DECONSTRUCTING NEURONAL AND ASTROGLIAL CONTRIBUTIONS TO SLEEP AND INFLAMMATORY IMMUNE RESPONSES

by

Ashley Miranda Ingiosi

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in The University of Michigan
2015

Doctoral Committee:

Professor Mark R. Opp, Co-Chair, University of Washington
Associate Professor Michael M. Wang, Co-Chair
Associate Professor Anuska V. Andjelkovic-Zochowska
Professor Horacio O. de la Iglesia, University of Washington
Regents Professor James M. Krueger, Washington State University
Assistant Professor Orie Shafer

DEDICATION

For the second fiddles

Garfunkel, Oates, Robin, Cooper Manning, glia

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Mark Opp. It was with him that I gained my first experience in the laboratory. From undergraduate technician to doctoral candidate, he largely shaped who I am today as a scientist while allowing me to explore my own research interests. I will always be grateful for your constant support and encouragement as I carry the skills you taught me to new endeavors. I also want to express my sincere appreciation for my committee members Drs. Michael Wang, Horacio de la Iglesia, James Krueger, Orie Shafer, and Anuska Andjelkovic-Zochowski. Your valued insights contributed to my growth as a researcher. I also want to extend a special thanks to Dr. Wang for his time and dedication for serving as my Co-chair from afar.

I owe an enormous debt of gratitude to both Michigan- and Washington-based Opp lab members. To Amrita George, Blair Sutton, Cailin Wilson, CC Angelakos, Francesca Baracchi, Hannah Thomasy, Heidi Febinger, Jacqueline Ho, Jenna Grillo, Jill Priestley, Kelley Jordan, Linella Gemma, Kristyn Ringgold, Marieke Hoekstra, Maria Pavlova, Melissa Olivadoti, Oleg Kritsky, Paulien Barf, Sara Waugh and Rick Raymond, thank you for all the support, numerous hours of sleep deprivation assistance, happy hours, coffee runs, and happy hours. Did I mention happy hours? I cannot make enough baked goods to thank you for all you have done. I especially thank Drs. Richard Raymond and Maria Pavlova for their

assistance and dedication in generating the transgenic mice used for this project. Without you, literally none of this would be possible.

Even though much of my time was spent away from the University of Michigan, I am so grateful to the Neuroscience Graduate Program, namely Dr. Edward Stuenkel, Valerie Smith, and Rachel Harbach, for their continued support and assistance even from 2345.3 miles away. To my amazing cohort: Ali, Blair, Graham, Megan, Seth, Shanna, Shawn, Stephanie, Trace, and Travis. You all made it very difficult to leave Michigan. I consider myself lucky to start this journey with such an awesome and talented group of people, people who encompass the wisdom and humor of the all-powerful anteater.

I want to thank my family who instilled in me the value of good work ethic as well as encouraged and supported me in *all* academic and non-academic endeavors alike. You brought balance to the force. I love you guys, and in everything I do, I remember, “Silverware.”

To my husband, Paul, who directly contributed to this project in more ways than I can possibly count. Thank you for being my undergrad, but most of all, I cannot adequately express how much your love and support has pulled me through the highs and the lows. Teamwork. Dreamwork. Finally, to our sweet Ava Jane. You help me keep everything in perspective, and I cannot wait to see what amazing things you will do. I love yous.

“For today, goodbye. For tomorrow, good luck. And forever, GO BLUE!”

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER II

CHAPTER III

CHAPTER IV

APPENDIX A

APPENDIX B

LIST OF APPENDICES

CHAPTER I

INTRODUCTION

Sleep and immune interactions

Chronic insufficient sleep is linked to inflammation, metabolic syndrome, cardiovascular disease, increased sensitivity to pain stimuli, fatigue, excessive daytime sleepiness, and impaired cognitive and physical performance. Symptoms of these pathologies and conditions are associated with increased levels of endogenous pro-inflammatory cytokines and can be experimentally elicited by peripheral or central exogenous administration of these cytokines (Dantzer, 2001; Imeri and Opp, 2009). Conversely, inhibition of inflammatory immunomodulators attenuates many sleep-loss associated symptoms. Additionally, reduction of endogenous expression of cytokines, whether in mutant mice or by use of soluble receptors, antibodies, or receptor antagonists, inhibits spontaneous sleep [reviewed by (Imeri and Opp, 2009; Krueger et al., 2008; Krueger, 2012)]. There is a wealth of evidence indicating that cytokines are also involved in physiological sleep regulation and that their amplification during pathology is causative of characteristic sleep disturbances associated with many pathological states (Krueger et al., 2001; Imeri and Opp, 2009; Krueger, 2012). Indeed, the brain, including regions associated with

* Portions of this chapter were adapted from a previous publication: Ingiosi AM, Opp MR, Krueger JM (2013) Sleep and immune function: glial contributions and consequences of aging. Curr Opin Neurobiol 23:806-811.

the regulation of sleep-wake behavior, produces and is responsive to cytokines (Imeri and Opp, 2009; Krueger et al., 2008; Dantzer, 2009). Furthermore, neuronal activity upregulates pro-inflammatory cytokines in brain regions implicated in sleep regulation (Hallett et al., 2010; Churchill et al., 2008). Impaired sleep also affects adaptive immune responses. Sleep deprivation attenuates antibody responses to vaccine (Lange et al., 2003; Spiegel et al., 2002), whereas good sleep imparts long-lasting immunoenhancing effects (Lange et al., 2011; Besedovsky et al., 2012). Moreover, sleep is a profound regulator of cellular immunity and formation of immunological memory critical for adaptive responses to immune challenges [reviewed by (Besedovsky et al., 2012)].

The Centers for Disease Control and Prevention declared insufficient sleep a public health epidemic (Centers for Disease Control and Prevention, 2014). Approximately 50 – 70 million American adults have a disorder of sleep or wakefulness. Sleep deficiency is associated with greater propensity to chronic illness, reduced quality of life, impaired cognition, and even increased mortality. Moreover, upwards of 100 billion dollars in direct medical costs associated with the evaluation and treatment of sleep disorders are spent every year (Committee on Sleep Medicine and Research, 2006). Whereas it is imperative to disseminate the importance of good sleep hygiene, investigation of mechanisms underlying sleep-immune interactions is imperative for determining effective therapeutic interventions to improve quality of life and decrease the economic burden in an increasingly 24-hour society.

Although we acquired substantial insight into sleep and immune system interactions during the last 30 years, the cellular substrates for these interactions are less well understood. Most of what we know regarding central nervous system (CNS)-mediate processes is founded on neuron-centric studies. However, understanding the role of glia in sleep and immune system function is crucial because research has started to shift the traditional view of these cells as passive constituents of the CNS to active contributors capable of mediating behavior (see below). This chapter highlights the contributions of the pro-inflammatory cytokine interleukin-1β (IL-1) to sleep and immune responses as well as recent findings implicating a role for astrocytes in sleep and immune function.

IL-1 and immune function

An immunomodulator widely known to mediate sleep and immune interactions is the pro-inflammatory cytokine IL-1. IL-1 is an endogenous pyrogen that is upregulated during innate immune responses to facilitate fever and create a less hospitable environment for invading pathogens. Central IL-1 expression is elevated in acute pathologies such as stroke and traumatic brain injury as well as neurodegenerative disorders like Alzheimer's disease and multiple sclerosis (Allan, 2003). Administration of IL-1 during the course of these pathologies can exacerbate centrally-mediated immune responses, whereas inhibition of IL-1 limits the detrimental effects (Rothwell and Luheshi, 2000; Allan, 2003). IL-1 activity stimulates the production of several secondary mediators including chemokines, growth factors, and prostaglandins as well as other cytokines. The effects of IL-1 on

sleep and fever, for example, are mediated, in part, by IL-6 (Olivadoti and Opp, 2008).

The mature, 17-kDa form of IL-1 is synthesized from the inactive, 31-kDa precursor, pro-IL-1, by caspase-1 (Dinarello, 1994; Thornberry et al., 1992). IL-1 exerts its effects through interleukin-1 receptor 1 (IL1R1) (Sims et al., 1993; O'Neill and Dinarello, 2000). Signal transduction via the IL-1 / IL1R1 complex requires the assistance of the adapter protein IL-1 receptor accessory protein (IL-1AcP) (Wesche et al., 1997; Greenfeder et al., 1995). IL-1 receptors are expressed in the CNS by neurons, astrocytes, oligodendrocytes, and endothelial cells (Cunningham, Jr. and DeSouza, 1993; Smith et al., 2009; Basu et al., 2004; Parnet et al., 1994). Furthermore, neurons and glia produce IL-1 in response to immune challenges (Bartfai and Schultzberg, 1993; Baumann et al., 1993; Dong and Benveniste, 2001).

Although IL-1 stimulates its own synthesis (Dinarello et al., 1987; Dinarello, 1988), the IL-1 system encompasses inherent negative regulatory mechanisms. IL1R type II (IL1R1II) is a decoy receptor that binds IL-1 but lacks a cytoplasmic signaling domain (McMahan et al., 1991; Colotta et al., 1994). Indeed, inhibition of IL1RII potentiates IL-1-mediated behavioral responses (Cremona et al., 2002; Cremona et al., 1998). IL-1 activity is also modulated by the naturally occurring IL-1 receptor antagonist (IL-1ra) that competitively binds to IL1R1 (Hannum et al., 1990). Administration or overexpression of IL-1ra confers neuroprotection from central infarct (Touzani et al., 1999). Additional modulation of IL-1 activity is provided by a brain-specific isoform of the accessory protein known as IL-1AcPb (Smith et al., 2009). Inhibition of IL-1AcPb exacerbates responses to lipopolysaccharide (LPS)

(Taishi et al., 2012), a component of Gram-negative bacterial cell walls that induces inflammatory immune challenges via cytokine production. Finally, IL-1 cannot pass freely across the blood-brain barrier (BBB), but instead shuttles across the BBB via a self-inhibitable, saturable transport mechanism (Banks et al., 1991).

IL-1 and sleep

IL-1 is an important mediator of physiological and pathological sleep-wake behavior. This cytokine is also one of few substances classified as an endogenous sleep regulatory substance (SRS). The distinction of SRS is assigned if several criteria are met, including 1) expression of the substance fluctuates with propensity for sleep, 2) time spent in sleep is sensitive to exogenous administration or inhibition of the substance, and 3) the substance exerts effects on recognized sleep-wake circuitry (Borbely and Tobler, 1989; Krueger et al., 2001).

In accordance with the SRS criteria, IL-1 expression follows a diurnal pattern that parallels sleep-wake behavior with the highest concentrations emerging during the sleep period (Moldofsky et al., 1986; Taishi et al., 1998; Lue et al., 1987). Indeed, peak oscillations of IL-1 mRNA are detected in sleep-associated brain regions when the occurrence of non-rapid eye movement sleep (NREMS) is greatest (Taishi et al., 1997). A similar rhythm is reported for IL-1 protein in brain (Nguyen et al., 1998). Circulating IL-1 also exhibits a diurnal rhythm in humans with the highest concentrations observed at sleep onset (Gudewill et al., 1992; Moldofsky et al., 1986). Consistent with the notion that SRS expression should vary with sleep

propensity, sleep deprivation elevates IL-1 (Mackiewicz et al., 1996; Taishi et al., 1998).

Central and peripheral exogenous administration of IL-1 alters sleep-wake behavior by increasing time spent in NREMS and suppressing rapid eye movement sleep (REMS) (Opp et al., 1991; Opp and Krueger, 1991; Fang et al., 1998; Olivadoti and Opp, 2008). The somnogenic effects of IL-1 were first demonstrated in rabbits (Krueger et al., 1984) and have since been replicated in various species (Opp and Toth, 2003). Pharmacological and genetic inhibition of IL-1 and IL1R1 decreases physiological sleep and attenuates pathological sleep responses. For example, mice lacking IL1R1 exhibit less spontaneous NREMS (Fang et al., 1998). Similarly, administration of IL-1ra in the absence of, or prior to, IL-1 administration reduces time spent in NREMS (Opp and Krueger, 1991). Furthermore, inhibition of IL-1 following sleep deprivation attenuates subsequent compensatory sleep responses (Opp and Krueger, 1994).

IL-1 exerts its effects on known sleep circuitry to manifest alterations in sleep-wake behavior (Alam et al., 2004; Brambilla et al., 2007; Brambilla et al., 2010; Breder et al., 1988). IL-1 inhibits wake-promoting neurons while also stimulating sleep-promoting neurons in the preoptic nucleus, basal forebrain, and anterior hypothalamus to enhance the appearance of NREMS (Alam et al., 2004). Microinjection of IL-1 into the dorsal raphe nucleus (DRN) of the brainstem also increases time spent in NREMS (Manfridi et al., 2003). IL-1-induced REMS suppression is manifest, in part, by inhibiting firing rates of wake-active, REM-OFF serotonergic neurons within the DRN via potentiation of evoked GABAergic inhibitory

responses (Brambilla et al., 2007; Manfridi et al., 2003). Additionally, microinjection of IL-1 into the laterodorsal tegmental nucleus (LDT) of the brainstem suppresses REMS (Brambilla et al., 2010). IL-1 inhibits firing rates of REM-active cholinergic neurons of the LDT presumably by reducing evoked glutamatergic responses (Brambilla et al., 2010). Administration of IL-1 also stimulates the production of adenosine, a recognized SRS (Sperlagh et al., 2004; Zhu et al., 2006). Adenosine exerts inhibitory effects on wake-promoting basal forebrain neurons (Basheer et al., 2004). Collectively, these findings demonstrate that IL-1 regulates sleep-immune interactions, but the mechanisms that underlie IL-1-mediated sleep-wake behavior and immunomodulation require further investigation.

Astrocytes and immune function

A traditional view of astrocytes was that they played a passive, supportive role for neurons. However, recent studies demonstrate that these cells are active contributors to complex behaviors and immune responses. Astrocytes are the most abundant glial cell type in the brain. In addition to their role as mediators of neuronal homeostasis, astrocytes respond rapidly to inflammation and express receptors for immunomodulators (Farina et al., 2007; Dong and Benveniste, 2001). These characteristics indicate astrocytes are well-poised for local mediation of immune responses and may serve as ideal targets for early intervention. However, studies investigating the role of astrocytes in innate immunity are generally lacking.

Astrocytes express IL1R1, and *in vitro* studies demonstrate that application of IL-1 to astroglial cultures induces astrogliosis (Giulian et al., 1994), produces

secondary inflammatory mediators such as IL-6 (Benveniste, 1997; Pinteaux et al., 2002), and increases adenosinergic activity (Narcisse et al., 2005). IL-1 also impairs the homeostatic function of astrocytes by inhibiting glutamatergic uptake, thereby increasing the propensity for neurotoxicity. This effect is abolished by administration of IL-1ra (Hu et al., 2000). Furthermore, astrocytes maintain functional integrity of the BBB which serves to limit the passage of blood-borne substances to the CNS (Bush et al., 1999). Astroglial end-feet surround the cerebrovasculature, and IL-1 activation of astrocytes compromises the cellular junctions that comprise the BBB (Chaitanya et al., 2011).

IL-1 signaling in astrocytes primarily triggers the classical pathway that induces nuclear factor-κB (NF-κB)-mediated transcription of inflammatory mediators (Parker et al., 2002). Astroglial signaling is attenuated by IL-1ra as well as by genetic ablation of IL1R1 (Parker et al., 2002) and IL-1AcP (Zetterstrom et al., 1998). Furthermore, selective inhibition of NF-κB in astrocytes facilitates functional recovery and reduces lesion volume and cytokine expression following spinal cord injury (Brambilla et al., 2005). Although these studies implicate astrocytes as active contributors of immune responses, much of our current understanding regarding astroglial immune contributions derives from *in vitro* studies. *In vivo* investigations of astroglial functions are needed to fully elucidate mechanisms of CNS-mediated processes.

Astrocytes and sleep

Thirty years ago, astrocytes were identified as a source of sleep regulatory

substances. Centrally administered IL-1 derived from astroglial cell cultures increased NREMS in rats (Tobler et al., 1984). However, until recently, few studies explored astroglial contributions to sleep-wake behavior. Current findings now demonstrate astrocytes play a role in sleep homeostasis. Selectively inhibiting astrocyte gliotransmission via conditional astrocyte-specific expression of the SNARE domain of synaptobrevin II (dnSNARE) in mice reduces electroencephalographic (EEG) slow wave delta activity during NREMS, a traditional measure of sleep pressure (Halassa et al., 2009). Inhibition of vesicular release from astrocytes also attenuates the compensatory increase of NREMS and cognitive deficits typically observed subsequent to 6 h of sleep deprivation (Halassa et al., 2009). These data suggest astrocytic gliotransmission contributes to the modulation of sleep need.

Because impaired gliotransmission of astrocytes results in reduced sleep pressure, studies have turned to astrocyte-derived adenosine as a potential molecular substrate of this effect. Adenosine accumulates in brain with increasing time awake (Schmitt et al., 2012), and extracellular elevation of adenosine concentrations is astrocyte dependent (Schmitt et al., 2012; Pascual et al., 2005). Indeed, inhibition of the adenosine 1 receptor (A_1R) in wild type mice recapitulates the reductions in baseline EEG slow wave activity and attenuated responses to sleep deprivation observed in gliotransmission-impaired dnSNARE mice (Halassa et al., 2009; Florian et al., 2011). Conditional CNS A_1R knockout mice also fail to demonstrate enhanced EEG delta power following intermittent sleep deprivation (Bjorness et al., 2009). Consistent with the notion that the A_1R mediates sleep

need, chronic sleep restriction increases A_1R mRNA expression in the wake-promoting basal forebrain in rats (Kim, 2012), but not in the sleep-promoting hypothalamic regions of mice (Zielinski et al., 2012). Taken together, these findings introduce an active role for astrocytes in sleep homeostasis. Continued investigation of astroglial contributions to sleep-wake behavior is crucial to understanding sleep regulatory mechanisms as well as determining the elusive function(s) of sleep.

Specific aims

The overall objective of the project described here was to determine a role for neuronal-glial interactions in sleep-wake behavior and immunomodulation. To accomplish this objective, we engineered two transgenic mouse lines that express IL1R1 only in the CNS and selectively on neurons or astrocytes. These mice express murine IL1R1 cDNA under transcriptional control of the neuron-specific enolase (NSE) or the human glial fibrillary acidic protein (gfa2) promoters. The transgenes were bred onto a genetic background lacking central and peripheral endogenous IL1R1 expression. Those mice with selective neuronal expression of IL1R1 are referred to as NSE-IL1R1, and mice expressing IL1R1 selectively on astrocytes are called GFAP-IL1R1.

The central hypothesis that neuronal-glial interactions, mediated by cytokines, contribute to altered sleep-wake behavior during immune challenge was tested within the context of the following aims: (1) to determine the roles of neurons and astrocytes in physiological sleep-wake behavior, (2) to determine the relative contribution of neurons and astrocytes in mediating responses to cytokines; and (3)

to compare neuronal- and astroglial-driven responses to systemic immune challenge. To address Aim 1, transgenic sleep-wake phenotypes were characterized under baseline conditions and in response to sleep deprivation via EEG recordings in Chapter II. Chapter III (Aim 2) describes the relative roles of neurons and astrocytes in sleep and immunomodulation in response to central immune challenge with intracerebroventricular administration of IL-1. Responses to IL-1 were assessed via EEG determination of sleep-wake behavior and quantification of cytokine expression in brain tissue as well as in neuronal and astroglial cell cultures. Finally, in Chapter IV (Aim 3), CNS modulation of behavioral and inflammatory responses to systemic immune challenge was investigated via peripheral injection of LPS in transgenic and wild type mice. As in Chapter III, EEG analyses and cytokine quantification in brain tissue were performed to determine effects of LPS on sleep and immunomodulation, respectively. The systematic and selective examination of neuronal- and astroglial-mediated cytokine activity detailed in later chapters will aid to expand our understanding of cellular and molecular contributions to sleep and immune interactions.

References

Alam MN, McGinty D, Bashir T, kumar S, Imeri L, Opp MR, Szymusiak R (2004) Interleukin-1b modulates state-dependent discharge activity of preoptic area and basal forebrain neurons: role in sleep regulation. Eur J Neurosci 20:207-216.

Allan SM (2003) The interleukin-1 system: an attractive and viable therapeutic target in neurodegenerative disease. Current drug targets CNS & neurological disorders 2:293-302.

Banks WA, Ortiz L, Platkin SR, Kastin AJ (1991) Human interleukin (IL) 1a, murine IL-1a and murine IL-1b are transported from blood to brain in the mouse by a shared saturable mechanism. J Pharmacol Exp Ther 259:988-996.

Bartfai T, Schultzberg M (1993) Cytokines in neuronal cell types. Neurochem Int 22:435-444.

Basheer R, Strecker RE, Thakkar MM, McCarley RW (2004) Adenosine and sleep-wake regulation. Prog Neurobiol 73:379-396.

Basu A, Krady JK, Levison SW (2004) Interleukin-1: A master regulator of neuroinflammation. J Neurosci Res 78:151-156.

Baumann N, Baron-Van Evercooren AF, Jacque CF, Zalc B (1993) Glial biology and disorders. Current Opinion in Neurology and Neurosurgery 6:27-33.

Benveniste EN (1997) Cytokines: influence on glial cell gene expression and function. Chemical immunology 69:31-75.

Besedovsky L, Lange T, Born J (2012) Sleep and immune function. Pflugers Arch 463:121-137.

Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW (2009) Control and Function of the Homeostatic Sleep Response by Adenosine A1 Receptors. The Journal of Neuroscience 29:1267-1276.

Borbely AA, Tobler I (1989) Endogenous sleep-promoting substances and sleep regulation. Physiol Rev 69:605-670.

Brambilla D, Barajon I, Bianchi S, Opp MR, Imeri L (2010) Interleukin-1 inhibits putative cholinergic neurons in vitro and REM sleep when microinjected into the rat laterodorsal tegmental nucleus. Sleep 33:919-929.

Brambilla D, Franciosi S, Opp MR, Imeri L (2007) Interleukin-1 inhibits firing of serotonergic neurons in the dorsal raphe nucleus and enhances GABAergic inhibitory post-synaptic potentials. Eur J Neurosci 26:1862-1869.

Brambilla R, Bracchi-Ricard V, Hu WH, Frydel B, Bramwell A, Karmally S, Green EJ, Bethea JR (2005) Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. The Journal of Experimental Medicine 202:145-156.

Breder CD, Dinarello CA, Saper CB (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240:321-324.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV (1999) Leukocyte Infiltration, Neuronal Degeneration, and Neurite Outgrowth after Ablation of Scar-Forming, Reactive Astrocytes in Adult Transgenic Mice. Neuron 23:297-308.

Centers for Disease Control and Prevention (2014) Insufficient Sleep Is a Public Health Epidemic.

Chaitanya GV, Cromer WE FAU - Wells S, Wells SR FAU - Jennings M, Jennings MH FAU, Couraud PO FAU - Romero I, Romero IA FAU - Weksler B, Weksler BF, Erdreich-Epstein AF, Mathis JM FAU - Minagar A, Minagar AF, Alexander JS (2011) Gliovascular and cytokine interactions modulate brain endothelial barrier in vitro. J Neuroinflammation 8.

Churchill L, Rector DM, Yasuda K, Fix C, Rojas MJ, Yasuda T, Krueger JM (2008) Tumor necrosis factor alpha: activity dependent expression and promotion of cortical column sleep in rats. Neuroscience 156:71-80.

Colotta F, Dower SK, Sims JE, Mantovani A (1994) The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. Immunol Today 15:562-566.

Committee on Sleep Medicine and Research BoHSP (2006) Sleep disorders and sleep deprivation: an unmet public health problem. Washington, DC: The National Academies Press.

Cremona S, Goujon E, Kelley KW, Dantzer R, Parnet P (2002) Brain type I but not type II IL-1 receptors mediate the effects of IL-1b on behavior in mice. Am J Physiol 274:R735-R740.

Cremona S, Layé S, Dantzer R, Parnet P (1998) Blockade of brain type II interleukin-1 receptors potentiates IL1beta-induced anorexia in mice. Neurosci Lett 246:101-104.

Cunningham ET, Jr., DeSouza EB (1993) Interleukin 1 receptors in the brain and endocrine tissues. Immunol Today 14:171-176.

Dantzer R (2001) Cytokine-induced sickness behavior: where do we stand? Brain Behav Immun 15:7-24.

Dantzer R (2009) Cytokine, Sickness Behavior, and Depression. Immunology and Allergy Clinics of North America 29:247-264.

Dinarello CA (1988) Biology of interleukin-1. FASEB J 2:108-115.

Dinarello CA (1994) The interleukin-1 family: 10 years of discovery. The FASEB Journal 8:1314-1325.

Dinarello CA, Ikejima T, Warner SJC, Orencole SF, Lonnemann G, Cannon JG, Libby P (1987) Interleukin 1 induces interleukin 1 I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. J Immunol 139:1902-1910.

Dong Y, Benveniste EN (2001) Immune function of astrocytes. GLIA 36:180-190.

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-R660.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends in Immunology 28:138-145.

Florian C, Vecsey CG, Halassa MM, Haydon PG, Abel T (2011) Astrocyte-Derived Adenosine and A1 Receptor Activity Contribute to Sleep Loss-Induced Deficits in Hippocampal Synaptic Plasticity and Memory in Mice. The Journal of Neuroscience 31:6956-6962.

Giulian D, Li J, Li X, George J, Rutecki PA (1994) The Impact of Microglia-Derived Cytokines upon Gliosis in the CNS. Developmental Neuroscience 16:128-136.

Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995) Molecular Cloning and Characterization of a Second Subunit of the Interleukin 1 Receptor Complex. J Biol Chem 270:13757-13765.

Gudewill S, Pollmächer T, Vedder H, Schreiber W, Fassbender K, Holsboer F (1992) Nocturnal plasma levels of cytokines in healthy men. Eur Arch Psychiat Clin Neurosci 242:53-56.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Hallett H, Churchill L, Taishi P, De A, Krueger JM (2010) Whisker stimulation increases expression of nerve growth factor- and interleukin-1beta-immunoreactivity in the rat somatosensory cortex. Brain Res 1333:48-56.

Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP, Thompson RC (1990) Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. Nature 343:336-340.

Hu S, Sheng WS, Ehrlich LC, Peterson PK, Chao CC (2000) Cytokine Effects on Glutamate Uptake by Human Astrocytes. Neuroimmunomodulation 7:153-159.

Imeri L, Opp MR (2009) How (and why) the immune system makes us sleep. Nat Rev Neurosci 10:199-210.

Kim Y (2012) Decoupling of sleepiness from sleep time and intensity during chronic sleep restriction: evidence for a role of the adenosine system. Sleep (New York, N Y) 35:861-869.

Krueger JM, Obal FJ, Fang J, Kubota T, Taishi P (2001) The role of cytokines in physiological sleep regulation. Ann N Y Acad Sci 933:211-221.

Krueger JM, Rector DM, Roy S, Van Dongen HP, Belenky G, Panksepp J (2008) Sleep as a fundamental property of neuronal assemblies. Nat Rev Neurosci 9:910-919.

Krueger JM, Walter J, Dinarello CA, Wolff SM, Chedid L (1984) Sleep-promoting effects of endogenous pyrogen (interleukin-1). Am J Physiol 246:R994-R999.

Krueger JM (2012) Translation of brain activity into sleep. Hirosaki igaku 63:S1-S16.

Lange T, Dimitrov S, Bollinger T, Diekelmann S, Born J (2011) Sleep after vaccination boosts immunological memory. J Immunol 187:283-290.

Lange T, Perras B, Fehm HL, Born J (2003) Sleep enhances the human antibody response to hepatitis A vaccination. Psychosom Med 65:831-835.

Lue FA, Bail M, Gorczynski R, Moldofsky H (1987) Sleep and interleukin-1-like activity in cat cerebrospinal fluid. pp 51.

Mackiewicz M, Sollars PJ, Ogilvie MD, Pack AI (1996) Modulation of IL-1b gene expression in the rat CNS during sleep deprivation. NeuroReport 7:529-533.

Manfridi A, Brambilla D, Bianchi S, Mariotti M, Opp MR, Imeri L (2003) Interleukin-1b enhances non-rapid eye movement sleep when microinjected into the dorsal raphe nucleus and inhibits serotonergic neurons in vitro. Eur J Neurosci 18:1041-1049.

McMahan CJ, Slack JL, Mosley B, Cosman D, Lupton SD, Brunton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan CI, Copeland NG, Huebner K, Croce CM, Cannizzarro LA, Benjamin D, Dower SK, Spriggs MK, Sims JE (1991) A novel

IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J 10:2821-2832.

Moldofsky H, Lue FA, Eisen J, Keystone E, Gorczynski RM (1986) The relationship of interleukin-1 and immune functions to sleep in humans. Psychosom Med 48:309-318.

Narcisse L, Scemes E, Zhao Y, Lee SC, Brosnan CF (2005) The cytokine IL-1beta transiently enhances P2X7 receptor expression and function in human astrocytes. GLIA 49:245-258.

Nguyen KT, Deak T, Owens SM, Kohno T, Fleshner M, Watkins LR, Maier SF (1998) Exposure to acute stress induces brain interleukin-1beta protein in the rat. J Neurosci 18:2239-2246.

O'Neill LAJ, Dinarello CA (2000) The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol Today 21:206-209.

Olivadoti MD, Opp MR (2008) Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. Neuroscience 153:338-348.

Opp MR, Krueger JM (1991) Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever. Am J Physiol 260:R453-R457.

Opp MR, Krueger JM (1994) Anti-interleukin-1b reduces sleep and sleep rebound after sleep deprivation in rats. Am J Physiol 266:R688-R695.

Opp MR, Obál F, Krueger JM (1991) Interleukin-1 alters rat sleep: temporal and dose-related effects. Am J Physiol 260:R52-R58.

Opp MR, Toth LA (2003) Neural-immune interactions in the regulation of sleep. Front Biosci 8:d768-d779.

Parker LC, Luheshi GN, Rothwell NJ, Pinteaux E (2002) IL-1beta signalling in glial cells in wildtype and IL-1RI deficient mice. Brit J Pharmacol 136:312-320.

Parnet P, Amindari S, Wu C, Brunke-Reese D, Goujon E, Weyhenmeyer JA, Dantzer R, Kelley KW (1994) Expression of type I and type II interleukin-1 receptors in mouse brain. Mol Brain Res 27:63-70.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic Purinergic Signaling Coordinates Synaptic Networks. Science 310:113-116.

Pinteaux E, Parker LC, Rothwell NJ, Luheshi GN (2002) Expression of interleukin-1 receptors and their role in interleukin-1 actions in murine microglial cells. J Neurochem 83:754-763.

Rothwell NJ, Luheshi GN (2000) Interleukin I in the brain: biology, pathology and therapeutic target. Trends Neurosci 23:618-625.

Schmitt LI, Sims RE, Dale N, Haydon PG (2012) Wakefulness Affects Synaptic and Network Activity by Increasing Extracellular Astrocyte-Derived Adenosine. The Journal of Neuroscience 32:4417-4425.

Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG, Colotta F, Re F, Mantovani A, Shanebeck K, Grabstein KH, Dower SK (1993) Interleukin 1 signaling occurs exclusively via the type I receptor. Proc Natl Acad Sci USA 90:6155-6159.

Smith DE, Lipsky BP, Russell C, Ketchem RR, Kirchner J, Hensley K, Huang Y, Friedman WJ, Boissonneault V, Plante MMl, Rivest S, Sims JE (2009) A Central Nervous System-Restricted Isoform of the Interleukin-1 Receptor Accessory Protein Modulates Neuronal Responses to Interleukin-1. Immunity 30:817-831.

Sperlagh B, Baranyi M, Hasko G, Vizi ES (2004) Potent effect of interleukin-1 beta to evoke ATP and adenosine release from rat hippocampal slices. J Neuroimmunol 151:33-39.

Spiegel K, Sheridan JF, Van Cauter E (2002) Effect of sleep deprivation on response to immunization. JAMA 288:1471-1472.

Taishi P, Bredow S, Guha-Thakurta N, Krueger JM (1997) Diurnal variations of interleukin-1b mRNA and b-actin mRNA in rat brain. J Neuroimmunol 75:69-74.

Taishi P, Chen Z, Hansen MK, Zhang J, Fang J, Krueger JM (1998) Sleep-associated changes in interleukin-1b mRNA in the brain. Journal of Interferon and Cytokine Research 18:793-798.

Taishi P, Davis CJ, Bayomy O, Zielinski MR, Liao F, Clinton JM, Smith DE, Krueger JM (2012) Brain-specific interleukin-1 receptor accessory protein in sleep regulation. J Appl Physiol (1985) 112:1015-1022.

Thornberry NA, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1[beta]processing in monocytes. Nature 356:768-774.

Tobler I, Borbély AA, Schwyzer M, Fontana A (1984) Interleukin-1 derived from astrocytes enhances slow wave activity in sleep EEG of the rat. Eur J Pharmacol 104:191-192.

Touzani O, Boutin H, Chuquet J, Rothwell N (1999) Potential mechanisms of interleukin-1 involvement in cerebral ischaemia. J Neuroimmunol 100:203-215.

Wesche H, Korherr C, Kracht M, Falk W, Resch K, Martin MU (1997) The Interleukin-1 Receptor Accessory Protein (IL-1RAcP) Is Essential for IL-1-induced Activation of Interleukin-1 Receptor-associated Kinase (IRAK) and Stress-activated Protein Kinases (SAP Kinases). J Biol Chem 272:7727-7731.

Zetterstrom M, Lundkvist J, Malinowsky D, Eriksson G, Bartfai T (1998) Interleukin-1-mediated febrile responses in mice and interleukin-1 beta activation of NFkappaB in mouse primary astrocytes, involves the interleukin-1 receptor accessory protein. European cytokine network (Montrouge) 9:131-138.

Zhu G, Okada M, Yoshida S, Mori F, Hirose S, Wakabayashi K, Kaneko S (2006) Involvement of Ca(2+)-induced Ca2+ releasing system in interleukin-1beta-associated adenosine release. Eur J Pharmacol 532:246-252.

Zielinski MR, Taishi P, Clinton JM, Krueger JM (2012) 5'-Ectonucleotidase-knockout mice lack non-REM sleep responses to sleep deprivation. Eur J Neurosci 35:1789-1798.

CHAPTER II

SPONTANEOUS SLEEP AND SLEEP DEPRIVATION RESPONSES OF MICE EXPRESSING INTERLEUKIN-1 RECEPTOR 1 SELECTIVELY ON NEURONS OR ASTROCYTES

Abstract

Interactions between sleep and immune function are bidirectional. Interleukin-1β (IL-1) is a pro-inflammatory cytokine that regulates sleep. However, the cellular substrates that mediate IL-1 regulation of sleep and immune interactions are not fully elucidated. To this end, we engineered two transgenic mouse lines that express interleukin-1 receptor 1 (IL1R1) only in the central nervous system and selectively on neurons or astrocytes via transcriptional control of the neuron-specific enolase (NSE) or human glial fibrillary acidic protein (gfa2) promoters, respectively. Animals with selective neuronal IL1R1 expression are referred to as NSE-IL1R1, and mice expressing IL1R1 on astrocytes are called GFAP-IL1R1. Spontaneous sleep and temperature phenotypes were determined in transgenic and wild type (WT) mice via electroencephalographic recordings and brain temperature measurements. Under baseline conditions, NSE-IL1R1 and GFAP-IL1R1 mice spend a comparable amount of time in non-rapid eye movement sleep (NREMS) but exhibit more rapid eye movement sleep (REMS) compared to WT. Transgenic mice also have reduced theta power in REMS, enhanced NREM delta power, and lower brain temperatures compared to WT mice. In response to 6 h of sleep deprivation,

transgenic mice sleep the same amount as WT mice but sleep is more consolidated. Additionally, the NREM delta power response is impaired in NSE-IL1R1 animals following sleep deprivation. These data suggest that neuronal- and astroglial-specific IL1R1 expression contributes to the modulation of spontaneous sleep and temperature. Furthermore, astroglial IL1R1 activity may contribute to homeostatic responses to sleep deprivation.

Introduction

The reciprocal interactions between sleep and immune function are extensively studied. We now know that insufficient sleep alters immune function and immune activation alters sleep [reviewed by (Imeri and Opp, 2009; Besedovsky et al., 2012; Krueger et al., 2011)]. Interleukin-1β (IL-1) is a well-characterized pro-inflammatory cytokine that plays critical roles in host defense and serves as a mutual mediator of sleep and immune responses.

Although IL-1 is commonly associated with pathological states, this cytokine is also involved in the regulation of physiological sleep. The diurnal peak of IL-1 is concurrent with the sleep period while lower levels are in accordance with waking hours (Moldofsky et al., 1986; Taishi et al., 1998; Lue et al., 1987). Central administration of IL-1 increases non-rapid eye movement sleep (NREMS) and suppresses rapid eye movement sleep (REMS) (Opp et al., 1991; Olivadoti and Opp, 2008), and these effects are attenuated by inhibition of IL-1 activity (Opp and Krueger, 1991; Imeri et al., 1993; Imeri and Opp, 2009). Furthermore, IL-1 exerts its effects on known sleep circuitry (Alam et al., 2004; Brambilla et al., 2007; Brambilla

et al., 2010; Breder et al., 1988). IL-1 binds to IL-1 receptor 1 (IL1R1) present on neurons and astrocytes (Smith et al., 2009), and these cells also produce IL-1 (Bartfai and Schultzberg, 1993; Baumann et al., 1993; Dong and Benveniste, 2001).

Much of our current understanding of sleep and immune function is based on neuron-centric studies. However, recent research now implicates astrocytes as critical, active mediators of central nervous system (CNS) processes. Astrocytes are the most abundant glial cell type in the brain, are responsible for homeostasis of neuronal functioning, express receptors for immunomodulators, respond rapidly to inflammation, and have a newly demonstrated role in sleep-wake processes (Aschner, 1998; Farina et al., 2007; Frank, 2011; Halassa et al., 2009). These qualities indicate astrocytes are poised for local mediation of immune responses and are important constituents of sleep-wake behavior and physiology. However, *in vivo* investigations of astroglial contributions to CNS-mediated process and behaviors are recent. Consequently, much remains to be elucidated regarding the role of astrocytes in sleep and immune responses.

To further our understanding of cellular contributions to sleep and immune interactions in the brain, we engineered two transgenic mouse lines that express IL1R1 only in the CNS and selectively on neurons or astrocytes. Although these mice offer a unique opportunity to dissect neuronal and astroglial roles in variety of processes, behaviors, and pathologies, this study focuses on sleep.

Methods

Engineering and breeding of transgenic mice

All protocols for creating transgenic mice were approved by the University of Michigan Committee on Use and Care of Animals. Full length *Il1r1* cDNA was generated by RT-PCR from a total RNA sample extracted from mouse liver using Trizol reagent. The left primer used was 5'-ATGGAGAATATGAAAGTGCTACTGG-3' and the right primer was 5'-CTAGCCGAGTGGTAAGTGTGTT-3'. The NSE-IL1R1 transgene was constructed by excising the rat neuron-specific enolase (NSE) promoter [described by (Forss-Petter et al., 1990 ; Mucke et al., 1994; Race et al., 1995; Kearney et al., 2001); gifted by Dr. Miriam Meisler, University of Michigan, Ann Arbor, MI, USA] using SalI and HindIII digests and ligating with the pBluescriptII SK vector (-). *Il1r1* cDNA was inserted into the NSE containing vector at the EcoRV site. The transgene fragment for microinjection was excised with SalI and SacII digests.

The GFAP-IL1R1 transgene was built in the pGfa2-cLac plasmid containing the promoter for human glial fibrillary acidic protein (GFAP) [described by (Brenner et al., 1994); gifted by Dr. Michael Brenner, University of Alabama, Birmingham, AL, USA]. The *lacZ* gene was excised via digestion with BamHI. BglII ends were added to *Il1r1* cDNA that was subsequently cloned into the BamHI site of the pGfa2 plasmid. The transgene fragment for microinjection was excised with BglII digest.

Transgenic mice were generated by the Transgenic Animal Model Core at the University of Michigan via pronuclear injection of the transgenes into oocytes obtained from (C57BL/6 X SJL)F2 mice. NSE-IL1R1 mice were identified via PCR of tail snip DNA using the forward primer 5'- GGCAAGGGAGAACCCCTTCTA-3' and reverse primer 5'- AATCTCCAGCGACAGCAGAGG-3' which produced a 433 bp

product. PCR of GFAP-IL1R1 tail snip DNA used the forward primer 5'-AGAGCCAGAGCAGGTTGGAGA-3' and reverse primer 5'-TGGGGGTCTTGCTGTCATTCT-3' yielding a 489 bp product. Primer pairs were complementary to the NSE and gfa2 promoter sequences and *Il1r1* sequence.

Transgenic founders were bred with mice null for *Il1r1* and *Tnfr1* genes (B6;129S-Tnfrsf1a^{tm1Imx} Il1r1^{tm1Imx}/J, stock #003244, The Jackson Laboratory, Bar Harbor, ME). *Il1r1*$^{-/-}$*Tnfr1*$^{-/-}$ mice were used for breeding because transgenic TNFR1 counterparts were simultaneously engineered with the transgenic IL1R1 mice. To generate transgenic IL1R1 strains null for the endogenous *Il1r1* gene and wild type for the endogenous *Tnfr1* gene, transgenic progeny of the founders were crossed with non-transgenic littermates (Tg(IL1R1)*Il1r1*$^{+/-}$*Tnfr1*$^{+/-}$ x *Il1r1*$^{+/-}$*Tnfr1*$^{+/-}$). Non-transgenic progeny carrying endogenous *Il1r1* and *Tnfr1* (*Il1r1*$^{+/+}$*Tnfr1*$^{+/+}$) were used as wild type (WT) controls. As per instructions of the Jackson Laboratory, mice were genotyped for wild type and mutant alleles of *Il1r1* and *Tnfr1* using custom primers. Wild type *Il1r1* was identified using the primer pair 5'-GGTTTGAATGTTGGGGTTTG-3' (forward) and 5'-CACCACCACCTGGCTACTTT-3' (reverse), whereas mutant *Il1r1* primer pairs were 5'-TCTGGACGAAGAGCATCAGGG-3' (forward) and 5'-CAAGCAGGCATCGCCATG-3' (reverse). Wild type *Tnfr1* was detected with the primer pair 5'-GGATTGTCACGGTGCCGTTGAAG-3' (forward) and 5'-TGACAAGGACACGGTGTGTGGC-3' (reverse), and mutant *Tnfr1* used primer pairs 5'-TGCTGATGGGGATACATCCATC-3' (forward) and 5'-CCGGTGGATGTGGAATGTGTG-3' (reverse).

Experimental animals express IL1R1 only in the CNS and selectively on neurons or astrocytes. Those mice with selective neuronal expression of IL1R1 are referred to as NSE-IL1R1, and mice expressing IL1R1 selectively on astrocytes are called GFAP-IL1R1. Figure 2.1 (A – C) shows co-localization of IL1R1 expression with neuronal staining in NSE-IL1R1 mouse brain. Conversely, IL1R1 expression specifically co-localizes with astrocytes in GFAP-IL1R1 mouse brain (Figure 2.1, D – F).

Animals

During the studies, adult male WT, NSE-IL1R1, and GFAP-IL1R1 mice (20 – 30 g, 8 – 12 weeks at time of surgery) were obtained from our breeding colony and individually housed in standard cages on a 12:12 h light:dark cycle at 29 ± 1ºC. Food and water were available *ad libitum*. All procedures involving the experimental use of animals were approved by the University of Washington Institutional Animal Care and Use Committee in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

In situ hybridization and immunofluorescence

Mice were lightly anesthetized, euthanized via bilateral pneumothorax, and transcardially perfused with 10% buffered formalin. Brains were paraffin embedded and cut sagittally on a sliding microtome in 10 µM sections.

Identification of IL1R1 in NSE-IL1R1 brain tissue was achieved via in situ hybridization using a digoxigenin (DIG)-labeled probe generated from murine IL1R1 cDNA using the AmpliScribe T7-Flash Transcription Kit (ASB71110, Epicentre, Madison, WI, USA). After in situ hybridization, tissue was blocked in 10% normal donkey serum (017-000-121, Jackson ImmunoResearch, Inc., West Grove, PA, USA) and incubated with anti-DIG made in sheep (11333089001, Roche Nutley, NJ, USA) for 1 h. DIG was the visualized via 1 h incubation with Alexa Fluor 488 donkey anti-sheep (A-11015) secondary antibody. Neurons were identified by sequential 1 h incubations with the primary antibody mouse anti-NeuN (MAB377, EMD Millipore, Temecula, CA, USA) and the secondary antibody Alexa Fluor 555 donkey anti-mouse (A-31570). All antibody solutions were made in 1% normal donkey serum at a concentration of 1:500. Secondary antibodies were purchased from Life Technologies (Carlsbad, CA, USA).

IL1R1 in GFAP-IL1R1 brain sections was detected with sequential 1 h incubations with the primary antibody rabbit anti-IL1R1 (sc-25775, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and secondary antibody Alexa Fluor 555 donkey anti-rabbit (A-31572). Astrocytes were identified via sequential treatment with the primary antibody anti-GFAP made in chicken (GFAP, Aves Labs, Inc., Tigard, OR, USA) and the secondary antibody Alexa Fluor 488 donkey anti-chicken (703-545-155, Jackson ImmunoResearch, Inc.). All antibody solutions were made in 1% normal donkey serum at a concentration of 1:500. Secondary antibodies were purchased from Life Technologies unless otherwise noted.

Surgical procedures

Electroencephalographic (EEG) recordings were obtained from mice stereotaxically instrumented under isoflurane anesthesia with three stainless steel screws (MPX-0080-02P-C, Small Parts Inc., Logansport, IN, USA) and a calibrated 10 kΩ thermistor (AB6E3-GC16KA103L, Thermometrics, Northridge, CA, USA) as previously described (Baracchi and Opp, 2008). Analgesia was provided at the time of surgery by subcutaneous (SC) injection of buprenorphine (0.05 mg/kg) as well as topical application of 4% lidocaine at the incision site. Penicillin (1,200,000 IU/kg; SC) and topical triple antibiotic treatment of the surgical site was also provided to minimize risk of infection. Additional buprenorphine (0.03 mg/kg; SC) was administered 24 h post-surgery. Animals were allowed at least seven days recovery prior to initiation of experimental protocols.

Data acquisition

EEG and temperature data were obtained by connecting the mice to a lightweight flexible tether that allowed unrestricted movement as previously described (Baracchi and Opp, 2008). Cage activity was monitored by an infrared sensor (BioBserve, GmbH, Bonn, Germany). Digitized EEG signals were stored as binary files for subsequent analysis.

Arousal state was determined by visual inspection of the EEG, theta-to-delta ratio, brain temperature, and cage activity with 10 s resolution using custom software (ICELUS, M. Opp, University of Washington) written in LabView for Windows (National Instruments, Austin, Texas, USA) based on previously published criteria

(Opp and Krueger, 1994; Opp, 1998). Epochs containing artifacts were excluded from subsequent spectral analyses. The EEG underwent fast Fourier transformation (FFT) to produce power spectra between 0.5 and 30 Hz in 0.5 Hz bins as previously described (Baracchi and Opp, 2008). Spectral bins within the delta (δ) and theta (θ) bands were normalized to the total state-specific power (delta in NREMS; theta in REMS) summed across all frequency bins from 0.5 to 30 Hz for the 12h light and dark periods and expressed as a percentage of total power. Hourly NREM delta power data from the light and dark periods were normalized to 12 h light and dark averages, respectively, derived from control conditions.

Determination of sleep phenotype and responses to sleep deprivation

After recovery from surgery and habituation to the recording environment, 48 h of undisturbed baseline recordings beginning at light onset (LO) were obtained from WT (n = 7), NSE-IL1R1 (n = 8), and GFAP-IL1R1 (n = 7). Animals were then subjected to 6 h sleep deprivation via gentle handling beginning at LO. Upon observation of behavioral sleep cues, laboratory personnel introduced arousing acoustic, tactile, or novel object stimuli. Following sleep deprivation, mice were allowed 18 h of undisturbed recovery sleep.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (IBM Corporation, Armonk, NY, USA). Data are presented as means ± standard error of the mean (SEM). Between strain comparisons for baseline sleep physiology,

temperature, cage activity, spectral analyses, and calculated difference scores for sleep deprivation effects were performed in 12 h and 6 h time blocks using one-way analysis of variance (ANOVA) with strain (WT, NSE-IL1R1, GFAP-IL1R1) as the independent variable. If statistically significant strain effects were revealed, post-hoc comparisons by Tukey's HSD test were used to determine differences among transgenic and WT mice.

Within strain comparisons for percentage of time in REMS and NREMS, normalized NREM delta power, and brain temperature were performed via a general linear model for repeated measures using time (hours) as the repeated measure and manipulation (undisturbed baseline vs. 6 h sleep deprivation) as the between-subjects factor.

An alpha level of $p < 0.05$ was accepted as indicating significant departures from control conditions or strain differences for all tests.

Results

Comparison of physiological sleep phenotypes and temperature

There were no differences among strains in the percentage of time spent in wakefulness or NREMS during the 24 h baseline recording period (Figure 2.2). However, NSE-IL1R1 and GFAP-IL1R1 mice had more wake bouts during the light period, and GFAP-IL1R1 mice also had more wake bouts during the dark period. Wake bouts during the dark period were shorter in GFAP-IL1R1 mice than in WT animals. NSE-IL1R1 mice had more bouts of NREMS during the light period that were of shorter duration than those of WT mice. Both transgenic lines spent more

time in REMS during the light period in comparison to WT counterparts. This increase in REMS is due, in part, to more REM bouts. Additionally, during the dark period, GFAP-IL1R1 mice had longer REM bouts than did WT mice. Sleep of GFAP-IL1R1 mice is fragmented during the dark period, likely as a consequence of an increased number of wake bouts.

Normalized EEG spectra obtained during NREM and REM sleep differed among strains (Figures 2.2 and 2.3). NREM delta power (0.5 – 4.5 Hz) was greater in NSE-IL1R1 and GFAP-IL1R1 mice than in WT mice. The REMS EEG of both transgenic strains contained less theta power (6.0 – 9.0 Hz) compared to WT counterparts, and REM theta power was reduced in GFAP-IL1R1 mice compared to NSE-IL1R1 animals.

Finally, brain temperature in WT mice was higher than NSE-IL1R1 and GFAP-IL1R1 animals for the entire 24 h period, whereas cage activity did not differ among strains (Figure 2.4).

Responses to 6-h sleep deprivation

The deprivation method was effective as REMS and NREMS were virtually abolished for all strains of mice during this 6 h period (Figure 2.5). During sleep deprivation, brain temperatures of WT and NSE-IL1R1 mice, but not GFAP-IL1R1 mice, were significantly increased.

There were significant increases in NREMS and REMS during the recovery period after deprivation ended (Figure 2.5). Sleep deprivation induced a statistically significant REMS rebound in the dark period for all strains. WT and transgenic mice

also exhibited a small but significant increase in NREMS during the first 6 h of recovery. Although the increased time spent in NREMS was modest, this was accompanied by a significant elevation of NREM delta power in all strains for the 6 h period post-deprivation. NSE-IL1R1 mice also exhibited a greater amount of time spent in NREMS in the early portion of the dark period. Sleep deprivation had no effect on brain temperature in the 18 h recovery period.

To determine if responses to sleep deprivation differed among WT and transgenic mice, difference scores were calculated by subtracting sleep deprivation values from baseline (Figure 2.6). No strain differences were detected for the amount of REMS or NREMS acquired during the 18 h recovery period following sleep deprivation. NSE-IL1R1 mice had significantly less NREM delta power compared to WT during the first 12 h post-deprivation, presumably indicating less restorative sleep. However, sleep of NSE-IL1R1 and GFAP-IL1R1 mice was more consolidated than that of WT mice during the first 6 h of recovery as evidenced by fewer sleep state transitions. Brain temperature during the recovery period did not differ among stains.

Discussion

Physiological sleep

There is an abundance of data demonstrating a role for IL-1 in the regulation of sleep [reviewed by (Krueger et al., 2011; Imeri and Opp, 2009)], yet the contributions of neurons and astrocytes to sleep-wake behavior remain largely unknown. This study aimed to establish a role for neurons and astrocytes in IL-1

regulation of sleep. To this end, we engineered two transgenic mouse lines that express IL1R1 only in the CNS, and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1). During spontaneous sleep, NSE-IL1R1 and GFAP-IL1R1 mice have more REMS during the light period that is characterized by reduced theta power in the EEG spectra. NREM delta power of transgenic mice is also elevated in comparison to WT animals. In response to 6 h of sleep deprivation, sleep of transgenic mice is more consolidated than that of WT animals. Although the NREM EEG of NSE-IL1R1 mice contains less delta power.

Previous findings demonstrate that mice lacking IL1R1 have normal REMS (Fang et al., 1998). Our data suggest neuronal and astroglial IL1R1 contribute to REMS regulation. Transgenic mice with IL1R1 selectively on neurons or on astrocytes have more REMS during the light period. Current knowledge regarding the brain regions and neurotransmitters involved in mediating effects of IL-1 on REMS is limited. Most studies demonstrate that IL-1 suppresses REMS (Opp et al., 1991; Opp and Toth, 1998; Fang et al., 1998; Olivadoti and Opp, 2008; Brambilla et al., 2010). However, microinjection of IL-1 into the rostral margins of the laterodorsal tegmental nucleus adjacent to the dorsal raphe nucleus (DRN) increase REMS (Brambilla et al., 2010). The DRN contains serotonergic REM-OFF neurons (McCarley, 2007), and IL-1 inhibits DRN serotonergic activity *in vitro* (Manfridi et al., 2003; Brambilla et al., 2007). IL-1 also induces adenosine release (Sperlagh et al., 2004; Zhu et al., 2006), and stimulation of adenosine A_1 receptors diminishes extracellular serotonin (Feuerstein et al., 1988; Okada et al., 1997). Therefore, enhanced REMS in transgenic mice during the light period, when the diurnal

expression of IL-1 is greatest (Moldofsky et al., 1986; Taishi et al., 1998; Lue et al., 1987), may be due to subsequent adenosinergic suppression of REM-OFF serotoninergic neurons in the DRN.

Several studies demonstrate that REMS is generated in the brainstem which contains cholinergic REM-ON neurons in laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) [reviewed by (Brown et al., 2012)]. Microinjection of IL-1 into the LDT reduces REMS (Brambilla et al., 2010). Similarly, IL-1 inhibits firing rates of cholinergic LDT neurons *in vitro*. Attenuation of cholinergic activity appears to be a consequence of inhibiting evoked glutamatergic responses and, thus, excitatory drive in cholinergic LDT neurons (Brambilla et al., 2010). An essential function of astrocytes is to regulate extracellular concentrations of glutamate. However, IL-1 impairs astroglial glutamate uptake (Ye and Sontheimer, 1996; Hu et al., 2000). Consequently, there may be an excess of glutamate in the extracellular space of transgenic mice due to disturbed IL-1-mediated glutamatergic dynamics of the tripartite synapse. This putative overabundance of glutamate may, in turn, stimulate cholinergic REM-ON neurons of the LDT resulting in the initiation and enhancement of REMS in transgenic mice during the light period. However, additional experiments are necessary to determine the mechanisms of neuronal and astroglial modulation of REMS via IL1R1.

In contrast to REMS, the percentage of time spent in NREMS is not altered in NSE-IL1R1 or GFAP-IL1R1 mice compared to WT counterparts. Mice lacking IL1R1 exhibit less NREMS (Fang et al., 1998), and antagonizing IL-1 with the IL-1 receptor antagonist (Opp and Krueger, 1991), antibodies (Opp and Krueger, 1994), or soluble

receptors (Imeri et al., 1993) or inhibiting its production (Imeri et al., 2006) reduces NREMS. Collectively, these data demonstrate a role for IL-1 in the regulation of spontaneous NREMS. Our new data suggest that selective IL1R1 expression on neurons or astrocytes is sufficient for spontaneous NREMS. Although the amount of time spent in NREMS is unchanged in transgenic animals, NREMS architecture during the light period is altered in NSE-IL1R1 mice, but not in GFAP-IL1R1 mice. Collectively, these results implicate a role for astroglial IL1R1 in the initiation and maintenance of NREMS.

The basal forebrain and preoptic areas of the anterior hypothalamus are primary regulatory nuclei of NREMS. In particular, the median preoptic nucleus (MnPO) contains a large number of GABAergic sleep-ON/wake-OFF neurons that send inhibitory projections to wake-active neurons of the ascending reticular activating system [reviewed by (Brown et al., 2012)]. Central administration of IL-1 in rats induces c-Fos protein expression in the MnPO with an associated increase of NREMS (Baker et al., 2005). Taken together with the results of this current study, astroglial IL1R1 expression in the MnPO may contribute to the configuration of NREMS architecture. Still, further experimentation is needed required.

Although the amount of NREMS is normal in transgenic animals, NSE-IL1R1 and GFAP-IL1R1 mice exhibit enhanced NREM delta power. NREM delta power is widely regarded as a marker of NREMS quality or depth (Borbély, 1982). These results are consistent with previous studies that demonstrate dissociation of NREMS delta power from time spent in NREMS (Halassa et al., 2009; Yasuda et al., 2005; Davis et al., 2011). Transgenic mice also have a reduction in the REM theta peak

compared to WT. Therefore, the demonstrated increase in transgenic REMS may be offset by attenuation of theta power. These effects on NREM delta and REM theta power may be mediated, in part, by the cholinergic basal forebrain (BF). Inhibition of cholinergic BF neurons increases delta power and suppresses theta activity (Cape and Jones, 2000). Indeed, IL-1 suppresses discharge activity of wake-active BF neurons (Alam et al., 2004). Likewise, adenosine inhibits cholinergic and non-cholinergic neurons of the BF (Basheer et al., 2004). Taken together, IL-1 may influence NREMS delta and REMS theta power through inhibition of cholinergic neurons in the BF. This effect may be modulated, to some extent, by adenosine. Furthermore, NREM delta and REM theta power may not be dependent specifically on neuronal or astroglial IL1R1 expression. However, neuronal IL1R1 activity may contribute more prominently to REM theta power as evidenced by greater suppression of the theta peak in GFAP-IL1R1 mice compared to WT and NSE-IL1R1 mice during the dark period.

Transgenic mice have lower brain temperatures than WT animals under physiological conditions, a result that does not appear to be neuron- or astrocyte-specific. Moreover, this temperature reduction is not due to diminished cage activity. Thermoregulatory centers reside in the preoptic area and anterior hypothalamus. Stimulation of cholinergic neurons in these nuclei lowers brain temperature (Imeri et al., 1995; Mallick and Joseph, 1997; Takahashi et al., 2001). Interestingly, hypothalamic thermoregulatory areas like the preoptic nucleus are innervated by the LDT/PPT (Chiba and Murata, 1985). In view of the fact that transgenic mice have more REMS which may be mediated, in part, by activation of LDT/PPT cholinergic

neurons at least in wild type animals, it is not unreasonable to postulate that transgenic brain temperature reductions may be a result of enhanced stimulation of preoptic areas via LDT/PPT projections.

6-h sleep deprivation

Responses to sleep deprivation are similar across all strains including REMS rebound, transient increase in NREMS, enhanced delta power, and increased sleep consolidation. Previous studies demonstrate that IL1R1 knockout mice have more wakefulness and less NREMS compared to wild type animals following sleep deprivation (Schmidt and Wisor, 2012). NSE-IL1R1 and GFAP-IL1R1 mice do not differ from WT in terms of percentage of times spent in REMS, NREMS, or wakefulness (data not shown) after 6 h sleep deprivation. These data suggest that neuronal and astroglial IL1R1 expression is sufficient for rebound sleep responses during the post-deprivation recovery period.

One of the major strain differences in response to sleep deprivation is that transgenic mice have more consolidated sleep during the first 6 h of the recovery period. This sleep consolidation is generally indicative of more efficient sleep [reviewed (Imeri and Opp, 2009)], and thus, transgenic mice may recover from insufficient sleep more quickly than WT animals. Central IL-1 administration fragments sleep (Olivadoti and Opp, 2008), and sleep deprivation induces elevations in IL-1 (Mackiewicz et al., 1996; Taishi et al., 1998). Therefore, IL1R1 expression limited to either neurons or astrocytes in transgenic mice may account for this increased sleep consolidation.

The two-process model of sleep regulation states that sleep is mediated, in part, by a homeostatic mechanism that increases propensity for sleep with increasing time awake (Borbély, 1982). In response to sleep deprivation, NSE-IL1R1 mice have less NREM delta power 12 h post sleep deprivation compared to WT. This result suggests that the sleep homeostat in NSE-IL1R1 is less affected by sleep loss under these conditions. Inhibition of gliotransmission by astrocytes attenuates enhancement of delta power following sleep deprivation. This altered homeostatic response is due to the inhibition of astrocyte-derived adenosine which signals via the adenosine A_1 receptor (A_1R) (Halassa et al., 2009). Furthermore, conditional knockout of the A_1R attenuates EEG delta power responses to intermittent sleep deprivation (Bjorness et al., 2009). As previously mentioned, IL-1 increases with prolonged time awake (Mackiewicz et al., 1996; Taishi et al., 1998), and IL-1 induces adenosine production (Sperlagh et al., 2004; Zhu et al., 2006). Therefore, IL-1 signaling via astroglial IL1R1 may be modulated by the production of adenosine. Additionally, lack of IL1R1 on astrocytes in NSE-IL1R1 mice could result in lower extracellular concentrations of adenosine, further indicating that neuronal IL1R1 activation may not stimulate as much adenosine release as astroglial IL1R1 activity. Alternatively, neuron-derived adenosine may act via mechanisms distinct from astroglial-derived adenosine (Lovatt et al., 2012).

Conclusions

Overall, these studies demonstrate the complexity of sleep and immune interactions and provide a foundational basis from which to build. Future studies

may aim to identify the contributions of other cell types including microglia. Discerning the molecular contributions of astrocytes and other neural cells will be vital to understanding a variety of CNS-mediated processes and behaviors.

Acknowledgements

The NSE promoter was gifted by Dr. Miriam H. Meisler from the University of Michigan, Ann Arbor. The gfa2 promoter was provided by Dr. Michael Brenner through the Alabama Neuroscience Blueprint Core (NIH grants NS39055 and NS057098).

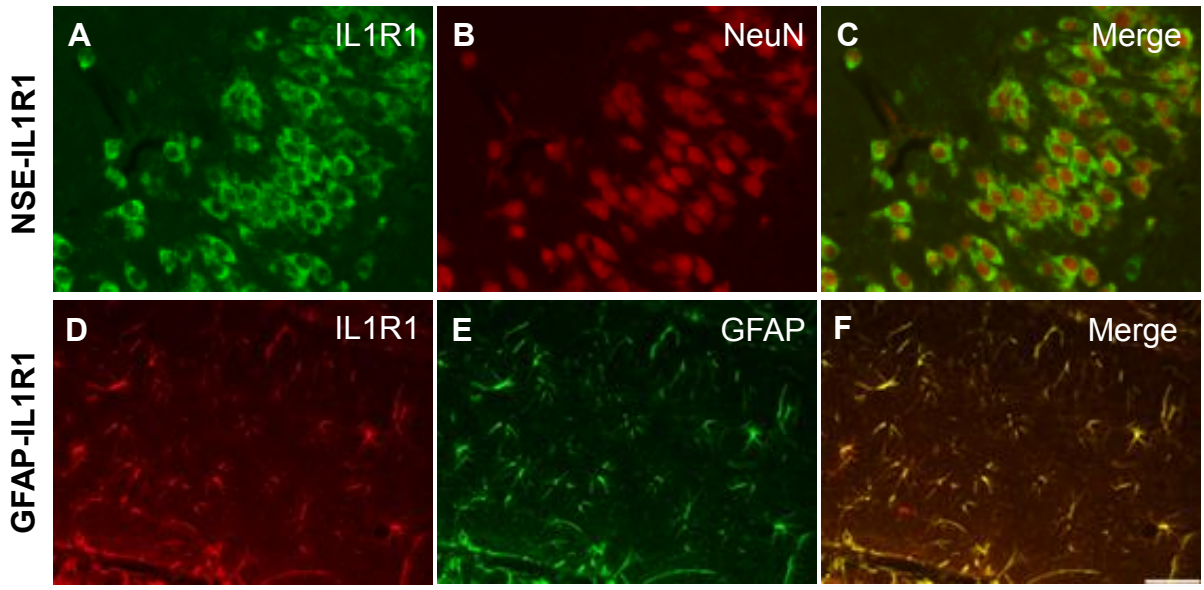


Figure 2.1: (**A**) In situ hybridization for IL1R1 and (**B**) immunofluorescent neuronal staining (NeuN) in NSE-IL1R1 brain tissue. (**C**) Merged image of IL1R1 (green) and NeuN (red) staining indicates IL1R1 is expressed on neurons in NSE-IL1R1 mice. (**D**) Immunofluorescent detection of IL1R1 and (**E**) astrocytes (GFAP) in GFAP-IL1R1 brain tissue. (**F**) Merged image shows IL1R1 (red) co-localizes with astrocytes (green) in GFAP-IL1R1 mice. Images are 40x; scale bar = 50 µM.

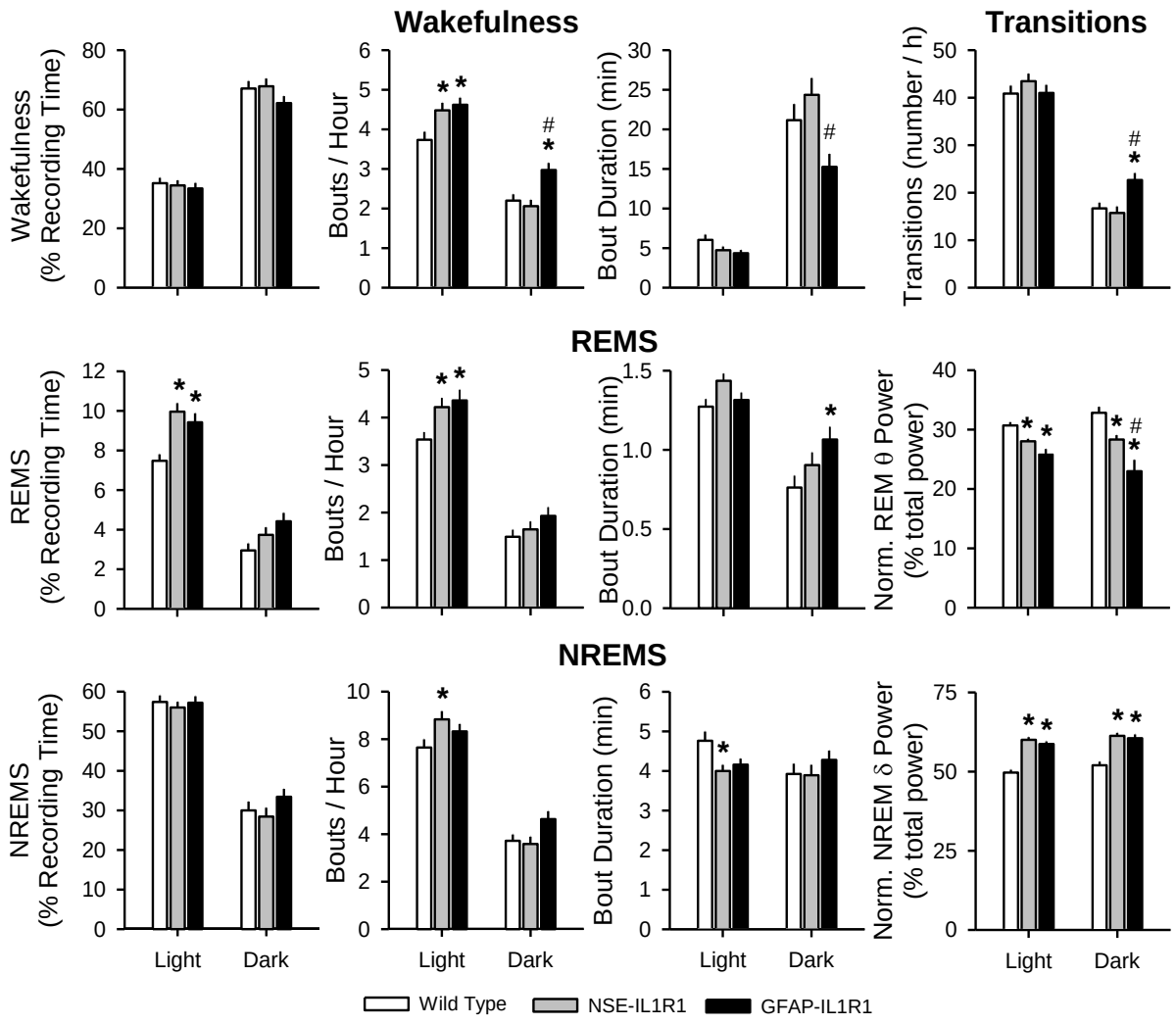


Figure 2.2: Sleep architecture parameters shown as 12 h averages derived from undisturbed baseline conditions during the light and dark periods for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Asterisk (*) indicates a statistical difference of $p < 0.05$ from WT. Pound sign (#) denotes a statistical difference of $p < 0.05$ from NSE-IL1R1.

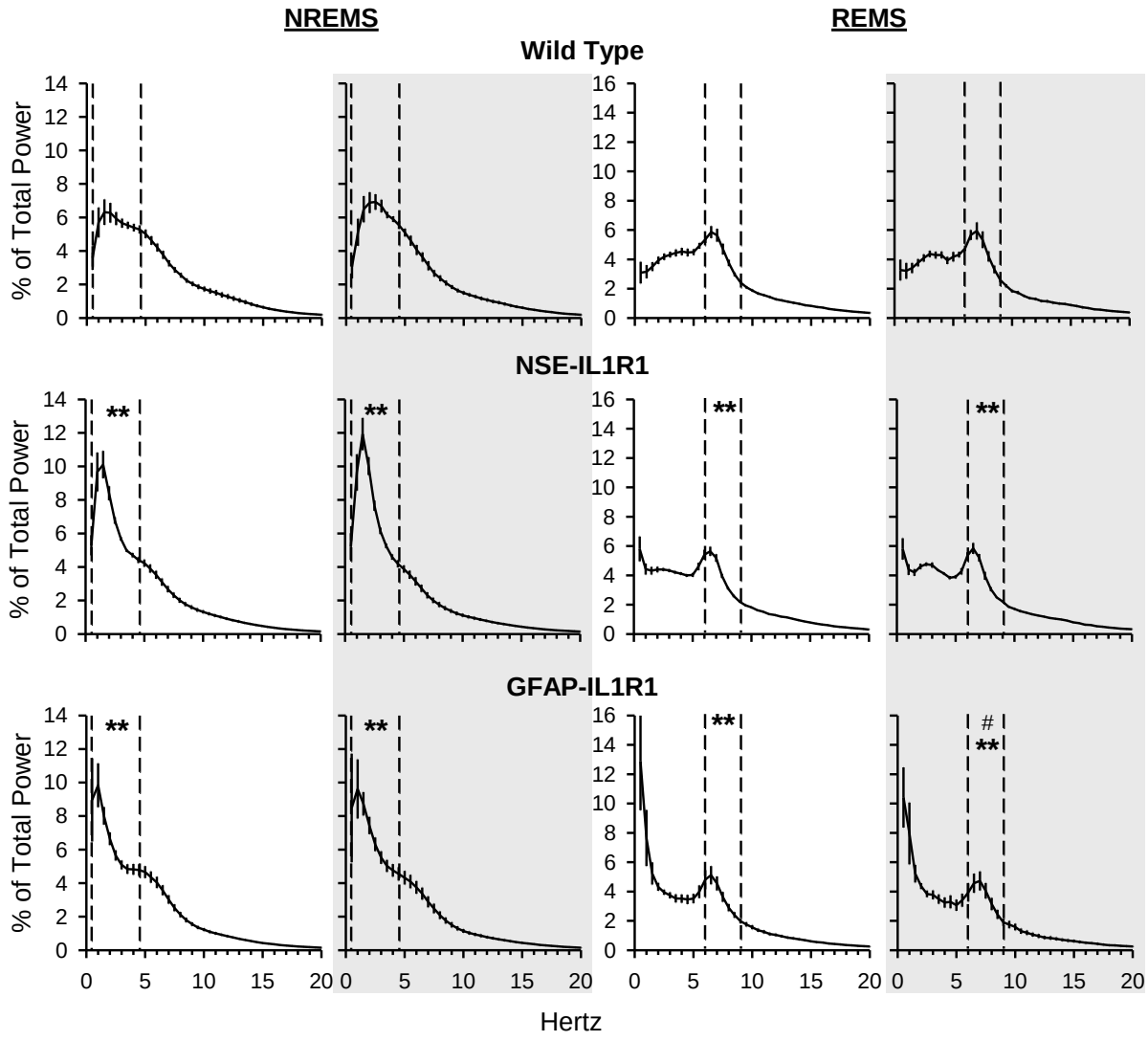


Figure 2.3: State-specific, normalized EEG power spectra for WT, NSE-IL1R1, and GFAP-IL1R1 mice obtained during 12 h light and dark periods of 24 h undisturbed baseline recordings. Spectral analyses were performed on frequencies 0.5 – 30.0 Hz, but data depicted are limited to 0.5 – 20.0 Hz. Spectra were normalized as a percentage of total power across all frequencies for specific sleep states within the 12 h light or dark periods. Values are plotted as means ± SEM for each frequency bin. Statistical analyses were conducted on the delta (0.5 – 4.5 Hz) frequency band for NREMS and the theta (6.0 – 9.0 Hz) frequency band for REMS. Double asterisk (**) indicates a statistical difference of $p < 0.01$ from WT. Pound sign (#) denotes statistical difference of $p < 0.05$ from NSE-IL1R1. Dark columns indicate spectra derived from the 12 h dark period.

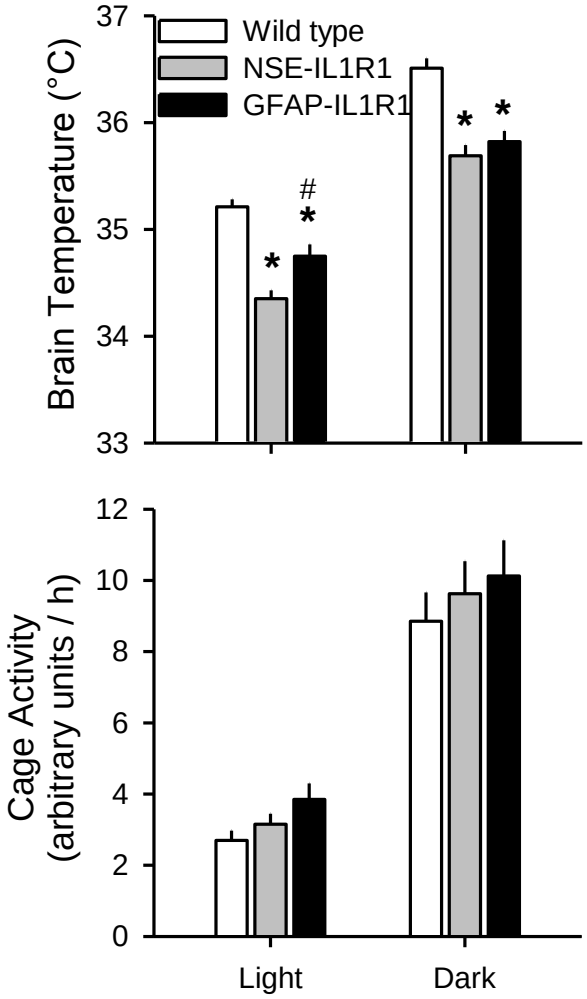


Figure 2.4: Brain temperature and cage activity reported as 12 h averages derived from undisturbed baseline conditions during the light and dark periods for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Asterisk (*) indicates a statistical difference of $p < 0.05$ from WT. Pound sign (#) denotes a statistical difference of $p < 0.05$ from NSE-IL1R1.

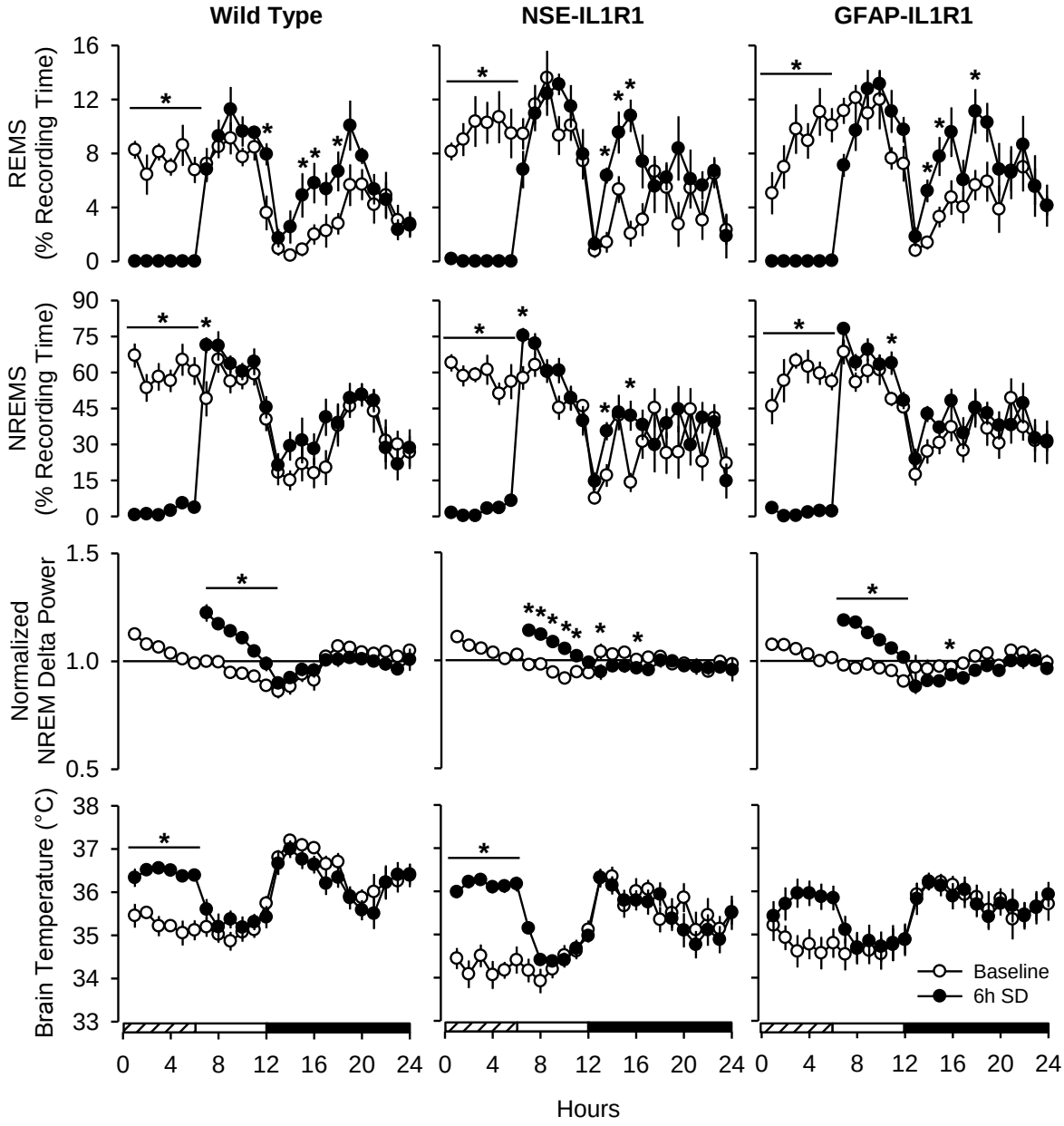
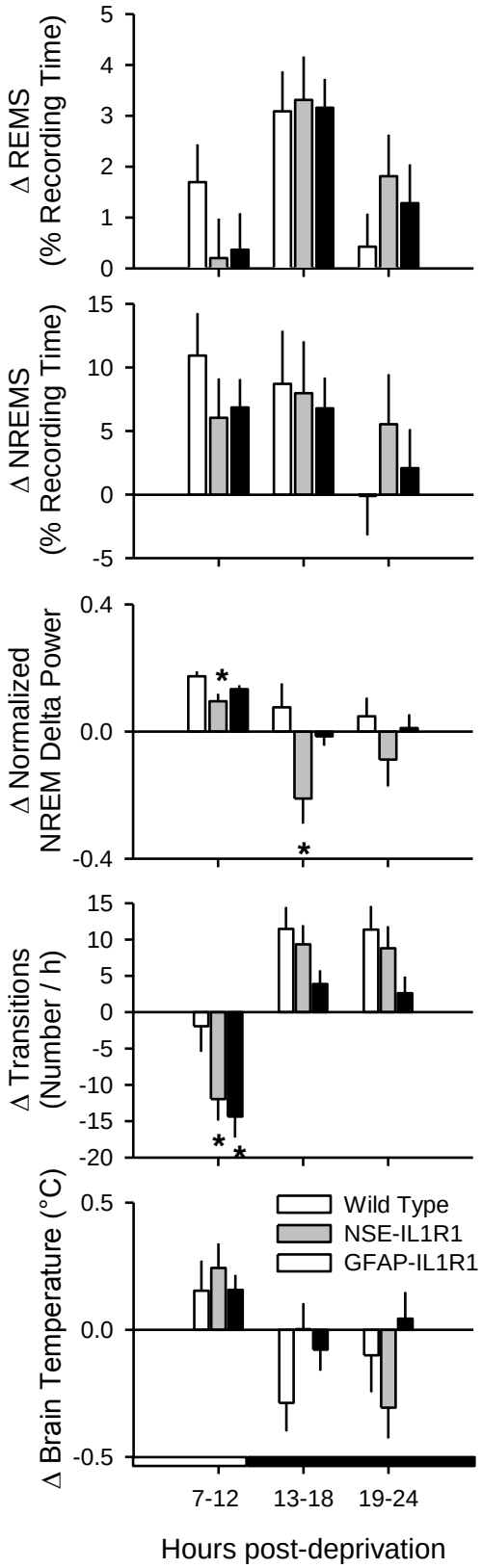


Figure 2.5: Percentage of recording time spent in REMS and NREMS, normalized NREM delta power, and brain temperature responses obtained under undisturbed baseline conditions (open symbols) and during and after 6 h of sleep deprivation (SD; filled symbols) for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Hourly data for EEG delta power were normalized to 12 h light and dark undisturbed baseline averages. Statistical analyses were conducted on 6 h time blocks. Asterisk (*) denotes statistical difference of $p < 0.05$ from undisturbed baseline conditions. The open and filled bars on the X-axis indicate light and dark periods of the light-dark cycle, respectively. The cross-hatched portion of the open bar on the X-axis denotes the 6 h sleep deprivation period.

Figure 2.6: Effects of 6 h of sleep deprivation shown as differences from undisturbed baseline conditions for percentage of recording time spent in REMS and NREMS, normalized NREM delta power, number of sleep state transitions, and brain temperature plotted in 6 h time blocks for the 18 h period following 6 h of sleep deprivation for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Asterisk (*) indicates statistical difference of $p < 0.05$ from WT. Open and filled bars on the X-axis denote light and dark portions of the light-dark cycle, respectively.


Δ REMS (% Recording Time)
5
4
3
2
1
0
Δ NREMS (% Recording Time)
15
10
5
0
-5
Δ Normalized NREM Delta Power
0.4
0.0
-0.4
*
*
Δ Transitions (Number / h)
15
10
5
0
-5
-10
-15
-20
*
*
Δ Brain Temperature (°C)
0.5
0.0
-0.5
Wild Type
NSE-IL1R1
GFAP-IL1R1
7-12
13-18
19-24
Hours post-deprivation

References

Alam MN, McGinty D, Bashir T, kumar S, Imeri L, Opp MR, Szymusiak R (2004) Interleukin-1β modulates state-dependent discharge activity of preoptic area and basal forebrain neurons: role in sleep regulation. Eur J Neurosci 20:207-216.

Aschner M (1998) Immune and inflammatory responses in the CNS: modulation by astrocytes. Toxicology Letters 102–103:283-287.

Baker FC, Shah S, Stewart D, Angara C, Gong H, Szymusiak R, Opp MR, McGinty D (2005) Interleukin 1beta enhances non-rapid eye movement sleep and increases c-Fos protein expression in the median preoptic nucleus of the hypothalamus. Am J Physiol Regul Integr Comp Physiol 288:R998-R1005.

Baracchi F, Opp MR (2008) Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1. Brain Behav Immun 22:982-993.

Bartfai T, Schultzberg M (1993) Cytokines in neuronal cell types. Neurochem Int 22:435-444.

Basheer R, Strecker RE, Thakkar MM, McCarley RW (2004) Adenosine and sleep-wake regulation. Prog Neurobiol 73:379-396.

Baumann N, Baron-Van Evercooren AF, Jacque CF, Zalc B (1993) Glial biology and disorders. Current Opinion in Neurology and Neurosurgery 6:27-33.

Besedovsky L, Lange T, Born J (2012) Sleep and immune function. Pflugers Arch 463:121-137.

Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW (2009) Control and Function of the Homeostatic Sleep Response by Adenosine A1 Receptors. The Journal of Neuroscience 29:1267-1276.

Borbély AA (1982) A two process model of sleep regulation. Hum Neurobiol 1:195-204.

Brambilla D, Barajon I, Bianchi S, Opp MR, Imeri L (2010) Interleukin-1 inhibits putative cholinergic neurons in vitro and REM sleep when microinjected into the rat laterodorsal tegmental nucleus. Sleep 33:919-929.

Brambilla D, Franciosi S, Opp MR, Imeri L (2007) Interleukin-1 inhibits firing of serotonergic neurons in the dorsal raphe nucleus and enhances GABAergic inhibitory post-synaptic potentials. Eur J Neurosci 26:1862-1869.

Breder CD, Dinarello CA, Saper CB (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240:321-324.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. The Journal of Neuroscience 14:1030-1037.

Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW (2012) Control of Sleep and Wakefulness. Physiol rev 92:1087-1187.

Cape EG, Jones BE (2000) Effects of glutamate agonist versus procaine microinjections into the basal forebrain cholinergic cell area upon gamma and theta EEG activity and sleep–wake state. Eur J Neurosci 12:2166-2184.

Chiba T, Murata Y (1985) Afferent and efferent connections of the medial preoptic area in the rat: A WGA-HRP study. Brain Res Bull 14:261-272.

Davis CJ, Clinton JM, Jewett KA, Zielinski MR, Krueger JM (2011) Delta wave power: an independent sleep phenotype or epiphenomenon? J Clin Sleep Med 7:S16-S18.

Dong Y, Benveniste EN (2001) Immune function of astrocytes. GLIA 36:180-190.

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-R660.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends in Immunology 28:138-145.

Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, Sutcliffe G (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron 5:187-197.

Frank MG (2011) Beyond the neuron: astroglial regulation of mammalian sleep. Current topics in medicinal chemistry 11:2452-2456.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Hu S, Sheng WS, Ehrlich LC, Peterson PK, Chao CC (2000) Cytokine Effects on Glutamate Uptake by Human Astrocytes. Neuroimmunomodulation 7:153-159.

Imeri L, Bianchi S, Angeli P, Mancia M (1995) Stimulation of cholinergic receptors in the medial preoptic area affects sleep and cortical temperature. Am J Physiol 269:R294-R299.

Imeri L, Bianchi S, Opp MR (2006) Inhibition of caspase-1 in rat brain reduces spontaneous nonrapid eye movement sleep and nonrapid eye movement

sleep enhancement induced by lipopolysaccharide. Am J Physiol Regul Integr Comp Physiol 291:R197-R204.

Imeri L, Opp MR (2009) How (and why) the immune system makes us sleep. Nat Rev Neurosci 10:199-210.

Imeri L, Opp MR, Krueger JM (1993) An IL-1 receptor and an IL-1 receptor antagonist attenuate muramyl dipeptide- and IL-1-induced sleep and fever. Am J Physiol 265:R907-R913.

Kearney JA, Plummer NW, Smith MR, Kapur J, Cummins TR, Waxman SG, Goldin AL, Meisler MH (2001) A gain-of-function mutation in the sodium channel gene Scn2a results in seizures and behavioral abnormalities. Neuroscience 102:307-317.

Krueger JM, Clinton JM, Winters BD, Zielinski MR, Taishi P, Jewett KA, Davis CJ (2011) Involvement of cytokines in slow wave sleep. Prog Brain Res 193:39-47.

Lovatt D, Xu Q, Liu W, Takano T, Smith NA, Schnermann J, Tieu K, Nedergaard M (2012) Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. Proceedings of the National Academy of Sciences 109:6265-6270.

Lue FA, Bail M, Gorczynski R, Moldofsky H (1987) Sleep and interleukin-1-like activity in cat cerebrospinal fluid. pp 51.

Mackiewicz M, Sollars PJ, Ogilvie MD, Pack AI (1996) Modulation of IL-1β gene expression in the rat CNS during sleep deprivation. NeuroReport 7:529-533.

Mallick BN, Joseph MM (1997) Role of cholinergic inputs to the medial preoptic area in regulation of sleepΓÇôwakefulness and body temperature in freely moving rats. Brain Res 750:311-317.

Moldofsky H, Lue FA, Eisen J, Keystone E, Gorczynski RM (1986) The relationship of interleukin-1 and immune functions to sleep in humans. Psychosom Med 48:309-318.

Mucke L, Masliah E, Johnson WB, Ruppe MD, Alford M, Rockenstein EM, Forss-Petter S, Pietropaolo M, Mallory M, Abraham CR (1994) Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. Brain Res 666:151-167.

Olivadoti MD, Opp MR (2008) Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. Neuroscience 153:338-348.

Opp MR (1998) Rat strain differences suggest a role for corticotropin-releasing hormone in modulating sleep. Physiol Behav 63:67-74.

Opp MR, Krueger JM (1991) Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever. Am J Physiol 260:R453-R457.

Opp MR, Krueger JM (1994) Anti-interleukin-1β reduces sleep and sleep rebound after sleep deprivation in rats. Am J Physiol 266:R688-R695.

Opp MR, Obál F, Krueger JM (1991) Interleukin-1 alters rat sleep: temporal and dose-related effects. Am J Physiol 260:R52-R58.

Opp MR, Toth LA (1998) Somnogenic and pyrogenic effects of interleukin-1β and lipopolysaccharide in intact and vagotomized rats. Life Sci 62:923-936.

Race RE, Priola SA, Bessen RA, Ernst D, Dockter J, Rall GF, Mucke L, Chesebro B, Oldstone MBA (1995) Neuron-specific expression of a hamster prion protein minigene in transgenic mice induces susceptibility to hamster scrapie agent. Neuron 15:1183-1191.

Schmidt MA, Wisor JP (2012) Interleukin 1 receptor contributes to methamphetamine- and sleep deprivation-induced hypersomnolence. Neurosci Lett 513:209-213.

Smith DE, Lipsky BP, Russell C, Ketchem RR, Kirchner J, Hensley K, Huang Y, Friedman WJ, Boissonneault V, Plante MMl, Rivest S, Sims JE (2009) A Central Nervous System-Restricted Isoform of the Interleukin-1 Receptor Accessory Protein Modulates Neuronal Responses to Interleukin-1. Immunity 30:817-831.

Sperlagh B, Baranyi M, Hasko G, Vizi ES (2004) Potent effect of interleukin-1 beta to evoke ATP and adenosine release from rat hippocampal slices. J Neuroimmunol 151:33-39.

Taishi P, Chen Z, Hansen MK, Zhang J, Fang J, Krueger JM (1998) Sleep-associated changes in interleukin-1β mRNA in the brain. Journal of Interferon and Cytokine Research 18:793-798.

Takahashi A, Kishi E, Ishimaru H, Ikarashi Y, Maruyama Y (2001) Role of preoptic and anterior hypothalamic cholinergic input on water intake and body temperature. Brain Res 889:191-199.

Yasuda T, Yoshida H, Garcia-Garcia F, Kay D, Krueger JM (2005) Interleukin-1beta has a role in cerebral cortical state-dependent electroencephalographic slow-wave activity. Sleep 28:177-184.

Ye ZC, Sontheimer H (1996) Cytokine modulation of glial glutamate uptake: a possible involvement of nitric oxide. NeuroReport 7:2181-2185.

Zhu G, Okada M, Yoshida S, Mori F, Hirose S, Wakabayashi K, Kaneko S (2006) Involvement of Ca(2+)-induced Ca2+ releasing system in interleukin-1beta-associated adenosine release. Eur J Pharmacol 532:246-252.

CHAPTER III

SELECTIVE CONTRIBUTIONS OF NEURONAL AND ASTROGLIAL INTERLEUKIN-1 RECEPTOR 1 TO INTERLEUKIN-1-INDUCED ALTERATIONS IN SLEEP AND IMMUNMODULATION

Abstract

Immune challenges alter central nervous system (CNS)-mediated processes and behaviors including immunomodulation and sleep. Interleukin-1β (IL-1) is a pro-inflammatory cytokine that mediates sleep and immune interactions. However, the mechanisms by which the CNS regulates behavior and physiology during immune responses are not fully understood. To this end, we used two transgenic mouse lines that express interleukin-1 receptor 1 (IL1R1) only in the CNS and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1). Mice were instrumented with cranial electroencephalographic electrodes, a thermistor, and a guide cannula positioned in the lateral ventricle. Intracerebroventricular injection of 50 ng IL-1 suppressed rapid eye movement sleep (REMS), increased non-rapid eye movement sleep (NREMS), decreased NREM delta power, and induced fever in wild type (WT) mice. Conversely, IL-1 did not alter sleep or temperature of NSE-IL1R1 or GFAP-IL1R1 mice. Likewise, IL-1 elevated IL-6 expression in brain tissue of WT mice but failed to do so in transgenic animals. However, *de novo* IL-1 was detected in brain tissue of all mouse strains in response to exogenous IL-1 administration. Furthermore, IL-1 induced IL-6 production in NSE-IL1R1 neuronal and GFAP-IL1R1

astroglial cell cultures. Collectively, these data suggest selective IL1R1 expression on neurons or astrocytes is not sufficient for sleep, temperature, or normal *in vivo* immunomodulatory responses to central administration of IL-1.

Introduction

Interactions between sleep and immune function are bidirectional. Insufficient sleep is associated with elevated expression of inflammatory mediators. Likewise, immune challenges alter sleep-wake behavior [reviewed by (Imeri and Opp, 2009; Besedovsky et al., 2012; Krueger et al., 2011)]. Interleukin-1β (IL-1) is pro-inflammatory cytokine that mediates interactions between sleep and immune function.

Central administration of IL-1 induces febrile responses, increases non-rapid eye movement sleep (NREMS), and suppresses rapid eye movement sleep (REMS) (Olivadoti and Opp, 2008; Opp et al., 1991). IL-1-induced alterations in sleep and temperature are attenuated by pharmacological and genetic manipulations of IL-1 activity (Opp and Krueger, 1991; Imeri et al., 1993; Fang et al., 1998). IL-1 exerts its effects via IL-1 receptor 1 (IL1R1) expressed on neurons and astrocytes (Smith et al., 2009), and these cells also produce IL-1 (Bartfai and Schultzberg, 1993; Baumann et al., 1993; Dong and Benveniste, 2001).

Traditionally, astrocytes were relegated to passive constituents of the central nervous system (CNS). However, recent findings demonstrate these cells are active mediators of CNS processes. Astrocytes express receptors for immunomodulators, rapidly respond to inflammation, and modulate sleep homeostasis (Aschner, 1998;

Farina et al., 2007; Frank, 2011; Halassa et al., 2009). The objective of this study was to determine a role for neurons and astrocytes in sleep and immune interactions. To this end, we evaluated sleep and immunomodulatory responses to IL-1 in two transgenic mouse lines that express IL1R1 only in the CNS and selectively on neurons or astrocytes. Understanding the contributions of astrocytes to sleep-immune interactions is important for elucidating the mechanisms that govern a variety of CNS-mediated processes and behaviors.

Methods

Animals

Transgenic and wild type (WT) mice used for these studies were engineered by our lab as previously described in Chapter II. Briefly, transgenic mice were designed on background lacking IL1R1. IL1R1 was rescued by driving expression to neurons with the rat neuron specific enolase [NSE; (Forss-Petter et al., 1990)] promoter or to astrocytes via the human glial fibrillary acidic protein [gfa2; (Brenner et al., 1994)] promoter. Therefore, mice express IL1R1 only in the CNS and selectively on neurons or astrocytes. Mice expressing IL1R1 specifically on neurons are called NSE-IL1R1, whereas mice with astroglial localized expression of IL1R1 are referred to as GFAP-IL1R1. Heterozygous *Il1r1*$^{+/-}$ non-transgenic littermates were mated to generate mice homozygous positive for endogenous *Il1r1* (*Il1r1*$^{+/+}$). These homozygous mice were used as wild type (WT) controls for NSE-IL1R1 and GFAP-IL1R1 mice.

During the studies, adult male WT, NSE-IL1R1, and GFAP-IL1R1 mice (20 – 30 g, 8 – 12 weeks at time of surgery) were obtained from our breeding colony and individually housed in standard cages on a 12:12 h light:dark cycle at 29 ± 1ºC. Food and water were available *ad libitum*. All procedures involving the experimental use of animals were approved by the University of Washington Institutional Animal Care and Use Committee in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

Surgical procedures

Mice used for electroencephalographic (EEG) recordings were stereotaxically instrumented under isoflurane anesthesia with three stainless steel screws (MPX-0080-02P-C, Small Parts Inc., Logansport, IN, USA) and a calibrated 10 kΩ thermistor (AB6E3-GC16KA103L, Thermometrics, Northridge, CA, USA) as previously described (Baracchi and Opp, 2008).

Mice receiving intracerebroventricular (i.c.v.) injections were stereotaxically instrumented with a chronic guide cannula (C315GS, Plastics One Inc., Roanoke, VA, USA) in the right lateral ventricle (AP: -0.5 mm, relative to Bregma; lateral: 1.25 mm relative to the central suture; DV: -2.0 mm relative to the dura). Patency of the cannula was assessed after recovery from surgery by central injection of 250 ng angiotensin II (H-1750, Bachem Americas, Inc., Torrance, CA, USA) in 0.5 µl pyrogen-free saline (PFS). If the cannula is patent and ventricular circulation unobstructed, angiotensin II stimulates a drinking response upon activation of pre-

optic structures (Epstein et al., 1970; Skott, 2003). Only mice with positive drinking responses were included in experimental analyses.

Analgesia for all surgical procedures was provided at the time of surgery by subcutaneous (SC) injection of buprenorphine (0.05 mg/kg) as well as topical application of 4% lidocaine at the incision site. Penicillin (1,200,000 IU/kg; SC) and topical triple antibiotic treatment of the surgical site was also provided to minimize risk of infection. Additional buprenorphine (0.03 mg/kg; SC) was administered 24 h post-surgery. Animals were allowed at least seven days recovery prior to initiation of experimental protocols.

Data Acquisition

EEG and temperature data were obtained by connecting the mice to a lightweight flexible tether that allowed unrestricted movement as previously described (Baracchi and Opp, 2008). Cage activity was monitored by an infrared sensor (BioBserve, GmbH, Bonn, Germany). Digitized EEG signals stored as binary files for subsequent analysis.

Arousal state was determined by visual inspection of the EEG, theta-to-delta ratio, brain temperature, and cage activity with 10 s resolution using custom software (ICELUS, M. Opp, University of Washington) written in LabView for Windows (National Instruments, Austin, Texas, USA) based on previously published criteria (Opp and Krueger, 1994; Opp, 1998). Epochs containing artifacts were excluded from subsequent spectral analyses. The EEG underwent fast Fourier transformation (FFT) to produce power spectra between 0.5 and 30 Hz in 0.5 Hz bins as previously

described (Baracchi and Opp, 2008). Spectral bins within the delta band (0.5 – 4.5 Hz) were normalized to the total NREM power summed across all frequency bins from 0.5 to 30 Hz for the 12h dark and light periods and expressed as a percentage of total power. Hourly NREM delta power data from the dark and light periods were normalized to 12 h dark and light averages, respectively, derived from control conditions.

Central administration of IL-1

After recovery from surgery and adaptation to the recording apparatus, WT (n = 4), NSE-IL1R1 (n = 5), and GFAP-IL1R1 (n = 5) mice were injected i.c.v. in a counterbalanced schedule with vehicle (PFS; 0.5 µl) and 50 ng recombinant mouse IL-1β (401-ML-005/CF, R&D Systems Minneapolis, MN, USA). All injections were given 15 min prior to dark onset, and recordings obtained for 48 h.

Cytokine quantification

Two groups of mice were used to determine the effects of IL-1 administration on cytokine protein concentrations in brain. The first group [WT (n = 6), NSE-IL1R1 (n = 7), GFAP-IL1R1 (n = 6)] received a single i.c.v. injection of vehicle (PFS; 0.5 µl). The second group [WT (n = 6), NSE-IL1R1 (n = 7), GFAP-IL1R1 (n = 7)] was injected with 50 ng IL-1β in 0.5 µl PFS. All injections were given at light onset in order to optimize protein detection. Mice were lightly anesthetized and sacrificed via decapitation 4 h post-injection. Brains were extracted, and the hypothalamus,

hippocampus, and brainstem dissected. Samples were snap frozen in liquid nitrogen and stored at -80°C for subsequent protein extraction.

Frozen tissues were thawed and lysed in a buffer containing cell lysis buffer from the Bio-Rad Cell Lysis Kit (171-304012, Bio-Rad, Hercules, CA, USA), 500 mM phenylmethanesulfonyl fluoride (P7626PMSF, Sigma-Aldrich, St. Louis, MO, USA), and 1% protease inhibitor (S8830, Sigma-Aldrich). Tissue was mechanically disrupted using an ultrasonicator. The resulting homogenate was centrifuged for 20 min at 8,000 rpm at 4°C and the supernatant removed.

The protein concentration of each sample was determined using the bicinchoninic acid (BCA) protein assay kit (23225, Thermo Scientific, Rockford, IL, USA). Bovine serum albumin (BSA) was used to generate the standard curve. Sample absorbances were read at 562 nm on a spectrophotometer.

IL-1 and IL-6 protein concentrations were quantified using a bead-based assay and Luminex xMAP technology (Luminex Corporation, Austin, TX, USA). Custom beads sets were developed in the lab using Luminex MagPlex microspheres (regions 34 and 38 for IL-1β and IL-6, respectively). Beads were conjugated to capture antibodies from IL-1β (DY401) and IL-6 DuoSets (DY406, R&D Systems) via the xMAP Antibody Coupling Kit (40-50016, Luminex Corporation).

Diluents were generated as appropriate for the sample type: cell lysis buffer (171-304012, Bio-Rad) for protein extracts and 0.1% BSA in phosphate buffered saline (PBS) for cell culture supernatant. IL-1 and IL-6 recombinant proteins were used to generate a 7-point standard curve (27, 81, 247, 741, 2,222, 6,667 and 20,000 pg/mL). Samples were loaded in duplicate, and IL-1 and IL-6 were detected

by sequential 30 min incubations with the respective DuoSet detection antibodies and streptavidin-phycoerythrin (S866, Life Technologies).

The plate was read on the Bio-Plex 200 system (Bio-Rad) and data analyzed using Bio-Plex Manager 4.1 software with five-parameter logistic regression (5PL) curve fitting. Observed concentrations were obtained from the Bio-Plex software for all sample types. The cutoff for level of detection was set at the lowest point on the standard curve with an observed concentration between 70-130% of the (observed/expected)*100 value, as suggested by the Bio-Plex 200 manufacturers. Observed concentrations obtained from brain tissue were adjusted for sample protein concentrations determined from BCA assay, and final are values expressed as pg/mL/µg of protein loaded.

Cell culture

Whole brains from NSE-IL1R1 P0 – 2 pups were harvested to generate neuronal cultures. Neurons were plated on poly-L-lysine coated coverslips at a density of 80,000 cells/cm^2 in 24-well plates in NbActiv4 media (Nb4-500, BrainBits, LLC, Springfield, IL, USA) containing 1% penicillin-streptomycin-glutamine (10378-016, Life Technologies). Cultures were incubated at 37°C with 5% CO_2 and received a 50% media change every 3 – 4 days. Neuronal cultures were ready for experimental use 12 – 14 days post-plating.

Astroglial cultures were obtained from whole brains of P0 – 2 GFAP-IL1R1 pups and cultured according to a protocol adapted from Schildege *et al.* (2013). Astrocytes were plated at 10 – 15 x 10^6 cells on poly-L-lysine coated T75 flask in

high glucose Dulbecco's Modified Eagle Medium (DMEM; 11965-092, Life Technologies) containing 10% heat inactivated fetal bovine serum (10082-147, Life Technologies) and 1% penicillin-streptomycin-glutamine. Once astrocytes were confluent, cultures were astrocyte enriched via agitation on an orbital shaker. Resulting astrocytes were re-plated in two poly-L-lysine coated T75 flasks. Approximately 14 days post-split when cells were confluent, cells were trypsinized and evenly distributed to poly-L-lysine-coated 24-well plates. Cultures were incubated at 37°C with 5% CO_2. Cells were ready for experimental use 24 – 48 h post-plating once confluent.

Mature neuronal and astroglial cultures were subjected to three conditions: untreated, vehicle (0.1% BSA in PFS), or IL-1 (neuronal: 50 ng/well; astroglial: 10 ng/well). After 24 h, cell culture supernatant was aliquoted and stored at -80°C until cytokine analysis. IL-6 protein was quantified via bead-based assay using Luminex xMAP technology as described above.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (IBM Corporation, Armonk, NY, USA). Data are presented as means ± standard error of the mean (SEM) or means ± standard deviation (SDEV), as appropriate. Within strain comparisons for percentage of time in REMS and NREMS, normalized NREM delta power, and brain temperature were performed via a general linear model for repeated measures using time (hours) as the repeated measure and manipulation (PFS vs. IL-1) as the between-subjects factor.

Between strain comparisons for cytokine protein quantification were performed using a mixed-effects analysis of variance (ANOVA) designating manipulation (PFS vs. IL-1) and strain (WT, NSE-IL1R1, or GFAP-IL1R1) as between-subjects factors. Post-hoc comparisons by Tukey's HSD test were used to determine differences among mouse strains where appropriate.

One-way ANOVA was used to determine IL-1 effects in cell culture using treatment (untreated, vehicle, IL-1) as the independent variable. If statistically significant treatment effects were revealed, post-hoc comparisons by Tukey's HSD test were used to determine differences among culture manipulations.

An alpha level of $p < 0.05$ was accepted as indicating significant departures from control conditions or strain differences for all tests.

Results

Effects of IL-1 on sleep and temperature

Intracerebroventricular administration of IL-1 (50 ng) altered sleep and brain temperature of WT mice but not of NSE-IL1R1 or GFAP-IL1R1 animals (Figure 3.1). IL-1 transiently increased NREMS, suppressed REMS and NREM delta power, and induced long-lasting fever in WT mice. Repeated measures analysis revealed a manipulation effect of IL-1 on REMS in WT animals, but no subsequent hourly differences were revealed.

IL-1-induced cytokine expression in brain

To determine impact of IL-1 on brain cytokine expression, mice were injected with either PFS or 50 ng IL-1, sacrificed 4 h later, and discrete brain regions dissected. Protein content for IL-1 and IL-6 was determined in hypothalamus, hippocampus, and brainstem (Figure 3.2). IL-1 administration induced IL-1 in hypothalamus and brainstem and tended to increase IL-1 in hippocampus of WT mice. IL-1 also significantly increased IL-1 in hippocampus of NSE-IL1R1 mice and in hippocampus and brainstem of GFAP-IL1R1 animals. Central administration of IL-1 increased IL-6 in all brain regions assayed in WT mice, but not in either of the transgenic mouse strains.

IL-1-induced cytokine expression in cell culture

In vitro responses of neurons and astrocytes to treatment with IL-1 were obtained from cultures of NSE-IL1R1 and GFAP-IL1R1 brains (Figure 3.3). IL-6 production by NSE-IL1R1 neurons and GFAP-IL1R1 astrocytes increased after 24-h challenge with 50 or 10 ng/well IL-1, respectively.

Discussion

Several lines of evidence demonstrate a role for IL-1 in sleep regulation [reviewed by [reviewed by (Krueger et al., 2011; Imeri and Opp, 2009)]. However, the cellular substrates of IL-1 effects on sleep are not fully elucidated. This study aimed to determine the contributions of neurons and astrocytes to IL-1-induced alterations of sleep-wake behavior and immunomodulation. To this end, we used

two transgenic mouse lines that express IL1R1 only in the CNS, and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1). Central administration of IL-1 does not alter sleep or temperature of transgenic mice. This effect may be due to impaired *in vivo* production of IL-6.

Responses of WT mice to central administration of IL-1 are typical of those previously observed in rodents, including suppressed REMS, increased NREMS, diminished NREM delta power, and a prolonged febrile response (Opp et al., 1991; Opp and Krueger, 1991; Olivadoti and Opp, 2008). Unexpectedly, IL-1 has no effect on sleep or temperature of the transgenic mice used in this study. These data indicate that IL1R1 expression specifically on neurons or astrocytes alones is not sufficient for mediating sleep or temperature responses to exogenous IL-1.

To determine potential mechanisms underlying these results, cytokine protein was quantified in brain tissue. IL-1 stimulates its own production (Dinarello, 1988; Dinarello et al., 1987), and the elimination half-life of exogenous IL-1 is approximately 40 - 60 min (Reimers et al., 1991; Loddick and Rothwell, 1996). Therefore, IL-1 detected at our 4 h post-injection collection timepoint is *de novo* IL-1 generated from exogenous IL-1 administration. Indeed, central injection of IL-1 induces *de novo* IL-1 production in all strains of mice. Importantly, elevated IL-1 is not detected in hypothalamic tissue from transgenic mice, which may explain, at least in part, lack of sleep and temperature responses in these strains.

In WT mice, IL-1 administration also increases IL-6 in brain, which is consistent with the literature (Romero et al., 1996; Tsakiri et al., 2008). Interestingly, IL-1 administration does not increase IL-6 protein concentrations in these transgenic

mice. Sleep and temperature responses to intracerebroventricular administration of IL-1 are attenuated in IL-6 knockout mice (Olivadoti and Opp, 2008). Collectively, previously published data and results reported in this chapter suggest that IL-1-induced fever and, to some extent, sleep responses are driven by subsequent production of IL-6.

Interestingly, NSE-IL1R1 neurons and GFAP-IL1R1 astrocytes produce IL-6 in response to IL-1 challenge in culture. IL-1 binding to IL1R1 on neurons and astrocytes induces IL-6 release via unique pathways (Tsakiri et al., 2008). It is unlikely that both neuronal- and astroglial-specific mechanisms of IL-1-induced *in vivo* IL-6 production are disrupted. Therefore, inhibition of *in vivo* IL-6 likely occurs upstream of these pathways. A potential source of inhibition may occur at the cellular level. Endothelial cells are significant cellular substrates of IL-1 effects (Konsman et al., 2004; Thornton et al., 2010). Fevers in response to central IL-1 administration are attenuated in mice with inducible inhibition of IL1R1 on endothelial cells, and endothelial-specific IL1R1 knockdown reduces IL-1-induced hypothalamic c-Fos expression (Ching et al., 2007). Therefore, as with fever, expression of IL1R1 on endothelial cells may be necessary for sleep responses to central administration of IL-1. Although additional experiments are necessary, endothelial IL1R1 might initiate IL-1-induced immune responses, whereas neuronal and astroglial IL1R1 may potentiate these effects. Alternatively, concurrent neuronal and astroglial expression of IL1R1 may be necessary for behavioral and *in vivo* immunomodulatory responses to IL-1. These cells do not work in isolation in the brain. Some neuronal functions are dependent on astrocytes and vice versa (Stevens, 2008; Ghosh et al., 2011).

Further experimentation is needed to determine the central mechanisms of IL-1 activity.

Overall, this study demonstrates that neuronal- or astroglial-specific expression of IL1R1 is not sufficient for IL-1-induced alterations of sleep and temperature. Future studies may aim to identify independent or collaborative contributions of neurons, astrocytes, or other cellular substrates of IL-1 effects in the brain. Continued investigation of the molecular contributions of astrocytes and other neural cells is crucial to fully elucidating the mechanistic underpinnings of a variety of CNS-mediated processes and behaviors.

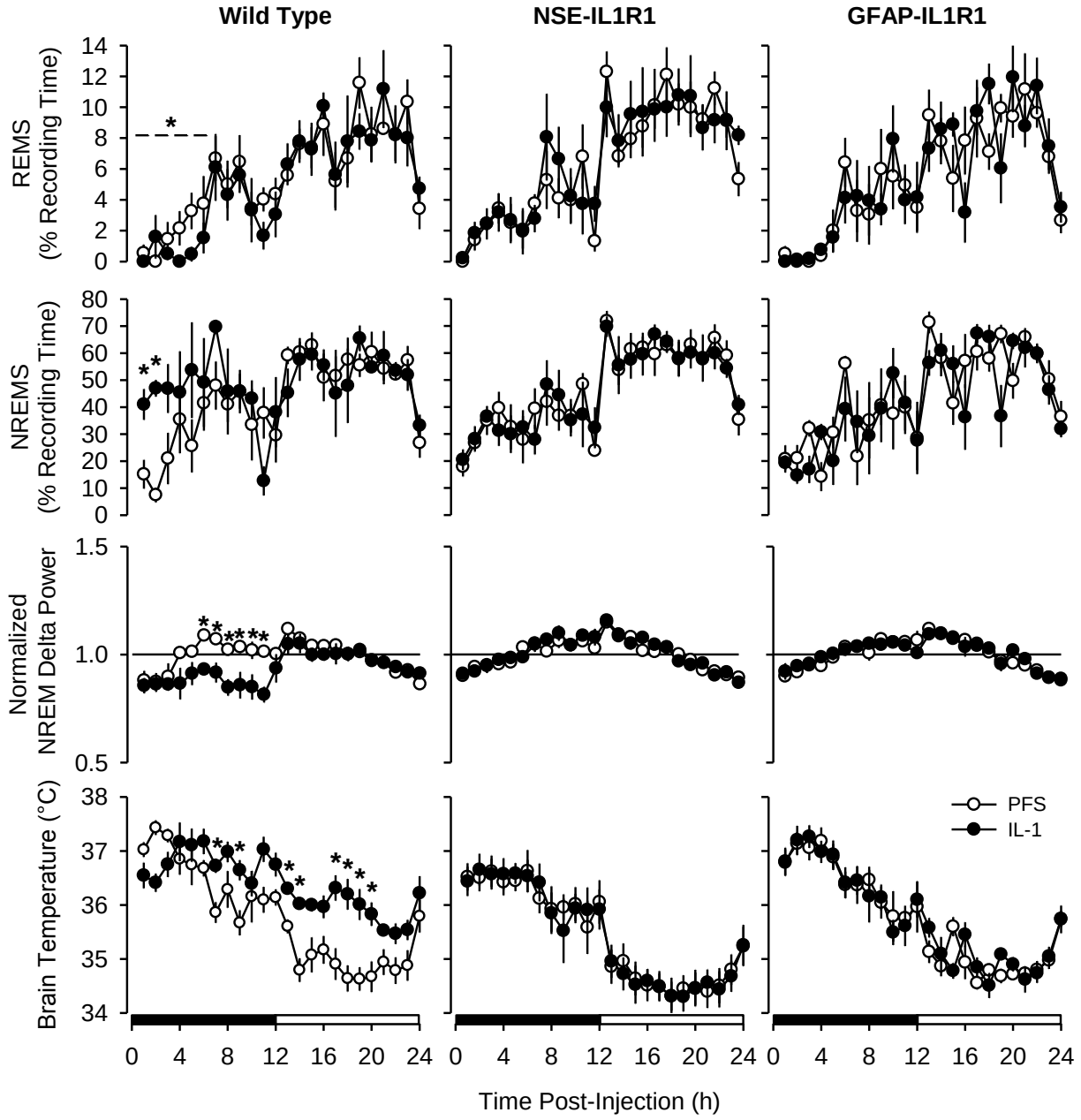


Figure 3.1: Percentage of recording time spent in REMS and NREMS, normalized NREMS delta power, and brain temperature responses obtained after central injection with PFS (open symbols) or IL-1 (filled symbols) for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Hourly data for EEG delta power were normalized to 12 h dark and light averages obtained from PFS data. Statistical analyses were conducted on 6 h time blocks. Asterisk (*) denotes statistical difference of $p < 0.05$ from undisturbed baseline conditions. Dashed line indicates a statistically significant manipulation effect but no post-hoc significance. The filled and open bars on the X-axis indicate dark and light periods of the light-dark cycle, respectively.

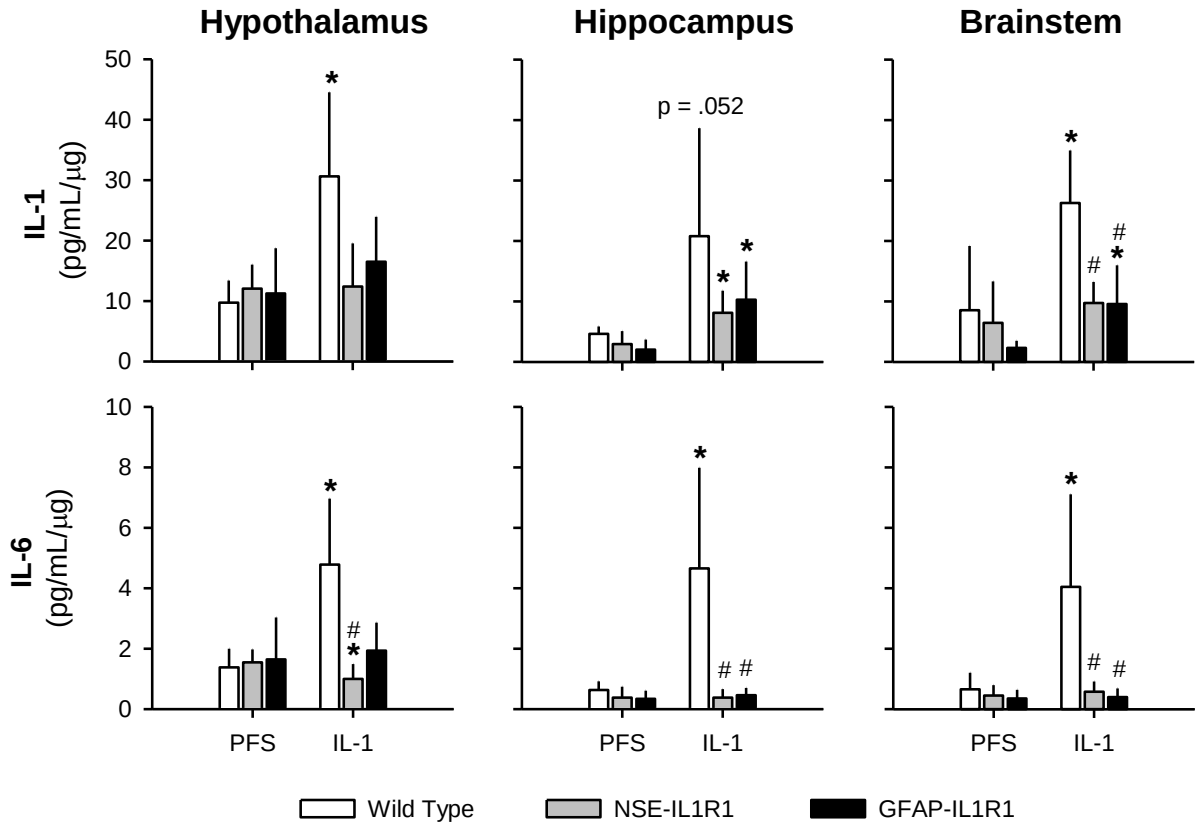


Figure 3.2: Quantification of IL-1 and IL-6 protein extracted from hypothalamus, hippocampus, and brainstem of WT, NSE-IL1R1, and GFAP-IL1R1 mice harvested 4 h post-i.c.v. injection with PFS or 50 ng IL-1. Values are means ± SDEV. Asterisk (*) indicates statistical difference of $p < 0.05$ from PFS. Pound sign (#) denotes statistical difference of $p < 0.05$ from WT IL-1 values.

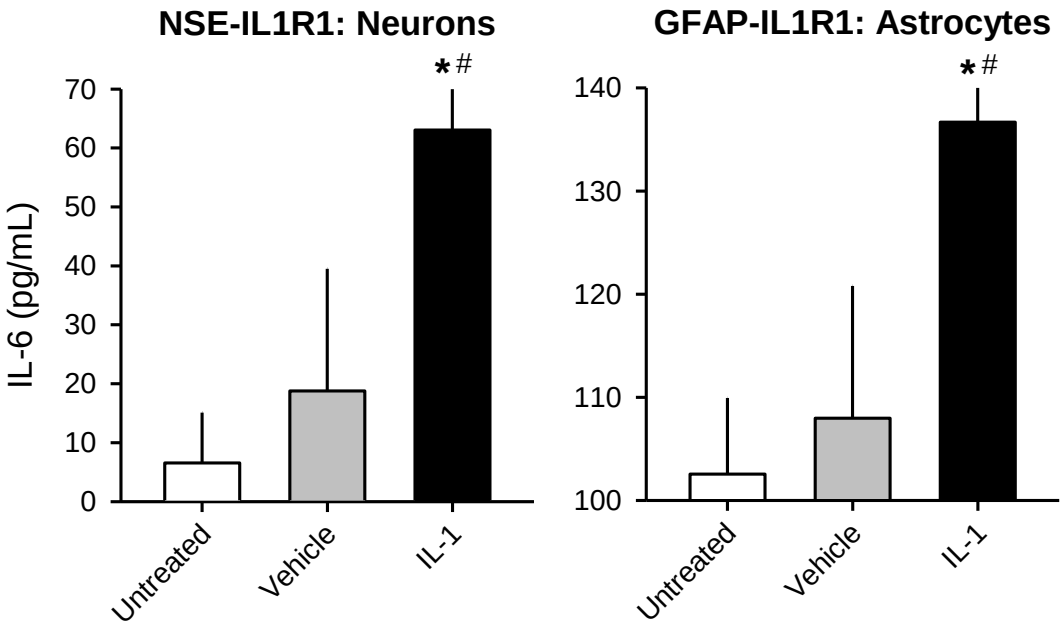


Figure 3.3: Quantification of IL-6 protein from cell culture supernatant of NSE-IL1R1 neuronal and GFAP-IL1R1 astroglial cultures that were untreated, treated with vehicle, or challenged with IL-1 (50 ng/well: neurons; 10 ng/well: astrocytes) for 24 h. Values are means ± SDEV. Asterisk (*) indicates statistical difference of $p < 0.05$ from untreated conditions. Pound sign (#) denotes statistical difference of $p < 0.05$ from vehicle.

References

Aschner M (1998) Immune and inflammatory responses in the CNS: modulation by astrocytes. Toxicology Letters 102–103:283-287.

Baracchi F, Opp MR (2008) Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1. Brain Behav Immun 22:982-993.

Bartfai T, Schultzberg M (1993) Cytokines in neuronal cell types. Neurochem Int 22:435-444.

Baumann N, Baron-Van Evercooren AF, Jacque CF, Zalc B (1993) Glial biology and disorders. Current Opinion in Neurology and Neurosurgery 6:27-33.

Besedovsky L, Lange T, Born J (2012) Sleep and immune function. Pflugers Arch 463:121-137.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. The Journal of Neuroscience 14:1030-1037.

Ching S, Zhang H, Belevych N, He L, Lai W, Pu Xa, Jaeger LB, Chen Q, Quan N (2007) Endothelial-Specific Knockdown of Interleukin-1 (IL-1) Type 1 Receptor Differentially Alters CNS Responses to IL-1 Depending on Its Route of Administration. The Journal of Neuroscience 27:10476-10486.

Dinarello CA (1988) Biology of interleukin-1. FASEB J 2:108-115.

Dinarello CA, Ikejima T, Warner SJC, Orencole SF, Lonnemann G, Cannon JG, Libby P (1987) Interleukin 1 induces interleukin 1 I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. J Immunol 139:1902-1910.

Dong Y, Benveniste EN (2001) Immune function of astrocytes. GLIA 36:180-190.

Epstein AM, Fitzsimons JT, Rolls BJ (1970) Drinking induced by injection of angiotensin into the brain of the rat. J Physiol 210:457-474.

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-R660.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends in Immunology 28:138-145.

Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, Sutcliffe G (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron 5:187-197.

Frank MG (2011) Beyond the neuron: astroglial regulation of mammalian sleep. Current topics in medicinal chemistry 11:2452-2456.

Ghosh M, Yang Y, Rothstein JD, Robinson MB (2011) Nuclear Factor-kappaB Contributes to Neuron-Dependent Induction of Glutamate Transporter-1 Expression in Astrocytes. The Journal of Neuroscience 31:9159-9169.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Imeri L, Opp MR (2009) How (and why) the immune system makes us sleep. Nat Rev Neurosci 10:199-210.

Imeri L, Opp MR, Krueger JM (1993) An IL-1 receptor and an IL-1 receptor antagonist attenuate muramyl dipeptide- and IL-1-induced sleep and fever. Am J Physiol 265:R907-R913.

Konsman JP, Vigues S, Mackerlova L, Bristow A, Blomqvist A (2004) Rat brain vascular distribution of interleukin-1 type-1 receptor immunoreactivity: Relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli. The Journal of Comparative Neurology 472:113-129.

Krueger JM, Clinton JM, Winters BD, Zielinski MR, Taishi P, Jewett KA, Davis CJ (2011) Involvement of cytokines in slow wave sleep. Prog Brain Res 193:39-47.

Loddick SA, Rothwell NJ (1996) Neuroprotective Effects of Human Recombinant Interleukin-1 Receptor Antagonist in Focal Cerebral Ischaemia in the Rat. J Cereb Blood Flow Metab 16:932-940.

Olivadoti MD, Opp MR (2008) Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. Neuroscience 153:338-348.

Opp MR (1998) Rat strain differences suggest a role for corticotropin-releasing hormone in modulating sleep. Physiol Behav 63:67-74.

Opp MR, Krueger JM (1991) Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever. Am J Physiol 260:R453-R457.

Opp MR, Krueger JM (1994) Anti-interleukin-1β reduces sleep and sleep rebound after sleep deprivation in rats. Am J Physiol 266:R688-R695.

Opp MR, Obál F, Krueger JM (1991) Interleukin-1 alters rat sleep: temporal and dose-related effects. Am J Physiol 260:R52-R58.

Reimers J, Wogensen LD, Welinder B, Hejnæs KR, Poulsen SS, Nisson P, Nerup J (1991) The pharmacokinetics and distribution of authentic human recombinant interleukin 1β (rhIL-1β) in normal rats. pp 514.

Romero LI, Kakucska I, Lechan RM, Reichlin S (1996) Interleukin-6 (IL-6) is secreted from the brain after intracerebroventricular injection of IL-1β in rats. Am J Physiol Regul Integr Comp Physiol 270:R518-R524.

Schildge S, Bohrer C, Beck K, Schachtrup C (2013) Isolation and Culture of Mouse Cortical Astrocytes.e50079.

Skott O (2003) Angiotensin II and control of sodium and water intake in the mouse. Am J Physiol Regul Integr Comp Physiol 284:R1380-R1381.

Smith DE, Lipsky BP, Russell C, Ketchem RR, Kirchner J, Hensley K, Huang Y, Friedman WJ, Boissonneault V, Plante MMl, Rivest S, Sims JE (2009) A Central Nervous System-Restricted Isoform of the Interleukin-1 Receptor Accessory Protein Modulates Neuronal Responses to Interleukin-1. Immunity 30:817-831.

Stevens B (2008) Neuron-Astrocyte Signaling in the Development and Plasticity of Neural Circuits. Neurosignals 16:278-288.

Thornton P, McColl BW, Cooper L, Rothwell NJ, Allan SM (2010) Interleukin-1 drives cerebrovascular inflammation via MAP kinase-independent pathways. Current neurovascular research 7:330-340.

Tsakiri N, Kimber I, Rothwell NJ, Pinteaux E (2008) Interleukin-1-induced interleukin-6 synthesis is mediated by the neutral sphingomyelinase/Src kinase pathway in neurones. Brit J Pharmacol 153:775-783.

CHAPTER IV

SLEEP AND IMMUNOMODULATORY RESPONSES TO SYSTEMIC LIPOPOLYSACCHARIDE IN MICE WITH SELECTIVE INTERLEUKIN-1 RECEPTOR 1 EXPRESSION ON NEURONS OR ASTROCYTES

Abstract

Sleep-wake behavior is altered in response to immune challenges. Although the mechanisms that govern sickness-induced sleep changes are not fully understood, interleukin-1β (IL-1) is a known mediator of these interactions. To further clarify the underlying substrates of sleep and inflammatory responses to immune challenge, we used two transgenic mouse strains that express interleukin-1 receptor 1 only in the central nervous system and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1). Electroencephalographic and temperature recordings from transgenic and wild type (WT) mice revealed systemic challenge via intraperitoneal administration of lipopolysaccharide (LPS) suppressed rapid eye movement sleep (REMS), increased non-REMS (NREMS), diminished NREM delta power, and induced febrile responses in all mouse strains. However, GFAP-IL1R1 mice had greater REMS suppression compared to NSE-IL1R1 animals and elevated NREM delta power compared to WT and NSE-IL1R1 mice. Quantification of cytokine protein showed that LPS induces IL-1 and IL-6 production in NSE-IL1R1 and GFAP-IL1R1 brain tissue but at concentrations generally less than those of WT mice. This effect was more evident in GFAP-IL1R1 brain tissue. However,

peripheral expression of these cytokines in transgenic plasma did not differ from WT. Taken together, these data suggest neuronal IL1R1 activity may modulate central aspects of pathological sleep and immunomodulatory responses to systemic challenge with LPS.

Introduction

Sleep and immune function are mutually influential. Sleep loss impairs immune function, and sleep-wake behavior is modified in response to immune challenges [reviewed by (Imeri and Opp, 2009)]. Nevertheless, the mechanistic underpinnings of these interactions are not well understood. However, the pro-inflammatory cytokine interleukin -1β (IL-1) is an immunomodulator extensively implicated in mediating sleep and immune interactions.

Systemic immune challenges triggers acute host defense responses that upregulate IL-1 in the brain and elicit clinical signs of illness (Van Dam et al., 1992). Indeed, symptoms of sickness, such as sleep-wake alterations, anorexia, loss of body weight, social withdrawal, and fever are induced by central administration of IL-1 (Opp, 2005; Dantzer and Kelley, 2007). Brain regions associated with the regulation of sleep-wake behavior, feeding, social behavior, and thermoregulation are immunoreactive for IL-1 (Breder et al., 1988; Konsman and Dantzer, 2001; Datta and Opp, 2008). IL-1 is also a recognized sleep regulatory substance. This distinction was earned, in part, by the ability of IL-1 administration to suppress rapid eye movement sleep (REMS) and increases non-REMS (NREMS) by acting on defined sleep circuitry (Olivadoti and Opp, 2008; Fang et al., 1998). Furthermore,

inflammatory-induced alterations in sleep are attenuated by inhibition of IL-1 activity (Baracchi and Opp, 2008; Fang et al., 1998). IL-1 exerts its effects via IL-1 receptor 1 (IL1R1) which is constitutively expressed in the brain and present on neurons and astrocytes (Smith et al., 2009; Farina et al., 2007).

Although neurons produce cytokines (Breder et al., 1988; Bartfai and Schultzberg, 1993), the majority of immunomodulator production and release is managed by glial cells such as astrocytes (Baumann et al., 1993; Dong and Benveniste, 2001; Mason et al., 2001). Astrocytes express receptors for immunomodulators, respond rapidly to inflammation, and produce sleep regulatory substances in response to immune challenges (Halassa et al., 2007; Pinteaux et al., 2002; Sugama et al., 2011; Farina et al., 2007; Ingiosi et al., 2013). Recent studies also report that astrocytes regulate sleep homeostasis and physiology (Halassa et al., 2009; Frank, 2013; Nadjar et al., 2013). However, it is unknown to what extent neuronal-glial interactions influence complex behavior and physiology during immune challenges.

The objective of this study was to determine cellular contributions to systemic immune challenge by targeting IL1R1 expression specifically to neurons or astrocytes. In these experiments, two lines of transgenic mice that express IL1R1 only in the central nervous system (CNS) and selectively on neurons or astrocytes were administered lipopolysaccharide (LPS) intraperitoneally to determine the extent to which neurons and astrocytes mediate immune challenges of peripheral origin. LPS is a component of the Gram-negative bacteria cell wall that induces inflammatory cascades which includes IL-1 production (Datta and Opp, 2008). The

behavioral and molecular analyses presented here provide foundational insights into the cellular intricacies of central responses to systemic immune challenge.

Methods

Animals

Transgenic and wild type (WT) mice used for these studies were engineered by our lab as previously described in Chapter II. Briefly, transgenic mice were designed on a background lacking IL1R1. IL1R1 was rescued by driving expression to neurons with the rat neuron specific enolase [NSE; (Forss-Petter et al., 1990)] promoter or to astrocytes via the human glial fibrillary acidic protein [gfa2; (Brenner et al., 1994)] promoter. Therefore, mice express IL1R1 only in the CNS and selectively on neurons or astrocytes. Mice expressing IL1R1 specifically on neurons are called NSE-IL1R1, whereas mice with astroglial localized expression of IL1R1 are referred to as GFAP-IL1R1. Heterozygous *Il1r1*$^{+/-}$ non-transgenic littermates were mated to generate mice homozygous positive for endogenous *Il1r1* (*Il1r1*$^{+/+}$). These homozygous mice served as WT controls for NSE-IL1R1 and GFAP-IL1R1 mice.

During the studies, adult male WT, NSE-IL1R1, and GFAP-IL1R1 mice (20 – 30 g, 8 – 12 weeks at time of surgery) were obtained from our breeding colony and individually housed in standard cages on a 12:12 h light:dark cycle at 29 ± 1ºC, an ambient temperature that is within the murine thermoneutral zone (Gordon and White, 1985; Rudaya et al., 2005). Food and water were available *ad libitum*. All procedures involving the use of animals were approved by the University of

Washington Institutional Animal Care and Use Committee in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals.

Surgical procedures

Mice used for electroencephalographic (EEG) recordings were stereotaxically instrumented under isoflurane anesthesia with three stainless steel screws (MPX-0080-02P-C, Small Parts Inc., Logansport, IN, USA) and a calibrated 10 kΩ thermistor (AB6E3-GC16KA103L, Thermometrics, Northridge, CA, USA) as previously described (Baracchi and Opp, 2008). Analgesia was provided at the time of surgery by subcutaneous (SC) injection of buprenorphine (0.05 mg/kg) as well as topical application of 4% lidocaine at the incision site. Penicillin (1,200,000 IU/kg; SC) and topical triple antibiotic treatment of the surgical site was also provided to minimize risk of infection. Additional buprenorphine (0.03 mg/kg; SC) was administered 24 h post-surgery. Animals were allowed at least seven days recovery prior to initiation of experimental protocols.

Data acquisition

EEG and temperature data were obtained using a lightweight tethered swinging arm system which allowed for unrestricted cage navigation as previously described (Baracchi and Opp, 2008). Cage activity was monitored by an overhead infrared sensor (BioBserve, GmbH, Bonn, Germany). Digitized EEG signals were

filtered using Chebyschev filters with third order coefficients into delta (0.5 – 4.5 Hz) frequency bands and stored as binary files for subsequent analysis.

Arousal state was determined by visual inspection of the EEG, theta-to-delta ratio, brain temperature, and cage activity with 10 s resolution using custom software (ICELUS, M. Opp, University of Washington) written in LabView for Windows (National Instruments) based on previously published criteria (Opp and Krueger, 1994; Opp, 1998). Epochs containing artifacts were excluded from subsequent spectral analyses. The EEG underwent fast Fourier transformation (FFT) to produce power spectra between 0.5 and 30.0 Hz in 0.5 Hz bins as previously described (Baracchi and Opp, 2008). Spectral bins within the delta band (0.5 – 4.5 Hz) were normalized to the total NREM power summed across all frequency bins from 0.5 to 30.0 Hz for the 12h dark and light periods and expressed as a percentage of total power. Hourly NREM delta power data from the dark and light periods were normalized to 12 h dark and light averages, respectively, derived from control conditions.

Systemic administration of LPS

After recovery from surgery and habituation to the recording apparatus, WT (n = 7), NSE-IL1R1 (n = 7), and GFAP-IL1R1 (n = 7) mice received an intraperitoneal (i.p.) injection of vehicle [0.2 mL, pyrogen-free saline (PFS)]. After 48 h, mice received 0.4 mg/kg LPS (Escherichia coli serotype 0111:B4, Sigma-Aldrich, St. Louis, MO, USA). All injections occurred 15 min prior to dark onset (DO). Although it would be ideal to counterbalance saline and LPS injections, LPS challenge

occurred subsequent to vehicle administration as it is unknown how long the effects of LPS persist. EEG and temperature data were collected for 48 h following each injection beginning at DO. Daily body weight, food consumption, and water intake data were obtained via scale measurements 15 min prior to DO.

Cytokine quantification

Two groups of WT and transgenic mice were used to determine the impact of LPS on cytokine expression. The first group [WT (n = 6), NSE-IL1R1 (n = 5), GFAP-IL1R1 (n = 5)] received a single i.p. injection of vehicle (PFS; 0.2 mL). The second group [WT (n = 7), NSE-IL1R1 (n = 7), GFAP-IL1R1 (n = 7)] was injected with 0.4 mg/kg LPS i.p. In order to improve probability of detection, injections occurred at light onset when the diurnal concentration of the proteins of interest are more elevated (Cearley et al., 2003; Guan et al., 2005). All mice were anesthetized 4 h post-injection. Blood was obtained via orbital bleed for subsequent plasma retrieval. Mice were then decapitated, and the hypothalamus, hippocampus, and brainstem were harvested and snap frozen in liquid nitrogen. Blood was centrifuged for 20 min at 3000 rpm at 4°C, and the resulting supernatant (plasma) was collected. All samples were stored at -80°C until subsequent analysis.

Protein extractions and quantification were performed as previously described in Chapter III. Briefly, frozen brain samples were transferred to lysis buffer (171-304012, Bio-Rad, Hercules, CA, USA) to thaw. Samples were then mechanically disrupted using an ultrasonicator, homogenates centrifuged, and the resulting supernatant stored at -80°C until further processing. The protein concentration of

each sample was determined using the bicinchoninic acid (BCA) protein assay kit (23225, Thermo Scientific, Rockford, IL, USA).

IL-1 and IL-6 protein concentrations were quantified using a bead-based assay and Luminex xMAP technology (Luminex Corporation, Austin, TX, USA) as previously described in Chapter III. Custom beads sets were developed using IL-1β (DY401, R&D Systems, Minneapolis, MN, USA) and IL-6 (DY406, R&D Systems) DuoSets via the xMAP Antibody Coupling Kit (40-50016, Luminex Corporation). Tumor necrosis factor α was also quantified but was not detected in WT or transgenic brain tissue samples (data not shown).

Diluents were generated as appropriate for the sample type: cell lysis buffer (171-304012, Bio-Rad) for protein extracts and 5% normal donkey serum (017-000-121, Jackson ImmunoResearch, West Grove, PA, USA) in phosphate buffered saline for plasma samples. Samples were quantified in duplicate via the Bio-Plex 200 system (Bio-Rad) and data analyzed using Bio-Plex Manager 4.1 software. Observed concentrations obtained from plasma samples are reported as pg/mL. Brain tissue values were adjusted for sample protein concentrations determined from BCA assay, and final are values expressed as pg/mL/µg of protein loaded.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (IBM Corporation, Armonk, NY, USA). Data are presented as means ± standard error of the mean (SEM) of means ± standard deviation (SDEV). Within strain comparisons for percent time in REMS and NREMS, normalized NREM delta power, sleep state

transitions, and brain temperature were performed via a general linear model for repeated measures using time (hours) as the repeated measure and manipulation (PFS vs. LPS) as the between-subjects factor. LPS effects on 12 h dark and light period NREM delta power and brain temperature means for each strain were analyzed with independent-samples t-test using manipulation (PFS vs. LPS) as the grouping variable.

Between strain comparisons for calculated difference scores for LPS effects and normalized NREM delta power were performed in 12 h time blocks using one-way analysis of variance (ANOVA) with strain (WT, NSE-IL1R1, GFAP-IL1R1) as the independent variable. If statistically significant strain effects were revealed, post-hoc comparisons by Tukey's HSD test were used to determine differences among mouse strains.

Within and between strain comparisons for body weight, food and water intake, and cytokine protein quantification were performed using a mixed-effects ANOVA designating manipulation (PFS vs. LPS) and strain (WT, NSE-IL1R1, or GFAP-IL1R1) as between-subjects factors. Post-hoc comparisons by Tukey's HSD test were used to determine differences among mouse strains where appropriate.

An alpha level of $p < 0.05$ was accepted as indicating significant departures from control conditions or strain differences for all tests.

Results

Effects of LPS on sleep and temperature

To determine the contributions of neuronal- and astroglial-driven IL1R1 modulation of systemic immune responses, transgenic and WT mice were injected intraperitoneally (i.p.) with 0.4 mg/kg LPS. In response to LPS, all mice exhibited REMS suppression during the dark and light periods, increased NREMS during the dark period, and a greater number of sleep state transitions 18 h post-injection compared to injection with PFS (Figure 4.1). Additionally, repeated measures analysis revealed LPS induced a febrile response in WT mice during the light period, whereas GFAP-IL1R1 animals had a hypothermic response during the dark period. NSE-IL1R1 mice did not exhibit a significant, hourly temperature response to LPS. However, analysis of mean brain temperature for the 12 h dark and light periods showed LPS induced hypothermic responses in the dark period for WT and GFAP-IL1R1 mice and light period febrile responses in all strains (Figure 4.1). The NSE-IL1R1 hypothermic response in the dark period trended towards significance.

The influence of neuronal and astroglial IL1R1 on LPS alterations of NREM EEG spectra were also evaluated as an additional measure of sleep quality (Borbély, 1982). Examination of the hourly responses demonstrated that LPS challenge diminished normalized NREM delta power for 18 h post-injection in WT mice which was followed by a significant rebound of NREM delta power (Figure 4.1). NSE-IL1R1 mice saw a small reduction in normalized NREM delta power at hour 12 post-LPS administration compared to PFS. Although LPS appeared to suppress NREM delta power on an hourly basis in GFAP-IL1R1 mice, these departures did not reach statistical significance. However, examination of the 12 h means during the dark and light period revealed NREM delta power of WT and NSE-IL1R1 mice

was reduced during the dark period, whereas GFAP-ILR1 delta power was enhanced during the light period (Figure 4.1).

In order to determine the magnitude of LPS effects on sleep-wake behavior and temperature among the WT and transgenic mice, differences scores from PFS conditions were calculated (Figure 4.2). LPS manipulated NREMS and brain temperature to the same extent in all mouse strains. However, GFAP-IL1R1 mice exhibited greater suppression of REMS 24 h post-injection compared to NSE-IL1R1 animals. Additionally, the NREM delta power of GFAP-IL1R1 mice saw an overall enhancement during the light period compared to WT and NSE-IL1R1 mice. Finally, the sleep of NSE-IL1R1 mice was more consolidated following LPS challenge compared to WT and GFAP-IL1R1 animals as evidenced by fewer sleep state transitions.

Impact of LPS on symptoms of clinical illness

LPS effects on body weight, food consumption, and water intake in WT and transgenic mice were analyzed as additional clinical measures of immune response (Figure 4.3). All mouse strains exhibited a reduction in body weight and food and water intake after LPS challenge. Calculated difference scores revealed these responses were of the same magnitude for all strains except for NSE-IL1R1 water consumption which was lesser than that of WT mice. Additionally, GFAP-IL1R1 mice drank less water under control conditions compared to WT and NSE-IL1R1 animals.

LPS-induced alterations of cytokine expression

To more fully understand neuronal and astroglial contributions to LPS-induced immune responses, cytokine proteins were quantified in discrete brains regions of WT and transgenic mice after i.p. administration of PFS and LPS (Figure 4.4). IL-1 and IL-6 protein concentrations were elevated after LPS challenge in hypothalamus, hippocampus, and brainstem of all mouse strains. The only exception was increased NSE-IL1R1 hypothalamic IL-1 protein did not achieve statistical significance. Although IL-1 and IL-6 protein concentrations did not differ among mouse strains under control conditions, NSE-IL1R1 production of IL-6 was blunted in hippocampus and brainstem compared to WT following LPS challenge. Attenuation of LPS-induced cytokine production in GFAP-IL1R1 brain regions was even more pronounced with statistically significant departures from WT in all conditions except for hypothalamic IL-1.

IL-1 and IL-6 protein concentrations were also quantified in plasma to assess the effects of LPS on peripheral immune responses of WT, NSE-IL1R1, and GFAP-IL1R1 mice (Figure 4.5). LPS administration increased plasma concentrations of IL-1 and IL-6 in all mouse strains. Furthermore, transgenic plasma cytokine concentrations did not differ statistically from WT conditions.

Discussion

The aim of this study was to determine the contributions of neurons and astrocytes to sleep-wake behavior and immunomodulation during systemic immune challenge. We used two transgenic mouse lines that express IL1R1 only in the

CNS, and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1) to dissect cellular modulation of LPS effects. In response to LPS, GFAP-IL1R1 mice have greater REMS suppression compared to NSE-IL1R1 mice and elevated NREM delta power compared to WT and NSE-IL1R1 mice. Additionally, NSE-IL1R1 mice exhibit more consolidated sleep during LPS challenge. Finally, LPS induces elevations in brain and plasma cytokine protein in WT and transgenic mice. However, transgenic cytokine expression in brain in many instances is blunted compared to WT conditions.

In accordance with previous studies (Morrow and Opp, 2005; Nadjar et al., 2013), systemic LPS administration decreases REMS in all mouse strains. This effect is more pronounced in GFAP-IL1R1 mice compared to NSE-IL1R1 animals. Although numerous studies detail the suppressive influence of LPS on REMS, the mechanisms that underlie this alteration in sleep behavior are not well understood. However, it is known that IL-1 administration suppresses REMS (Olivadoti and Opp, 2008; Fang et al., 1998), and this response is mediated, in part, by the serotonergic system (Brambilla et al., 2007; Manfridi et al., 2003). Serotonergic neurons in dorsal raphe nucleus of the brainstem activate REM-OFF neurons to inhibit the occurrence of REMS (McCarley, 2007). In genetically intact animals, LPS and IL-1 induce serotonin reuptake via stimulation of the serotonin transporter (SERT). Quantification of brainstem cytokine protein reveals LPS-induced IL-1 expression is attenuated in GFAP-IL1R1 mice. The lack of IL-1 expression in GFAP-IL1R1 mice may limit SERT activity resulting in a greater concentration of serotonin in the extracellular space, and thus, prolonged activation of REM-OFF neurons. However,

further experimentation is needed. Whatever the mechanism may be, it is interesting to note that IL-1 expression in NSE-IL1R1 brainstem following LPS does not differ from GFAP-IL1R1 mice, yet REMS suppression is more pronounced in GFAP-IL1R1 animals. Therefore, the variance of LPS-induced REMS suppression between GFAP-IL1R1 and NSE-IL1R1 mice despite similar central expression of IL-1 suggests differential effects of neuronal- and astroglial- derived IL-1 on REMS circuitry during LPS immune challenge.

Although NREMS is elevated to the same extent in WT and transgenic mice following challenge with LPS, alterations to the NREM EEG spectra differ among strains. LPS reduces the percentage of the total NREM spectra distributed to the delta frequency band during the dark period in WT and NSE-IL1R1, but the magnitude of this effect is similar among WT and transgenic mice. In the light period, however, LPS induced an overall elevation of NREM delta power in GFAP-IL1R1 mice, an effect that was greater than WT and NSE-IL1R1 responses. NREM delta power is considered a measure of sleep quality and depth (Borbély, 1982). Therefore, despite increased time spent in NREMS, these data suggest NREMS is less restorative in WT and NSE-IL1R1 mice compared to GFAP-IL1R1 mice in response to LPS. Furthermore, neuronal IL1R1 activity may be driving LPS-induce reductions in NREM delta power.

The mechanisms that govern NREM spectral characteristics have yet to be fully identified. However, a recent study demonstrated that astrocyte-derived adenosine mediates LPS-induced alterations of delta power, and LPS effects on delta power are ameliorated by inhibition of astroglial gliotransmission (Nadjar et al.,

2013). Data from this current study are not entirely consistent with these findings. In contrast to the experiments presented here, LPS administration occurred at light onset in the study by Nadjar and colleagues which elevates NREM delta power. Therefore, the discrepancy may be due to the diurnal timing of immune challenge (Morrow and Opp, 2005). Alternatively, LPS-induced gliotransmission may not be dependent on activation of astroglial IL1R1.

An additional measure of sleep quality and efficiency is sleep state consolidation [reviewed by (Imeri and Opp, 2009)]. LPS increases the number of sleep state transitions in all mice, a response generally indicative of poorer sleep. However, sleep fragmentation is less robust in NSE-IL1R1 mice. This study and others demonstrate LPS upregulates IL-1 in brain (Datta and Opp, 2008; Zielinski et al., 2013), and intracerebroventricular administration of IL-1 fragments sleep (Olivadoti and Opp, 2008). Collectively, these data suggest that IL1R1 activation on astrocytes may modulate sleep state maintenance and/or transitions during LPS immune challenge.

LPS effects on temperature were small on an hourly scale, but these responses accumulate during the dark and light periods for an overall hypothermic and febrile response, respectively, in WT and transgenic mice. Furthermore, LPS alters temperature to the same extent across all mouse strains. Considering that inhibition of IL-1 and IL1R1 activity does not eliminate fever in response to LPS (Lundkvist et al., 1999; Alheim et al., 1997; Leon, 1996), the data indicate that LPS-induced temperature responses are not dependent on neuronal- or astroglial-specific expression of IL-1.

Consistent with the literature, LPS induces weight loss and attenuates feeding and drinking behavior in all mouse strains. The magnitude of LPS effects on body weight and food intake do not differ among WT and transgenic mice. This result is not entirely surprising as LPS induces weight loss and anorexia even in IL1R1 knockout mice, effects that may be mediated more so by the periphery (Bluthé et al., 2000). In terms of drinking behavior, the effect of LPS on NSE-IL1R1 adipsia is attenuated compared WT suggesting astroglial IL1R1 activity may play a role in water intake. Indeed, immunoreactivity of glial fibrillary acidic protein, an astroglial marker, fluctuates in accordance with hydration status in the supraoptic nucleus of the hypothalamus (Hawrylak et al., 1998). Although the LPS-induced reduction of water intake GFAP-IL1R1 mice does not differ from WT or NSE-IL1R1 animals, GFAP-IL1R1 mice drink less water under control conditions. These data suggest that neurons mediate physiological drinking responses, whereas astrocytes modulate water intake under pathological conditions.

In order to better understand the underlying immune responses mediating behavioral alterations to LPS, cytokine protein was quantified. LPS upregulates IL-1 and IL-6 expression in brain and plasma of all mouse strains. This effect is in many cases is less robust in transgenic brain tissue compared to WT. Although the IL-6 system is intact, IL-1 mediates IL-6 expression post-LPS (Luheshi et al., 1996; Kuida et al., 1995). Therefore, reduced IL-1 protein expression likely accounts for the accompanying attenuation of IL-6 expression.

Unlike brain cytokine expression, there were no statistically significant differences among the strains regarding IL-1 and IL-6 plasma concentrations in

response to LPS. LPS actions are initiated by activation of toll-like receptor 4 resulting subsequent production of cytokines including tumor necrosis factor α (TNF), IL-1, and IL-6. Despite attenuated brain IL-1 and IL-6 expression in transgenic mice, NSE-IL1R1 and GFAP-IL1R1 animals still manifest behavioral responses to LPS challenge. Taken together, these data may indicate that peripheral cytokine expression is driving the behavioral alterations to LPS, and IL-1 and IL-6 may not be crucial mediators of these responses. However, this is not likely the case as blockade of central responses to peripheral challenge can diminish manifestation of symptoms of sickness induced by LPS (Imeri et al., 2006; Luheshi et al., 1996; Morrow and Opp, 2005). Although peripheral challenge with a low dose of LPS alters sleep and central immunomodulation through stimulation of vagal afferents, the high dose of LPS used in this study may affect central responses via a different mechanism (Zielinski et al., 2013) such as increasing blood-brain barrier (BBB) permeability (Xaio et al., 2001; Minami et al., 1998). In fact, LPS itself can cross the BBB when administered peripherally at high doses (Banks and Robinson, 2010). Therefore, LPS may be acting directly at TLR4 receptors in the brain to alter sleep and temperature of the WT and transgenic mice.

Behavioral responses to LPS in transgenic mice may also be modulated by compensatory actions of other cytokines such as TNF. IL1R1 knockout mice are responsive to LPS challenge (Leon, 1996; Bluthé et al., 2000), but LPS effects are inhibited by antagonism of TNF in these animals (Bluthé et al., 2000). Interestingly, TNF inhibition in wild type mice does not attenuate LPS-induced symptoms of sickness, thus indicating compensation by TNF in the absence of IL1R1 (Bluthé et

al., 2000). Although in the current study IL1R1 in not entirely absent and central TNF expression was not detected in any condition (data not shown), these factors do not discount the possibility of some degree of immunomodulatory compensation of the IL-1 system.

Overall, these studies demonstrate the complexity of cellular mechanisms that underlie behavioral and immune responses to inflammatory challenge. To our knowledge, these are the first studies to investigate the sufficiency of neuronal- and astroglial-specific contributions to peripheral immune challenge. Continued study of non-neuronal cells will be invaluable to better understanding a variety of CNS-mediated processes and behaviors as they relate to sickness and disease.

Figure 4.1: Percentage of recording time spent in REMS and NREMS, normalized NREM delta power, sleep state transitions, and brain temperature responses obtained after i.p. injection with PFS (open symbols) or LPS (filled symbols) for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Hourly data for EEG delta power were normalized to 12 h PFS averages for the dark and light periods. Statistical analyses were conducted on 6 and 12 h time blocks. Graphical insets for NREM delta power (Δ power) and brain temperature (Tbr) depict 12 h mean comparisons between PFS (open bars) and LPS (filled bars) effects for the dark (D) and light (L) periods. Asterisk (*) denotes statistical difference of $p < 0.05$ from PFS. The filled and open bars on the X-axis indicate dark and light periods of the light-dark cycle, respectively.

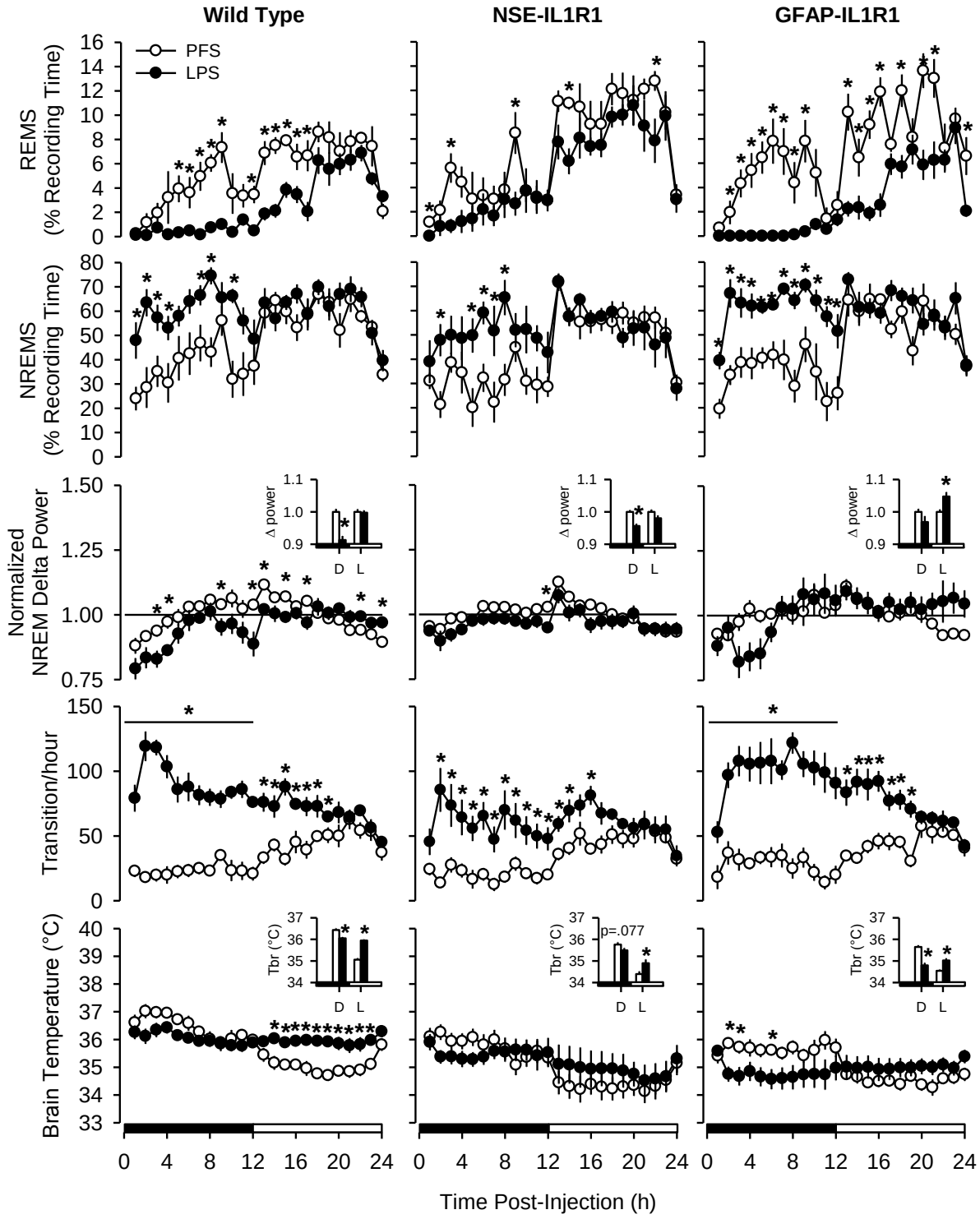
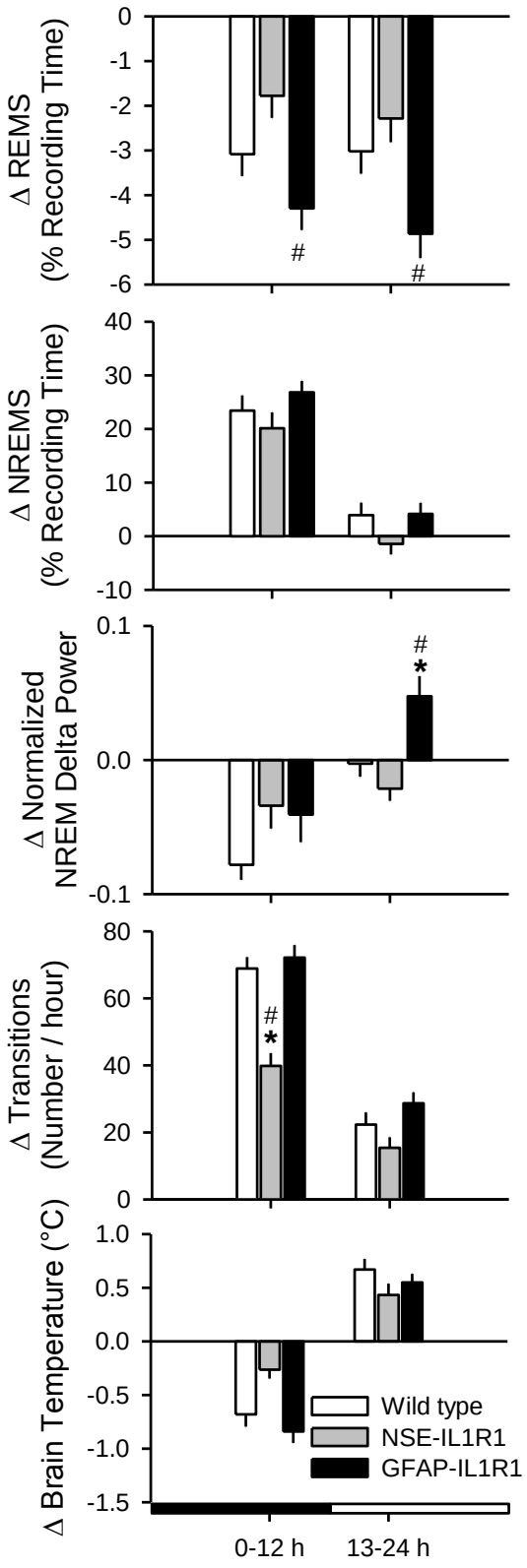

Wild Type
NSE-IL1R1
GFAP-IL1R1
PFS
LPS
REMS (% Recording Time)
NREMS (% Recording Time)
Normalized NREM Delta Power
Δ power
Transition/hour
Brain Temperature (°C)
Tbr (°C)
p=.077
D L
Time Post-Injection (h)

Figure 4.2: Effects of intraperitoneal injection with LPS shown as differences from PFS for percent recording time spent in REMS and NREMS, normalized EEG delta power during NREMS, number of sleep state transitions, and brain temperature plotted in 12 h time blocks for WT, NSE-IL1R1, and GFAP-IL1R1 mice. Values are means ± SEM. Asterisk (*) indicates a statistical difference of $p < 0.05$ from WT. Pound symbol (#) denotes a statistical difference of $p < 0.05$ from NSE-IL1R1. Filled and open bars on the X-axis indicate dark and light portions of the light-dark cycle, respectively.


Δ REMS (% Recording Time)
0
-1
-2
-3
-4
-5
-6
#
#
Δ NREMS (% Recording Time)
40
30
20
10
0
-10
Δ Normalized NREM Delta Power
0.1
0.0
-0.1
#
*
Δ Transitions (Number / hour)
80
60
40
20
0
#
*
Δ Brain Temperature (°C)
1.0
0.5
0.0
-0.5
-1.0
-1.5
Wild type
NSE-IL1R1
GFAP-IL1R1
0-12 h
13-24 h
Time Post-Injection

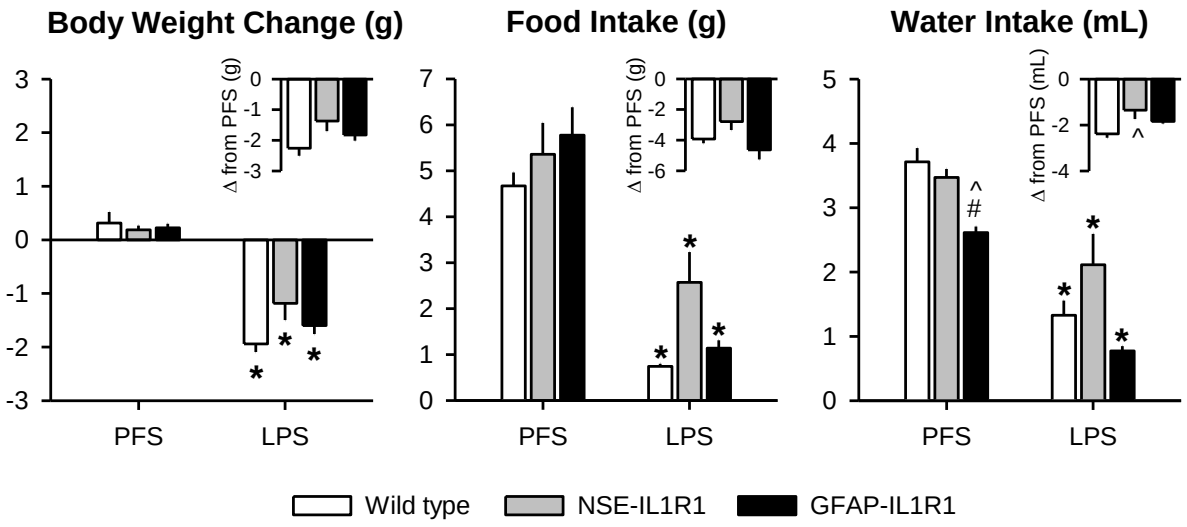


Figure 4.3: Effects of intraperitoneal administration of PFS and LPS on body weight, food intake, and water intake for WT, NSE-IL1R1, and GFAP-IL1R1 over a 24 h period. Values are means ± SEM. Graphical insets depict differences from PFS. Asterisk (*) denotes a statistical difference of $p < 0.05$ from PFS. Pound sign (#) indicates a statistical difference of $p < 0.05$ from NSE-IL1R1 under the same condition. Carrot symbol (^) shows statistical difference of $p < 0.05$ from WT difference scores.

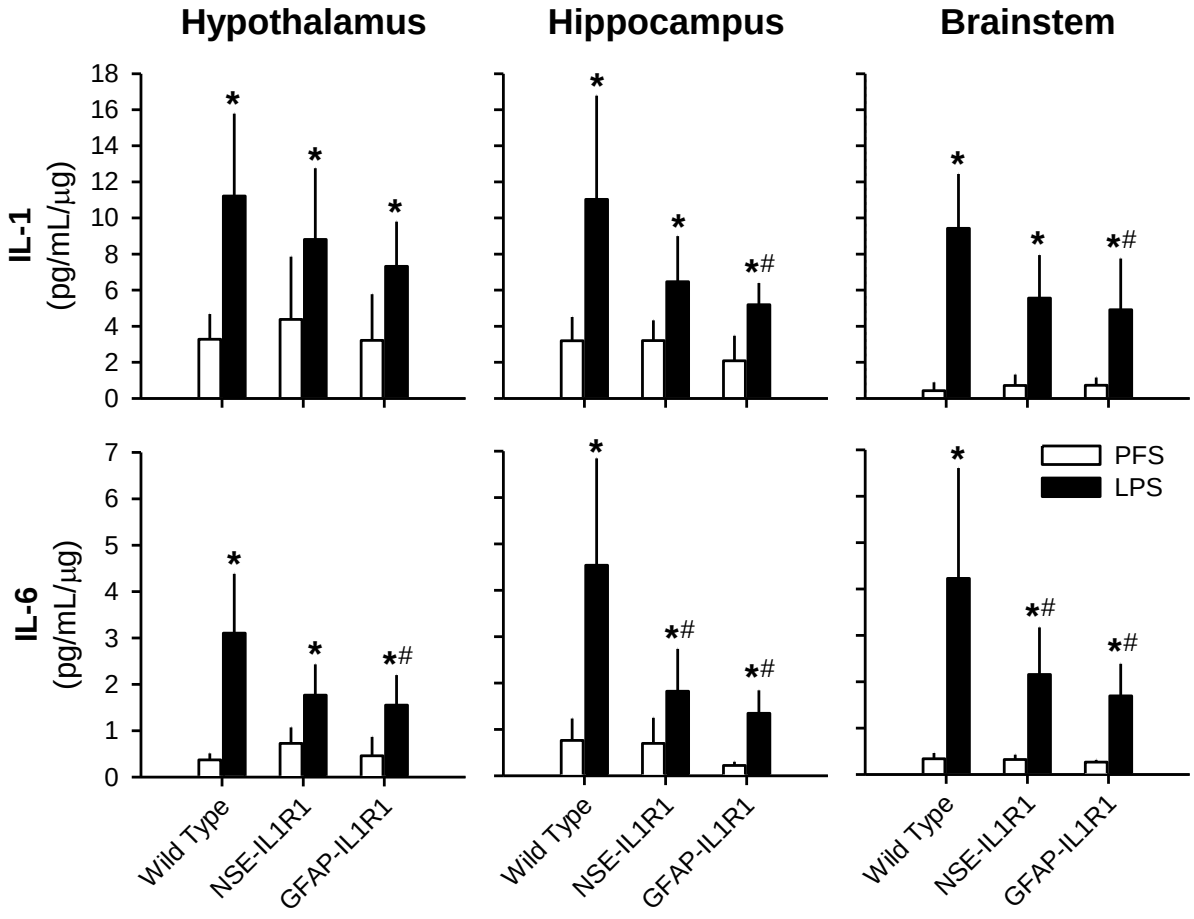


Figure 4.4: Quantification of IL-1 and IL-6 protein extracted from hypothalamus, hippocampus, and brainstem of WT, NSE-IL1R1, and GFAP-IL1R1 mice harvested 4 h post-intraperitoneal injection with PFS or LPS. Values are means ± SEM. Asterisk (*) indicates a statistical difference of $p < 0.05$ from PFS. Pound sign (#) denotes a statistical difference of $p < 0.05$ from WT LPS values.

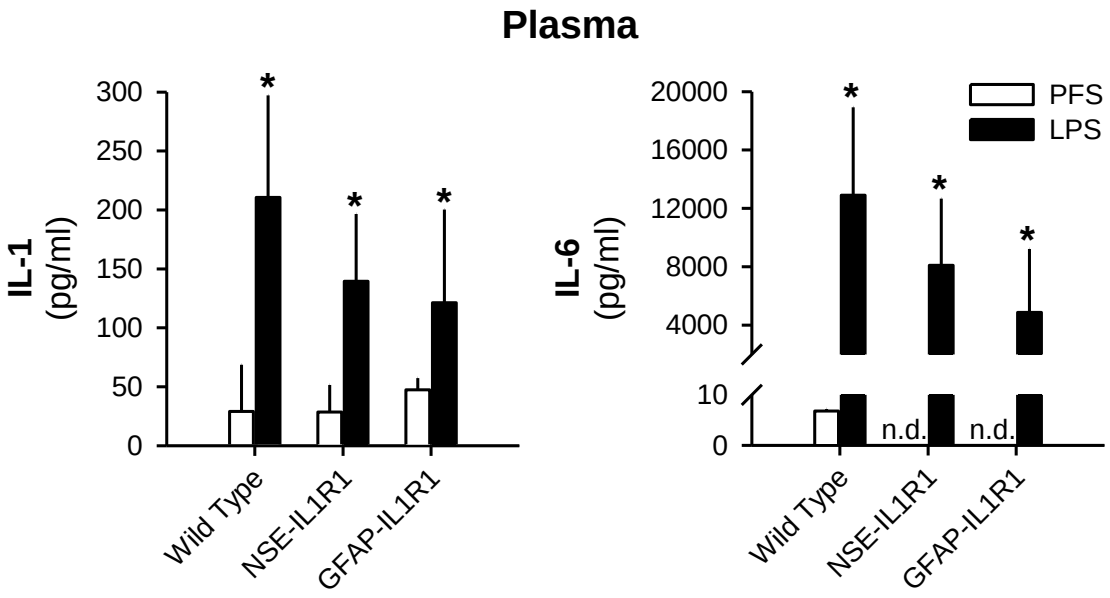


Figure 4.5: Quantification of IL-1 and IL-6 protein in plasma from WT, NSE-IL1R1, and GFAP-IL1R1 mice collected 4 h post-intraperitoneal injection with PFS or LPS. Values are means ± SDEV. Asterisk (*) denotes a statistical difference of $p < 0.05$ from PFS. n.d. indicates where values were not detected.

References

Alheim K, Chai Z, Fantuzzi G, Hasanvan H, Malinowsky D, Di Santo E, Ghezzi P, Dinarello CA, Bartfai T (1997) Hyperresponsive febrile reactions to interleukin (IL) 1alpha and IL-1beta, and altered brain cytokine mRNA and serum cytokine levels, in IL-1beta-deficient mice. Proceedings of the National Academy of Sciences 94:2681-2686.

Banks WA, Robinson SM (2010) Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. Brain, Behavior, and Immunity 24:102-109.

Baracchi F, Opp MR (2008) Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1. Brain Behav Immun 22:982-993.

Bartfai T, Schultzberg M (1993) Cytokines in neuronal cell types. Neurochem Int 22:435-444.

Baumann N, Baron-Van Evercooren AF, Jacque CF, Zalc B (1993) Glial biology and disorders. Current Opinion in Neurology and Neurosurgery 6:27-33.

Bluthé R-M, Layé S, Michaud B, Combe C, Dantzer R, Parnet P (2000) Role of interleukin-1b and tumour necrosis factor-a in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. Eur J Neurosci 12:4447-4456.

Borbély AA (1982) A two process model of sleep regulation. Hum Neurobiol 1:195-204.

Brambilla D, Franciosi S, Opp MR, Imeri L (2007) Interleukin-1 inhibits firing of serotonergic neurons in the dorsal raphe nucleus and enhances GABAergic inhibitory post-synaptic potentials. Eur J Neurosci 26:1862-1869.

Breder CD, Dinarello CA, Saper CB (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240:321-324.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A (1994) GFAP promoter directs astrocyte-specific expression in transgenic mice. The Journal of Neuroscience 14:1030-1037.

Cearley C, Churchill L, Krueger JM (2003) Time of day differences in IL1beta and TNFalpha mRNA levels in specific regions of the rat brain. Neurosci Lett 352:61-63.

Dantzer R, Kelley KW (2007) Twenty years of research on cytokine-induced sickness behavior. Brain, Behavior, and Immunity 21:153-160.

Datta SC, Opp MR (2008) Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology. J Neurosci Methods 175:119-124.

Dong Y, Benveniste EN (2001) Immune function of astrocytes. GLIA 36:180-190.

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-R660.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends in Immunology 28:138-145.

Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, Sutcliffe G (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. Neuron 5:187-197.

Frank MG (2013) Astroglial regulation of sleep homeostasis. Curr Opin Neurobiol 23:812-818.

Gordon CJ, White EC (1985) Temporal response of neurons to ambient heating in the preoptic and septal area of the unanesthetized rabbit. Comp Biochem Physiol A 82:879-884.

Guan Z, Vgontzas AN, Omori T, Peng X, Bixler EO, Fang J (2005) Interleukin-6 levels fluctuate with the light-dark cycle in the brain and peripheral tissues in rats. Brain Behav Immun 19:526-529.

Halassa MM, Fellin T, Haydon PG (2007) The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol Med 13:54-63.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Hawrylak N, Fleming JC FAU, Salm AK (1998) Dehydration and rehydration selectively and reversibly alter glial fibrillary acidic protein immunoreactivity in the rat supraoptic nucleus and subjacent glial limitans. GLIA 22:260-271.

Imeri L, Bianchi S, Opp MR (2006) Inhibition of caspase-1 in rat brain reduces spontaneous nonrapid eye movement sleep and nonrapid eye movement sleep enhancement induced by lipopolysaccharide. Am J Physiol Regul Integr Comp Physiol 291:R197-R204.

Imeri L, Opp MR (2009) How (and why) the immune system makes us sleep. Nat Rev Neurosci 10:199-210.

Ingiosi AM, Opp MR, Krueger JM (2013) Sleep and immune function: glial contributions and consequences of aging. Curr Opin Neurobiol 23:806-811.

Konsman JP, Dantzer R (2001) How the immune and nervous systems interact during disease-associated anorexia. Nutrition 17:664-668.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science 267:2000-2003.

Leon LR (1996) IL-1 type I receptor mediates acute phase response to turpentine, but not lipopolysaccharide, in mice. American journal of physiology Regulatory, integrative and comparative physiology 271:R1668-R1675.

Luheshi G, Miller AJ, Brouwer S, Dascombe MJ, Rothwell NJ, Hopkins SJ (1996) Interleukin-1 receptor antagonist inhibits endotoxin fever and systemic interleukin-6 induction in the rat. Am J PhysiolE91-E95.

Lundkvist J, Sundgren-Andersson A, Tingsborg S, Engfors C, Alheim K, Bartfai T, Iverfeldt K, Schultzberg M (1999) Acute-phase responses in transgenic mice with CNS overexpression of IL-1 receptor antagonist. Am J Physiol 276:R644-R651.

Manfridi A, Brambilla D, Bianchi S, Mariotti M, Opp MR, Imeri L (2003) Interleukin-1b enhances non-rapid eye movement sleep when microinjected into the dorsal raphe nucleus and inhibits serotonergic neurons in vitro. Eur J Neurosci 18:1041-1049.

Mason JL, Suzuki K, Chaplin DD, Matsushima GK (2001) Interleukin-1beta Promotes Repair of the CNS. The Journal of Neuroscience 21:7046-7052.

McCarley RW (2007) Neurobiology of REM and NREM sleep. Sleep Med 8:302-330.

Minami T, Okazaki J, Kawabata A, Kawaki H, Okazaki Y, Tohno Y (1998) Roles of nitric oxide and prostaglandins in the increased permeability of the blood-brain barrier caused by lipopolysaccharide. Environmental Toxicology and Pharmacology 5:35-41.

Morrow JD, Opp MR (2005) Diurnal variation of lipopolysaccharide-induced alterations in sleep and body temperature of interleukin-6-deficient mice. Brain Behav Immun 19:40-51.

Nadjar A, Blutstein T, Aubert A, Laye S, Haydon PG (2013) Astrocyte-derived adenosine modulates increased sleep pressure during inflammatory response. GLIA 61:724-731.

Olivadoti MD, Opp MR (2008) Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. Neuroscience 153:338-348.

Opp MR (1998) Rat strain differences suggest a role for corticotropin-releasing hormone in modulating sleep. Physiol Behav 63:67-74.

Opp MR (2005) Cytokines and sleep. Sleep Med Rev 9:355-364.

Opp MR, Krueger JM (1994) Anti-interleukin-1b reduces sleep and sleep rebound after sleep deprivation in rats. Am J Physiol 266:R688-R695.

Pinteaux E, Parker LC, Rothwell NJ, Luheshi GN (2002) Expression of interleukin-1 receptors and their role in interleukin-1 actions in murine microglial cells. J Neurochem 83:754-763.

Rudaya AY, Steiner AA, Robbins JR, Dragic AS, Romanovsky AA (2005) Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. Am J Physiol Regul Integr Comp Physiol 289:R1244-R1252.

Smith DE, Lipsky BP, Russell C, Ketchem RR, Kirchner J, Hensley K, Huang Y, Friedman WJ, Boissonneault V, Plante MMl, Rivest S, Sims JE (2009) A Central Nervous System-Restricted Isoform of the Interleukin-1 Receptor Accessory Protein Modulates Neuronal Responses to Interleukin-1. Immunity 30:817-831.

Sugama S, Takenouchi T, Sekiyama K, Kitani H, Hashimoto M (2011) Immunological responses of astroglia in the rat brain under acute stress: interleukin 1 beta co-localized in astroglia. Neuroscience 192:429-437.

Van Dam A-M, Brouns M, Louisse S, Berkenbosch F (1992) Appearance of interleukin-1 in macrophages and in ramified microglia in the brain of endotoxin-treated rats: A pathway for the induction of non-specific symptoms of sickness? Brain Res 588:291-296.

Xaio H, Banks WA, Niehoff ML, Morley JE (2001) Effect of LPS on the permeability of the blood-brain barrier to insulin. Brain Res 896:36-42.

Zielinski MR, Dunbrasky DL, Taishi P, Souza G, Krueger JM (2013) Vagotomy attenuates brain cytokines and sleep induced by peripherally administered tumor necrosis factor-alpha and lipopolysaccharide in mice. Sleep 36:1227-1238.

CHAPTER V

GENERAL DISCUSSION

Introduction

The findings presented in this dissertation suggest that neurons and astrocytes impart differential effects on sleep-wake behavior and immunomodulation as mediated by interleukin-1 receptor 1 (IL1R1). These studies utilized novel strains of transgenic mice that were engineered to express IL1R1 only in the central nervous system (CNS) and selectively on neurons (NSE-IL1R1) or astrocytes (GFAP-IL1R1). The transgenic mice described here allow for *in vivo* investigations of neuronal- and astroglial-driven modulation of physiology and behavior. This final chapter will briefly summarize the findings and their implications within a broader context.

Spontaneous sleep

The experiments in Chapter II demonstrate that selective expression of IL1R1 on neurons or astrocytes modulates physiological sleep. Compared to wild type (WT) mice, transgenic mice spend a comparable amount of time in non-rapid eye movement sleep (NREMS) but exhibit more rapid eye movement sleep (REMS). Interestingly, IL1R1 deficient mice spend less time in NREMS but display normal amounts of REMS (Fang et al., 1998). These data suggest neuronal and astroglial

IL1R1 modulate spontaneous sleep responses which is characterized, in part, by altered sleep architecture.

Although our lab generally views NREM delta power as a measure of sleep quality and depth, others consider it an indicator of sleep pressure (Franken et al., 2001). Based on this latter distinction, baseline data indicate that transgenic mice, which exhibit elevated NREM delta power, are “sleepier” despite spending comparable amounts of time in NREMS and wakefulness relative to WT animals. The increased time spent in REMS, therefore, may reflect an intrinsic REM rebound in transgenic mice. Nevertheless, these data suggest neuronal- and astroglial-specific IL1R1 expression contributes to electroencephalographic (EEG) spectral characteristics under physiological conditions.

Sleep deprivation

Unlike IL1R1 knockout mice (Schmidt and Wisor, 2012), the percentage of time transgenic mice spend in REMS, NREMS, and wakefulness after sleep deprivation does not differ from WT. These data (presented in Chapter II) suggest that neuronal or astroglial IL1R1 expression is sufficient for normal spontaneous sleep duration during the post-deprivation recovery period. Consistent with previous findings demonstrating astrocytes mediate EEG slow-wave delta activity following sleep deprivation (Halassa et al., 2009), NSE-IL1R1 delta power response to 6 h of sleep deprivation is impaired compared to WT mice. Therefore, astroglial IL1R1 activity may modulate sleep homeostasis.

Response to central IL-1

The most surprising result of this dissertation is the lack of response to interleukin-1β (IL-1) in transgenic mice (Chapter III). Intracerebroventricular administration of IL-1 fails to alter sleep or temperature in NSE-IL1R1 and GFAP-IL1R1 mice, indicating that expression of IL1R1 on neurons or astrocytes alone is not sufficient to manifest responses to central challenge with IL-1. This effect may be mediated, in part, by the inability of NSE-IL1R1 and GFAP-IL1R1 mice to produce IL-6 in brain subsequent to IL-1 challenge. Indeed, effects of central IL-1 administration on NREMS and fever are attenuated in IL-6 deficient mice (Olivadoti and Opp, 2008). However, NSE-IL1R1 neuronal and GFAP-IL1R1 astroglial cell cultures produce IL-6 in response to IL-1. These data suggest additional mechanisms or substrates are required for *in vivo* IL-1 activity. For example, simultaneous neuronal and astroglial expression of IL1R1 may be required to elicit responses to IL-1, or other cell types, such as endothelial cells, might initiate central responses to IL-1 (see below).

Response to systemic LPS

In Chapter IV, responses to lipopolysaccharide (LPS) are largely preserved in transgenic mice, as evidenced by decreased REMS, increased NREMS, elevated NREM delta power, and fever. However, GFAP-IL1R1 mice exhibit greater REMS suppression compared to NSE-IL1R1 animals and have more NREM delta power than WT and NSE-IL1R1 mice. These data suggest neuronal IL1R1 activity may drive aspects of pathological responses to LPS. These data stand somewhat in

contrast to previous findings that astrocyte gliotransmission mediates slow-wave delta activity in response to LPS (Nadjar et al., 2013). Our data do not discount the possibility that astrocytes modulate NREM delta power responses to LPS but merely suggest that LPS-induced gliotransmission may not be dependent on activation of astroglial IL1R1.

Although transgenic mice exhibit behavioral responses to LPS, cytokine production is impaired. LPS induces IL-1 and IL-6 production in NSE-IL1R1 and GFAP-IL1R1 brain tissue but at concentrations generally less than those observed in WT samples. However, peripheral expression of these cytokines is unaffected in transgenic mice. The differential immunomodulatory responses to LPS may be due to reduced IL1R1 expression at the blood-brain barrier in transgenic mice, whereas sustained behavioral responses to LPS may result from compensatory actions of other cytokines such as tumor necrosis factor α (TNF) (see below).

Mediators of sleep and immune responses

The data presented in this body of work exemplify the complexity of sleep and immune interactions. Although these studies demonstrate neurons and astrocytes are active contributors to sleep and immunomodulation, the results also implicate the contributions of additional substrates that were beyond the scope of these experiments. These putative downstream and upstream mediators of neuronal and astroglial modulation of sleep and immune interactions may exist at both molecular and cellular levels.

Adenosine

Adenosine is a sleep regulatory substance that serves to promote sleep, in part, by inhibiting wake-promoting neurons (Alam et al., 1999; Basheer et al., 2004). For example, adenosine inhibits cholinergic and non-cholinergic neurons of the wake-promoting basal forebrain (Basheer et al., 2004), an effect linked to increased delta power and suppressed theta activity (Cape and Jones, 2000). Furthermore, microinjections of an adenosine agonist into the brainstem increases REMS (Marks and Birabil, 1998), and systemic administration of adenosinergic agonists increases time spent in NREMS and REMS (Radulovacki et al., 1984). Recently, Halassa *et al.* (2009) reported that impaired astrocytic gliotransmission reduces compensatory EEG delta power responses to sleep deprivation and that this effect is mediated by adenosine. In Chapter II, NSE-IL1R1 mice were found to have less NREM delta power following 6 h of sleep deprivation. This finding raises the possibility that astroglial-specific IL1R1 signaling facilitates gliotransmission of adenosine. Indeed, IL-1 induces adenosine production (Sperlagh et al., 2004; Zhu et al., 2006). Likewise, adenosine triggers signaling via nuclear factor-κB (NF-κB) (Basheer et al., 2001), a transcription factor that induces IL-1 expression. Furthermore, LPS-induced elevations of EEG slow wave activity are attenuated in gliotransmission-impaired mice (Nadjar et al., 2013). This effect is mimicked by central inhibition of the adenosine 1 receptor (Nadjar et al., 2013). Taken together, adenosine may be a downstream mediator of neuronal- and astroglial-driven IL1R1 modulation of sleep and immune responses. These findings demonstrate a need for further investigation of adenosine in sleep-immune interactions.

Interleukin-6

IL-6 is pro-inflammatory cytokine that is an important downstream mediator of IL-1 activity (Tsakiri et al., 2008). IL-6 deficiency attenuates IL-1- and LPS-induced alterations in NREMS and febrile responses (Olivadoti and Opp, 2008; Morrow and Opp, 2005a). As with transgenic animals, IL-6 knockout mice spend more time in REMS but do not differ from WT with respect to spontaneous NREMS duration (Morrow and Opp, 2005b). In Chapters III and IV, IL-6 production was impaired in transgenic brain tissue in response to immune challenge. Although IL-6 protein was not quantified under baseline conditions, IL-6 production may be impaired in transgenic mice, thus contributing to the spontaneous sleep phenotypes described in Chapter II. Interestingly, cultured NSE-IL1R1 neurons and GFAP-IL1R1 astrocytes produce IL-6 in response to IL-1. These data indicate that, in transgenic mice, some *in vivo* inhibitory factor is in place. A potential candidate for this IL-6 inhibition is the endothelial cell.

Endothelial cells

Endothelial cells are the primary cellular constituent of the blood-brain barrier (BBB). These cells express IL-1 receptors (Konsman et al., 2004) and are activated by IL-1 (Thornton et al., 2010). Endothelial-specific knockdown of IL1R1 abolishes IL-1-induced fever (Ching et al., 2007). Furthermore, inducible inhibition of endothelial IL1R1 inhibits elevation of IL-1 concentrations and anxiety responses due to repeated social defeat (Wohleb et al., 2014). Although endothelial cells are widely implicated in immune responses, we are not aware of any studies that

investigate endothelial-specific modulation of sleep-wake behavior. On the other hand, studies report that sleep affects endothelial cells. Chronic sleep restriction impairs BBB function and increases its permeability (He et al., 2014). REMS restriction also increases BBB permeability, and BBB integrity can be restored by sleep recovery (Gómez-González et al., 2013). Taken together, expression of IL1R1 on endothelial cells may be necessary to initiate sleep responses to central administration of IL-1 and could be a source of IL-1-induced IL-6 production. In light of the findings presented in Chapter III, futures studies should explore endothelial-specific contributions to sleep-immune interactions.

Neuronal-astroglial interactions

Although this dissertation investigates the extent to which IL1R1 signaling in neurons or astrocytes is sufficient for the regulation of sleep-wake behavior and immunomodulation, these cells do not work in isolation. *In vitro* studies show that some neuronal functions are dependent on astrocytes and vice versa. Astrocytes mediate synaptic development and plasticity (Stevens, 2008), confer neuroprotection against immune effector cells (Darlington et al., 2008), and regulate hippocampal neurogenesis (Song et al., 2002). Likewise, neurons influence astroglial expression of glutamate transporters (Swanson et al., 1997) via activation of NF-κB (Ghosh et al., 2011). Neurons also mediate astroglial proliferation (Hatten, 1987). Some of these interactions are modulated by secreted factors (Seil et al., 1992; Hayashi et al., 1988), whereas others manifest from direct cellular contact (Hatten, 1987). To

address this concern, NSE-IL1R1 and GFAP-IL1R1 mice could be bred to produce progeny that express IL1R1 on both neurons and astrocytes.

Methodological considerations

To our knowledge, these are the first *in vivo* studies to investigate the sufficiency of neuronal and astroglial contributions to sleep and immune interactions. As such, data derived from the novel transgenic mouse strains introduced here are met with some unknowns. Due to the nature of the transgenic mechanism, IL1R1 may be overexpressed on neurons and astrocytes of transgenic mice. Although IL1R1 expression in brain is constitutive and widespread (Breder et al., 1988; Farrar et al., 1987; Takao et al., 1990; Yabuuchi et al., 1994), it is currently unclear whether IL1R1 distribution differs from wild type conditions. Additionally, the IL-1 system is self-mediated by a number of negative regulatory factors including the decoy receptor IL1R type II (McMahan et al., 1991), the endogenous IL-1 receptor antagonist (Hannum et al., 1990), and the IL-1 receptor brain-specific accessory protein (Taishi et al., 2012). The impact of transgenic IL1R1 expression on these system components is currently unknown.

IL-1 also produces a number of secondary mediators that exhibit inhibitory autocrine and paracrine effects on IL-1 activity. These factors include anti-inflammatory cytokines such as IL-4 and IL-10 (Pousset et al., 1999; Fiorentino et al., 1991). Although IL-4 and IL-10 were not detected at our experimental timepoint, this does not discount the potential influence of these and other anti-inflammatory cytokines on IL-1 activity in NSE- and GFAP-IL1R1 mice. Additionally, IL-1 activates

the hypothalamic-pituitary-adrenal (HPA) axis including corticotropin-releasing hormone expression (Opp and Imeri, 2001) and glucocorticoid release (Knudsen et al., 1987; Lee et al., 1988) that subsequently inhibits IL-1. The impact of IL-1 on the HPA axis and its mediators in these transgenic mice has not been investigated. Lastly, due to the redundancy of the cytokine cascade of which IL-1 is a component, TNF is known to impart compensatory actions in IL1R1 deficient mice during challenge with LPS (Bluthé et al., 2000). Although IL1R1 expression is rescued in neurons and astrocytes in transgenic mice, it is unclear if this pattern of receptor expression triggers compensatory responses of other cytokines.

Non-neuronal contributions

A primary objective at the onset of this project was to contribute information regarding the role of non-neuronal cells, particularly glia, in physiology and behavior. Glia were traditionally relegated to supportive, passive constituents of the CNS. However, recent studies are emphasizing the active contributions of these cells to physiology, behavior, and disease. As such, these investigations reveal the extent of our lack of understanding regarding CNS-mediated processes and the associated mechanistic underpinnings. Although the studies presented in previous chapters explore the role of astrocytes in sleep and immune function, these cells and others are implicated in variety of CNS-mediated process. For example, astrocytes actively contribute to synaptic plasticity (Araque et al., 1999; Stevens, 2008) as well as learning, memory, and cognition (Gerlai et al., 1995; Halassa et al., 2009; Han et al., 2013). Furthermore, astroglial impairments are implicated in disorders of

metabolism [reviewed by (Yi et al., 2011)], aging [reviewed by (Ingiosi et al., 2013)], and depression [reviewed by (Smialowska et al., 2013)], as well as neurodegenerative disorders such as Alzheimer's disease [reviewed by (Avila-Muñoz and Arias, 2014)] and Parkinson's disease [reviewed by (Niranjan, 2014)]. Additional glial cells such as microglia also actively contribute to these and other CNS processes and disorders.

Microglia are touted as resident immune cells of the CNS that are mobilized and activated in response to an immune challenge. The role of microglia in mediating responses to immune challenge has been studied exhaustively. However, microglia are now implicated as active contributors to physiological processes and behavior as well. Intraperitoneal administration of minocycline, an inhibitor of microglial activation, induces an acute increase in wakefulness and significantly reduces NREMS compared to saline-treated mice (Wisor et al., 2011). Minocycline also reduces EEG slow wave delta activity (Kim and Suh, 2009). In response to sleep deprivation, microglia assume a deramified morphology, a marker of activation (Hsu et al., 2003). In addition to their role in sleep, microglia also contribute to other physiological processes such as aging [reviewed by (Ingiosi et al., 2013)], as well as synaptic plasticity, learning, and memory [reviewed by (Morris et al., 2013; Schafer et al., 2013)]. These findings offer just a sampling of topics that merit further investigation of non-neuronal contributions to CNS-mediated processes and behaviors.

Concluding remarks

The studies that comprise this dissertation demonstrate, through the use of novel transgenic mice, that neurons and astrocytes differentially mediate aspects of sleep and immunomodulation under physiological and pathological conditions. To our knowledge, these experiments are the first to explore the sufficiency of neuronal and astroglial contributions to sleep and immune interactions *in vivo*. Investigations into the role of astrocytes in sleep-wake behavior are in their infancy, and these studies contribute to a limited, but growing, body of work exploring active glial contributions to complex physiology and behavior. Overall, these studies illustrate the complexity of cellular mechanisms that underlie behavioral and immune interactions. Continued study of non-neuronal cells will be crucial to better understanding a variety of CNS-mediated processes and behaviors as they relate to physiology, sickness, and disease.

References

Alam MN, Szymusiak R, Gong H, King J, McGinty D (1999) Adenosinergic modulation of rat basal forebrain neurons during sleep and waking: neuronal recording with microdialysis. J Physiol 521.3:679-690.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208-215.

Avila-Muñoz E, Arias C (2014) When astrocytes become harmful: Functional and inflammatory responses that contribute to Alzheimer's disease. Ageing Research Reviews 18:29-40.

Basheer R, Rainnie DG, Porkka-Heiskanen T, Ramesh V, McCarley RW (2001) Adenosine, prolonged wakefulness, and A1-activated NF-kB DNA binding in the basal forebrain of the rat. Neuroscience 104:731-739.

Basheer R, Strecker RE, Thakkar MM, McCarley RW (2004) Adenosine and sleep-wake regulation. Prog Neurobiol 73:379-396.

Bluthé R-M, Layé S, Michaud B, Combe C, Dantzer R, Parnet P (2000) Role of interleukin-1b and tumour necrosis factor-a in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. Eur J Neurosci 12:4447-4456.

Breder CD, Dinarello CA, Saper CB (1988) Interleukin-1 immunoreactive innervation of the human hypothalamus. Science 240:321-324.

Cape EG, Jones BE (2000) Effects of glutamate agonist versus procaine microinjections into the basal forebrain cholinergic cell area upon gamma and theta EEG activity and sleep-wake state. Eur J Neurosci 12:2166-2184.

Ching S, Zhang H, Belevych N, He L, Lai W, Pu Xa, Jaeger LB, Chen Q, Quan N (2007) Endothelial-Specific Knockdown of Interleukin-1 (IL-1) Type 1 Receptor Differentially Alters CNS Responses to IL-1 Depending on Its Route of Administration. The Journal of Neuroscience 27:10476-10486.

Darlington PJ, Podjaski C, Horn KE, Costantino S, Blain M, Saikali P, Chen Z, Baker KA, Newcombe J, Freedman M, Wiseman PW, Bar-Or A, Kennedy TE, Antel JP (2008) Innate Immune-Mediated Neuronal Injury Consequent to Loss of Astrocytes. Journal of Neuropathology & Experimental Neurology 67.

Fang J, Wang Y, Krueger JM (1998) Effects of interleukin-1 beta on sleep are mediated by the type I receptor. Am J Physiol 274:R655-R660.

Farrar WL, Kilian PL, Ruff MR, Hill JM, Pert CB (1987) Visualiztion and characterization of interleukin 1 receptors in brain. J Immunol 139:459-463.

Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A (1991) IL-10 inhibits cytokine production by activated macrophages. J Immunol 147:3815-3822.

Franken P, Chollet D, Tafti M (2001) The homeostatic regulation of sleep need is under genetic control. The Journal of Neuroscience 21:2610-2621.

Gerlai R, Wojtowicz JM, Marks A, Roder J (1995) Overexpression of a calcium-binding protein, S100 beta, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learning & Memory 2:26-39.

Ghosh M, Yang Y, Rothstein JD, Robinson MB (2011) Nuclear Factor-kappaB Contributes to Neuron-Dependent Induction of Glutamate Transporter-1 Expression in Astrocytes. The Journal of Neuroscience 31:9159-9169.

Gómez-González B, Hurtado-Alvarado G, Esqueda-León E, Rojas-Zamorano J, Velázquez-Moctezuma J (2013) REM sleep loss and recovery regulates blood-brain barrier function. Current neurovascular research 10:197-207.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Han X, Chen M, Wang F, Windrem M, Wang S, Shanz S, Xu Q, Oberheim N, Bekar L, Betstadt S, Silva A, Takano T, Goldman S, Nedergaard M (2013) Forebrain Engraftment by Human Glial Progenitor Cells Enhances Synaptic Plasticity and Learning in Adult Mice. Cell Stem Cell 12:342-353.

Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP, Thompson RC (1990) Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. Nature 343:336-340.

Hatten ME (1987) Neuronal inhibition of astroglial cell proliferation is membrane mediated. The Journal of Cell Biology 104:1353-1360.

Hayashi M, Hayashi R, Tanii H, Hashimoto K, Patel AJ (1988) The influence of neuronal cells on the development of glutamine synthetase in astrocytes in vitro. Brain Res 469:37-42.

He J, Hsuchou H, He Y, Kastin AJ, Wang Y, Pan W (2014) Sleep restriction impairs blood-brain barrier function. The Journal of Neuroscience 34:14697-14706.

Hsu JC, Lee YS, Chang CN, Chuang HL, Ling EA, Lan CT (2003) Sleep deprivation inhibits expression of NADPH-d and NOS while activating microglia and astroglia in the rat hippocampus. Cells Tissues Organs 173:242-254.

Ingiosi AM, Opp MR, Krueger JM (2013) Sleep and immune function: glial contributions and consequences of aging. Curr Opin Neurobiol 23:806-811.

Kim HS, Suh YH (2009) Minocycline and neurodegenerative diseases. Behav Brain Res 196:168-179.

Knudsen PJ, Dinarello CA, Strom TB (1987) Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U937 cells. J Immunol 139:4129-4134.

Konsman JP, Vigues S, Mackerlova L, Bristow A, Blomqvist A (2004) Rat brain vascular distribution of interleukin-1 type-1 receptor immunoreactivity: Relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli. The Journal of Comparative Neurology 472:113-129.

Lee SW, Tsou A-P, Chan H, Thomas J, Petrie K, Eugui EM, Allison AC (1988) Glucocorticoids selectively inhibit the transcription of the interleukin 1b gene and decrease the stability of interleukin 1b mRNA. Proc Natl Acad Sci USA 85:1204-1208.

Marks GA, Birabil CG (1998) Enhancement of rapid eye movement sleep in the rat by cholinergic and adenosinergic agonists infused into the pontine reticular formation. Neuroscience 86:29-37.

McMahan CJ, Slack JL, Mosley B, Cosman D, Lupton SD, Brunton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan CI, Copeland NG, Huebner K, Croce CM, Cannizzarro LA, Benjamin D, Dower SK, Spriggs MK, Sims JE (1991) A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J 10:2821-2832.

Morris GP, Clark IA, Zinn R, Vissel B (2013) Microglia: A new frontier for synaptic plasticity, learning and memory, and neurodegenerative disease research. Neurobiology of Learning and Memory 105:40-53.

Morrow JD, Opp MR (2005a) Diurnal variation of lipopolysaccharide-induced alterations in sleep and body temperature of interleukin-6-deficient mice. Brain Behav Immun 19:40-51.

Morrow JD, Opp MR (2005b) Sleep-wake behavior and responses of interleukin-6-deficient mice to sleep deprivation. Brain Behav Immun 19:28-39.

Nadjar A, Blutstein T, Aubert A, Laye S, Haydon PG (2013) Astrocyte-derived adenosine modulates increased sleep pressure during inflammatory response. GLIA 61:724-731.

Niranjan R (2014) The Role of Inflammatory and Oxidative Stress Mechanisms in the Pathogenesis of Parkinson's Disease: Focus on Astrocytes. Mol Neurobiol 49:28-38.

Olivadoti MD, Opp MR (2008) Effects of i.c.v. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. Neuroscience 153:338-348.

Opp MR, Imeri L (2001) Rat strains that differ in corticotropin-releasing hormone production exhibit different sleep-wake responses to interleukin 1. Neuroendocrinology 73:272-284.

Pousset F, Cremona S, Dantzer R, Kelly K, Parnet P (1999) Interleukin-4 and interleukin-10 regulate IL1-beta induced mouse primary astrocyte activation: a comparative study. GLIA 26:12-21.

Radulovacki M, Virus RM, Djuricic-Nedelson M, Green RD (1984) Adenosine analogs and sleep in rats. J Pharmacol Exp Ther 228:268-274.

Schafer DP, Lehrman EK, Stevens B (2013) The "quad-partite" synapse: Microglia-synapse interactions in the developing and mature CNS. GLIA 61:24-36.

Schmidt MA, Wisor JP (2012) Interleukin 1 receptor contributes to methamphetamine- and sleep deprivation-induced hypersomnolence. Neurosci Lett 513:209-213.

Seil FJ, Eckenstein FP, Reier PJ (1992) Induction of dendritic spine proliferation by an astrocyte secreted factor. Experimental Neurology 117:85-89.

Smialowska M, Szewczyk B, Wozniak M, Wawrzak-Wlecial A, Domin H (2013) Glial degeneration as a model of depression. Pharmacol Rep 65:1572-1579.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Sperlagh B, Baranyi M, Hasko G, Vizi ES (2004) Potent effect of interleukin-1 beta to evoke ATP and adenosine release from rat hippocampal slices. J Neuroimmunol 151:33-39.

Stevens B (2008) Neuron-Astrocyte Signaling in the Development and Plasticity of Neural Circuits. Neurosignals 16:278-288.

Swanson RA, Liu J, Miller JW, Rothstein JD, Farrell K, Stein A, Longuemare MC (1997) Neuronal Regulation of Glutamate Transporter Subtype Expression in Astrocytes. The Journal of Neuroscience 17:932-940.

Taishi P, Davis CJ, Bayomy O, Zielinski MR, Liao F, Clinton JM, Smith DE, Krueger JM (2012) Brain-specific interleukin-1 receptor accessory protein in sleep regulation. J Appl Physiol (1985) 112:1015-1022.

Takao T, Tracey DE, Mitchell WM, De Souza EB (1990) Interleukin-1 receptors in mouse brain: characterization and neuronal localization. Endocrinology 127:3070-3078.

Thornton P, McColl BW, Cooper L, Rothwell NJ, Allan SM (2010) Interleukin-1 drives cerebrovascular inflammation via MAP kinase-independent pathways. Current neurovascular research 7:330-340.

Tsakiri N, Kimber I, Rothwell NJ, Pinteaux E (2008) Interleukin-1-induced interleukin-6 synthesis is mediated by the neutral sphingomyelinase/Src kinase pathway in neurones. Brit J Pharmacol 153:775-783.

Wisor JP, Schmidt MA, Clegern WC (2011) Evidence for neuroinflammatory and microglial changes in the cerebral response to sleep loss. Sleep 34:261-272.

Wohleb ES, Patterson JM, Sharma V, Quan N, Godbout JP, Sheridan JF (2014) Knockdown of Interleukin-1 Receptor Type-1 on Endothelial Cells Attenuated Stress-Induced Neuroinflammation and Prevented Anxiety-Like Behavior. The Journal of Neuroscience 34:2583-2591.

Yabuuchi K, Minami M, Katsumata S, Satoh M (1994) Localization of type I interleukin-1 receptor mRNA in the rat brain. Mol Brain Res 27:27-36.

Yi CX, Habegger KM, Chowen JA, Stern J, Tschop MH (2011) A Role for Astrocytes in the Central Control of Metabolism. Neuroendocrinology 93:143-149.

Zhu G, Okada M, Yoshida S, Mori F, Hirose S, Wakabayashi K, Kaneko S (2006) Involvement of Ca(2+)-induced Ca2+ releasing system in interleukin-1beta-associated adenosine release. Eur J Pharmacol 532:246-252.

APPENDIX A

SLEEP AND IMMUNE FUNCTION: GLIAL CONTRIBUTIONS AND CONSEQUENCES OF AGING*

Presented here is a published review that discusses recent findings that demonstrate a role for glia, specifically astrocytes and microglia, in sleep and immune interactions with an emphasis on cytokines. The conversion continues in regards to how sleep, immune function, and cellular phenotypes change as a function of aging.

Abstract

The reciprocal interactions between sleep and immune function are well-studied. Insufficient sleep induces innate immune responses as evidenced by increased expression of pro-inflammatory mediators in the brain and periphery. Conversely, immune challenges upregulate immunomodulator expression, which alters central nervous system-mediated processes and behaviors, including sleep. Recent studies indicate that glial cells, namely microglia and astrocytes, are active contributors to sleep and immune system interactions. Evidence suggests glial regulation of these interactions is mediated, in part, by adenosine and adenosine 5'-

* This review was previously published: Ingiosi AM, Opp MR, Krueger JM (2013) Sleep and immune function: glial contributions and consequences of aging. Curr Opin Neurobiol 23:806-811.

triphosphate actions at purinergic type 1 and type 2 receptors. Furthermore, microglia and astrocytes may modulate declines in sleep-wake behavior and immunity observed in aging.

Introduction

Chronic insufficient sleep is associated with inflammation, metabolic syndrome, cardiovascular disease, increased sensitivity and to pain stimuli, fatigue, excessive daytime sleepiness, and impaired cognitive and physical performance. Symptoms of these pathologies are associated with increased levels of endogenous pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), and can be experimentally elicited by peripheral or central exogenous administration of these cytokines to subjects (Dantzer, 2001, Imeri and Opp, 2009). Conversely, inhibition of IL-1β or TNFα attenuates many sleep-loss associated symptoms. Additionally, reduction of endogenous levels of IL-1β or TNFα, whether in mutant mice or by use of soluble receptors, antibodies, or receptor antagonists, inhibits spontaneous sleep (reviewed (Imeri and Opp, 2009), (Krueger et al., 2008), and (Krueger, 2011)). There is a wealth of evidence indicating that IL-1β and TNFα are also involved in physiological sleep regulation and that their amplification during pathology is causative of characteristic sleep disturbances associated with many pathological states (Krueger et al., 2008, Imeri and Opp, 2009, Krueger, 2011). Indeed, the brain, including regions associated with the regulation of sleep-wake behavior, produces and is responsive to cytokines (Krueger et al., 2008, Dantzer, 2009, Imeri and Opp, 2009). Furthermore, neuronal activity upregulates these

cytokines in brain regions implicated in the regulation of sleep (Churchill et al., 2008, Hallett et al., 2010). Impaired sleep also affects adaptive immune responses. Sleep deprivation attenuates antibody responses to vaccine (Spiegel et al., 2002, Lange et al., 2003), whereas good sleep imparts long-lasting immunoenhancing effects (Lange et al., 2011•, Besedovsky et al., 2012). Furthermore, sleep is a profound regulator of cellular immunity and formation of immunological memory critical for adaptive responses to immune challenges (reviewed by (Besedovsky et al., 2012)).

We acquired substantial insight into sleep and immune system interactions during the last 30 years. However, the cellular substrates for these interactions are less well understood. Understanding the role of glia in sleep and immune system functioning is crucial because research has started to shift the traditional view of these cells as passive constituents of the central nervous system (CNS) to active contributors capable of mediating behavior (see below). Furthermore, because the aforementioned inflammatory pathologies are common in elderly individuals, identifying age-related changes in glial cell functioning may be critical in elucidating the mechanisms driving senescence of sleep and immune networks in aging. This review highlights recent findings implicating a role for glia in sleep and immune interactions and aging.

Microglia in sleep and immune function

Microglia are the resident immune cells of the CNS that are mobilized and activated in response to an immune challenge. The role of microglia in mediating

responses to immune challenge has been studied exhaustively. Microglial influences on sleep-wake behavior are not extensively studied although recent data implicate microglia in sleep regulation. Microglia assume a deramified morphology, a marker of activation, in response to sleep deprivation (Hsu et al., 2003). In addition, slow wave activity is reduced following administration of minocycline, an inhibitor of microglial activation (Kim and Suh, 2009). Minocycline inhibits microglial production of immunomodulators including cytokines and nitric oxide (Kim and Suh, 2009). Intraperitoneal minocycline administration induces an acute increase in wakefulness and significantly reduces non-rapid eye movement sleep (NREMS) compared to saline-treated mice (Wisor et al., 2011••). Furthermore, minocycline inhibits sleep deprivation-induced augmentation of NREMS delta power, a surrogate indicator of sleep depth (Wisor et al., 2011••). Although data are limited, recent studies indicate microglia are potentially critical components of sleep regulatory mechanisms.

A possible effector of microglial influences on sleep-wake behavior may be extracellular adenosine-5'-triphosphate (ATP) acting at the purinergic type 2 receptor $P2X_7$ ($P2X_7R$). The $P2X_7R$ links increased cellular activity during waking to adenosine and cytokine sleep modulation. Activation of glial $P2X_7R$ by extracellular ATP mediates post-translational processing of sleep regulatory substances including IL-1β, TNFα, and IL-6 (Choi et al., 2007, Krueger et al., 2008, Mingam et al., 2008). $P2X_7R$ expression in brain is most prominent on microglia (Choi et al., 2007, Mingam et al., 2008), and neurons and glia release ATP into the extracellular space in response to cellular activity (Krueger et al., 2008). Administration of $P2X_7R$ agonists

increases time spent in NREMS and enhances electroencephalographic (EEG) delta power (Krueger et al., 2010). Conversely, $P2X_7R$ inhibition reduces NREMS in rats (Krueger et al., 2010). Furthermore, mice lacking the $P2X_7R$ exhibit less robust increases in NREMS and EEG delta power in response to sleep deprivation compared to wild type animals (Krueger et al., 2010). Collectively, the data suggest a role for microglia and purinergic receptors as one component of systems and networks that mediate sleep and immune interactions (Figure A.1).

Astrocytes in sleep and immune function

A traditional view of astrocytes was that they played a passive, supportive role for neurons. However, recent studies demonstrate that these cells are active contributors to complex behaviors and immune responses. Astrocytes are the most abundant glial cell type in the brain, respond rapidly to inflammation, express receptors for immunomodulators, and produce sleep regulatory substances in response to immune challenge (Dong and Benveniste, 2001, Farina et al., 2007). Selectively inhibiting astrocyte gliotransmission via the dominant negative (dnSNARE) mouse reduces EEG slow wave activity during NREMS, a traditional measure of sleep pressure (Halassa et al., 2009)[1]. Inhibition of vesicular release from astrocytes also attenuates the increase of NREMS and cognitive deficits typically observed subsequent to 6 h of sleep deprivation (Halassa et al., 2009).

[1] EEG slow wave activity is regulated independently from duration of NREMS [Davis CJ, Clinton JM, Jewett KA, Zielinski MR, Krueger JM (2011) Delta wave power: an independent sleep phenotype or epiphenomenon? Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine 7:S16-18. There is good evidence for the involvement of extracellular adenosine in its regulation; this might occur via vasodilation induced by adenosine since cerebral blood flow alters EEG slow wave power.

These data suggest astrocytic gliotransmission contributes to the modulation of sleep need.

Because altered gliotransmission of astrocytes results in reduced sleep pressure, studies have turned to astrocyte-derived adenosine, an ATP metabolite, as a potential molecular substrate of this effect. Adenosine accumulates in brain with increasing time awake (Schmitt et al., 2012••), and extracellular elevation of adenosine concentrations is astrocyte dependent (Pascual et al., 2005, Schmitt et al., 2012••). Indeed, inhibition of the adenosine 1 receptor (A_1R) in wild type mice recapitulates the reductions of baseline EEG slow wave activity and responses to sleep deprivation observed in gliotransmission-impaired dnSNARE mice (Halassa et al., 2009, Florian et al., 2011). Conditional CNS A_1R knockout mice also fail to demonstrate enhanced EEG delta power following intermittent sleep deprivation (Bjorness et al., 2009). Conversely, mice lacking CD73, an ectonucleotidase that converts extracellular ATP to adenosine, have more spontaneous NREMS than wild type controls (Zielinski et al., 2012•). Consistent with the notion that the A_1R mediates sleep need, chronic sleep restriction increases A_1R mRNA expression in the wake-promoting basal forebrain in rats (Kim et al., 2012) although not in the sleep-promoting hypothalamus of mice (Zielinski et al., 2012•).

In response to immune challenge, A_1R activity is upregulated to impart neuroprotection via generating neurotrophic factors, downregulating excitotoxicity, preventing excessive astrogliosis, and inhibiting pro-inflammatory cytokines (Barrie and Nicholls, 1993, Ciccarelli et al., 2001, Jhaveri et al., 2006, Lauro et al., 2010). Blockade of this receptor increases hippocampal injury in response to hypoxia

(Boissard et al., 1992) and mortality to infectious disease (Gallos et al., 2005). Furthermore, lipopolysaccharide (LPS)-induced elevations of EEG slow wave activity are attenuated in gliotransmission-impaired dnSNARE mice, an effect mimicked by central inhibition of the A_1R in wild type mice (Nadjar et al., 2013••). Although, studies regarding the impact of inhibiting the A_1R or gliotransmission on sleep-immune interactions are generally lacking, current data suggest astroglial modulation of sleep and immune function is mediated, in part, by astrocyte-derived ATP and/or adenosine and subsequent activation of purine type 1 and 2 receptors (Figure A.1).

Sleep, immune function, and aging

Sleep alterations are a well-documented feature of aging. Sleep in old age is characterized by more fragmentation, less rapid eye movement sleep (REMS), reduced time in deeper stages of NREMS (i.e. stages N2 and N3), decreased EEG delta power, and more time spent in lighter stages of NREMS which results in more nighttime awakenings. Furthermore, sleep onset is progressively earlier and is accompanied by early morning wake time and more frequent daytime napping (for review see (Singletary and Naidoo, 2011) and (Rolls, 2012)). Although healthy aging need not be associated with sleep complaints, the elderly frequently indicate they have difficulty initiating or maintaining sleep (Espiritu, 2008). Increased severity of these alterations in sleep can increase susceptibility to disease and predict age-related disease onset (Gibson et al., 2009, Postuma and Montplaisir, 2009). Indeed,

several brain regions associated with sleep-wake behavior are impacted by neurodegenerative disease (Singletary and Naidoo, 2011).

Poor sleep can also exacerbate the age-related changes in immune system functioning. “Inflammaging” is the term used to describe the homeostatic shift to a chronic, low-grade inflammatory state in aged individuals (Franceschi et al., 2007). This change is manifest by increased inflammatory mediators centrally and peripherally (Ye and Johnson, 1999), enhanced production of reactive oxygen and nitrogen species (Vishwas et al., 2012), as well as suppression of anti-inflammatory mediators and antioxidants (Ye and Johnson, 2001). A similar syndrome is elicited by chronic sleep loss in younger subjects. This shift predisposes one to exacerbated responses to immune challenge compared to that of normal, younger individuals. Indeed, the severity of this low-grade inflammatory state is predictive of all-cause mortality in the elderly (Bauer, 2008). Alternatively, longevity is inversely correlated with plasma concentrations of IL-6 (Maggio et al., 2006), a mediator of chronic inflammation in aging (Freund et al., 2010). Inflammation is also implicated in contributing to poor sleep observed in the elderly. However, recent data demonstrate that moderate exercise decreases pro-inflammatory cytokines IL-1β and TNFα and increases the anti-inflammatory cytokine IL-10 in aged individuals (Santos et al., 2012•, Speisman et al., 2012•). This effect is associated with improved sleep maintenance and enhanced quality of life (Santos et al., 2012•). Exercise also increases circulating concentrations of neurotrophic factors which are associated with increased temporal lobe connectivity indicative of improved

neurocognition (Voss et al., 2013). Lastly, better sleep efficiency is associated with lower concentrations of IL-6 in elderly women (Friedman et al., 2005).

Although poor sleep in aged individuals increases immunomodulators and, likewise, inflammation alters sleep, the dynamics differ from those observed in younger counterparts. For example, pre-clinical data demonstrate that intracerebroventricular administration of IL-1β reduces REMS to a similar extent in aged and middle-aged rats. However, in aged rats, there is no observed increase in NREMS characteristic of IL-1β challenge in younger animals (Imeri et al., 2004). These data indicate a potential dysregulation of interactions between sleep and immune networks in aging. Furthermore, the lack of NREMS response may contribute to increased morbidity and mortality in response to immune challenge in the elderly (Yoshikawa, 2000). Studies regarding the cumulative effects of aging on sleep and immune function are generally lacking. However, understanding these interactions is increasingly important with the rapid growth of the 65 years and older population.

Glial contributions to aging

Age-related changes of the CNS are not attributed to neuronal loss per se, but are a consequence of synaptic alterations, which may be due to decreased synaptic contacts (reduced dendritic spines) or molecular alterations of intact synapses (Hof and Morrison, 2004). With age, there is a progressive decline in gene expression relating to vesicular function, receptor trafficking, post-synaptic density scaffolding, and neurotrophic systems (Berchtold et al., 2012). Glia are

strong candidates for effectors of age-related alterations in sleep and immune function as these cells are known mediators of synaptic homeostasis (Wake et al., 2009). One of the characteristics of aging is a morphological shift of microglia and astrocytes to a primed or activated state (Franceschi et al., 2007, Dilger and Johnson, 2008). Excessive and prolonged production of pro-inflammatory cytokines in aged CNS in response to systemic immune challenge with LPS is a result of primed microglia (Godbout et al., 2005, Henry et al., 2009). Also, $P2X_7R$ expression increases with age in mouse brain, an effect associated with damage to the post-synaptic density (Lee et al., 2011••). Furthermore, astrocytes become hypersensitive after exposure to microglial-conditioned media, which may perpetuate the chronic inflammatory state in aging (Henn et al., 2011•). Aging brain is associated with increased astrogliosis (Godbout et al., 2005), and astrocytes produce 10-fold more IL-6 in aged patients relative to younger counterparts (Bhat et al., 2012•). Interestingly, peripheral concentrations of cytokines do not necessarily reflect neuroinflammatory states (Godbout et al., 2005). Collectively, these data suggest that aging may be a centrally mediated process driven by glial alterations of the CNS milieu.

Conclusions

Reciprocal influences between sleep and immune system are well-documented, but the cellular and molecular substrates modulating in these interactions are not completely understood. Current data demonstrating glial contributions to sleep regulation suggests further investigation is warranted as these

cells may be critical mechanistic components of sleep and immune interactions throughout the lifespan. Future studies might aim to address the immune consequences of inhibiting glial activity and how this relates to sleep alterations. Also, the mechanisms driving dysregulation of sleep and central immune responses in aging are not well defined. Answering these, and other questions, is necessary before network components of sleep-immune interactions and their role in healthy aging may be fully elucidated.

Acknowledgements

This work was supported in part by the National Institutes of Health, grants NS025378, NS031453 and HD036520 to JMK and AG041827 and MH064843 to MRO.

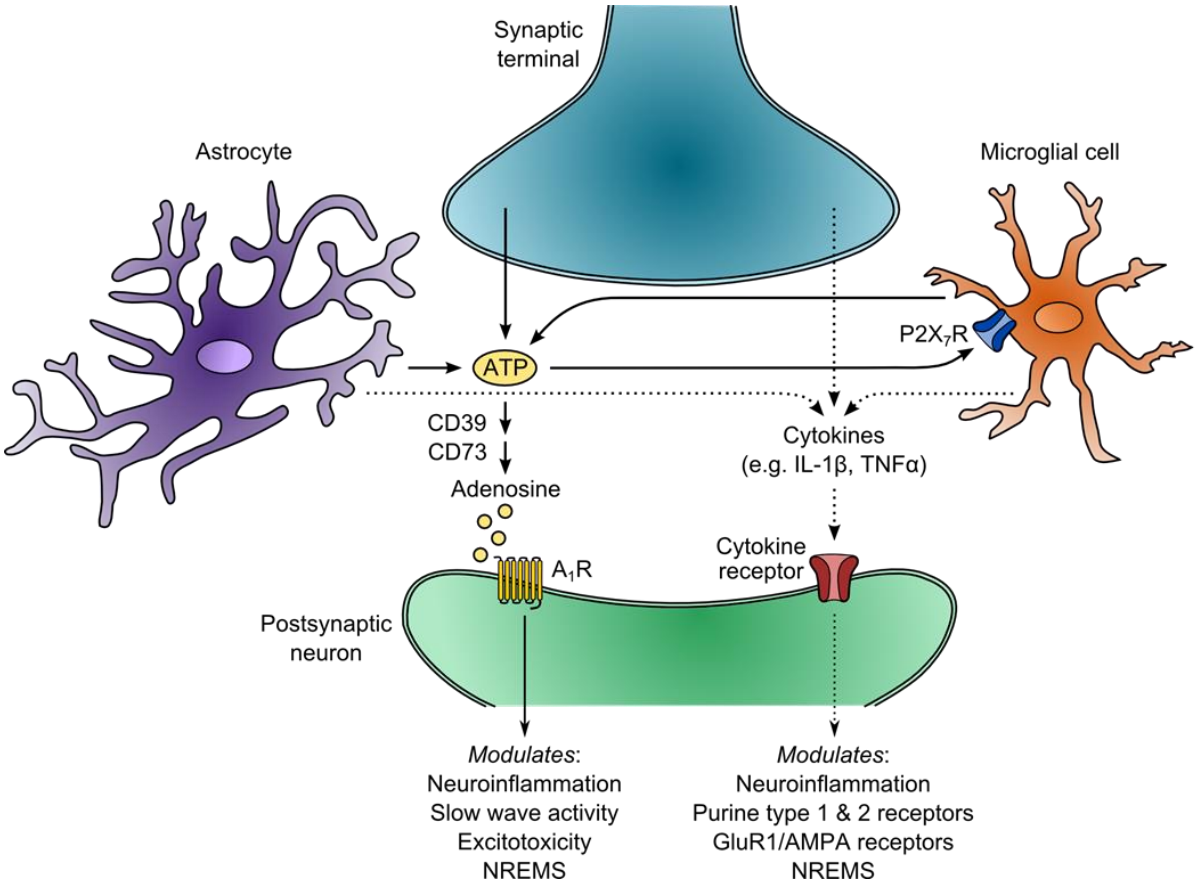


Figure A.1: Glial modulation of sleep and immune interactions. Cellular activity causes ATP release into the extracellular space via neurotransmission and gliotransmission. Extracellular ATP induces rapid effects once metabolized to adenosine by ectonucleotidases such as CD39 and CD73. Adenosine binds to purine type 1 receptors like the A1R to modulate EEG slow wave activity and NREMS, as well as local neuroinflammation and excitotoxicity. Slower effects of extracellular ATP occur through direct activation of purine type 2 receptors such as the P2X7R prominently expressed on microglia. P2X7R activation induces processing and release of cytokines including, but not limited to, IL-1β and TNFα. Cytokines subsequently act on their respective receptors to activate transcription factors like nuclear factor-κB (not shown) which modulates neuroinflammation, physiological and pathological sleep, and gene transcription of receptors such as the A1R, AMPA, and the AMPA subunit GluR1. This overly simplified schematic focuses on the featured topics of this mini-review, and thus, not all cellular and molecular components are fully represented. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors; GluR1, glutamate receptor 1.

References and recommended reading

• of special interest
•• of outstanding interest

Barrie AP, Nicholls DG (1993) Adenosine A1 receptor inhibition of glutamate exocytosis and protein kinase C-mediated decoupling. J Neurochem 60:1081-1086.

Bauer ME (2008) Chronic stress and immunosenescence: a review. Neuroimmunomodulation 15:241-250.

Berchtold NC, Coleman PD, Cribbs DH, Rogers J, Gillen DL, Cotman CW (2012) Synaptic genes are extensively downregulated across multiple brain regions in normal human aging and Alzheimer's disease. Neurobiology of Aging.

Besedovsky L, Lange T, Born J (2012) Sleep and immune function. Pflugers Arch 463:121-137.

•Bhat R, Crowe EP, Bitto A, Moh M, Katsetos CD, Garcia FU, Johnson FB, Trojanowski JQ, Sell C, Torres C (2012) Astrocyte senescence as a component of Alzheimer's disease. PLoS One 7:e45069.

Senescent astrocytes produce more IL-6 and accumulate in greater numbers with age. This accumulation of senescent astrocytes is further exacerbated in Alzheimer's disease patients.

Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW (2009) Control and function of the homeostatic sleep response by adenosine A1 receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:1267-1276.

Boissard CG, Lindner MD, Gribkoff VK (1992) Hypoxia produces cell death in the rat hippocampus in the presence of an A1 adenosine receptor antagonist: an anatomical and behavioral study. Neuroscience 48:807-812.

Choi HB, Ryu JK, Kim SU, McLarnon JG (2007) Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. J Neurosci 27:4957-4968.

Churchill L, Rector DM, Yasuda K, Fix C, Rojas MJ, Yasuda T, Krueger JM (2008) Tumor necrosis factor alpha: activity dependent expression and promotion of cortical column sleep in rats. Neuroscience 156:71-80.

Ciccarelli R, Ballerini P, Sabatino G, Rathbone MP, D'Onofrio M, Caciagli F, Di Iorio P (2001) Involvement of astrocytes in purine-mediated reparative processes in the brain. Int J Dev Neurosci 19:395-414.

Dantzer R (2001) Cytokine-induced sickness behavior: where do we stand? Brain, Behavior and Immunity 15:7-24.

Dantzer R (2009) Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 29:247-264.

Davis CJ, Clinton JM, Jewett KA, Zielinski MR, Krueger JM (2011) Delta wave power: an independent sleep phenotype or epiphenomenon? Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine 7:S16-18.

Dilger RN, Johnson RW (2008) Aging, microglial cell priming, and the discordant central inflammatory response to signals from the peripheral immune system. Journal of Leukocyte Biology 84:932-939.

Dong Y, Benveniste EN (2001) Immune function of astrocytes. GLIA 36:180-190.

Espiritu JR (2008) Aging-related sleep changes. Clin Geriatr Med 24:1-14, v.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends in Immunology 28:138-145.

Florian C, Vecsey CG, Halassa MM, Haydon PG, Abel T (2011) Astrocyte-derived adenosine and A1 receptor activity contribute to sleep loss-induced deficits in hippocampal synaptic plasticity and memory in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:6956-6962.

Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S (2007) Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mechanisms of Ageing and Development 128:92-105.

Freund A, Orjalo AV, Desprez PY, Campisi J (2010) Inflammatory networks during cellular senescence: causes and consequences. Trends in molecular medicine 16:238-246.

Friedman EM, Hayney MS, Love GD, Urry HL, Rosenkranz MA, Davidson RJ, Singer BH, Ryff CD (2005) Social relationships, sleep quality, and interleukin-6 in aging women. Proc Natl Acad Sci U S A 102:18757-18762.

Gallos G, Ruyle TD, Emala CW, Lee HT (2005) A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic injury in murine septic peritonitis. Am J Physiol Renal Physiol 289:F369-376.

Gibson EM, Williams WP, 3rd, Kriegsfeld LJ (2009) Aging in the circadian system: considerations for health, disease prevention and longevity. Exp Gerontol 44:51-56.

Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW, Johnson RW (2005) Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19:1329-1331.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:213-219.

Hallett H, Churchill L, Taishi P, De A, Krueger JM (2010) Whisker stimulation increases expression of nerve growth factor- and interleukin-1beta-immunoreactivity in the rat somatosensory cortex. Brain Research 1333:48-56.

•Henn A, Kirner S, Leist M (2011) TLR2 hypersensitivity of astrocytes as functional consequence of previous inflammatory episodes. J Immunol 186:3237-3247.

Under baseline conditions, astroglial cultures are unresponsive to TLR2 ligands. However, following pre-stimulation with microglial-condition media or inflammatory substances, astrocytes become hypersensitive and demonstrate a prolonged response to TLR2 ligands.

Henry CJ, Huang Y, Wynne AM, Godbout JP (2009) Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. Brain, Behavior, and Immunity 23:309-317.

Hof PR, Morrison JH (2004) The aging brain: morphomolecular senescence of cortical circuits. Trends in Neurosciences 27:607-613.

Hsu JC, Lee YS, Chang CN, Chuang HL, Ling EA, Lan CT (2003) Sleep deprivation inhibits expression of NADPH-d and NOS while activating microglia and astroglia in the rat hippocampus. Cells, tissues, organs 173:242-254.

Imeri L, Ceccarelli P, Mariotti M, Manfridi A, Opp MR, Mancia M (2004) Sleep, but not febrile responses of Fisher 344 rats to immune challenge are affected by aging. Brain, Behavior and Immunity 18:399-404.

Imeri L, Opp MR (2009) How (and why) the immune system makes us sleep. NatRevNeurosci 10:199-210.

Jhaveri KA, Toth LA, Sekino Y, Ramkumar V (2006) Nitric oxide serves as an endogenous regulator of neuronal adenosine A1 receptor expression. J Neurochem 99:42-53.

Kim HS, Suh YH (2009) Minocycline and neurodegenerative diseases. Behav Brain Res 196:168-179.

Kim Y, Bolortuya Y, Chen L, Basheer R, McCarley RW, Strecker RE (2012) Decoupling of sleepiness from sleep time and intensity during chronic sleep restriction: evidence for a role of the adenosine system. Sleep 35:861-869.

Krueger JM (2011) Translation of brain activity into sleep. Hirosaki Med J 62:S1-S16.

Krueger JM, Rector DM, Roy S, Van Dongen HP, Belenky G, Panksepp J (2008) Sleep as a fundamental property of neuronal assemblies. NatRevNeurosci 9:910-919.

Krueger JM, Taishi P, De A, Davis CJ, Winters BD, Clinton J, Szentirmai E, Zielinski MR (2010) ATP and the purine type 2 X7 receptor affect sleep. J Appl Physiol 109:1318-1327.

•Lange T, Dimitrov S, Bollinger T, Diekelmann S, Born J (2011) Sleep after vaccination boosts immunological memory. Journal of Immunology 187:283-290.

Normal sleep, as opposed to sleep deprivation, during the night following vaccination against hepatitis A enhances Th1 cytokine activity, an indicator of cellular adaptive immune responses. In addition, EEG slow wave activity the night after vaccination is predictive of the subsequent T-helper cell response even at 1 year post-vaccination.

Lange T, Perras B, Fehm H, Born J (2003) Sleep enhances the human antibody response to hepatitis A vaccination. Psychosom Med 65:831-835.

Lauro C, Cipriani R, Catalano M, Trettel F, Chece G, Brusadin V, Antonilli L, van Rooijen N, Eusebi F, Fredholm BB, Limatola C (2010) Adenosine A1 receptors and microglial cells mediate CX3CL1-induced protection of hippocampal neurons against Glu-induced death. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:1550-1559.

••Lee HG, Won SM, Gwag BJ, Lee YB (2011) Microglial P2X(7) receptor expression is accompanied by neuronal damage in the cerebral cortex of the APPswe/PS1dE9 mouse model of Alzheimer's disease. Experimental & molecular medicine 43:7-14.

Microglial $P2X_7R$ expression increases with age in wild type mice, and this increase is accelerated in a mouse model of Alzheimer's disease. Elevated

$P2X_7R$ also precedes increases in reactive oxygen species and amyloid β peptide.

Maggio M, Guralnik JM, Longo DL, Ferrucci L (2006) Interleukin-6 in aging and chronic disease: a magnificent pathway. J Gerontol A Biol Sci Med Sci 61:575-584.

Mingam R, De Smedt V, Amedee T, Bluthe RM, Kelley KW, Dantzer R, Laye S (2008) In vitro and in vivo evidence for a role of the P2X7 receptor in the release of IL-1 beta in the murine brain. Brain Behav Immun 22:234-244.

••Nadjar A, Blutstein T, Aubert A, Laye S, Haydon PG (2013) Astrocyte-derived adenosine modulates increased sleep pressure during inflammatory response. GLIA 61:724-731.

Systemic immune challenge with LPS increases EEG slow wave activity in wild type mice but not in a mouse model of impaired astrocyte gliotransmission. This effect is mediated by astrocyte-derived adenosine activation of the A_1R. However, other mechanisms are implicated in modulating LPS-induced alterations of NREMS and REMS duration.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113-116.

Postuma RB, Montplaisir J (2009) Predicting Parkinson's disease - why, when, and how? Parkinsonism Relat Disord 15 Suppl 3:S105-109.

Rolls A (2012) Hypothalamic control of sleep in aging. Neuromolecular medicine 14:139-153.

•Santos RV, Viana VA, Boscolo RA, Marques VG, Santana MG, Lira FS, Tufik S, de Mello MT (2012) Moderate exercise training modulates cytokine profile and sleep in elderly people. Cytokine 60:731-735.

Moderate exercise in previously sedentary elderly adults improves sleep, reduces inflammation, and increases anti-inflammatory mediators compared to baseline measurements.

••Schmitt LI, Sims RE, Dale N, Haydon PG (2012) Wakefulness affects synaptic and network activity by increasing extracellular astrocyte-derived adenosine. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:4417-4425.

Using a biosensor-based analysis of adenosine, this study demonstrates that extracellular adenosine increases with wakefulness. This accumulation of adenosine is dependent on astrocytic gliotransmission and mediates activity of the A_1R as demonstrated by genetic inhibition of gliotransmission.

Singletary KG, Naidoo N (2011) Disease and Degeneration of Aging Neural Systems that Integrate Sleep Drive and Circadian Oscillations. Front Neurol 2:66.

•Speisman RB, Kumar A, Rani A, Foster TC, Ormerod BK (2012) Daily exercise improves memory, stimulates hippocampal neurogenesis and modulates immune and neuroimmune cytokines in aging rats. Brain, Behavior, and Immunity.

Daily exercise in aged rats reduces IL-1β concentrations and increases neurotrophic factors in hippocampus as well as improves cognitive function compared to non-running controls. Additionally, exercise increases neurogenesis in aged rats. This study also demonstrates that circulating cytokines do not necessarily reflect neuroinflammatory conditions.

Spiegel K, Sheridan JF, Van Cauter E (2002) Effect of sleep deprivation on response to immunization. Journal of the American Medical Association 288:1471-1472.

Vishwas DK, Mukherjee A, Haldar C, Dash D, Nayak MK (2012) Improvement of oxidative stress and immunity by melatonin: An age dependent study in golden hamster. Exp Gerontol.

Voss MW, Erickson KI, Prakash RS, Chaddock L, Kim JS, Alves H, Szabo A, Phillips SM, Wojcicki TR, Mailey EL, Olson EA, Gothe N, Vieira-Potter VJ, Martin SA, Pence BD, Cook MD, Woods JA, McAuley E, Kramer AF (2013) Neurobiological markers of exercise-related brain plasticity in older adults. Brain, Behavior, and Immunity 28:90-99.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974-3980.

••Wisor JP, Schmidt MA, Clegern WC (2011) Evidence for neuroinflammatory and microglial changes in the cerebral response to sleep loss. Sleep 34:261-272.

Microglial inhibition by the anti-inflammatory minocycline induces a reduction in NREMS and attenuates increases in NREM delta power normally observed after sleep deprivation. These data suggest microglia contribute to the modulation of sleep need.

Ye SM, Johnson RW (1999) Increased interleukin-6 expression by microglia from brain of aged mice. Journal of Neuroimmunology 93:139-148.

Ye SM, Johnson RW (2001) An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. Neuroimmunomodulation 9:183-192.

Yoshikawa TT (2000) Epidemiology and unique aspects of aging and infectious diseases. Clinical Infectious Diseases 30:931-933.

•Zielinski MR, Taishi P, Clinton JM, Krueger JM (2012) 5'-Ectonucleotidase-knockout mice lack non-REM sleep responses to sleep deprivation. Eur J Neurosci 35:1789-1798.

Mice lacking CD73, an ectonucleotidase that aids in the metabolism of ATP to adenosine, exhibit more spontaneous NREMS and attenuated response to sleep deprivation compared to wild type controls. These data suggest extracellular adenosine regulation of sleep is mediated, in part, by CD73.

APPENDIX B

LIPOPOLYSACCHARIDE-INDUCED BEHAVIORAL RESPONSES OF MICE EXPRESSING INTERLEUKIN-1 RECEPTOR 1 OR TUMOR NECROSIS FACTOR RECEPTOR 1 SELECTIVELY ON NEURONS OR ASTROCYTES

INTRODUCTION

At the time we engineered the NSE-IL1R1 and GFAP-IL1R1 mice, we concurrently developed transgenic counterparts for tumor necrosis factor α (TNF) that express TNF receptor 1 (TNFR1) only in the central nervous system and selectively on neurons (NSE-TNFR1) or astrocytes (GFAP-TNFR1). Similar to interleukin-1β (IL-1), TNF is a pro-inflammatory cytokine that mediates sleep and multiple aspects of the innate immune response. Febrile responses and symptoms of sickness, such as sleep-wake alterations, decreased feeding and water intake, loss of body weight, and social withdrawal can also be induced by central administration of TNF (Bluthe et al., 1994, Dantzer, 2004, Opp, 2005, Chida and Iwakura, 2007). TNF exerts its effects through two receptors: TNF p55 (TNFR1) and TNF p75 (TNFR2). However, most biologic activity of TNF is mediated via TNFR1 which is expressed on neurons and astrocytes (Vandenabeele et al., 1995).

Prior to deciding to direct the focus of this dissertation to IL-1 contributions to sleep and immune responses, we ran a pilot study aimed at determining the effects of lipopolysaccharide (LPS) on symptoms of sickness in wild type (B6129SF2/J), NSE-IL1R1, NSE-TNFR1, and GFAP-TNFR1 mice. Founders for GFAP-IL1R1 mice

were being generated at the time of this study, and are not included in the following dataset. The transgenes for all transgenic mice were expressed on a genetic background lacking endogenous expression for both IL1R1 and TNFR1. Below is a brief summary of the pilot study that demonstrates the relative contributions of neurons and astrocytes to behavioral response to LPS as modulated by IL1R1 and TNFR1.

METHODS

Adult male B6129SF2/J (n = 4), NSE-IL1R1 (n = 3), NSE-TNFR1 (n = 4), and GFAP-TNFR1 (n = 3) mice were surgically instrumented with electroencephalographic (EEG) electrodes to determine arousal state. EEG data were obtained via a lightweight, tethered swinging arm system. Mice were housed with running wheels in recording chambers maintained at 29 ± 1°C under a 12:12 h light:dark cycle. All mice received pyrogen-free saline (PFS; 0.2 ml) intraperitoneally (i.p.) at dark onset. The next day mice were challenged at dark onset with LPS (0.4 mg/kg; i.p.). EEG, wheel running, body weight, and food and water intake data were collected for 24 h following each injection.

RESULTS AND DISCUSSION

LPS suppressed rapid eye movement sleep (REMS) and increased non-REMS (NREMS) of B6129SF2/J, NSE-IL1R1, and NSE-TNFR1 mice, whereas GFAP-TNFR1 mice exhibited no sleep alterations in response to LPS (Figure B.1). LPS also decreased wheel running activity of B6129SF2/J, NSE-IL1R1, and NSE-

TNFR1 animals (Figure B.2). However, wheel running was preserved in GFAP-TNFR1 mice following challenge with LPS. Finally, LPS decreased body weight and food and water intake of B6129SF2/J, NSE-IL1R1, and NSE-TNFR1 mice (Figure B.3). Body weight, feeding, and drinking was maintained in GFAP-TNFR1 mice after LPS. These data suggest that selective expression of TNFR1 on astrocytes is not sufficient for behavioral immune responses to systemic challenge with LPS.

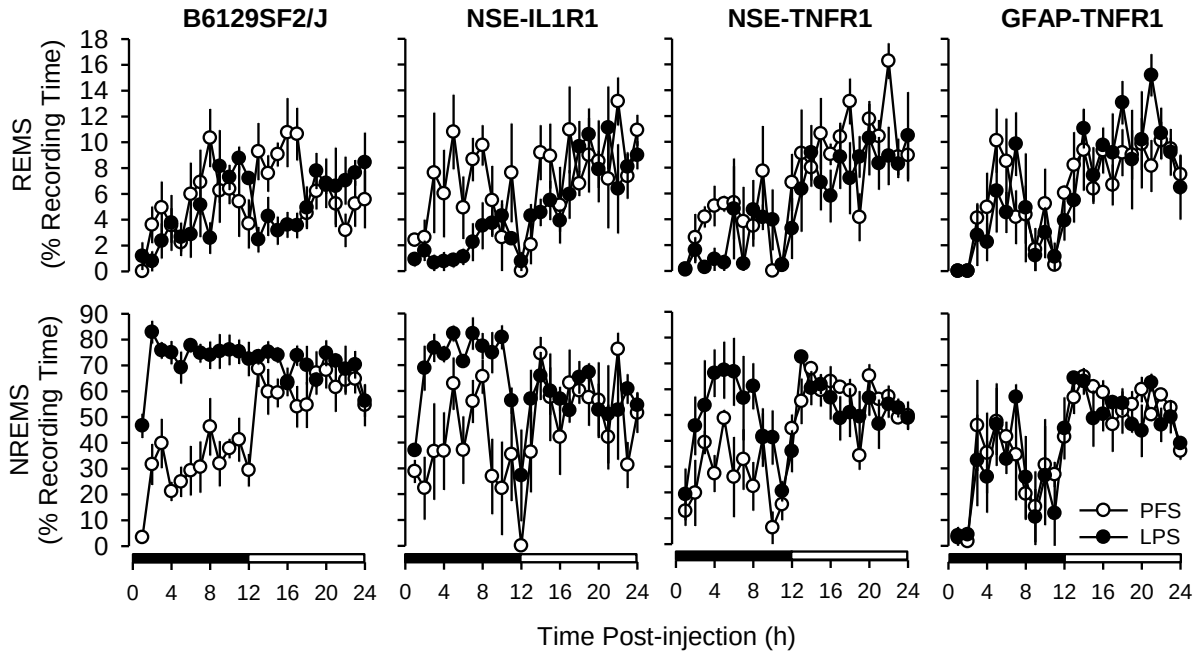
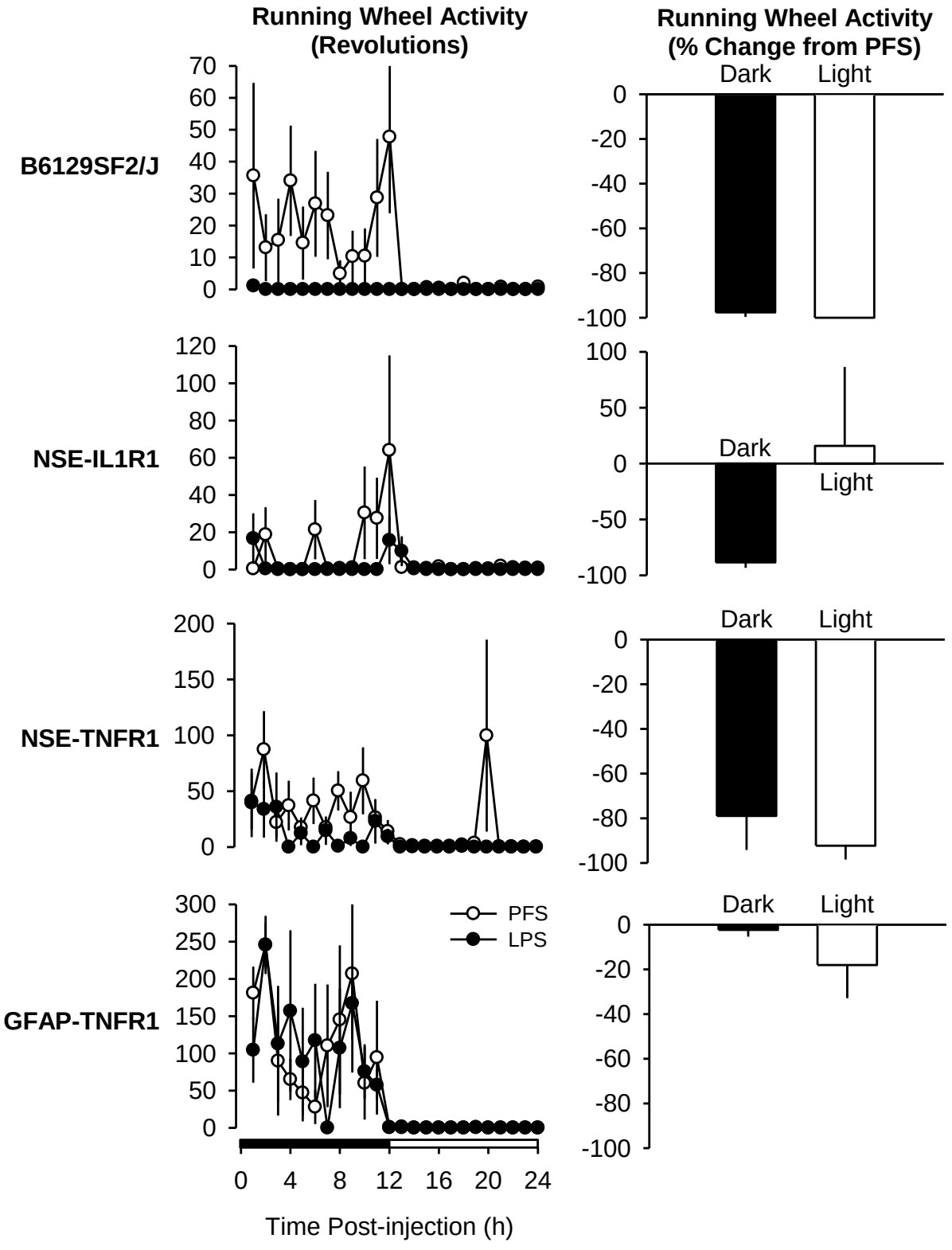


Figure B.1: Effects of intraperitoneal injection of PFS and LPS on percentage of time spent in REMS and NREMS for B6129SF2/J, NSE-IL1R1, NSE-TNFR1, and GFAP-TNFR1 mice. Values are hourly averages expressed as means ± S.E.M. PFS (0.2 ml; i.p.) was injected at dark onset followed by LPS (0.4 mg/kg; i.p.) at dark onset the next day. EEG was recorded and sleep states determined. The filled and open bars on the X-axis indicate dark and light periods of the light-dark cycle, respectively.

Figure B.2: LPS-induced alterations in wheel running activity of B6129SF2/J, NSE-IL1R1, NSE-TNFR1, and GFAP-TNFR1 mice. Values are hourly and 12 h averages expressed as means ± S.E.M. PFS (0.2 mL; i.p.) was administered at dark onset followed by LPS (0.4 mg/kg; i.p.) at dark onset the next day. Running wheel activity was recorded. The filled and open bars on the X-axis indicate dark and light periods of the light-dark cycle, respectively.


Running Wheel Activity
(Revolutions)
Running Wheel Activity
(% Change from PFS)
B6129SF2/J
NSE-IL1R1
NSE-TNFR1
GFAP-TNFR1
Dark
Light
PFS
LPS
Time Post-injection (h)

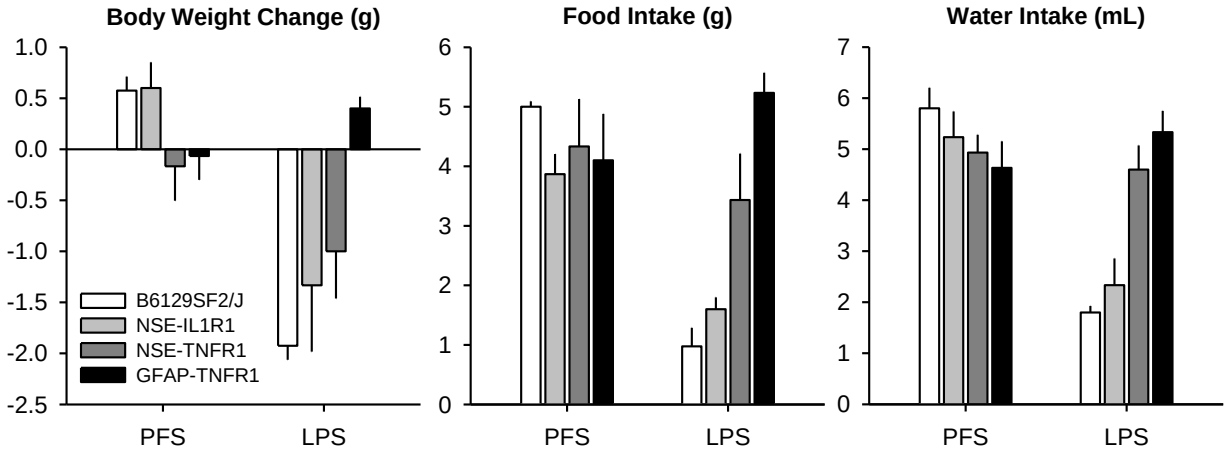


Figure B.3: Body weight, feeding, and drinking responses to LPS of B6129SF2/J, NSE-IL1R1, NSE-TNFR1, and GFAP-TNFR1 mice. Values are 24 h averages expressed as means ± S.E.M. PFS (0.2 mL; i.p.) was administered at dark onset followed by LPS (0.4 mg/kg; i.p.) at dark onset the next day. Body weight, food intake, and water intake data were collected daily.

References

Bluthe RM, Pawlowski M, Suarez S, Parnet P, Pittman Q, Kelley KW, Dantzer R (1994) Synergy between tumor necrosis factor alpha and interleukin-1 in the induction of sickness behavior in mice. Psychoneuroendocrinology 19:197-207.

Chen G, Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. Science 296:1634-1635.

Chida D, Iwakura Y (2007) Peripheral TNFalpha, but not peripheral IL-1, requires endogenous IL-1 or TNFalpha induction in the brain for the febrile response. Biochem Biophys Res Commun 364:765-770.

Dantzer R (2004) Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. European Journal of Pharmacology 500:399-411.

Dantzer R (2006) Cytokine, sickness behavior, and depression. NeurolClin 24:441-460.

Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GH, Chen EY, Goeddel DV (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci U S A 88:2830-2834.

MacEwan DJ (2002) TNF ligands and receptors--a matter of life and death. Br J Pharmacol 135:855-875.

Opp MR (2005) Cytokines and sleep. Sleep MedRev 9:355-364.

Tartaglia LA, Pennica D, Goeddel DV (1993) Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem 268:18542-18548.

Vandenabeele P, Declercq W, Beyaert R, Fiers W (1995) Two tumour necrosis factor receptors: structure and function. Trends Cell Biol 5:392-399.