, 5.3C, 5.3D). A trend of a significant LNFPIII main effect on dH LTP magnitude was also observed ($p=0.076$) and it was due to the LNFPIII's ability to ameliorate the effect of GWIR exposure (Fig. 5.3D). An interaction effect between GWIR X LNFPIII on LTP magnitude also approached significance ($p=0.096$). A *post hoc* comparison revealed that the decrease in dH LTP magnitude of GWI-vehicle animals compared to vehicle-vehicle animals was significant ($p=0.0029$). Importantly, dH LTP magnitude of GWI-LNFPIII animals was significantly greater compared to the dH LTP magnitude of GWI-vehicle animals ($p=0.016$; Fig. 5.3C, 5.3D). No significant differences in LTP magnitude between vehicle-vehicle, vehicle-LNFPIII, and GWI-LNFPIII animals were observed in the dH ($p's>0.05$).

In contrast to our findings 48 h and seven months post GWIR exposure, a significant reduction in dH basal synaptic transmission was detected 11 months post-exposure (Fig. 5.3E). Specifically, a significant main effect of GWIR exposure on dH basal synaptic transmission was found at stimulation intensities of 75-180 μA ($p's<0.05$). A main effect of GWIR exposure on basal synaptic transmission also approached significance at a stimulus intensity of 60 μA ($p<0.1$). A *post hoc* analysis indicated that decreases in dH basal synaptic transmission in GWI-vehicle mice compared to vehicle-vehicle mice at stimulus intensities of 60, 75, 90, 105, 120, 135, 150, 165, and 180 μA were significant ($p<0.05$). Moreover, a significant increase in dH basal synaptic transmission in GWI-LNFPIII mice was displayed compared to GWI-vehicle mice at stimulus intensities of 120, 135, and 180 μA ($p<0.05$); at 165 μA stimulus intensity, this effect was trending ($p<0.1$). No significant difference in dH basal synaptic transmission between vehicle-vehicle and vehicle-LNFPIII-treated animals was detected ($p>0.05$). Similar to the other time points, there were no significant main effects of GWIR exposure ($p=0.31$) or LNFPIII treatment ($p=0.75$) on PPF in the dH (Fig. 5.3F).

5.3.4 Ventral hippocampal basal synaptic transmission is reduced 11 months after GWIR exposure, an effect that is ameliorated by delayed LNFPIII treatment

Interestingly, there was no significant main effect of GWIR exposure on ventral hippocampal (vH) LTP magnitude ($p=0.75$); however, a significant main effect of LNFPIII led to an increase in vH LTP magnitude ($p=0.019$; Fig. 5.4A, 5.4B, 5.4C). Notably, due to a numeric decrease in GWI-vehicle and an increase in GWI-LNFPIII animals, vH LTP magnitude of the GWI-LNFPIII group was significantly greater compared to GWI-vehicle animals ($p=0.0028$; Fig. 5.4C). No significant differences in LTP magnitude between vehicle-vehicle, vehicle-LNFPIII, GWI-LNFPIII animals were observed in the vH ($p's>0.05$).

Additionally, a significant main effect of GWIR exposure on basal synaptic transmission was found in the vH at stimulus intensities of 135-180 μA ($p's<0.05$; Fig. 5.4D). A main effect of GWIR exposure on basal synaptic transmission also approached significance at stimulus intensities of 105 and 120 μA ($p<0.1$). A *post hoc* analysis found a significant decrease in vH basal synaptic transmission in GWI-vehicle animals compared to vehicle-vehicle animals at stimulus intensities of 135, 150, 165, and 180 μA ($p<0.01$). Compared to vehicle-vehicle animals, a trend of a decrease in vH basal synaptic transmission in GWI-vehicle animals that approached significance ($p=0.055$) was detected at 120 μA. A significant increase in the maximal basal synaptic response elicited in the vH of GWI-LNFPIII mice compared to GWI-vehicle mice was also found ($p=0.035$). No significant difference in vH basal synaptic transmission between vehicle-vehicle and vehicle-LNFPIII treated animals was present ($p>0.05$). Similar to the dH, no significant main effects of GWIR exposure ($p=0.81$) or LNFPIII treatment ($p=0.22$) on PPF were detected in the vH (Fig. 5.4E).

5.3.5 Coadministration of LNFPIII with GWIR treatment or delayed LNFPIII treatment enhanced the production of BDNF in the ventral hippocampus

The expression of key inflammatory mediators (caspase 1, IL-6, IL-1β, and TNFα) and trophic factors (brain-derived neurotrophic factor [BDNF], nerve growth factor [NGF], and ciliary neurotrophic factor [CNTF]) were assessed in the vH 48 h post GWIR exposure ± LNFPIII coadministration (Tables 5.1 and 5.2). No significant effects of GWIR exposure or LNFPIII treatment on vH inflammatory markers were detected via qPCR, but there were numerical increases of IL-6, IL-1□ and TNF□ in the GWI-vehicle group at this time point ($p>0.05$; Table 5.2). Interestingly, both BDNF and NGF expression increases were detected in GWI-LNFPIII animals compared to vehicle-vehicle animals 48 h post-exposures, with the effect on BDNF being significant ($p=0.04$). While the NGF increase in the GWI-LNFPIII group compared to the vehicle-vehicle was only numeric, there was a stronger trend for an elevation in vH NGF expression when GWI-LNFPIII NGF was compared to GWI-vehicle NGF 48 h post-exposure ($p=0.08$).

Additionally, trophic factor expression was evaluated in both dH and the vH 11 months post GWIR exposure with LNFPIII treatment beginning eight months post-exposure (Table 5.3). A trend for a significant increase in dH BDNF expression in GWI-LNFPIII animals compared to vehicle-vehicle animals was revealed ($p=0.10$). Importantly, there was a trend for enhancement of dH BDNF expression in GWI-LNFPIII animals compared to GWI-vehicle animals that approached significance ($p=0.06$). Notably, significant elevations in dH NGF levels in vehicle-LNFPIII animals compared to vehicle-vehicle animals ($p=0.03$) as well as in GWI-LNFPIII animals compared to vehicle-vehicle animals ($p=0.001$) were observed. A trend of a significant increase in dH NGF levels was detected in GWI-vehicle animals compared to vehicle-vehicle animals ($p=0.08$); however, importantly, a strong trend of a significant increase in GWI-LNFPIII animals compared to GWI-vehicle animals was present ($p=0.06$). In the vH, there were no significant differences in BDNF or NGF expression between treatment groups, although all three trophic factors were numerically reduced in GWI-vehicle animals compared to vehicle-vehicle animals, something not seen in the vH of the GWI-LNFPIII mice. Of the numeric decreases of

vH trophic factors in the GWI-vehicle group, the decrease of CNTF was most pronounced (p=0.07).

5.4 Discussion

GWI models have been developed in the mouse (Abdullah et al., 2011; O'Callaghan et al., 2015) and the rat (Abdel-Rahman et al., 2002; Macht et al., 2018). Acute studies employing the model used in the present report (or a modification of it) described neurochemical abnormalities within six h after GWIR exposure such as dyshomeostasis of monoaminergic neurotransmitters in limbic structures (Carpenter et al., 2020) and notable reductions in brain AChE specific activity that led to enhanced brain ACh levels (Locker et al., 2017). In general, cholinergic transmission is impacted by acute exposure to AChE inhibitors, such as PB and DFP, resulting in learning and memory modulation (Hasselmo, 2006). Acute PB and DEET exposure has also been shown to increase hippocampal pyramidal cell excitability, an effect attributed to enhanced cholinergic transmission (Friedman et al., 1996; Corbel et al., 2009). Additionally, mice that received repeated exposure to chlorpyrifos, an OP that shares the same mechanism as DFP and has been implicated in GWI etiology, exhibited enhanced hippocampal basal synaptic transmission one-week post-chlorpyrifos exposure (Speed et al., 2011). Chlorpyrifos exposure increased phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2) and enhanced the secretion of neurotrophic factors such as BDNF (Betancourt et al., 2007; Tan et al., 2009). Phosphorylation of ERK-1/2 is essential for facilitating the signaling cascade that results in BDNF-induced increases in hippocampal spine density (Alonso et al., 2004) and synaptic transmission (Kang and Schuman, 1995). The present report demonstrated a significant increase in hippocampal basal synaptic transmission 48 h post GWIR exposure, which is consistent with the aforementioned studies. We postulate that this increase in basal synaptic transmission is due to elevated cholinergic activity, enhanced ERK-1/2 activation, and increased hippocampal BDNF, all of which are supported by the observed numerical and significant enhancement of vH BDNF mRNA levels in GWI-LNFPIII and GWI-vehicle animals,

respectively. The numerical increase in LTP magnitude in GWI-vehicle and GWI-LNFPIII animals detected 48 h post-GWIR exposure is also consistent with an enhanced cholinergic tone as well as ERK-dependent BDNF expression.

Acute neuroimmune abnormalities induced by the GWI exposure model used in the present study have been characterized. Specifically, an increase of multiple inflammatory cytokines in the brain (Miller et al., 2018), detrimental effects on prefrontal cortex glia functionality (Belgrad et al., 2019), and neuroinflammatory-related structural anomalies in the hippocampus (Koo et al., 2018) are among the acute (6 h) GWI-related effects in this model. These data are in line with acute neuroimmune dysfunction reported in other GWI animal models (Abdel-Rahman et al., 2004; Ojo et al., 2014; Zakirova et al., 2015), consistent with the hypothesis of GWI being a neuroimmune disease (O'Callaghan et al., 2016). Notably, some studies have found beneficial effects of immunoactive therapies on adverse components of GWI (O'Callaghan et al., 2015; Golier et al., 2016). The potential therapeutic, LNFPIII, investigated in the present study utilizes the CD14/TLR-MAPK (ERK) axis to induce anti-inflammatory chemokine and cytokine production, facilitating ERK-1/2 phosphorylation (Tundup et al., 2015). In the current report, we did not detect significant differences in key inflammatory markers in the vH 48 h post-exposure, a finding that is consistent with investigations demonstrating that inflammatory cytokine production peaks within six h after exposure and subsequently returns to baseline levels (O'Callaghan et al., 2015; Michalovicz et al., 2019; Carpenter et al., 2020). However, coadministration of LNFPIII throughout GWIR exposure resulted in a significant enhancement of synaptic transmission and LTP, which is in line with a direct (Tundup et al., 2015), likely BDNF-mediated effect. These results would also be consistent with LNFPIII shaping synaptic plasticity via diffuse, anti-inflammatory effects (Prieto and Cotman, 2017). Finally, as we found no changes in paired-pulse facilitation (PPF) at the acute time point, a postsynaptic mechanism was likely responsible for the observed enhancement. These electrophysiological data are consistent with an increase in synaptic density, suggesting that

LNFPIII-mediated enhancement of trophic factor production may also enhance synaptic transmission and LTP via modulation of structural plasticity 48 h post-exposure.

While our findings at the acute time point indicate LNFPIII can affect measures of synaptic plasticity and transmission and trophic factor levels, the results reported at the persisting time points underscore the potential therapeutic efficacy of LNFPIII for the treatment of GWI. Importantly, the 11-month duration of rodent aging between GWI induction and electrophysiology recordings recapitulates the aging process of GW veterans (Sengupta, 2013), suggesting translational relevance of our results. The acute neuroinflammatory response in GWI models is short-lived; however, months after exposure, re-emerging neuroinflammatory aberrations are present and associate with GWI symptomology (Alshelh et al., 2020; Cheng et al., 2020). Notably, a delayed progressive worsening of clinical GWI symptoms has been established (Zundel et al., 2019; Porter et al., 2020) and it is attributed, at least in part, to neuroimmune dysfunction (O'Callaghan et al., 2016). As it relates to the focus of our study, aberrant activation of microglia impairs hippocampal synaptic plasticity and transmission, resulting in deficits in learning and memory (Maggio et al., 2013; Prieto and Cotman, 2017). Moreover, abnormal microglia activation facilitates an undesirable activation of astrocytes, further modulating synaptic responses (Pascual et al., 2012). Persisting neuroinflammation is associated with biasing microglia to a pro-inflammatory, M1, activation state (Cherry et al., 2014), leading to an enhanced inflammatory tone that hinders hippocampal synaptic transmission (Maher et al., 2006; Kelly et al., 2013). Enhanced neuroinflammation also reduces the secretion of hippocampal trophic factors (Barrientos et al., 2004; Yirmiya and Goshen, 2011; Littlefield et al., 2015). Reports of delayed neuroimmune dysfunction in GWI (Coughlin, 2017) may be particularly relevant to hippocampal synaptic plasticity and transmission as aging alone leads to neuroinflammatory aberrations and reductions of hippocampal trophic factor production (Kelly et al., 2013; Littlefield et al., 2015), underscoring the necessity to evaluate GWI animal models and the efficacy of GWI therapeutics at clinically relevant time points.

In the present report, following an initial enhancement in basal synaptic transmission 48 h after GWIR exposure, no change was found seven months post-exposure, and a reduction was observed 11 months after exposure. This delayed reduction in hippocampal basal synaptic transmission is consistent with the expected synaptic effects induced by the aforementioned delayed GWIR exposure-induced neuroinflammatory phenotype. These findings are in line with a preclinical study demonstrating that exposure to an OP implicated in the etiology of GWI, chlorpyrifos, leads to an initial enhancement of hippocampal synaptic transmission followed by a reduction three months later (Speed et al., 2011). This delayed reduction was attributed to a decrease in CA1 apical dendritic spine density. In addition, we found a delayed decrease in LTP magnitude 11 months post-GWIR exposure. As we did not detect any significant changes in PPF at any time point, a postsynaptic, structure-specific effect, such as decreased synaptic density following GWI exposure, is indicated. This is further supported by the apparent reduction in all trophic factors assessed in the vH of GWI-vehicle animals 11 months post-GWIR exposure. Taken together, the findings in the present study are consistent with a GWIR exposure-induced delayed reduction in CA1 synaptic density, but this structural change has to be verified in the future.

Importantly, delayed LNFPIII treatment restored hippocampal synaptic transmission and LTP seven and 11 months after GWIR exposure. The prolonged impact of delayed LNFPIII treatment on microglia, astrocytes, and/or the CD14/TLR-MAPK (ERK) axis may be responsible for restoring hippocampal synaptic plasticity and transmission. This is supported by studies in which LNFPIII treatment reduced the severity of experimental autoimmune encephalomyelitis by modulating innate and T cell immune responses (Zhu et al., 2012), which are both dysfunctional in GWI (Coughlin, 2017). LNFPIII also enhanced IL-10 levels, mitigated chronic inflammation, and restored metabolic function associated with diet-induced obesity (Bhargava et al., 2012). Recent reports by our group demonstrated that LNFPIII treatment rebalanced neurochemical and neuroimmune functionality in multiple GWI animal models, acutely and chronically

(Carpenter et al., 2020; Mote et al., 2020). Therefore, we postulate that one likely reason for the restoration of synaptic transmission and LTP months after GWIR exposure is a result of LNFPIII-mediated anti-inflammatory:inflammatory milieu rebalancing. We observed an enhancement of hippocampal BDNF and NGF expression in GWI-LNFPIII-treated animals, suggesting that recovery of synaptic transmission and LTP months after GWIR exposure may also be a result of a direct, LNFPIII-induced increase in trophic factor production. Most notably, the efficacy of delayed LNFPIII treatment on ameliorating GWIR exposure-induced aberrations in hippocampal synaptic measurements and trophic factor levels is particularly encouraging as potential clinical treatment of GWI with LNFPIII would also follow a delayed timeline (i.e., beginning LNFPIII treatment decades after exposure).

The dH and the vH have been increasingly appreciated as functionally distinct structures (Fanselow and Dong, 2010; Papatheodoropoulos, 2018). Monitoring the persisting/delayed dorsoventral-specific impact of neurotoxin overexposures on measurements of hippocampal synaptic plasticity and transmission may be essential for interpreting subtle behavioral changes described in disease states, such as GWI, especially early in the course of the disease. We observed no distinct dorsoventral-specific effects of GWIR exposure on measurements of synaptic plasticity and transmission at the 48 h and seven-month time points; however, we detected differential responses at the 11-month time point. For instance, we found a significant main effect of GWIR exposure on LTP magnitude in the dH, but not in the vH. We also detected a differential impact of GWIR exposure on trophic factor production in the dH compared to the vH. These findings may provide insight into reports of impairments in dH-dependent spatial memory tasks (Parihar et al., 2013; Hattiangady et al., 2014; Joshi et al., 2018; Madhu et al., 2019). Abnormal vH-dependent behavior (i.e., anxiety), while reported, might be less pronounced (Parihar et al., 2013; Hattiangady et al., 2014; Zakirova et al., 2016), at least in GWI models and veterans with GWI that have experienced GWIR exposures in line with the model we used in the current study. Similar to the delayed effects we observed, clinical GWI learning

and memory deficits appear to become more marked over time (Zundel et al., 2019; Porter et al., 2020). Studies describing hippocampal sector- and subfield-specific structural abnormalities in GW veterans have also been reported (Vythilingam et al., 2005; Chao et al., 2014; Chao et al., 2017). As we detected biphasic impairments in synaptic plasticity and transmission that were differentially manifested along the hippocampal dorsoventral axis, accounting for such hippocampal sector-specific effects may be essential for interpreting subtle, progressive neurological alterations presented in GWI.

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Figures and tables

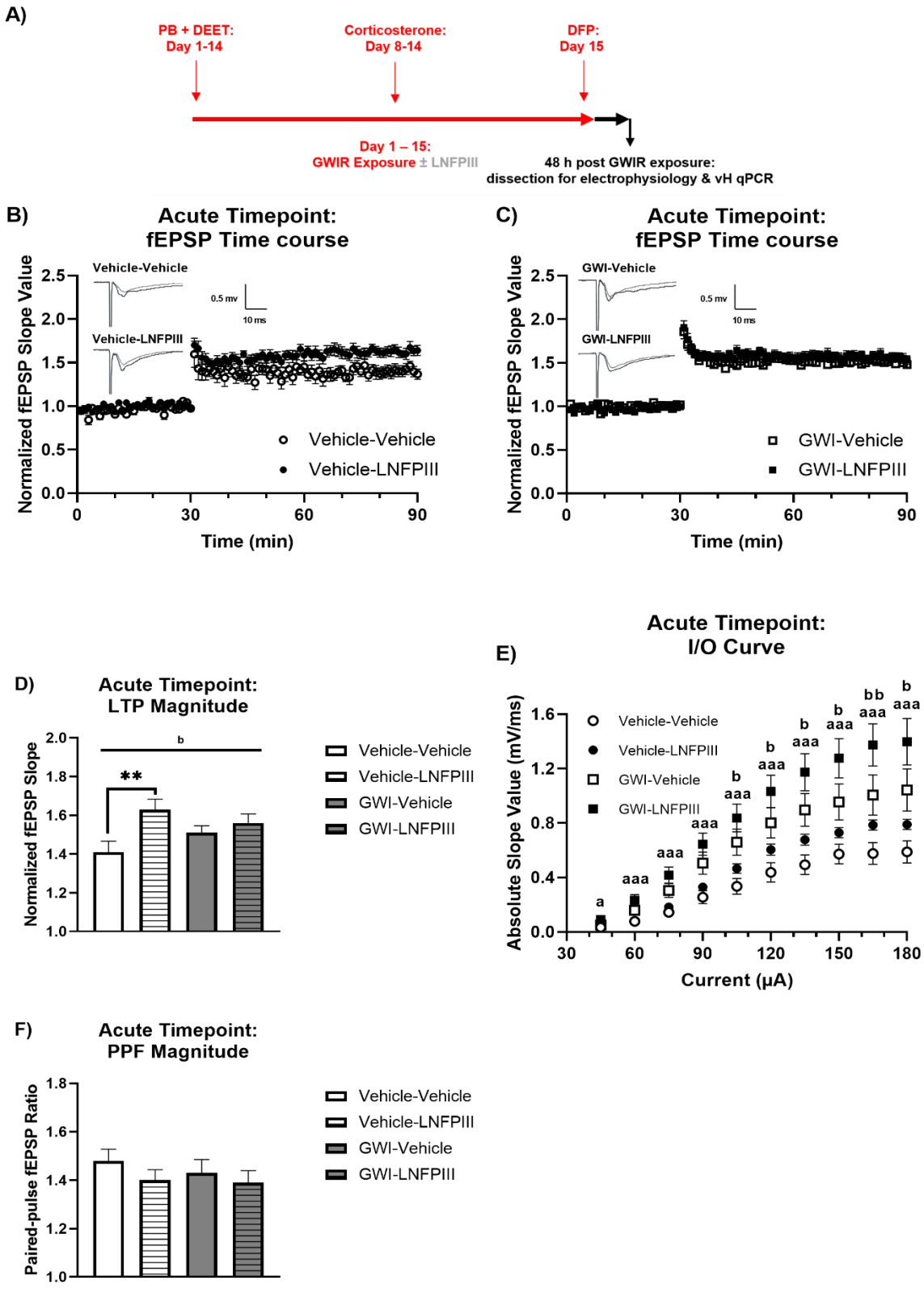
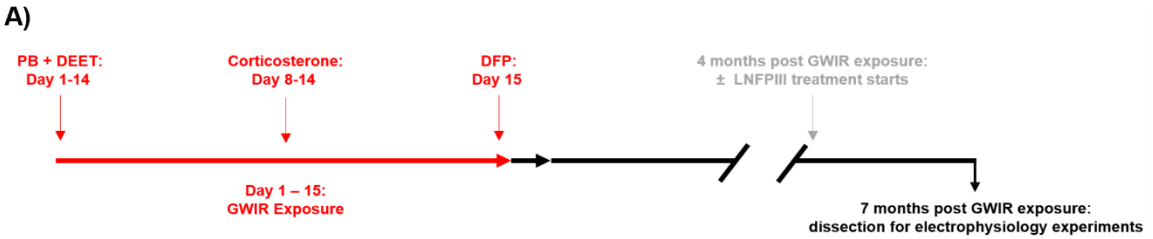
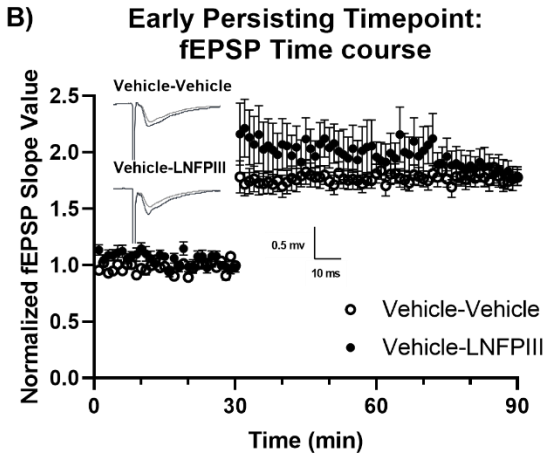
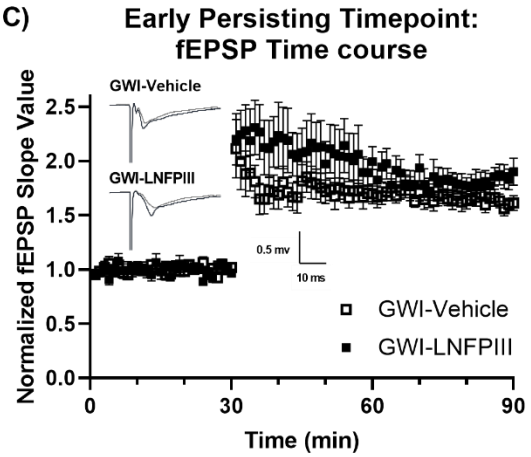
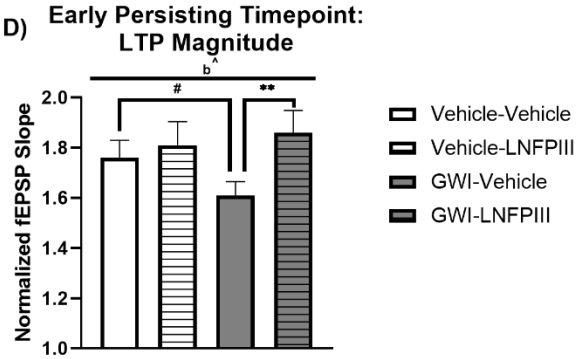


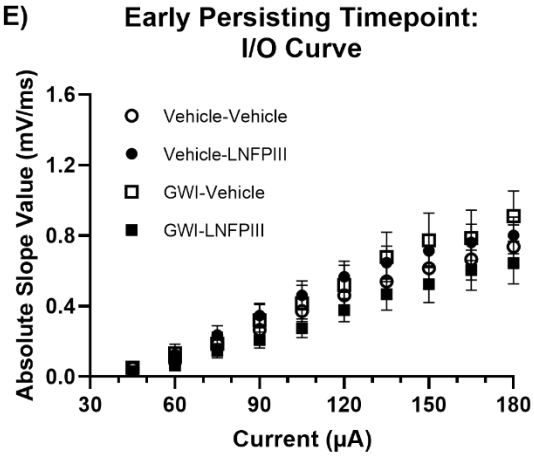
Figure 5.1. The effect of Gulf War Illness-related (GWIR) exposure ± LNFPIII coadministration on measurements of hippocampal synaptic plasticity and transmission 48 h post GWIR/LNFPIII treatment. A. Timeline of GWIR exposure and LNFPIII treatment for animals that were dissected at the acute electrophysiological timepoint. **B and C.** Summary plots of normalized fEPSP values recorded from either vehicle-treated (**B**) or GWIR-exposed (**C**) animals 48 h post GWIR exposure. (**B**) compares Vehicle-Vehicle (○) and Vehicle-LNFPIII (●) treated mice whereas (**C**) compares GWI-Vehicle (□) and GWI-LNFPIII (■) treated mice. Insets show traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post tetanus (black). **D.** Summary quantification of LTP magnitude recorded 48 h after GWIR exposure from Vehicle-Vehicle (1.41 ± 0.06, n=11(4)), Vehicle-LNFPIII (1.63 ± 0.05, n=18(4)), GWI-Vehicle (1.51 ± 0.04, n=21(4)), and GWI-LNFPIII (1.56 ± 0.05, n=17(4)) treated animals. **E.** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities from Vehicle-Vehicle (○), Vehicle-LNFPIII (●), GWI-Vehicle (□), and GWI-LNFPIII (■) treatment groups recorded 48 h after GWIR exposure. **F.** The paired-pulse ratio for Vehicle-Vehicle (1.48 ± 0.05), Vehicle-LNFPIII (1.40 ± 0.04), GWI-Vehicle (1.43 ± 0.06), and GWI-LNFPIII (1.39 ± 0.05) treatment groups recorded 48 h after GWIR exposure (interpulse interval = 50 ms). ^{a} denotes main effect of GWIR exposure ($p < 0.05$, ^{aa} denotes $p < 0.01$, ^{aaa} denotes $p < 0.001$) whereas ^{b} denotes main effect of LNFPIII ($p < 0.05$, ^{bb} denotes $p < 0.01$, ^{bbb} denotes $p < 0.001$). * denotes $p < 0.05$; ** denotes $p < 0.01$. Data are expressed as mean ± SEM.


A)
PB + DEET:
Day 1-14
Corticosterone:
Day 8-14
DFP:
Day 15
4 months post GWIR exposure:
± LNFPIII treatment starts
Day 1 – 15:
GWIR Exposure
7 months post GWIR exposure:
dissection for electrophysiology experiments


B)
Early Persisting Timepoint:
fEPSP Time course
Normalized fEPSP Slope Value
Vehicle-Vehicle
Vehicle-LNFPIII
0.5 mv
10 ms
Vehicle-Vehicle
Vehicle-LNFPIII
Time (min)


C)
Early Persisting Timepoint:
fEPSP Time course
Normalized fEPSP Slope Value
GWI-Vehicle
GWI-LNFPIII
0.5 mv
10 ms
GWI-Vehicle
GWI-LNFPIII
Time (min)


D)
Early Persisting Timepoint:
LTP Magnitude
Normalized fEPSP Slope
b^
#
**
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII


E)
Early Persisting Timepoint:
I/O Curve
Absolute Slope Value (mV/ms)
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII
Current (µA)

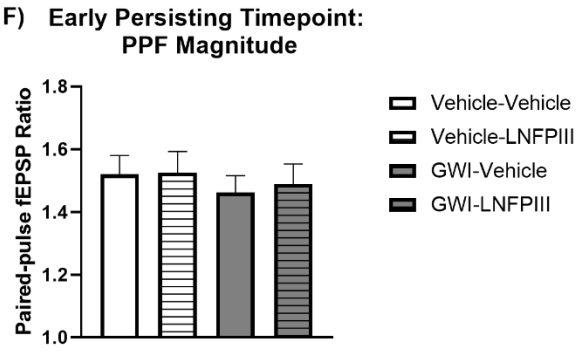
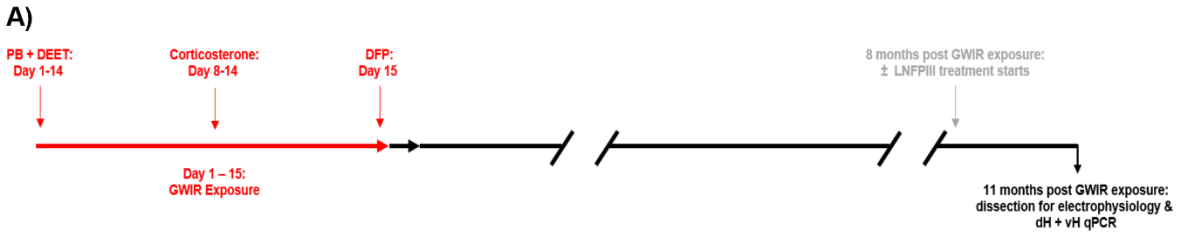
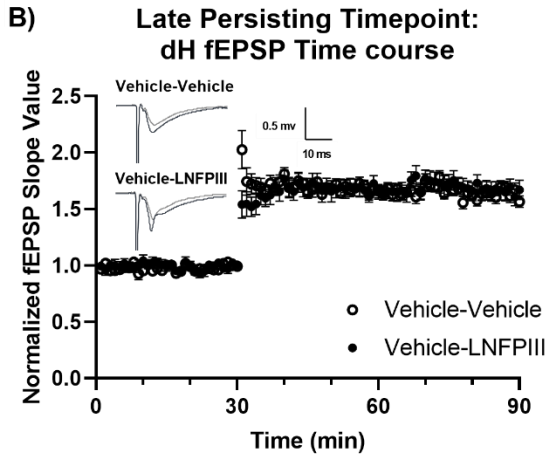
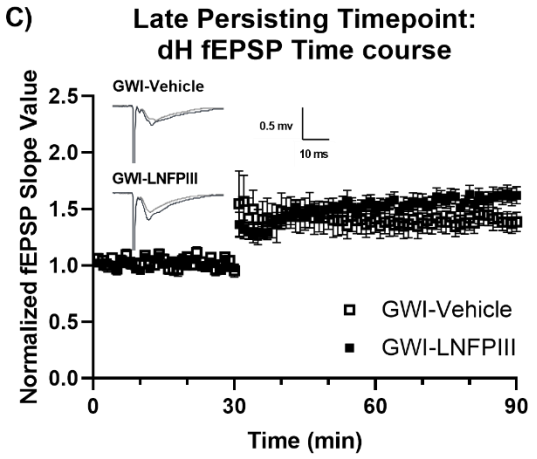
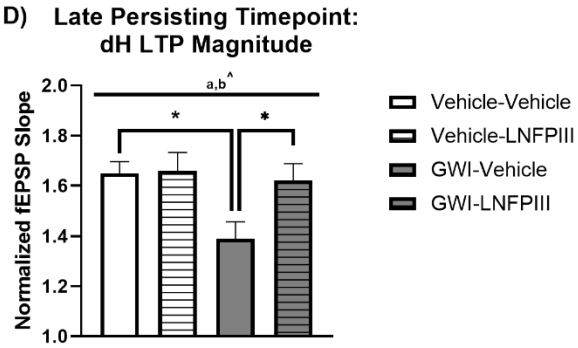

F)
Early Persisting Timepoint:
PPF Magnitude
Paired-pulse fEPSP Ratio
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII

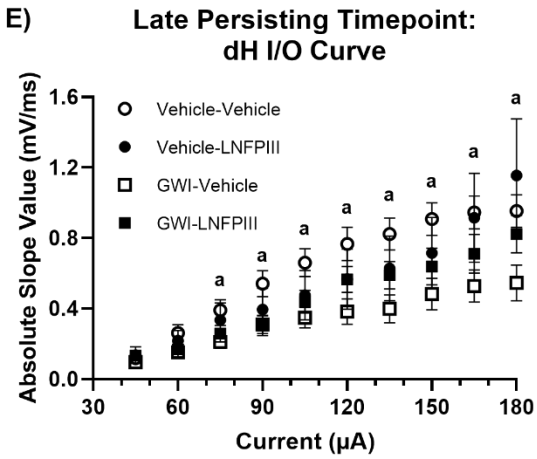
Figure 5.2. The effect of GWIR exposure ± delayed LNFPIII treatment on measurements of hippocampal synaptic plasticity and transmission 7 months post GWIR treatment. A. Timeline of GWIR exposure and LNFPIII treatment for animals that were dissected at the early persisting electrophysiological timepoint. **B and C.** Summary plots of normalized fEPSP values recorded from either vehicle-treated (**B**) or GWIR-exposed (**C**) animals 7 months post GWIR exposure. (**B**) compares Vehicle-Vehicle (○) and Vehicle-LNFPIII (●) treated mice whereas (**C**) compares GWI-Vehicle (□) and GWI-LNFPIII (■) treated mice. Insets show traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post tetanus (black). **D.** Summary quantification of LTP magnitude recorded 7 months after GWIR exposure from Vehicle-Vehicle (1.76 ± 0.07, n=16(3)), Vehicle-LNFPIII (1.81 ± 0.10, n=15(3)), GWI-Vehicle (1.61 ± 0.06, n=14(3)), and GWI-LNFPIII (1.86 ± 0.09, n=12(3)) treated animals. **E.** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities from Vehicle-Vehicle (○), Vehicle-LNFPIII (●), GWI-Vehicle (□), and GWI-LNFPIII (■) treatment groups recorded 7 months after GWIR exposure. **F.** The paired-pulse ratio for Vehicle-Vehicle (1.52 ± 0.06), Vehicle-LNFPIII (1.53 ± 0.07), GWI-Vehicle (1.46 ± 0.05), and GWI-LNFPIII (1.49 ± 0.06) treatment groups recorded 7 months after GWIR exposure (interpulse interval = 50 ms). ^{a} denotes main effect of GWIR exposure ($p < 0.05$) whereas ^{b} denotes main effect of LNFPIII ($p < 0.05$, $b^{\wedge}$ denotes $p < 0.1$). # denotes $p < 0.1$; * denotes $p < 0.05$; ** denotes $p < 0.01$. Data are expressed as mean ± SEM.


A)
PB + DEET:
Day 1-14
Corticosterone:
Day 8-14
DFP:
Day 15
Day 1 – 15:
GWIR Exposure
8 months post GWIR exposure:
± LNFPIII treatment starts
11 months post GWIR exposure:
dissection for electrophysiology &
dH + vH qPCR


B)
Late Persisting Timepoint:
dH fEPSP Time course
Vehicle-Vehicle
Vehicle-LNFPIII
0.5 mv
10 ms
Normalized fEPSP Slope Value
Time (min)
Vehicle-Vehicle
Vehicle-LNFPIII


C)
Late Persisting Timepoint:
dH fEPSP Time course
GWI-Vehicle
GWI-LNFPIII
0.5 mv
10 ms
Normalized fEPSP Slope Value
Time (min)
GWI-Vehicle
GWI-LNFPIII


D)
Late Persisting Timepoint:
dH LTP Magnitude
Normalized fEPSP Slope
a,b^
*
*
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII


E)
Late Persisting Timepoint:
dH I/O Curve
Absolute Slope Value (mV/ms)
Current (µA)
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII

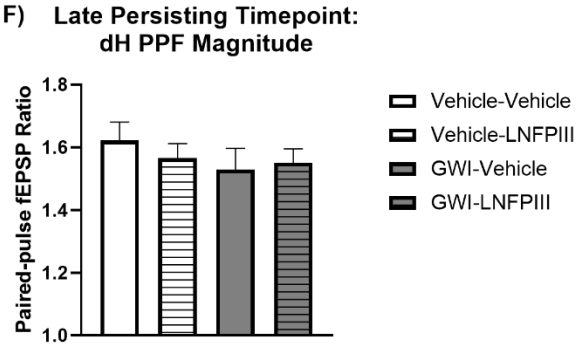

F)
Late Persisting Timepoint:
dH PPF Magnitude
Paired-pulse fEPSP Ratio
Vehicle-Vehicle
Vehicle-LNFPIII
GWI-Vehicle
GWI-LNFPIII

Figure 5.3. The effect of GWIR exposure ± delayed LNFPIII treatment on measurements of dorsal hippocampal (dH) synaptic plasticity and transmission 11 months post GWIR treatment. A. Timeline of GWIR exposure and LNFPIII treatment for animals that were dissected at the late persisting electrophysiological timepoint. This timeline was also employed for the data presented in Fig. 5.4. **B and C.** Summary plots of normalized fEPSP values recorded in the dH from either vehicle-treated (**B**) or GWIR-exposed (**C**) animals 11 months post GWIR exposure. (**B**) compares Vehicle-Vehicle (○) and Vehicle-LNFPIII (●) treated mice whereas (**C**) compares GWI-Vehicle (□) and GWI-LNFPIII (■) treated mice. Insets show traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post tetanus (black). **D.** Summary quantification of LTP magnitude recorded in the dH 11 months after GWIR exposure from Vehicle-Vehicle (1.65 ± 0.05, n=15(7)), Vehicle-LNFPIII (1.66 ± 0.07, n=13(5)), GWI-Vehicle (1.39 ± 0.07, n=14(6)), and GWI-LNFPIII (1.62 ± 0.07, n=14(5)) treated animals. **E.** Stimulus-response relationship of the average dH fEPSP slopes elicited by increasing stimulus intensities from Vehicle-Vehicle (●), Vehicle-LNFPIII (■), GWI-Vehicle (○), and GWI-LNFPIII (□) treatment groups recorded in the dH 11 months after GWIR exposure. **F.** The paired-pulse ratio for Vehicle-Vehicle (1.62 ± 0.06), Vehicle-LNFPIII (1.57 ± 0.05), GWI-Vehicle (1.53 ± 0.07), and GWI-LNFPIII (1.55 ± 0.05) treatment groups recorded in the dH 11 months after GWIR exposure (interpulse interval = 50 ms). [a] denotes main effect of GWIR exposure ($p < 0.05$) whereas [b] denotes main effect of LNFPIII ($p < 0.05$, b^ denotes $p < 0.1$). * denotes $p < 0.05$. Data are expressed as mean ± SEM.

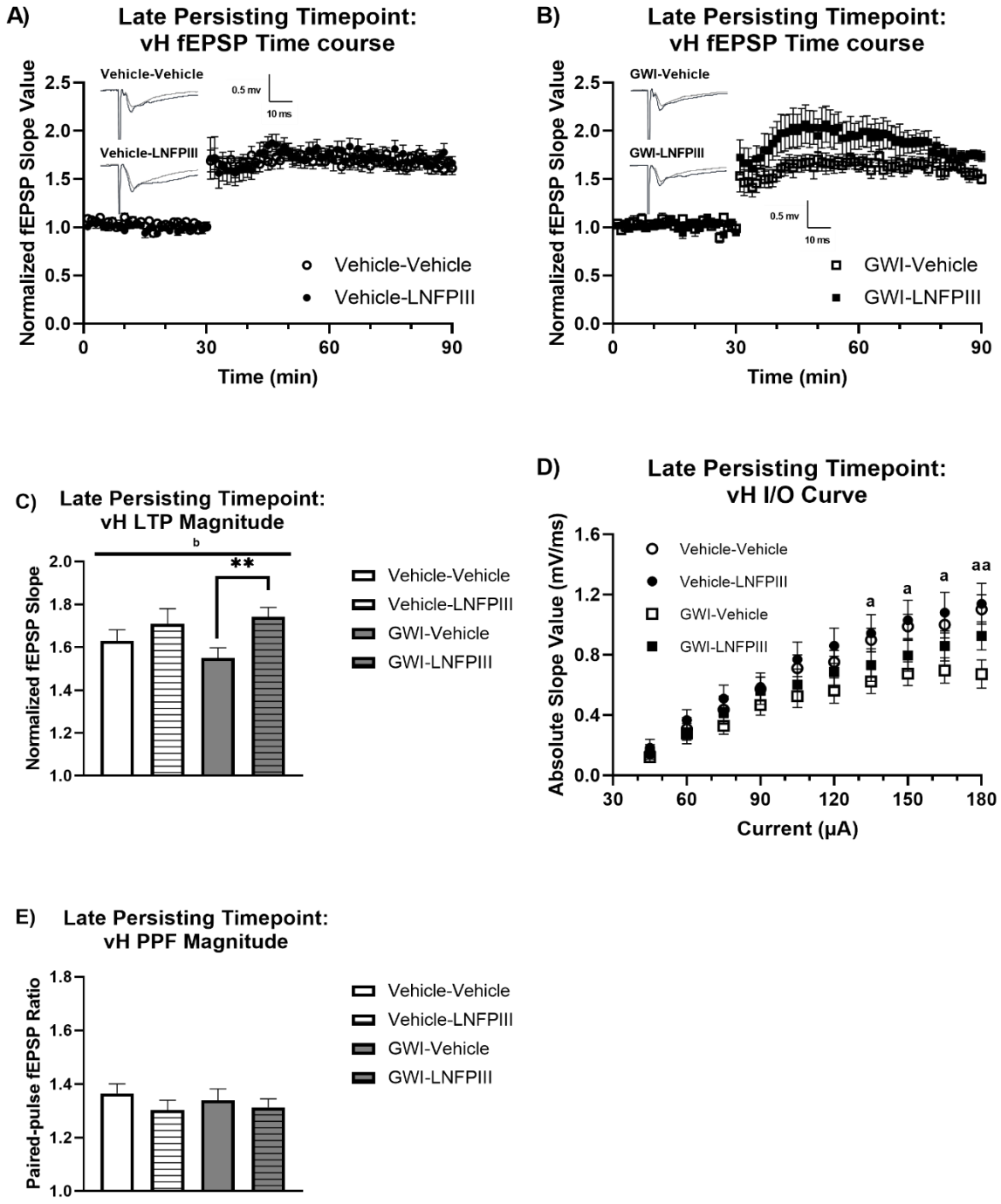


Figure 5.4. The effect of GWIR exposure ± delayed LNFPIII treatment on measurements of ventral hippocampal (vH) synaptic plasticity and transmission 11 months post GWIR treatment. A and B. Summary plots of normalized fEPSP values recorded in the vH from either vehicle-treated (**A**) or GWIR-exposed (**B**) animals 11 months post GWIR exposure. (**A**)

compares Vehicle-Vehicle (○) and Vehicle-LNFPIII (●) treated mice whereas (**B**) compares GWI-Vehicle (□) and GWI-LNFPIII (■) treated mice. Insets show traces composed of average sweeps from 5 min pre-tetanus (grey) and 56–60 min post tetanus (black). **C.** Summary quantification of LTP magnitude recorded in the vH 11 months after GWIR exposure from Vehicle-Vehicle (1.63 ± 0.05, n=15(7)), Vehicle-LNFPIII (1.71 ± 0.07, n=14(5)), GWI-Vehicle (1.55 ± 0.05, n=12(6)), and GWI-LNFPIII (1.74 ± 0.04, n=12(5)) treated animals. **D.** Stimulus-response relationship of the average dH fEPSP slopes elicited by increasing stimulus intensities from Vehicle-Vehicle (●), Vehicle-LNFPIII (■), GWI-Vehicle (○), and GWI-LNFPIII (□) treatment groups recorded in the vH 11 months after GWIR exposure. **E.** The paired-pulse ratio for Vehicle-Vehicle (1.36 ± 0.04), Vehicle-LNFPIII (1.30 ± 0.04), GWI-Vehicle (1.34 ± 0.04), and GWI-LNFPIII (1.31 ± 0.03) treatment groups recorded in the vH 11 months after GWIR exposure (interpulse interval = 50 ms). [a] denotes main effect of GWIR exposure ($p < 0.05$, [aa] denotes $p < 0.01$) whereas [b] denotes main effect of LNFPIII ($p < 0.05$). * denotes $p < 0.05$; ** denotes $p < 0.01$. Data are expressed as mean ± SEM.

Table 5.1. List of primers used in the quantitative PCR (qPCR) analysis

	CTGAGCGTGTGTGACAGTATTA	CTTTGGATACCGGGACTTTCTC
	GAGATGGTGAAAGAGGTGAA	GTGTTGAAGAGCAGAAAGCA
	ACAAGGTCTCCATAAGTGTCAGC	CTGCCATTGGTCCAGGATGA
	GAGGACATGAGCACCTTCTTT	GCCTGTAGTGCAGTTGTCTAA
	ATGGATGCTACCAAACTGGAT	TGAAGGACTCTGGCTTTGTCT
	CAGTGAGGTGCATAGCGTAAT	CTCCTTCTGGGACATTGCTATC
	GCAGGTCTACTTTGGAGTCATTGC	TCCCTTTGCAGAACTCAGGAATGG

Table 5.2. The effect of GWIR exposure ± LNFPIII on the expression of key inflammatory cytokines and growth factors in the ventral hippocampus (vH) measured by qPCR 48 h post GWIR exposure. vH samples from mice (n=6 per treatment) were used for RNA isolation and subsequent qPCR analyses. Data were analyzed by the $2^{-\Delta\Delta}$Ct method with 18S as the housekeeping gene and are expressed as a mean fold change from the Vehicle-Vehicle control ± SEM. Gene abbreviations: IL-6, Interleukin 6; IL-1β, Interleukin 1 beta; TNFα, Tumor necrosis factor alpha; BDNF, Brain-derived neurotrophic factor; NGF, Nerve growth factor; CNTF, Ciliary neurotrophic factor.

Table 5.2. qPCR assessment of cytokines and trophic factors in the ventral hippocampus 48 h post GWIR exposure

	Fold Change			p-values			
Gene	Veh. – LNFPIII vs. control	GWI – Veh. vs. control	GWI – LNFPIII vs. control	Veh. – LNFPIII vs. control	GWI – Veh. vs. control	GWI – LNFPIII vs. control	GWI – Veh. vs. GWI – LNFPIII
Casp1	0.54 ± 0.04	0.70 ± 0.05	0.67 ± 0.05	0.21	0.28	0.19	0.93
IL-6	1.09 ± 0.13	1.19 ± 0.14	0.97 ± 0.12	0.86	0.52	0.93	0.52
IL-1β	0.50 ± 0.15	1.31 ± 0.41	0.64 ± 0.20	0.23	0.64	0.29	0.13
TNFα	1.32 ± 0.40	1.78 ± 0.54	0.94 ± 0.28	0.51	0.26	0.90	0.26
BDNF	0.88 ± 0.07	1.39 ± 0.11	**1.87 ± 0.14**	0.71	0.28	**0.04**	0.43
NGF	1.06 ± 0.14	0.92 ± 0.12	1.29 ± 0.17	0.90	0.80	0.39	**0.08**
CNTF	1.07 ± 0.04	0.92 ± 0.03	0.82 ± 0.03	0.83	0.73	0.12	0.66

Table 5.3. The effect of GWIR exposure ± LNFPIII on the expression of growth factors in the dorsal (dH) and ventral hippocampus (vH) measured by qPCR post GWIR exposure. dH and vH samples from mice (n=7, 4, 7, 6, for the, respectively, Vehicle-Vehicle, Vehicle-LNFPIII, GWI-Vehicle, GWI-LNFPIII groups) were used for RNA isolation and subsequent qPCR analyses. Data were analyzed by the $2^{-\Delta\Delta}$Ct method with 18S as the housekeeping gene and are expressed as a mean fold change from the Vehicle-Vehicle control ± SEM. Gene abbreviations: BDNF, Brain-derived neurotrophic factor; NGF, Nerve growth factor; CNTF, Ciliary neurotrophic factor.

Table 5.3. qPCR assessment of trophic factors in the hippocampus 11 months post GWIR exposure

		Fold Change			p-values			
Region	**Gene**	Veh. – LNFPIII vs. control	GWI – Veh. vs. control	GWI – LNFPIII vs. control	Veh. – LNFPIII vs. control	GWI – Veh. vs. control	GWI – LNFPIII vs. control	GWI – Veh. vs. GWI – LNFPIII
Dorsal Hippocampus	BDNF	0.67 ± 0.09	0.90 ± 0.12	**1.55 ± 0.20**	0.13	0.69	**0.10**	**0.06**
	NGF	**1.48 ± 0.10**	**1.42 ± 0.09**	**2.18 ± 0.15**	**0.03**	**0.08**	**0.001**	**0.06**
	CNTF	0.97 ± 0.05	0.98 ± 0.05	1.03 ± 0.05	0.84	0.93	0.74	0.84
Ventral Hippocampus	BDNF	1.05 ± 0.07	0.87 ± 0.31	1.01 ± 0.07	0.82	0.54	0.95	0.56
	NGF	0.83 ± 0.08	0.67 ± 0.001	0.89 ± 0.09	0.48	0.11	0.61	0.19
	CNTF	0.79 ± 0.08	**0.72 ± 0.09**	0.96 ± 0.10	0.28	**0.07**	0.86	0.11

CHAPTER 6

SUMMARY AND CONCLUSION

The hippocampus is a bilateral structure located in the medial temporal lobe of mammalian organisms and is fundamentally associated with behavioral responses such as learning and memory. While the responsibility of the hippocampus in phases of learning and memory such as encoding, consolidation, and retrieval has been established for decades, emerging evidence continues to illuminate differential functionality between the dorsal hippocampus (dH) and the ventral hippocampus (vH; Fanselow and Dong, 2010). Studies indicate that unique roles of the dH sector compared to the vH sector may be due to distinctions at the intrinsic and extrinsic levels. For instance, differences in local circuitry and receptor densities between the dH and the vH have been proposed as a basis for sector-specific synaptic plasticity and transmission (Papatheodoropoulos, 2018). Projections from the dH to brain regions linked to higher-order cognitive processes whereas vH fibers predominately sent to neuroanatomical structures related to affective responses may also underlie a rudimental reason for these sectors diverging roles in behavioral responses (Fanselow and Dong, 2010). Accordingly, monitoring dH- and vH-specific synaptic and behavioral responses in complex preclinical models of neurological disease states such as Gulf War Illness (GWI) may provide valuable insight into clinical presentation of GWI.

GWI is a chronic multisymptom condition that affects approximately one-third of the 700,000 U.S. personnel deployed in the 1990-1991 Persian Gulf War (White et al., 2016). GWI presents with a constellation of symptoms, but neurological (i.e., cognitive) and immunological (i.e., inflammation) symptoms are consistently reported (Coughlin, 2017). While the initiating factors related to the etiology of GWI are unknown, overexposures to neurotoxic pesticides,

organophosphate (OP) nerve agents, nerve agent prophylactics, and wartime stress have been associated with GWI symptoms (Cherry et al., 2001; Winkenwerder, 2003; Steele et al., 2012). GWI symptom severity is correlated to factors of GWI-related exposures such as the degree, duration, and type of exposures (Steele et al., 2012; Sullivan et al., 2018). Increases in the reported incidence (Zundel et al., 2019) and symptom severity (Porter et al., 2020; Zundel et al., 2020) of GWI suggests disease progression. The previous two sentences underscore the critical need to employ translationally relevant doses and timelines of GWIR exposures in animal models that reflect the progressive presentation of GWI to identify the pathobiology of the disease as well as efficacious GWI therapeutics. The hippocampus has been implicated as a site of pathology in GWI as GWI-specific molecular, cellular, anatomical, and behavioral aberrations within and related to this structure have been consistently reported (White et al., 2016). Preclinical and clinical investigations of GWI therapeutics have found some success in ameliorating GWI-specific hippocampal abnormalities (Dickey et al., 2020; Nugent et al., 2020). Nonetheless, there are a limited number of reports detailing hippocampal synaptic abnormalities presented in GWI and even fewer studies that assessed the beneficial effects of therapeutics on GWI-related hippocampal synaptic anomalies. The objective of the present document was to ameliorate GWI-specific impairments in cognitive and synaptic function using an immunotherapeutic therapeutic, lacto-N-fucopenatose-III (LNFPIII). The current chapter will provide a summary of our novel findings and review some of challenges currently facing GWI researchers. A description of how our results address some of the obstacles and knowledge gaps within the field of GWI research will also be discussed.

6.1 Review of Experimental Results

6.1.1 Acute effects of the nerve agent surrogate diisopropylfluorophosphate on hippocampal synaptic transmission along the dorsoventral axis

The impact of low-level exposures to GWIR chemicals employed in GWI (O'Callaghan et al., 2015; Phillips et al., 2019) and status epilepticus (Deshpande et al., 2010; Pouliot et al.,

2016) animal models, such as the organophosphate alkylphosphate diisopropylfluorophosphate (DFP), on hippocampal functionality have been studied in a preclinical setting (White et al., 2016); however, publications investigating the mechanisms that underlie the aberrant effects of DFP on hippocampal synaptic transmission along the dorsoventral axis are limited. Therefore, we aimed to contribute to the understanding of these animal models by evaluating the cholinergic and noncholinergic mechanisms associated with the effect of bath-applied DFP on excitatory and inhibitory hippocampal synaptic transmission in dH and vH slices. DFP inhibited excitatory transmission in the dH via a muscarinic acetylcholine receptor M2 (M_2R)-dependent mechanism, a mechanism that was not observed in the vH. Rather, blockade of the M_2R, nicotinic acetylcholine receptor (nAChRs), and N-methyl-D-aspartate receptor (NMDAR) were required to prevent the inhibitory effect of DFP on excitatory transmission in the vH. Therefore, DFP-induced inhibition of excitatory neurotransmission was produced via a cholinergic mechanism in the dH while a noncholinergic mechanism was indispensable in mediating the inhibitory effect of DFP on excitatory transmission in the vH. Interestingly, blockade of the NMDAR, but not cholinergic receptors, with the NMDAR antagonist D-APV prevented DFP-induced impairments in inhibitory transmission in both the dH and the vH. To the best of our knowledge, this was the first study to demonstrate dorsoventral-specific effects of an OP on excitatory and inhibitory hippocampal synaptic transmission. The novel noncholinergic mechanisms underlying the observed effects of DFP on synaptic responses may also provide important insight into OP overexposure cases as well. Of particular relevance to this document, these findings indicated that we may detect unique responses in the dH compared to the vH in our studies assessing abnormalities in hippocampal synaptic plasticity and transmission in GWI animal models.

6.1.2 The beneficial effects of lacto-n-fucopentaose-III on acute and persisting neurological impairments in a pyridostigmine bromide/permethrin Gulf War Illness animal model

Investigating the impact of GWIR chemicals on hippocampal-related responses in a bath preparation was extended to an in vivo setting by employing a well-established GWI animal model consisting of 10 days of exposure to a reversible acetylcholinesterase inhibitor nerve agent prophylactic, pyridostigmine bromide (PB), and a type II pyrethroid insecticide, permethrin (PM; Zakirova et al., 2015). The efficacy of an immunomodulatory glycan conjugate, LNFPIII, on ameliorating GWIR neurological abnormalities was assessed. Notably, LNFPIII was previously shown to be beneficial for short-term and long-term GWI-specific neurochemical and neuroimmune impairments (Carpenter et al., 2020; Mote et al., 2020). A subset of animals was coadministered LNFPIII during the duration of GWIR exposure and was sacrificed 48 h post-exposure for assessment of synaptic plasticity and transmission as well as trophic factor/cytokine expression. Another subset of animals began receiving delayed LNFPIII treatment four months after exposure and was utilized for electrophysiology, neurobehavioral assays, and immunohistochemistry beginning one month after initiation of LNFPIII treatment and up to eight months post-PB-PM-exposure. Overall, we observed PB-PM-related behavioral deficits in the motor, mood, and cognitive domains, impairments in hippocampal synaptic responses, and elevated hippocampal glial activation, abnormalities that were partly ameliorated by delayed LNFPIII treatment. Specifically, numerous motor tasks revealed sensorimotor, locomotor, and neuromuscular deficits following PB-PM exposure. Mood tests also demonstrated modest anxiety-like behavior in PB-PM animals. Subtle PB-PM-induced learning and memory impairments were detected in cognitive tasks, particularly during the initial days of training in hippocampal-dependent tasks. Performance in tasks related to motor and cognitive domains was notably benefited by delayed LNFPIII treatment following PB-PM exposure. DH and vH basal synaptic transmission was impaired 48 h and seven months post-PB-PM exposure. Coadministered LNFPIII ameliorated basal synaptic transmission deficits, particularly

in the dH, whereas delayed LNFPIII treatment was beneficial for vH, but not dH, synaptic transmission. Interestingly, LTP magnitude was unaffected by PB-PM exposure at both time points but coadministered and delayed LNFPIII treatment enhanced LTP magnitude uniquely along the dorsoventral axis in animals exposed to PB-PM. LNFPIII treatment also resulted in distinct enhancements in basal synaptic transmission and LTP magnitude at both time points along the dorsoventral axis in vehicle animals, indicating potential efficacy of the immunotherapeutic in non-GWI disease states. Furthermore, vH brain-derived neurotrophic factor (BDNF) expression was enhanced in PB-PM-exposed animals that received LNFPIII coadministration. Of particular interest to this document, no observable effects of PB-PM exposure were detected on LTP or paired-pulse facilitation magnitude, indicating PB-PM may be impacting synaptic responses via a postsynaptic-specific effect (i.e., decreased synaptic density). This proposed effect on synaptic density is supported by beneficial effects of LNFPIII on synaptic transmission and plasticity as well as trophic factor expression (the latter shown in chapter five as well). The prolonged impact of PB-PM exposure and delayed LNFPIII treatment on RAM performance suggest modest aberrant and ameliorative effects, respectively, on dH and vH-dependent behavior, indicating that distinguishing the distinct impact of previous PB-PM exposures and delayed LNFPIII treatment on synaptic plasticity and transmission along the dorsoventral axis may be critical for interpreting subtle GWI-specific cognitive impairments. Additionally, evidence of progressively worsened neuroinflammation was detected where key inflammatory cytokine expression in the vH was unaffected 48 h post-PB-PM exposure whereas delayed LNFPIII treatment ameliorated prolonged activation of hippocampal GFAP+ astrocytes and IBA-1+ microglia months after PB-PM exposure. Overall, these findings suggest that PB-PM exposure results in persisting neurological abnormalities consistent with GWI. Importantly, delayed LNFPIII treatment may hold potential as a therapeutic in cases involving PB-PM overexposures, such as GWI.

6.1.3 Lacto-n-fucopentaose-III ameliorates short-term and long-term aberrations in hippocampal synaptic plasticity and transmission in a pyridostigmine bromide/N,N-diethyl-meta-toluamide/diisopropylfluorophosphate/corticosterone Gulf War Illness animal model

The efficacy of LNFPIII in ameliorating acute and persisting hippocampal synaptic, trophic factor, and cytokine aberrations in a different GWI animal model consisting of PB, N,N-diethyl-meta-toluamide (DEET), DFP, and corticosterone (cort; O'Callaghan et al., 2015) exposures was also investigated. During a 15 day period, animals were exposed to PB and DEET on days 1-14, cort on days 6-14, and DFP on day 15. A subset of animals was coadministered LNFPIII during the duration of GWIR exposure (sacrificed 48 h post-exposure), another subset began receiving LNFPIII four months after exposure (sacrificed seven months post-exposure), and another subset began LNFPIII treatment eight months after exposure (sacrificed 11 months post-exposure). Basal synaptic transmission was increased by GWIR exposure 48 h post-exposure, an effect that was further enhanced by LNFPIII coadministration. LNFPIII treatment also numerically enhanced LTP magnitude 48 h post-exposure. Key inflammatory cytokine expression was unaffected by GWIR exposure and LNFPIII treatment at this early time point; however, trophic factor expression (i.e., BDNF) was enhanced by LNFPIII coadministration. Basal synaptic transmission was unaffected by either GWIR exposure or LNFPIII treatment seven months post-exposure whereas a GWIR-induced reduction in LTP magnitude was ameliorated by delayed LNFPIII treatment. A progressively worsened impairment in LTP magnitude was detected in GWIR-exposed animals 11 months post-exposure, an effect that differentially manifested in the dH compared to the vH. A significant reduction in basal synaptic transmission was also detected in both sectors at this late time point. Strikingly, delayed LNFPIII treatment ameliorated these deficits in synaptic plasticity and transmission. The beneficial effects of delayed LNFPIII treatment on synaptic responses were also accompanied by improvements or enhancements in trophic factor expression in both the dH and the vH, results that may partly explain the ameliorative effects of the glycan conjugate

on synaptic plasticity and transmission. Collectively, GWIR exposures resulted in biphasic effects on basal synaptic transmission as well as a delayed reduction in LTP magnitude, a finding that is consistent with the progressively worsened phenotype of clinical GWI. Dorsoventral-specific impairments in synaptic plasticity and transmission also became more pronounced over time, a response that is consistent with clinical and preclinical data. Importantly, the ameliorative effects of delayed LNFPIII treatment on abnormalities in hippocampal synaptic responses and trophic factor expression in GWIR exposed-animals are especially promising as potential LNFPIII treatment for clinical GWI may also utilize a delayed timeline.

6.2 Current Challenges in Gulf War Illness Research

6.2.1 Overview

The present document has highlighted substantial advancement in GWI research over the past decade; however, many challenges persist. Some of these challenges include utilizing a single diagnosis criterion for GWI, contextualizing older GWI literature in which increasingly relevant subpopulation features (i.e., deployment status, symptomatic, etc.) are accounted for, balancing experimental designs with both male and female GWV, considering ethnic and racial disparities in the GWI population, and ensuring GWI animal model exposure paradigms and endpoints are relevant for GWI. The following section will review these clinical and preclinical challenges.

6.2.2 Clinical challenges

6.2.2.1 Clinical challenges: GWI diagnosis criteria

The National Academy of Medicine recommended that the CDC definition of GWI be employed for clinical GWI diagnoses and the Kansas criteria be utilized for research, facilitating the consistent use of two GWI diagnosis criteria rather than using other criteria like the Haley definition (Shine et al., 2014); however, the lack of a single, consistent GWI case definition is still an obstacle at multiple levels in a clinical setting. For instance, recent reports found that

many health care providers were unaware of GWI symptomology in addition to differential symptom severity based on distinct levels of exposure, resulting in inconsistent diagnoses and treatment plans for GWI veterans (Baldwin et al., 2019; Kaimal and Dieterich-Hartwell, 2020). Further, another study described the likelihood of ensuring approval of disability compensation programs for GWI claims was low and inconsistent as a result of an absence of a single GWI case definition (GAO, 2017). Not only will the absence of a consistent, single case definition obscure accurate diagnosis of GWI and limit financial support for continued treatment of the disease, but lacking an individual case definition will continue to influence the reproducibility of clinical GWI studies as well as cast doubt on the translational relevance of GWI animal models. Accordingly, a recent report by DDGWIRP (2018) highlighted an effort spearheaded by the U.S. Department of Veterans Affairs to develop a single case definition of GWI.

6.2.2.2 Clinical challenges: contextualizing outdated GWI literature with recent advancements

The lack of a single case definition of GWI coupled with an increasing understanding of the intricacy of GWI has led to an interest in contextualizing older GWI literature. For instance, decades ago there was less appreciation for the impact of causal factors in shaping GWI symptom severity such as pattern, degree, or frequency of exposure to individual/multiple GWIR exposures, deployment status, deployment location in the Gulf War theater, history of mild traumatic brain injury, genetic susceptibility, and variability in enzymes responsible for the metabolism of GWIR chemicals (Steele et al., 2012; Steele et al., 2015; James and Georgopoulos, 2018; Janulewicz et al., 2018). Additionally, researchers are increasingly taking into consideration the general progressive nature of GWI coupled with the additive toxicological effect of aging on symptom severity. Disparities in subpopulations utilized in GWI studies have also led to inconsistencies between older and more recent literature. Some of the discrepancies in subpopulations employed in GWI studies include comparing dissimilar domains of neuropsychological function, employing different variations of experimental instruments and test batteries to evaluate the same function within a neuropsychological domain, not considering

dose-response effects, inconsistent definitions of symptomatic GWI as a result of lacking an individual case definition of the disease, juxtaposing the behavioral performance of GWV with GWIR chemical exposure to GWV that were unexposed, comparing deployed and non-deployed GWV, and using non-veteran population as controls. These variations in experimental design, coupled with irregular sample sizes, provided an unclear representation of GWI-specific neuropsychological dysfunction in the early years of GWI research (Axelrod and Milner, 2000), stalling the formation of not only a clear, single case definition of GWI, but also the development of objective biomarkers and therapies for GWI. Recent meta-analyses and reviews have attempted to assess emerging evidence of the complexities of GWI, contextualizing these complexities with older findings by determining reproducible GWI-specific impairments in neuropsychological domains that can be detected via sensitive and robust behavioral tasks (Janulewicz et al., 2017; Jeffrey et al., 2019). Namely, a meta-analysis by Janulewicz et al. (2017) surveyed GWI neuropsychological publications from 1992-2015 based on two factors within subpopulations: deployment status or presentation of GWI-specific symptomology. By establishing strict cognitive criteria for GWI-specific deficits, the result of this approach provided a thorough illustration of impairments in a variety of neuropsychological domains presented in GWI subpopulations. Further, the meta-analysis approach led to the suggestion that future clinical GWI behavioral studies aiming to develop objective biomarkers as well as therapies include tasks representing domains of visuospatial capacity, executive function, attentional abilities, and learning and memory. Block Design from the Wechsler Adult Intelligence Scale-Third Edition, Trail-making Test Digit Span, Continuous Performance Task, and California Verbal Learning Task were sensitive and robust tests that detected GWI-specific impairments in the suggested cognitive domains, leading to their recommended use in GWI studies by the recently published Common Data Elements (CDEs; CDMRP, 2019). Another review of neuropsychological GWI studies found that early studies framed their experimental design around a limited number of factors while more recent reports have considered multiple GWI

parameters and increasingly utilized a uniform GWI case definition (Jeffrey et al., 2019). This review calls for the investigation of the four cognitive domains impaired in GWI suggested by the aforementioned meta-analysis via the use of the CDE-recommended behavioral batteries. Additionally, the review suggested the utilization of objective and robust immunological, genetic, or neuroanatomical GWI biomarkers that are sensitive to dose-response effects. This is in line with findings of the present literature review. The field of GWI research may also greatly benefit from guidance that continues to refine approaches for incorporation of appropriate baseline controls when considering complex factors such as deployment status and chemical exposure. Additionally, expanded recommendation of behavioral batteries and instruments for investigating reproducible GWI-specific deficits in particular neuropsychological domains may also facilitate the production of reproducible findings.

6.2.2.3 Clinical challenges: heterogenous GWI populations

6.2.2.3.1 Clinical challenges: sex-specific presentation of GWI

Increasing evidence suggests that GWI manifests in a sex-specific manner (Coughlin, 2016; Coughlin et al., 2017; Brown et al., 2019; Dursa et al., 2019; Heboyan et al., 2019; Porter et al., 2020; Sullivan et al., 2020; Abou-Donia et al., 2021). There has been augmented interest in studying sex-specific manifestations of GWI over the past five years, resulting from the variable presentation of GWI in women due to a paucity of females included in GWI epidemiological studies and a litany of sex-related confounding factors presented in these studies i.e., comparing male and female GWV or juxtaposing female GWV with healthy controls (Coughlin, 2016; Coughlin et al., 2017; Danan et al., 2017; Brown et al., 2019). A few recent reports studied GWI in women GWV while also considering sex-specific confounding factors (Brown et al., 2019; Heboyan et al., 2019; Porter et al., 2020; Sullivan et al., 2020). Heboyan et al. (2019) reanalyzed the CDC Air Force study dataset that was used in Fukuda et al. (1998) to evaluate sex disparities presented in a large GWI population. Using either the CDC or Kansas case definition of GWI, deployed female veterans had a greater GWI risk and exhibited GWI at

greater rates than their male counterparts. Additionally, female GWI veterans presented severe GWI symptomology at a significantly elevated rate compared to male GWI veterans. A fundamental drawback of this investigation is that it lacks the ability to assess the current health of veterans. Addressing the obstacle of assessing current health of female GWV while also analyzing sex-specific effects, Brown et al. (2019) found that although the percentage of male and females that were deployed and utilized the Veterans Health Administration were similar, female GWV were significantly more likely to report adverse health conditions than males. Specifically, female GWV were more likely to present health problems such as asthma, irritable bowel syndrome, nervous system disorders like migraines, musculoskeletal abnormalities such as osteoarthritis and osteoporosis, and mental health disorders like bipolar disorder, depression, anxiety/panic disorder, and eating disorders. On the contrary, men were more likely to report diabetes, tinnitus, hearing loss, hypertension, and spinal cord dysfunction. A limitation of this study is that while the authors investigated aberrations that are in line with GWI symptomology, the investigation did not determine if sex-specific distinctions manifested as defined by GWI case definitions like the Kansas or CDC criteria. A recent report by Sullivan et al. (2020) assessed the current health of female GWV and evaluated how their current symptoms relate to GWI case definitions and if sex-specific disparities are present. This study also observed that deployed female GWV exhibited significantly greater GWI-related symptoms than nondeployed female GWV as defined by CDC and Kansas criteria. Further, the results of this report were in line with female GWV being more likely to meet GWI-specific criterion than their male counterparts as defined by the Kansas definition of GWI, particularly respiratory abnormalities. A study by Porter et al. (2020) also indicated that women had a greater adjusted odds for being diagnosed with GWI by the CDC criteria compared to males. Collectively, these studies suggest that GWI presents in a sex-specific manner. It is currently unclear why GWI presents in female GWV at an elevated rate than male GWV. Notably, female veterans that served during the 1990-1991 Gulf War era comprise 56% of all living women U.S. veterans (VA, 2011),

underscoring the fact that more American women served in the 1991 Persian Gulf War than any previous war zone in U.S. history due to expanded military roles (Coughlin et al., 2017). As a result of the uniquely high levels of exposure to neurotoxicants in the Persian Gulf War, distinct sex-related differences in enzymes related to the metabolism of GWIR neurotoxicants may have contributed to the vulnerability of females to developing GWI (Heboyan et al., 2019). Increased female exposure to neurotoxicants as a result of deployment location may also shape GWI symptom severity. Additionally, persisting differences in GWI symptomology may be related to distinct proinflammatory cytokine signaling stemming from sex-specific differences in sex hormone profiles (Smylie et al., 2013; Coughlin, 2017). For instance, a recent investigation by Abou-Donia et al. (2021) identified plasma autoantibodies that distinctly presented in females with GWI compared to male GWI veterans. Future studies of sex-specific effects are warranted and would be strengthened by using a longitudinal approach to assess how women's general health, overall GWI symptoms, and diseases that disproportionately affect female GWI veterans change over time. Considering factors such as aging and menopause as well as comorbid conditions like PTSD may also be fruitful. Further, evaluating GWI-related teratogenicity may be beneficial for post-9/11 veterans. These future studies will aid in the development of robust and sensitive objective GWI biomarkers and facilitate the development of safe and efficacious GWI therapeutics.

6.2.2.3.2 Clinical challenges: race-specific presentation of GWI

Emerging evidence of GWI presenting in a race-specific manner has also been described (Coughlin and Heboyan, 2018). For instance, studies have revealed that GWI is more common in African American GWV than their white counterparts (Steele, 2000; Steele et al., 2012; Porter et al., 2020; Sullivan et al., 2020). Within a decade after the 1990-1991 Persian Gulf War, Steele (2000) found that the odds of black GWV to develop GWI were 23% greater than their white counterparts, even when their analysis was adjusted for the duration in theater, deployment location, service branch, rank, educational background, sex, and economic income.

A later study that considered several confounding factors reported that the unadjusted odds of black GWV developing GWI was 243% (Steele et al., 2012). A recent study by Sullivan et al. (2020) discovered that American Indian/Alaska Native, Asian/Pacific Islander, and all additional women defined as “nonwhite-other racial groups” were almost four times as likely to report more GWI-related symptoms than white GWV women. On the contrary, a recent study by Porter et al. (2020) found mixed results of race-specific presentation of GWI. It is presently unclear why race-specific disparities in GWI incidence and symptom severity exist. Race-specific differences in enzymes related to metabolism of GWIR chemicals such as paraoxonase-1 may underlie differences in the onset of GWI (Golomb, 2008; Davis et al., 2009). Further, the persisting phenotype of GWI may be related to distinct frequency and type of HLA allele expression across races (Allele-Frequencies, 2020). Collectively, these data suggest that future investigations into race-specific differences in GWI incidence and symptom severity are warranted. This is underscored by the paucity of reports that have investigated racial disparities in the occurrence of GWI (Coughlin and Heboyan, 2018). Accordingly, future longitudinal studies evaluating the current and altered prevalence of GWI-specific symptoms across racial groups are needed. Additionally, longitudinal studies assessing race-related distinctions in factors influencing GWI symptom severity such as deployment duration as well as location may be fruitful for understanding race-specific differences in GWI. Continued research into differential expression of protective factors like HLA alleles and the activity of enzymes that interact with GWIR chemicals will facilitate interpretation of race-related differences in patterns of GWI, which may potentially fill knowledge gaps required to define a single definition of GWI. Further, continued studies evaluating race-specific dissimilarities in GWI prevalence will aid not only in the production of robust, sensitive, and objective GWI-specific biomarkers but also in assessing safe and efficacious therapeutics for diverse GWI veteran populations.

6.2.3 Preclinical challenges

This review has established that complex factors of sex and race in an aging GWV population that can be subdivided by features such as deployment location and type of chemical exposure influences the prevalence and patterns of GWI symptomology. This heterogeneous population presents health care providers and clinical researchers with a substantial challenge. Accordingly, animal models utilized to study the clinical condition of GWI must be translationally relevant. Some considerations for preclinical GWI researchers that may be beneficial in addressing the relevance of animal models include the following: 1) determining if the ROA and dose of GWIR exposures are relevant to GWI; 2) establishing if the duration of aging an animal before beginning molecular, cellular, and behavioral experiments is translational to the current age of GWI veterans; 3) evaluating if the abnormalities presented in a GWI animal model are consistent with those observed in a clinical setting; 4) resolving if the delay prior to the assessment of the safety and efficacy of a potential therapeutic agent in treating GWI-specific aberrations is clinically relevant; 5) and validating the predictive validity of a GWI therapeutic by ensuring the assessed ROA has high patient compliance as well as translationally-relevant endpoints.

6.2.3.1 Preclinical challenges: GWI animal model exposure paradigms

We will highlight three exposure paradigms that are widely employed in preclinical studies to illustrate the importance of translationally-relevant ROA and doses of GWIR chemical and stress exposure (Abdel-Rahman et al., 2002; O'Callaghan et al., 2015; Zakirova et al., 2015). These paradigms induce a range of GWI-related pathological effects as a result of different concentrations and durations of GWIR chemical exposure and distinct methods of inducing stress; nevertheless, the GWIR chemical regimen and stress experience is based upon estimated Gulf War exposure levels to ensure construct and etiological validity of the animal model. Abdel-Rahman et al. (2002) utilized a daily exposure paradigm consisting of DEET, PB, PM, and restraint stress over 28 days in Sprague-Dawley rats to simulate the experience of

GWV. Specifically, the concentration of DEET, PB, and PM was derived from exposure estimated by IOM (1995) whereas five min restraint stress was selected after evaluating dose-response effects of the synergistic impact of differing restraint stress times and GWIR chemical exposure on neuropathology. The ROA of DEET, PB, and PM exposure was dermal, oral, and dermal, respectively, routes that are consistent with those experienced by GWV in the combat arena. Another widely-employed animal model consisting of a 10-day exposure paradigm of PB and PM was developed in CD1 (Abdullah et al., 2011) and C57BL/6J (Zakirova et al., 2015) mice. The authors justify the exposure paradigm by using a concentration of PB that is in line with a probable high-level daily dosage consumed by GWV and a concentration of PM that is consistent with previous studies showing pathology induced by the pesticide; however, while the i.p. ROA may share a similar general pharmacokinetic profile to the PB (oral) and PM (dermal) exposure experience of GWV, there are clearly limitations to the i.p. ROA used in this model. O'Callaghan et al. (2015) also recapitulated GW conditions by employing a 15-day exposure paradigm where animals were exposed to GW-relevant concentrations of PB (Friedman et al., 1996) and DEET (IOM, 1995) during day 1-14, corticosterone via drinking water on days 8-15, and DFP on day 15. While the concentration and ROA utilized for corticosterone exposure was selected to simulate the immunosuppressive phenotype presented in GWI (O'Callaghan, 1991), the justification for selecting 4 mg/kg DFP, a dose that is employed in status epilepticus animal models (Pouliot et al., 2016), is unclear. Additionally, the ROA of PB (s.c.), DEET (s.c.), and DFP (i.p.) share similar challenges as described in the PB-PM model. Recent work by this group used 1.5 mg/kg DFP (i.p.) and corticosterone exposure in Sprague-Dawley rats without PB and DEET to study the effects of GWIR chemicals and stress, an exposure paradigm that may be more translationally relevant to GWI (Belgrad et al., 2019). Future studies employing novel GWI animal models should strongly consider the translational relevance of the dose, ROA, and duration of GWIR chemical and stress exposure to ensure effective construct and etiological validity. Further, development and validation of female GWI animal models is warranted. These

considerations will aid in the development of GWI-specific objective biomarkers as well as safe and efficacious therapies for GWI.

6.2.3.2 Preclinical challenges: aging animals following GWIR exposure(s)

Evidence increasingly suggests that GWI symptomology progressively worsens (Porter et al., 2020; Zundel et al., 2020), and aging GWV are at a greater risk for developing other adverse health conditions compared to the general public (Zundel et al., 2019). Accordingly, ensuring that GWI animal models are aged to a time point that is relevant to the current age of GWV is critical for investigating the persisting phenotype of GWI. For instance, a recent study by Madhu et al. (2019) aged animals for 10 months (i.e., approximately 29 human years (Sengupta, 2013)) after exposure to GWIR chemicals and stress before beginning behavioral, histopathological, and molecular experiments. As the Persian Gulf War occurred 30 years ago, the results of this report may be more transitionally relevant than studies that aged animals for too short or too long of a period. Specifically, using the same animal model as Madhu et al. (2019), Carreras et al. (2018) did not observe consistent pathology with the former report when animals were only aged for three months post-GWIR chemical and stress exposure. On the contrary, a study found behavioral and pathological abnormalities that were in line with GWI six months after PB-PM exposure (i.e., approximately 17 human years (Sengupta, 2013; Zakirova et al., 2015)), but not in the same animal model 22 months post-PB-PM exposure (Zakirova et al., 2016). These data suggest that confounding effects of aging can prevent the observation of GWI-specific pathology. Accordingly, future GWI preclinical studies should ensure appropriate aging occurs in animals to confirm construct and etiological validity of the investigated animal model, which will maximize translational relevance of such a study's findings and contribute to the development of objective GWI-specific biomarkers as well as safe and efficacious GWI therapies.

6.2.3.3 Preclinical challenges: are abnormalities in GWI models consistent with clinical GWI symptoms?

It has been established that GWV were exposed to a variety of GWI-specific etiological agents at varying durations and concentrations as a result of factors such as deployment location (Steele et al., 2012). Therefore, GWV present varying GWI-specific symptom severity, which should be accounted for by the use of different GWI models to ensure face validity. These GWI models may employ different etiological agents at varying concentrations over time, resulting in distinct pathology. For instance, the model developed by Abdel-Rahman et al. (2002) consistently results in more severe and wide-spread GWI-related pathology and symptoms than the model developed by O'Callaghan et al. (2015). This may be due to the use of a longer exposure protocol or a uniquely stressful experience in the former model compared to the latter. Nevertheless, these two models still result in the development of detrimental neuroimmune signaling and other underlying factors of GWI pathophysiology, suggesting that they are both clinically-relevant GWI models that recapitulate differing degrees of symptom severity. Therefore, it is critical that future researchers developing or selecting a GWI animal model consider the construct, etiological, and face validity of the proposed animal model in relation to the subpopulation of GWI veterans of interest. The recently published CDEs (CDMRP, 2019) and general GWI landscape (DDGWIRP, 2018) should serve as guideposts for preclinical researchers in selecting particular tasks to investigate distinct neuropsychological domains as well as underlying neuropathological mechanisms consistently associated with GWI. This coherent approach may lead to not only advancement in developing a single case definition and animal model of GWI but also the development of objective biomarkers and therapeutics for GWI.

6.2.3.4 Preclinical challenges: delayed therapeutic intervention in GWI animal models

We have previously described the importance of aging animals following GWIR chemical and stress exposure to ensure clinically-relevant translation of GWI progression. Delaying the

initiation of GWI treatment with a novel therapeutic in animal models is also essential as 30 years have passed since the instigation of GWI. For instance, Laferriere et al. (2019) aged animals for nine months following GWIR exposures before beginning treatment with a therapeutic, an excellent delay period that substantially enhances the clinical relevance of the report's findings. Therefore, future studies investigating the safety and efficacy of therapies for the treatment of GWI should critically examine the delay between initial GWIR chemical exposure and the initiation of treating animals with a therapeutic to maximize the predictive validity of their results.

6.2.3.5 Preclinical challenges: Route of administration in assessing preclinical GWI therapies

Another factor that must be considered during preclinical investigations of the safety and efficacy of a novel therapeutic for the treatment of GWI includes the use of a translational ROA with likely high patient compliance. An example of a recent study using a translational ROA to assess the safety and efficacy of a novel therapeutic can be observed in Joshi et al. (2020) where the researchers introduced a GWI therapeutic into the chow of animals six months after PB-PM exposure and found amelioration of a variety of underlying GWI pathophysiological features. Some preclinical studies have reported promising results in treating GWI-specific aberrations but have employed a ROA that cannot be used in a clinical setting. For instance, Laferriere et al. (2019) detected an efficacious effect of a miRNA inhibitor on GWI-related abnormalities nine months after GWIR chemical and stress exposure; however, animals received an intracerebroventricular infusion of the inhibitor via an osmotic pump, a ROA that the authors note in their manuscript is impractical for a clinical setting. Indeed, this approach resulted in the amelioration of GWI-related pathology, but developing a miRNA inhibitor formulation that uses a ROA with greater patient compliance will likely be necessary for clinical trials.

6.2.3.6 Preclinical challenges: GWI animal model endpoints

A final consideration for future researchers investigating the safety and efficacy of potential therapeutics for GWI is the use of clinically relevant endpoints that are in line with GWI-specific symptomology. GWI symptomology is diverse and complex, leading to a myriad of endpoints used in previous studies. The lack of a unified approach to determining the safety and efficacy of therapeutics has led to slow progress in the development of a GWI therapy (Chester et al., 2019). Accordingly, the development of consistent endpoints to facilitate the production of clinically relevant and translational data from investigations of GWI animal models is essential for sustained progress in the production of a GWI therapeutic. Chester et al. (2019) recommended following the National Institute of Health roadmap to curtail many inconsistencies and advance the development of clinical GWI therapeutics. The application of this perspective to preclinical studies may also be fruitful. For instance, the CDEs established by CDMRP (2019) recommended that future clinical GWI neuropsychological studies include tasks that evaluate the cognitive domains of visuospatial capacity, executive function, attentional abilities, and learning and memory. Sensitive and robust tasks that were recommended by this report to evaluate these domains include the Block Design from the Wechsler Adult Intelligence Scale-Third Edition, Trail-making Test Digit Span, Continuous Performance Task, and California Verbal Learning Task. Validation of preclinical tasks that are similar to the aforementioned clinical tests and assess the same cognitive domains recommended by the CDEs would likely augment the impact of studies investigating potential therapeutics in animal models. Collectively, defining consistent, clinically relevant endpoints to assess the safety and efficacy of therapies in treating GWI-specific molecular, cellular, and behavioral aberrations may greatly benefit the rate and magnitude of success in developing a therapy for GWI.

There are many challenges in the field of GWI research. Moving towards a single case definition of GWI that considers the diverse subpopulations of GWV is critical. Additionally, ensuring that endpoints used to validate the dose and ROA of GWIR chemical and stress

exposure in an age-relevant animal model is crucial for recapitulating GWI. Assessing a ROA with high patient compliance and clinically relevant endpoints in preclinical investigations of GWI therapies will also accelerate progress in developing a treatment for this disease.

6.3 Implications for Clinical Gulf War Illness

Clinical GWI is a disease state that presents with a constellation of neurological symptoms that differ between individuals due to inherent physiological characteristics of the veteran (Coughlin, 2017) as well as the frequency, type, and duration of exposures to GWI causal factors (White et al., 2016). Therefore, preclinical evaluation of multiple forms of GWIR exposures (i.e., in vitro vs. in vivo) and presentation of their detrimental effects within distinct systems (i.e., ex vivo slice preparation vs. in vivo) may address knowledge gaps within the field of GWI research. The findings in the current document have addressed knowledge gaps related to preclinical, progressive pathological mechanisms initiated by GWIR exposures in vitro and in vivo as well as the efficacy of a coadministered and delayed immunotherapy in ameliorating neurological aberrations in multiple GWI animal models.

Several research groups have published a myriad of publications highlighting the pathological mechanisms initiated by GWIR exposures in vitro (Friedman et al., 1996; Pavlovsky et al., 2003; Grigoryan et al., 2008; Corbel et al., 2009; Grigoryan et al., 2009a; Grigoryan et al., 2009b; Ferchmin et al., 2015) as well as exposures in vivo including DEET, PB, PM, and restraint stress (Abdel-Rahman et al., 2002; Abdullah et al., 2012; Carreras et al., 2018; Shetty et al., 2020), variations of PB, DEET, DFP, and corticosterone (O'Callaghan et al., 2015; Koo et al., 2018; Michalovicz et al., 2019), PB-PM (Abdullah et al., 2011; Zakirova et al., 2015; Bose et al., 2020; Joshi et al., 2020), and PB along with restraint stress (Macht et al., 2018; Macht et al., 2020). These laboratories and many others have extensively investigated acute as well as persisting neurological aberrations and the pathological mechanisms that underlie these aberrations; however, the number of published studies available that employed electrophysiological techniques to investigate the effects of GWIR exposures on mammalian

neurophysiology is noticeably limited. As common neurophysiological measurements such as LTP serve as a synaptic substrate for learning and memory (Whitlock et al., 2006), a component of the cognitive domain that is consistently impaired in GWI (White et al., 2016), preclinical interrogation of synaptic circuits with electrophysiology approaches may provide insight into subtle and profound behavioral deficits that present in a heterogeneous disease state such as GWI. For instance, chapter two highlighted distinct cholinergic and noncholinergic mechanisms involved in mediating abnormalities in excitatory and inhibitory neurotransmission along the hippocampal dorsoventral axis following in vitro DFP exposure. While this work was completed in vitro and via an acute exposure paradigm, the finding of a unique response to a GWIR exposure along the dorsoventral axis was consistent with the reported differential presentation of pathology along the hippocampal longitudinal axis in GWV (Vythilingam et al., 2005; Odegard et al., 2013), indicating to our group that we may find dorsoventral-specific effects on synaptic plasticity and transmission responses following in vivo GWIR exposures. Indeed, we also detected diverging effects of GWIR exposures on hippocampal synaptic plasticity and transmission in two different GWI animal models in chapters three-five. Although the finding of dissimilar adverse effects on synaptic responses in two GWI animal models is consistent with the heterogeneity of clinical GWI, and we speculate that the abnormalities in synaptic activity in both models and sectors converge around a mechanism involving a reduction in CA1 subfield synaptic density, the dorsoventral-specific effects within each model became more pronounced over time. Delayed presentation of dorsoventral-specific effects was particularly noteworthy in the animal model described in chapter five where we did not detect significant differences in dH and vH synaptic plasticity and transmission until 11 months post-GWIR exposures. The detection of delayed and worsened dorsoventral-specific effects of GWIR exposures as described in chapter five is also consistent with the growing consensus that GWI becomes worse over time (Coughlin, 2017), underscoring the importance of assessing experimental endpoints at translationally relevant time points in GWI animal models. Additionally, the

progressive dorsoventral-specific effects of GWIR exposures on hippocampal synaptic responses may underlie differential, subtle impairments in learning and memory tasks described in chapter four as well as experiments (i.e., the 8-arm radial arm maze and the Barnes maze) with the animal model described in chapter five that are not shown here. Collectively, employing powerful techniques underused in GWI preclinical research (i.e., electrophysiology) on physiological systems that provide insight into modest or profound behavioral alterations implicated in the GWI phenotype (i.e., hippocampal dorsoventral axis) may shed light on heterogeneous and complex neuropsychological aberrations presented by GWI veterans (Jeffrey et al., 2019).

The present document highlighted some of the preclinical candidates currently being assessed for the treatment of GWI. Clinical GWI therapeutics are targeted and symptomatic, resulting in limitations for efficacious treatment of GWI in a diverse veteran population. As increasing evidence implicates neuroimmune dysfunction as one of the major underlying components of the GWI phenotype (O'Callaghan et al., 2016; Coughlin, 2017; Georgopoulos et al., 2017), we specifically aimed to assess if a coadministered immunotherapeutic, LNFPIII, would ameliorate neurological aberrations in multiple GWI animal models 48 h after GWIR exposures. Additionally, we investigated if LNFPIII treatment, initiated months after GWIR exposures, would ameliorate neurological abnormalities in two GWI animal models. Indeed, chapters three and five illustrated that coadministered LNFPIII led to enhancements in hippocampal synaptic plasticity and transmission 48 h post-GWIR exposure, results that coincided with elevated hippocampal trophic factor expression. Moreover, chapters four and five revealed that delayed LNFPIII treatment enhanced and ameliorated, respectively, deficits in LTP magnitude in both hippocampal sectors months after GWIR exposures. Deficits in synaptic transmission were similarly ameliorated in both animal models following delayed LNFPIII treatment. Chapter four describes further beneficial effects of delayed LNFPIII treatment on motor and cognitive function as well as glial activation months after PB-PM exposure whereas

chapter five illustrates ameliorative and enhancing effects of delayed LNFPIII administration on trophic factor production. However, there are some limitations of these studies. Chapter one discussed the emerging evidence of sex-specific GWI presentation (Dursa et al., 2019; Sullivan et al., 2020), underscoring the need to incorporate female rodents in preclinical GWI investigations. The studies that comprise the present document only employed male mice, limiting the scope of our findings. The work described in chapter four utilized a battery of behavioral tasks, which may have introduced an undesirable stress effect on the behavioral performance of animals. While previous work by our group established an ideal LNFPIII treatment dose (Atochina et al., 2008; Bhargava et al., 2012; Tundup et al., 2012; Zhu et al., 2012; Tundup et al., 2015; Carpenter et al., 2020; Mote et al., 2020), utilization of multiple LNFPIII concentrations to generate a dose-response curve in the context of GWI may have been fruitful. No evidence of adverse drug effects have been reported following LNFPIII treatment; however, the use of toxicological assays to assess the safety of LNFPIII within the examined GWI animal models may have improved the present work. On the other hand, the studies described in the current document have many strengths. For instance, the randomization procedure and blinded design reduced experimental bias introduced by researchers. The number of animals used in the included reports resulted in sufficient experimental power, minimizing type II statistical error. The experimental design of chapter five incorporated multiple time points to track the progressive effects of GWIR exposures until the animals reached an age that was translationally relevant to the current age of GWI veterans (Sengupta, 2013), substantially broadening the impact of our findings. Chapters three and four also used acute and persisting time points (i.e., 48 h up until eight months post-PB-PM exposure) that enhanced the translational relevance of those particular reports' results as well. While chapter two utilized an acute exposure paradigm that resulted in limited relevance to the delayed presentation of GWI, the data in that chapter may provide particular insight into the general, short-term mechanisms that underlie the detrimental effects of OP overexposures on

hippocampal synaptic transmission. This document has consistently emphasized the heterogenous nature of clinical GWI, and the use of multiple animal models to assess the efficacy of LNFPIII in treating this disease state extended the impact of our findings. We previously stated that the behavioral test battery used in chapter four may have introduced an undesirable stress effect on our data, but this approach allowed us to rigorously assess the effect of GWIR exposures on multiple cognitive domains in two animal models. The utilization of an interdisciplinary approach to assess the efficacy of LNFPIII in the context of GWI (i.e., neurochemistry, neuroimmunology, and neurophysiology) also expanded the scope of our results. Importantly, we used a powerful technique underrepresented in GWI reports, electrophysiology, to address substantial knowledge gaps within the field of GWI research. Collectively, our findings demonstrate the beneficial effects of LNFPIII on neurological abnormalities in multiple GWI animal models, underscoring the potential of LNFPIII as a therapeutic for treating GWI in a clinical setting.

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