The Cytokine-Induced Sickness Response: Investigations of How the Central Nervous System Controls the Biological Response to Disease

by

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A DISSERTATION

Presented to the Neuroscience Graduate Program

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

March 2017

CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

3V	Third Ventricle
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related peptide
AHA	Anterior hypothalamic area
AHN	Anterior hypothalamic nucleus
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Area postrema
ARC	Arcuate nucleus of the hypothalamus
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMR	Basal metabolic rate

BW	Body weight
CBT	Core body temperature
CCK	Cholesystokinin
Cd	Cluster of differentiation protein, applies to 11b and 45
CHF	Congestive heart failure
ChP	Choroid Plexus
cMCS	Central melanocortin system
CNS	Central nervous system
CNTF	Cilliary neurotrophic factor
COX-1	Cyclooxygenase-1
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CVO	Circumventricular organ
DLPO	Dorsolateral pre-optic nucleus of the hypothalamus
DMN	Dorsomedial nucleus of the hypothalamus
eGFP	Enhanced green-fluorescent protein
EP3	E-prostaglandin receptor 3
FACS	Fluorescence-activated cell sorting

FFA	Free fatty acid
FI	Food intake
FSH	Follicle-stimulating hormone
GC	Glucocorticoid
GFAP	Glial fibrillary acidic protein
GFP	Green-fluorescent protein
GH	Growth hormone
GHIH	Growth hormone-inhibiting hormone
GHRH	Growth hormone-releasing hormone
GHSR	Growth hormone secretagogue receptor
GLP-1	Glucagon-like peptide-1
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
H&E	Hematoxylin and eosin
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPT	Hypothalamic-pituitary-thyroid axis
IACUC	Institutional animal care and use committee

ICV	Intracerebroventricular
IGF1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL-1	Interleukin-1, applies to both α and β
IL1R1	Interleukin-1 receptor
IL1RAcP	Interleukin-1 receptor accessory protein
IP	Intraperotineal
IR	Immunoreactivity
KO	Knockout
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
MB	Mamillary body
MC	Mineralocorticoid
MCR	Melanocortin receptor, applies to 1-5
MCS	Melanocortin system
ME	Median eminence
MHC	Major histocompatibility complex

MnPO	Median pre-optic nucleus of the hypothalamus
MPO	Medial pre-optic nucleus of the hypothalamus
Myd88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	Neuropeptide-Y
OHSU	Oregon Health and Science University
OVLT	Organum vasculosum lamina terminalis
OXT	Oxytocin
PBS	Phosphate-buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGE2	Prostaglandin-E2
PHA	Posterior hypothalamic area
PHN	Posterior hypothalamic nucleus
PNS	Parasympathetic nervous system
POA	Pre-optic area of the hypothalamus
POMC	Pro-opiomelanocortin
PON	Pre-optic nucleus of the hypothalamus

PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide-YY
RQ	Respiratory quotient
SCN	Suprachiasmatic nucleus of the hypothalamus
SEM	Standard error of the mean
SFO	Subfornical organ
SNS	Sympathetic nervous system
SON	Supraoptic nucleus of the hypothalamus\
STAT3	Signal transducer and activator of transcription-3
SV	Subdiaphagmatic vagotomy
T3	Tri-iodothyronine
T4	Thyroxine
TNFα	Tumor necrosis factor
TRH	Thryrotropin-releasing hormone
TRP	Transient receptor potential
TSH	Thyroid-stimulating hormone
Tx	Triton-X100
UCP-1	Uncoupling protein-1

Veh	Vehicle
VLA	Voluntary locomotor activity
VLPO	Ventrolateral pre-optic nucleus of the hypothalamus
VMN	Ventromedial nucleus of the hypothalamus
WAT	White adipose tissue
WT	Wild-type
YFP	Yellow-fluorescent protein
α-MSH	α-melanocyte stimulating hormone

ACKNOWLEDGEMENTS

There are many people deserving of thanks for their help and support, without which I could never have completed graduate school. Education and science are team sports, and in order to do well you have to have good people around you to accomplish your goals. I would like to start by thanking Daniel Marks, my coach and mentor through this entire process. Without his guidance and knowledge none of this would have been possible. Thank you for giving me a home where I felt free to pursue my interests without hindrance and for all the helpful and insightful discussions along the way.

The other members of my dissertation advisory committee—Sergio Ojeda, Charles Rosseli, and Jae Lee—were very generous with their time and advice. They helped me shape a vague idea into a project and gave excellent suggestions when I struggled.

I would also like to thank my teammates—the former and present members of the Marks lab—who have been through all of this with me. Special thanks go to Stephanie Krasnow, who has been here the whole time, and not only helped with experiments but also with advice and discussions. Stephanie generated and analyzed all of the data related to the Tie2-Cre mice presented in Chapter 3 of this thesis. She also provided all of the microglia cultures used in Chapter 2 and helped me figure out how to make that system work. I am thankful for all of her time and help; I couldn't have done this without her. Aaron Grossberg and Ted Braun, two former MD/PhD graduates from the lab who were there in the beginning to show me the ropes and get me started as a graduate student, also deserve particular notice. Nearly everything I did in the lab was based on the foundations laid by Ted and Aaron. Xin Xia Zhu is probably the best laboratory scientist I have met. There is no surgical procedure or technique that she has

not mastered. She taught me how to do things right and therefore deserves credit for whatever I have managed to accomplish. Katy Michaelis and Kevin Burfiend, the newest graduate students in the lab, are rapidly overtaking my progress and deserve thanks for conversation and support. Peter Levasseur has also been here the whole time and was always there when I needed advice on anything molecular biology. Thank you all for being on my team.

I would also like to thank my mom, Diane Sparks, for everything she has done for me. There is no way that I could list everything, so I will simply say that her love, support and encouragement got me through a lot. I couldn't have done any of this without her.

I would like to thank everyone in the NGP who has been there for me whenever I needed it. Gary Westbrook was always there to talk and has the best interest of all graduate students as his first priority. Liz Lawson-Webber helped me figure out how to navigate through every step of grad school; I'm pretty sure if Liz wasn't around everything would fall apart. My classmates deserve recognition for friendship and companionship throughout this process.

Finally, I would like to thank my girlfriend, Caitlin Monaghan. She has provided love, support and comfort through it all. She is also an excellent scientist and deserves credit for many mistakes I didn't make by listening whenever I was struggling and helping me figure out better ways of approaching scientific problems. Without her I would have failed miserably.

ABSTRACT

Disease is an ever-present threat to the survival of the individual. To meet these challenges, higher organisms have developed complex mechanisms designed to heal and fight for survival. These mechanisms manifest as physiologic and behavioral alterations including fever, anorexia, malaise and loss of body weight (collectively the sickness response). Previous research has demonstrated that these effects are mediated by cytokine signaling. Our lab has contributed to this body of knowledge by showing that inflammatory cytokines directly modulate the activity of the central melanocortin and orexin signaling systems. However, much remains unknown about the role of central nervous system inflammation and how cytokine signaling is transmitted from the periphery to the central nervous system.

In the first part of this thesis we tested the hypothesis that central nervous system production of the pro-inflammatory cytokine interleukin-1β (IL-1β) is involved in initiating or prolonging sickness behaviors. Our results show that elimination of IL-1 signaling by germline deletion does not alter the initiation or duration of sickness behaviors. We also examined whether the response to IL-1β in microglia alone was sufficient to trigger sickness behaviors. Using a technique for harvesting and transplanting microglia we created chimeric animals in which only microglia possess the capability of responding to IL-1β. We found that chimeric mice did not exhibit sickness responses, demonstrating that transplanted microglia were insufficient to trigger sickness behaviors.

In the second part of this thesis we examined the cellular signaling components that are required for the sickness response based on the hypothesis that these responses are triggered by vascular endothelial cells or microglia. We first established a protocol for identifying which cell types within the brain show an immediate response to

intracerebroventricular (ICV) administration of IL-1β. In agreement with previously published results, we found that endothelial cells, astrocytes, microglia and ependymal cells (including choroid plexus and some tanycytes) all demonstrate a clear response to ICV IL-1β. We then eliminated IL-1β signaling capability from defined populations of cells to determine which cellular compartments are responsible for triggering the sickness response. We found that sickness responses were eliminated only when IL-1β signaling was disrupted in all endothelium and microglia. By comparison with other cellular knockout models (endothelium that does not include fenestrated capillaries, microglia only, or a combination of the two) our results indicate that central nervous system inflammation triggers sickness responses dependent on IL-1β signaling specifically in fenestrated capillaries.

The data presented in this thesis contribute to the understanding of how cytokines act to stimulate physiologic and behavioral modifications that promote survival and healing. The novel implication that fenestrated capillary-specific signaling mechanisms are responsible for these behaviors represents a new potential for therapeutic interventions.

Chapter 1:

Introduction

1. Significance and Rationale

"A healthy body is a guest chamber for the soul;
a sick body is a prison."

—Sir Francis Bacon
English philosopher, statesman, scientist

"I am sick and tired of being sick and tired."

—Fannie Lou Hamer
American civil rights leader

From the first tickle at the back of the throat, the first sense that something is wrong, or off, we know that we are getting sick. We dread what is coming: the aches and pains, the nausea, tiredness and generalized discomfort. We all know what it is to feel sick; illness is a part of life that we have all experienced. The feelings associated with being sick are also a reflection of the body's efforts to heal, to be well again. While the symptoms of sickness stem from processes that are generally beneficial, they can be extremely unpleasant and their impacts on quality of life are often detrimental, in some cases far worse than the disease itself.

Although often viewed as merely a nuisance, something to be endured but largely ignored, the physical and emotional aspects of sickness are now recognized as important medical considerations.(1) How we feel can impact how we heal, and those symptoms that limit the ability or desire for exercise and enjoyment can hamper the healing process. More, in situations of prolonged or chronic illness, the same processes that prove beneficial in the short term can turn deadly in the long run.

Cachexia, or disease-associated wasting, is a serious complication of multiple chronic illnesses including cancer, congestive heart failure (CHF) and infectious diseases such as HIV/AIDS. It manifests as a significant loss of body weight due

increased catabolism of lean body mass, complicated by anorexia and lethargy.[2] The severity and duration of cachexia are primary predictors of disease outcome, with increased morbidity and mortality associated with persistent cachexia.[3, 4] In one study on the effects of cachexia in CHF, the presence of cachexia was found to correlate with an increase in mortality from 17% (non-cachectic CHF) to 50% (for patients with cardiac cachexia).[5] Despite the prevalence of this condition and the severity of its impact, little is known about its causes and there are no effective treatment strategies. The importance of understanding cachexia is such that the National Cancer Institute included it in the list of provocative questions for 2012.[6] Advances in medical science have contributed to extending the lifespan of patients suffering from what have historically been fatal diseases and have thus prolonged the duration of the cachectic state. While these advances have allowed people to survive the immediate insult, a better understanding of the nature of cachexia is needed to improve the overall outcome.

In order to survive, an organism must be able to react to adversity and overcome challenges to its health and well-being; life is a struggle, often with other forms of life, sometimes with itself. The biological response to illness and injury is a complex, multi-faceted program involving changes to physiologic and behavioral parameters that likely evolved as mechanisms to promote survival of the individual. While the exact nature of the response depends in part on the type of illness, many features of the program are common to multiple different insults. These features include alterations in body temperature (fever), metabolism (energy homeostasis), appetite (anorexia), emotions (anhedonia and malaise) and arousal (sleep disturbances and lethargy).[7] Whether in response to common viral or bacterial infections, traumatic injury or chronic disease, the immediate actions of the body involve collaboration of the immune, nervous and endocrine systems to confront and respond to internal threats.

Alternatively referred to as the acute-phase response or sickness behaviors, in reality all of these symptoms represent aspects of a coordinated response to threats to the well-being of the organism. The first stage of this process involves detection of the threat or damage by components of the immune system. In addition to regulating the local response, the immune system also communicates this information to the rest of the body, in particular to the central nervous system (CNS). The CNS serves as the compiler of information from the peripheral body and also as the regulator of homeostasis and responses to differing circumstances, whether internal or external. Part of this regulatory response involves direct control of the endocrine system and autonomic nervous system (ANS). The endocrine system, via the release of hormones into the general circulation, controls body-wide changes in metabolism, growth and reproduction. The ANS also regulates diverse actions throughout the body via diffuse innervations of visceral organs, glands and blood vessels.

While an in-depth description of any one aspect of the sickness response can be, and has been, the subject of an entire body of work, a brief introduction to some of the components will assist in the assimilation of the whole as relates to the work of this thesis. The focus this thesis was to determine how cytokine signaling within the central nervous system results in the sickness response. We first examined whether interleukin-1 (IL-1) signaling was necessary for the sickness response and whether microglia are sufficient to trigger such responses. We then tested which cell types are responsible for generating the sickness response. The results of these studies advance our understanding of how cytokine signaling results in physiologic and behavioral modifications in response to disease. The discovery that fenestrated capillaries represent a unique signaling niche within the brain has the potential to provide new strategies for targeted treatments of sickness responses.

2. The Sickness Response

2.1 Physiology and Behavior

Before beginning a discussion of the mechanisms of sickness response generation, it is useful to first have a definition of what constitutes the sickness response. A considerable amount of research effort was dedicated to unraveling the nature of the biological response to disease. Much of this has been done by the group headed by Robert Dantzer. This group has focused primarily on the behavioral aspects of sickness, and their work has had a beneficial impact by highlighting the negative impacts on quality of life that accompanies the specific symptoms of sickness.[8] While animals, including humans, demonstrate similar behavioral responses to sickness, there are also physiologic responses that might not manifest as a change in behavior. For this reason, the term "sickness response," incorporating both physiology and behavior, will be used in this thesis. Since the most common and reproducible signs of sickness include fever, anorexia, decreased movement and loss of body weight, these are the focus of this thesis.

2.2 Animal Models of Disease

There are essentially as many models of disease as there are actual diseases; most naturally occurring disease states can be replicated in a controlled setting to study their effects, and for some of those that cannot, ingenuity on the part of researchers has provided for a host of methods that closely replicate the symptoms of disease. Our lab has used a multitude of techniques including surgical cardiac restriction (to simulate heart failure), surgical removal of most of the kidneys (for studies on renal failure) and multiple cancer cell lines (causing cancerous tumor formation when implanted in mice for

studying cancer cachexia).[9-12] All of these models cause severe disease in experimental animals, often leading to death, and interpretation of the results of such experiments can be difficult. In an effort to simplify conditions and study the specific mechanisms that drive the sickness response, the research presented in this thesis focuses on two commonly used models: intraperitoneal (IP) injection of lipopolysaccharide (LPS, a component of the cell wall gram-negative bacteria) to stimulate a peripheral immune response, and, primarily, intracerebroventricular (ICV) administration of interleukin-1β (IL-1β) to simulate CNS production of inflammatory cytokines.

The results discussed in this introduction relate to studies using rabbits, cats, ferrets, mice, rats, monkeys and, occasionally, humans. The point of these experiments is to understand not only how the sickness response is orchestrated in animals, but also how parallel processes occur in humans. If a given stimulus causes the same effect in multiple animal species, there is a good probability that there are conserved neural mechanisms driving these responses. Even so, it is important to refrain from anthropomorphizing or over-interpreting behavioral results.

While there is little doubt as to what an elevation of body temperature means (a treatment either is, or has caused the production of, a pyrogen that triggers the febrile response), the cause of other symptoms are not so concrete. For example, although decreased locomotor activity is one of the most reliable measures of sickness, there are multiple possible explanations for why sick animals move less and the exact cause(s) remains unknown. An incomplete list of possible explanations includes: physical discomfort that makes movement unpleasant; fever-associated behaviors (e.g. shivering, hunching or huddling that generate heat or reduce heat loss); reduction of food-seeking exploratory behavior caused by decreased appetite; or alterations to the activity of neural circuits that modulate sleep/wake or arousal/lethargy levels. Finally, despite the

fact that sickness responses seem to be evolutionarily conserved, there is evidence that major differences at the level of genetic regulation exist between different species.[13] Because of this uncertainty, this introduction will first describe how the immune system communicates the presence of disease, how the hypothalamus coordinates endocrine and autonomic activity to generate the sickness response and then focus on what is known about the circuitry involved in regulating energy homeostasis and body temperature.

3. The Immune System

3.1 The Immune System: Sentinels, Soldiers and Signalers of Distress

The vertebrate immune system is a highly complex network of cells whose function is defense of the body against foreign invaders and internal malfunction. Considering the array of threats that an organism is continuously exposed to, the importance of the immune system is difficult to overstate: without constant defense against bacterial and viral invaders (including those which symbiotically inhabit the intestinal cavities) and cellular malfunction (including oncogenic mutation and cell death) no complex organism could survive for long. Through the process of evolution the immune system has developed an array of defense mechanisms, both passive and active.

3.2 Passive Defense Mechanisms: Barriers to the Outside and Inside Worlds

The first line of immune defense is the system of passive barriers that prevent foreign organisms from entering the body. These are composed of the integumentary system (skin, hair and nails) which covers the external surfaces and the mucosal system

which lines the internal cavities.[14] The outer layers of these defensive barriers are composed of non-living material (epithelial cells and mucus) that act as a physical boundary between the body and its surroundings. While these barriers are passive in nature (meaning that they do not actively respond to the presence pathogens) they do possess antimicrobial properties (including acidic pH, glycoproteins and enzymes), and so represent more than simple walls that prevent entry into the body. In addition to their role as barricades, these systems also serve homeostatic functions including preventing dehydration and thermoregulation. Moreover, integrated within these structures are cells of the active immune system constantly on the lookout for infiltrating hazards. A growing body of evidence supports an active role for these cells in communicating immune information to the brain.[15]

3.3 Active Defense Mechanisms: The Innate and Acquired Immune Systems

Classically, the active immune system is divided into two categories: the innate immune system and the acquired immune system.[16, 17] The innate immune system is that which responds to a given stimulus in the same way regardless of how many times it has been encountered.[18] The acquired immune system, in contrast, stores information about a given pathogen to assist in a more effective response upon re-exposure. Another way to state this relationship is that the acquired immune system establishes immunity (a targeted defense based on recognition of a previously encountered pathogen) while the innate immune system is responsible for executing an immune response (actively eliminating pathogens). For the purpose of this thesis the functions of the innate immune system are most relevant, yet due to significant overlap, incomplete segregation and reciprocal stimulation both systems will be discussed briefly.

3.4 The Acquired Immune System: Catalogue of Challenges

The acquired immune system is responsible for recognizing specific antigens that are associated with specific pathogens. It is also the storehouse of information about previously encountered threats allowing for a more rapid and precise response upon re-exposure.[16] Composed of lymphatic cells—the antibody producing B cells and the antigen recognizing T Cells—the acquired immune system works to identify and eliminate anything that is not recognized as part of the "self." Both B and T cells go through a process of maturation that includes genetic rearrangement which results in a staggering variety of antigen binding molecules.[19] In B cells this manifests in the random assembly of antibodies that recognize specific antigens, while T cells generate cell surface receptors that serve a similar function, but are not secreted into the circulation. Lymphatic cells work together and independently to recognize threats and stimulate an amplified immune response through the production of epitope-specific antibodies.

Recognizing specific epitope shapes, antibodies provide a mechanism of labeling specific molecules to identify them for targeting by cells of the innate immune system. In addition to labeling threats, antibodies also stimulate the cells of the innate immune system through membrane bound antibody receptors. This causes an increase in activities such as phagocytosis that are discussed below. Finally, even though the primary role of the adaptive immune system is antigen recognition, the lymphocytes also secrete a variety of cytokines that contribute to the immune and inflammatory responses.[20]

3.5 The Innate Immune System: Effectors of the Immune Response

The cellular components of the innate immune system include monocytes, macrophages, neutrophils, eiosinophils, basophils, mast cells, dendritic cells and natural killer cells.[21] Although the innate immune response tends to be generalized, in the sense that different stimuli trigger the same responses, some specific pathogens are handled by different cells. Natural killer cells, sometimes called the pit bulls of the immune system for their tenacity in killing anything that does not present the correct cellular identification, are responsible for destroying virus-infected or oncogenetically mutated cells that no longer express the correct major histocompatibility complex (MHC) molecules that identify them as "self."[22, 23] Eosinophils, on the other hand, seem to be primarily involved in killing parasites such as nematodes.[24] Because these pathogens are too large for phagocytosis, eosinophils utilize stores of toxic compounds including neurotoxins to kill parasites. Eosinophils also signal the presence of infection by releasing a variety of cytokines and inflammatory mediators, and so likely play a role in the sickness response (as will be discussed later). Basophils and mast cells, similar in both appearance and function, react to antibody-bound pathogens by releasing stores of inflammatory mediators.[25] Although mast cells are best known for their release of histamine that contributes to allergic reactions, new evidence suggests that they are capable of releasing a broad spectrum of inflammatory mediators and neurohormones and might play a role in the development of other diseases such as atherosclerosis.[26]

3.6 Mononuclear phagocytes: One name, many cells

Macrophages are an integral component of the immune system and are directly responsible for the generation of the sickness response. As such, a more in-depth discussion of these cells is warranted. One of the first things that should be noted is that there are multiple different types of cells that are all called macrophages.[27] Among these are the macrophages found throughout the body that probably represent tissue

infiltrating monocytes.[28] There are also specialized macrophages found only within certain organs or tissues. These include the Langerhans cells of the skin, Kupffer cells of the liver and microglia of the brain.[29] While historically all of these cells were thought to arise from the same bone marrow progenitor pool, recent research has called this into question.

Within the last twenty years new genetic and technological tools have emerged that allow for detailed analysis of increasingly more precise aspects of biology. The development of Cre-recombinase expressing transgenic animals has provided a mechanism not only for genetic deletion of specific sequences of DNA, but also for lineage tracing. Another widely used tool is the fluorescence-activated cell sorting (FACS) technique. Together these tools allow for micro-dissection of closely related populations of cells. Unfortunately, the results are not always definitive and have led to disputes within many different fields, including immunology. It is now generally accepted that microglia, the resident macrophages of the CNS, originate from yolk-sac precursors, self-renew from an unidentified population of stem cells and are not replenished by bone marrow derived monocytes.[30-33] The same cannot be said for other populations of mononuclear phagocytes, as there is still considerable debate regarding the origins of other macrophages.[33-35] Regardless of their specific origins, the mononuclear phagocyte system represents the front line of defense against invading pathogens, largely responsible for the production of pro-inflammatory cytokines that trigger the sickness response.[36] Considering the importance of macrophages to a variety of biological functions, this will likely remain an active area of investigation.

4. The Inflammatory response

4.1 The Inflammatory Response: Lighting the Fire

The first step of the immune response is identification of a problem. This is primarily the job of the macrophages, which are large, long-lived, mobile, phagocytic cells that are present both in the circulation and as permanent resident cells within specific tissues.[28] Macrophages patrol their territory, constantly looking for threats and disposing of the by-products of normal cell death. Equipped with an array of cell surface receptors that bind to extracellular components of bacterial and fungal organisms, macrophages recognize and destroy foreign invaders through the process of phagocytosis.[37] Receptor activation, such as LPS binding toll-like receptor 4 (TLR4), causes macrophages to release cytokines and chemokines that signal the presence of infection and begin the inflammatory response.[38]

The main cytokines employed by macrophages are the interleukins (IL), IL-1β and IL-6, and tumor-necrosis factor α (TNFα).[36] These molecules serve multiple functions, both locally and systematically. As signals of local infection, cytokines and chemokines released by macrophages act in a paracrine manner, attracting other immune cells to the site of infection. These cells, particularly neutrophils, assist in pathogen elimination through phagocytosis and also secrete cytokines, thus amplifying the inflammatory response. As the immune response grows and more cells are recruited to fight infection, the magnitude of cytokine release multiplies exponentially. The increase in production and release of cytokines causes effects in more distal locations, leading to systemic inflammation and the sickness response.

This process of amplification is one of the central themes of the immune system, whether acquired or innate. Each of the cell types mentioned above is capable of

releasing signaling molecules that participate in feed-forward amplification. For example, IL-1β released by macrophages stimulates the production and release of IL-1β from monocytes and neutrophils. This leads to ever expanding production and rising levels of IL-1β in the circulation. In addition, cytokine signaling causes immune cell proliferation leading to an expansion of the available pool of potential targets and sources of cytokine signaling. Furthermore, although individual cytokines have specific effects on certain cell types, they also have overlapping and sometimes redundant properties. For example, both IL-1β and TNF-α are produced by macrophages, and while each activates distinct intracellular signaling pathways, they can individually trigger the same systemic responses, as evidenced by the fact that administration of either directly into the CNS elicits the full spectrum of sickness responses.[39] Finally, IL-1β, IL-6 and TNF-α act synergistically, creating a greater response upon co-stimulation than alone, and can compensate for one another in situations where one signal is absent (such as genetic knockout).[40-43] This complicated relationship is a reflection of the general tendency of the immune system to mount a seemingly exaggerated response (which can be detrimental and even lethal in the case of allergic or autoimmune conditions) to ensure threat elimination, but makes it difficult to ascribe any particular systemic response to an individual cytokine.

4.2 IL-1β: The Canonical Inflammatory Cytokine

IL-1 was the name given to the first interleukin to be identified. Named for its function in communicating information between leukocytes, the number of IL-1 family members has expanded since that first discovery. As discussed in more detail in chapter 2, IL-1β is the most potent family member, and seems to serve only inflammatory functions. Both IL-1α and IL-1β signal by binding to a receptor complex composed of the

IL-1 receptor 1 (IL1R1) and IL-1 receptor accessory protein (IL1RAcP).[44] Binding of IL-1 initiates a signaling cascade that begins with recruitment of the essential adapter protein myeloid differentiation primary response gene 88 (Myd88). Once initiated, the canonical IL-1 signaling cascade culminates in activation and nuclear localization of the transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). NF-κB activation ultimately results in changes in gene transcription. An alternative signaling pathway has been described that involves a novel isoform of IL1RAcP (IL1RAcPb) found exclusively in neurons.[45] Rather than activating NF-κB, the IL1RAcPb pathways activates Src kinase. This discovery was followed up with the discovery of an alternative form of IL1R1 (termed IL1R3) specifically in neurons that dimerizes with IL1RAcPb.[46] While the role of canonical, IL1R1/Myd88-mediated signaling in sickness responses is evident from their absence in Myd88 knockout mice, the significance of neuron-specific IL-1β signaling has yet to be determined.[47, 48]

4.3 Systemic Inflammation: The Immune-Neurologic Link

One of the common threads found in conditions that result in sickness responses is the presence of increased levels of circulating pro-inflammatory cytokines. This is true for a broad array of disease states including traumatic injury[49], bacterial and viral infections[50], organ failure[51], and chronic neurodegenerative diseases such as Alzheimer's[52]. As previously mentioned, these different conditions also share common physiologic and behavioral symptoms. That each of these disparate conditions share signaling mechanisms and symptoms suggests that there is a common neuronal mechanism for generating these responses. Cytokines (and other acute-phase proteins such as those of complement system and C-reactive protein) have been demonstrated to be the immune portion of this communication method.

As previously mentioned, central administration of individual cytokines causes all of the sickness responses including fever, anorexia, decreased movement and weight loss. This demonstrates, at the minimum, that these signals are one mechanism of generating sickness responses. However, cytokines such as IL-1β are prevented from freely entering the brain by the presence of the blood-brain barrier (BBB).[53] This has led to a considerable amount of research aimed at identifying how systemic inflammatory signals are transmitted to the central nervous system to elicit sickness responses. This research has identified three main mechanisms: 1) direct signaling via diffusion within the circumventricular organs (CVOs); 2) direct neural communication from the periphery to the CNS via the vagus nerves; and 3) indirect stimulation in response to signals produced by vascular endothelial cells (discussed extensively in Chapter 3). One other mechanism that circumvents the need to cross the BBB is production of pro-inflammatory cytokines within the CNS. Previous research shows that inflammatory stimuli trigger the expression of cytokines, particularly IL-1β, within the CNS in a pattern that is restricted to the CVOs.[54-57] The fact that there are so many avenues of action is yet another demonstration of functional redundancy related to the immune system and the degree to which the CNS is isolated by the BBB.

5. Inflammation and the CNS

5.1 The Blood-Brain Barrier

The extracellular environment of the CNS is highly regulated. In order to function properly, neurons require specific concentrations of ions in the extracellular fluid. In addition, many of the molecules present in the general circulation are either neurotoxic or are used as neurotransmitters within the CNS. Since most neurons are not replaced

throughout the lifetime of the organism, it is also especially important to protect them from pathogens that might cause damage or cell death. At the same time, the brain is an incredibly energy intensive organ that requires constant input of oxygen and nutrients, and as such is highly vascularized. To balance these differing requirements, the CNS is protected by the BBB.

Composed of multiple layers of fortifications not found in the peripheral vasculature, the BBB tightly regulates the extracellular fluid environment of the CNS by restricting the diffusion of most components of the blood into the brain.[58] The first and most critical layer of protection is inherent in the endothelial cells that form the wall of blood vessels. During development, a complex and incompletely understood system of signals from neurons, astrocytes and pericytes induce endothelial cells within the CNS to express tight junction proteins that prevent free diffusion between adjoining cells.[59-61] The next component of the BBB is the system of pericytes that reside within the vascular basement membrane. Pericytes are contractile cells that wrap long processes around microvessels and contribute to blood flow regulation, serve as stem cells and regulate permeability in direct proportion to the extent of pericyte coverage of vessels. CNS vessels are extensively covered by pericytes that arise from neuroectoderm (unlike the mesynchymal origin of peripheral pericytes) and are essential for both the development and maintenance of the BBB.[62-65] Surrounding both of these levels of defense, astrocytes form the final component of the BBB.[66] Most of the blood vessels in the CNS are completely ensheathed by astrocytic end-feet, extensions of the cell-bodies of astrocytes that wrap around the vasculature. Together, the elements of the BBB essentially prohibit the free flow of all components of the blood other than dissolved gasses; everything else must be actively transported by specific mechanisms.

5.2 The Circumventricular Organs: Exceptions to the Rule

In a healthy brain, the blood-brain barrier (BBB) effectively isolates most of the CNS from the general circulation and prevents circulating cytokines from entering the brain by simple diffusion.[53] However, the circumventricular organs (CVOs) have unique anatomical features that support their role as regulated gateways to the CNS.[67] Located adjacent to the ventricles of the brain, the CVOs share three defining features: 1) they contain loops of fenestrated capillaries, which allow increased diffusion into the surrounding tissue through large endothelial pores and altered tight junctions[68-70]; 2) they are surrounded tanycytes, specialized ependymal cells that regulate communication between peripheral and central compartments[71-73]; and 3) they contain neurons capable of responding to circulating signaling molecules and transmitting this information to deeper CNS structures responsible for modulating autonomic and homeostatic functions important for sickness responses.[74-77] These features combine to form small pockets within the CNS that are effectively outside of the BBB, isolated from the rest of the CNS by a diffusion barrier, and capable of relaying information to areas where diffusion is more restricted.

Despite similarities in structure, the CVOs are functionally distinct and generally divided into two categories. The sensory CVOs sense and respond to components of the blood, and include the organum vasculosum lamina terminalis (OVLT), sub-fornical organ (SFO) and area postrema (AP). The secretory CVOs include the median eminence (ME) and the pineal gland, both sites where neurohormones are secreted into the general circulation.[67, 75] The arcuate nucleus and median eminence (ARC/ME) region is commonly classified as a secretory CVO, but it possesses characteristics of both categories. The secretory component consists of hypophyseal releasing hormones that are secreted into the hypothalamic-pituitary portal vessels from the axon termini of

gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) neurons. These in turn modulate the hypothalamic-pituitary-gonadal, -thyroid, and -adrenal axes, all of which contribute to aspects of the sickness response.[71, 78-83] As a sensory structure, the ARC/ME contains the cell bodies of pro-opiomelanocortin (POMC) and agouti-related peptide/neuropeptide-Y (AgRP/NPY) neurons that directly respond to a large variety of circulating signals of nutritional status and inflammation to modulate feeding and energy balance (discussed in detail below).[84-89] One other structure, the choroid plexus (ChP), contains both fenestrated capillaries and specialized ependymal cells but is generally not considered a CVO due to its lack of neural components and its predominant role in the production of cerebrospinal fluid (CSF).[67] Despite the lack of synaptic signaling capability, recent evidence demonstrates that the ChP contributes to inflammatory signaling through the production of CSF released inflammatory signaling molecules and secreted vesicles.[90, 91] Overall, the CVOs are a unique niche where peripheral immune signals interact with elements of the CNS to generate the sickness response.

5.3 The Vagus Nerve: Direct Neural Communication of Abdominal Inflammation

The Vagus nerve (cranial nerve X) is a mixed sensory and motor nerve that serves to provide bi-directional communication between the brain visceral organs. The vagus carries approximately 90% of the parasympathetic pre-ganglionic motor neurons, including those that innervate the heart, lungs and digestive tract. Afferent axons within the vagus carry sensory information from the digestive tract, pancreas, liver, heart and airways.[92] Vagus afferent cell bodies are located in the nodose ganglion and synapse directly onto neurons in the medullary nucleus of the solitary tract, from which information is conveyed to other areas of the brain.

Some research suggests that vagal afferent fibers convey inflammatory signals from the abdominal cavity directly into the CNS. As discussed above, injection of IL-1β or LPS causes sickness responses and the production of IL-1β within the CVOs of rats and mice. To determine the contribution of direct vagal communication to this process and sickness responses, investigators have used subdiaphragmatic vagotomy (SV; cutting of the subdiaphragmatic trunk of the vagus, which innervates the liver, pancreas and digestive tract) to eliminate the neural abdominal information pathway. In mice, SV was found to attenuate the effects of IP LPS or IL-1β on food motivated behavior, general activity and brain IL-1β expression.[93, 94] Later reports, using rats as the experimental model, did not support these findings; SV did not affect IP IL-1β- or LPS-induced reduction in food intake, fever or brain IL-1β production.[95-97] One possible explanation for these differences is that the treatment doses were not consistent and it is possible that higher doses cause increased circulating levels of cytokines while lower doses remain confined to the abdomen. This is supported by reports which found that SV attenuated the effects of IP low-dose IL-1β but not higher doses in rats and that SV only attenuated the effects of IP but not subcutaneous or intravenous IL-1β in mice.[98, 99]

Whether inflammatory signals reach the brain via diffusion or direct neural communication, the previously described results clearly indicate that inflammatory cytokines induce sickness responses. The nature of these responses indicates that they are generated in the hypothalamus. Before beginning a discussion of the circuitry involved in these responses, a general description of the hypothalamus and its functions is warranted.

6. The Hypothalamus

6.1 The Hypothalamus: Introduction and Structure

The hypothalamus is an evolutionarily ancient neural structure found in all vertebrates. Although relatively small in size—the human hypothalamus is less than 0.3% of the total brain weight—the hypothalamus has an enormous variety of functions. In the most general sense, the hypothalamus functions as the integrator of information about the state of the body (both internally and in relation to external world) and the central regulator of biological responses to situational demands. It controls nearly every aspect of visceral function and body homeostasis. Through its control of the autonomic nervous system (ANS) the hypothalamus controls heart activity, blood pressure, body temperature and water balance. It is an endocrine organ, secreting the hormones vasopressin (AVP, for arginine-vasopressin) and oxytocin (OXT) directly into the circulation, and the master regulator of nearly every other endocrine organ through its releasing hormone control of the pituitary. As a component of the limbic system it also influences emotional states like pleasure and rage. These functions are regulated by different populations of cells located throughout the hypothalamus.

In early examinations it was noted that the structure of the hypothalamus more closely resembles that of the midbrain and brainstem than cortical structures, with groups of neuronal cell bodies clustered in nuclei. The main nuclei of the hypothalamus are the preoptic nucleus (PON, subdivided into the medial, MPO, median, MnPO, ventrolateral, VLPO, and dorsolateral, DLPO, nuclei), supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), anterior hypothalamic nucleus (AHN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), arcuate nucleus (ARC), posterior hypothalamic nucleus (PHN) and mammillary body (MB). The

hypothalamus also contains a more diffuse population of cells scattered around its periphery in preoptic area (POA), the anterior hypothalamic area (AHA), lateral hypothalamic area (LHA) and the posterior hypothalamic area (PHA).

Most of these nuclei are obvious under histological examination using non-specific labeling techniques (such as hematoxylin and eosin, H&E, staining) as dense groups of cell bodies surrounded by regions of relatively sparse cell density.[100] Closer examination using methods that distinguish specific types of neurons or synaptic connectivity reveals that many of these nuclei are composed of neurons that have diverse, and sometimes unrelated, functions. For example, one recent study identified 50 transcriptionally distinct types of cells located within the ARC/ME alone.[101] Furthermore, because of the degree of interconnectivity between hypothalamic nuclei and the variety of effects of individual neuropeptides, it is difficult to assign individual functions to any nucleus. Classical techniques used to assess connections between the hypothalamus and other regions of the CNS struggled due to the fact that most hypothalamic axons are unmyelinated. Despite this difficulty, a high degree of interconnectivity was postulated, with some researchers claiming evidence of hypothalamic connections to "every region of the brain."[102] While this might be an overstatement, it is true that the hypothalamus influences, or is influenced by, many different processes.

6.2 The Paraventricular Nucleus: Microcosm of the Hypothalamus

The PVN provides an excellent example of such complexity. Within the PVN H&E staining can distinguish differences based on the size of neuronal cell bodies. In the more lateral regions of the PVN are the larger magnocellular neurosecretory neurons that project axons through the infundibulum to the posterior pituitary. The magnocellular

neurons can be further divided into those that make OXT (implicated in such diverse functions as lactation, pair and group bonding and possibly even orgasm), and those that make AVP (mostly involved in water balance, but also pair bonding and many other functions).[103-107] Generally located more medially (closer to the third ventricle) are the smaller parvocellular neurons. Many of these neurons produce releasing hormones (parvocellular neurosecretory cells) that regulate the production and release of pituitary hormones from the anterior pituitary.

The two main types of PVN parvocellular neurosecretory cells are those that make thyrotropin-releasing hormone (TRH; stimulates pituitary thyrotrope release of thyroid-stimulating hormone) and those that make corticotrophin-releasing hormone (CRH; stimulates corticotropes to release adrenocorticotropic hormone, ACTH).[108, 109] Other populations of PVN parvocellular neurons, including cells that make AVP, OXT and neurotensin, project to the ME and to neural targets within the CNS. Many of behavioral effects of OXT and AVP are likely mediated by parvocellular neurons, which also regulate ANS function through projections to the brainstem and spinal cord.[110, 111] Thus, the PVN encapsulates the nature of the hypothalamus as a whole, exhibiting both endocrine and autonomic functions.

6.3 The Endocrine Hypothalamus

A complete description of the endocrine system is beyond the scope of this thesis. However, because sickness responses both influence, and are influenced by, circulating hormones and their actions, a brief discussion of the endocrine functions of the hypothalamus is necessary. In general, these functions can be categorized as either direct (release of hormones) or indirect (release of releasing hormones).

As described above, the hypothalamus is itself an endocrine organ, releasing OXT and AVP into the general circulation. In addition to the PVN, there are populations of magnocellular neurons that produce OXT and AVP located in the supraoptic nucleus (SON). Like the PVN populations, these SON neurons project axons to the posterior pituitary where they store and release their hormones. In this manner the hypothalamus directly (as opposed to indirectly, through regulation of the anterior pituitary functions described below) controls milk release from the mammary glands and cervical contractions during labor (OXT) and regulates water retention and blood pressure (AVP). The systemic effects of these two hormones are not likely involved in the sickness response, but they might play a role via their effects on regulating CNS circuits involved in social behaviors and autonomic function. For example, both OXT and AVP have been implicated in complex social behaviors that are suppressed by inflammatory stimuli.[41, 111] Through either inhibition or decreased stimulation of the neural circuitry that drives exploratory behavior, OXT and AVP could potentially contribute to the sickness-induced decrease in voluntary locomotor activity (VLA; a measure of how much an animal moves).

The indirect endocrine functions of the hypothalamus are extensive and regulate most other endocrine organs through the control of pituitary tropic hormones. The hypothalamus manages this through discreet populations of neurons that produce “releasing hormones” and “release inhibiting hormones” which are secreted into the hypothalamic-pituitary portal vasculature at the base of the ME and carried to the anterior pituitary where they exert their principal influence. These releasing hormones represent the pinnacle of the hypothalamic-pituitary-gonadal, -adrenal and -thyroid axes (HPG, HPA and HPT, respectively).

6.4 The HPG Axis

The HPG axis is mostly involved in controlling the circulating levels of sex steroids (androgens and estrogens) regulating reproductive function. In females, this system is responsible for controlling ovulation and implantation of fertilized zygotes, while in males it is most involved in sperm production.[112, 113] Ultimate control of the HPG axis resides in the gonadotropin-releasing hormone (GnRH) synthesizing neurons located in the basal forebrain and hypothalamus, although GnRH neuron activity is regulated by a variety of factors including inhibitory GABAergic synapses and stimulatory kisspeptin synapses.[114, 115] GnRH neurons send long processes to the ME where they release GnRH into the portal vasculature to stimulate anterior pituitary gonadotropes to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH act at the level of the gonads to regulate the release of sex-steroids and control gamete production (males) and maturation and release (females). Sex-steroids released from the gonads regulate the activity of the HPG axis through feedback inhibition at the level of the brain and pituitary.

Although not likely directly involved in the sickness response, the HPG axis does play a role in immune system development. The activity of the HPG is also modulated by inflammation.[116] It also appears that elements of the HPG axis (specifically a subpopulation of the ARC kisspeptin-expressing neurons) can play a role in thermoregulation, a process which might be involved in the manifestation of menopausal hot-flashes.[117] This presents the possibility that the HPG axis could contribute, at least under some circumstances, to the febrile response.

6.5 The HPA Axis

The main components of the HPA axis are the corticotropin-releasing hormone (CRH) producing parvocellular neurons in the PVN, the corticotropes of the anterior pituitary and the corticosteroid producing cells of the adrenal cortex. CRH released at the ME stimulates adrenocorticotropic hormone (ACTH) synthesis and release into the general circulation which, in turn, stimulates the production and release of corticosteroids from the adrenal cortex. Corticosteroids regulate the HPA via feedback inhibition at the level of the hypothalamus and pituitary, inhibiting CRH and ACTH release.

The primary purpose of the HPA axis (also called the stress axis) is to prepare the body to respond to adverse stimuli through corticosteroid-induced modulation of energy balance, immune function and arousal. There are two classes of corticosteroids produced in the adrenal cortex: mineralocorticoids (MC, e.g. aldosterone), produced in the zona glomerulosa, and glucocorticoids (GC, principally cortisol in humans and corticosterone in rodents), produced in the zona fasiculata. Of the two, GC signaling is the most relevant to the stress and sickness responses (MC signaling is mostly involved in water and electrolyte homeostasis). Elevated circulating levels of GC results in increased blood glucose—through inhibiting glucose sequestration, increased gluconeogenesis and insulin resistance—providing a ready source of energy for rapid responses to stressful situations.[118] The substrates for gluconeogenesis, free amino acids and fats, are produced by GC-induced breakdown of muscle proteins and adipose lipid stores.[119] These effects are directly relevant to the sickness response: to compensate for decreased food intake the body must provide for alternative sources of fuel, and muscle/adipose catabolism is directly reflected in the loss of body weight associated with disease. Multiple disease models activate the HPA and our lab has demonstrated that disease-associated muscle wasting is dependent upon HPA axis activation and GC receptor expression in muscle tissue.[12, 120-123] The HPA also

regulates the immune system through a form of feedback inhibition: inflammatory cytokines, particularly IL-1β, stimulate the HPA and GC signaling inhibits the production of inflammatory mediators such as prostaglandins and IL-1β.[124-126] Together with a direct action of GC on thymocytes, decreased IL-1 production results in decreased T-cell activation and proliferation, thereby reducing circulating antibody concentrations.[127] These features combine such that the overall effect of GC signaling on the immune system itself is anti-inflammatory.

6.6 The HPT Axis

The HPT axis is composed of thyrotropin-releasing hormone (TRH) producing parvocellular neurons within the PVN, thyrotropes of the anterior pituitary and the thyroid gland. TRH release at the ME stimulates thyroid-stimulating hormone (TSH) release into the general circulation, resulting in increased production and release of thyroid hormones thyroxine (T4) and the more potent tri-iodothyronine (T3). As with all HP axes, T3 and T4 down-regulate the HPT axis via negative feedback at the level of the hypothalamus and pituitary, reducing TRH and TSH release.[128]

Both T3 and T4 signal through thyroid hormone (TH) receptors (TR) located in the nucleus of target cells. Most of the effects of TR signaling are due to T3, produced by enzymatic deiodination (removal of one iodine atom) of T4. The location and expression of specific deiodinases, including the brain, determines site-specific activity. As with GC signaling, TRs are expressed by nearly every cell of the body. The overall effect of TH signaling is to increase basal metabolic rate (BMR). BMR is a measure of the amount of energy expended while at rest and not digesting and is expressed as heat generation.[129] Therefore a consequence of increased TH signaling is increased heat

generation, a factor that is important for thermoregulation and likely contributes to the febrile response. Furthermore, TH directly activates brown adipose tissue and contributes to the browning of white adipose tissue by inhibiting adenosine monophosphate-activated protein kinase in the VMN.[130] The overall result of these actions is increased heat production and loss of body weight, two cardinal sickness responses.

6.7 Growth Hormone

The hypothalamus also regulates pituitary production and release of growth hormone (GH). Separate populations of neurons within the ARC produce growth hormone-releasing hormone (GHRH) and somatostatin (also called growth hormone-inhibiting hormone, GHIH), which increase and decrease GH, respectively.[131] Both hormones are released into the ME portal vasculature to modulate the activity of pituitary somatotropes which control the circulating levels of GH. As with the previously described hypothalamic endocrine systems, GH exerts negative feedback regulation on its own release at the level of the hypothalamus and pituitary.[131] GH release is also increased by GC and ghrelin and decreased by TH, demonstrating cross-talk between different metabolic endocrine systems.[132]

The principle targets of GH are muscles, bones and the liver. GH is an anabolic peptide hormone that causes longitudinal bone growth and cell proliferation, effects which are most evident from disorders involving either over-production leading to excess growth (acromegaly) or under-production leading to short stature (pituitary dwarfism). GH also has a number of metabolic effects that are not so evident. GH causes increased circulating levels of glucose, predominantly driven by increased production in the liver,

and decreased insulin sensitivity.[133] GH also causes the liver to produce insulin-like growth factor 1 (IGF1), which mediates many of the effects of GH.[134] Together IGF-1 and GH signaling lead to breakdown of adipose stores, causing elevated circulating levels of lipids that are used by the liver as a substrate for glucose production and as a source of energy by muscles, where GH induces the uptake of fat.[135-137] The combined effects of GH on glucose and fat increase energy availability independent of food intake, and could contribute to the weight loss seen in sick animals. Finally, GH causes increased utilization of lipid by brown adipose tissue (BAT) and increased thermogenesis, an effect which is contributes to the febrile response (discussed in more detail in section 10).[138, 139]

6.8 The Autonomic Hypothalamus

One approach to functional organization is to divide the hypothalamus into columns. In the coronal plane the hypothalamus can be segregated into the periventricular column, closest to the third ventricle, the medial column and the lateral region. Because these columns extend for the entire length of the hypothalamus, the hypothalamus can be further divided along the rostro-caudal axis into three parts in the saggital plane: the anterior or preoptic area (containing the PON, and SCN) located above the optic chiasm and below the anterior commissure; the medial or tuberal region (containing the AHN, PVN, SON, DMN, VMN and ARC) centered above the infundibulum and extending to the top of the 3V; and the posterior region (containing the tuberomamillary, mammillary and supramammillary nuclei) above the mammilary body.

According to this classification system, the more medial and anterior portions of the hypothalamus are associated with parasympathetic tone, while the more lateral and posterior portions control sympathetic tone (as will be demonstrated in the following

section). The principal autonomic output nuclei are the PVN, DMN and LH. Early examinations, using retrograde transport of horseradish peroxidase and anterograde transport of radio-labeled amino acids demonstrated that these nuclei contain neurons that have direct, monosynaptic connections to the autonomic pre-ganglionic nuclei in the brainstem and spinal cord.[140] Activity of these output neurons is affected by input from most of the other hypothalamic nuclei as well as cortical and limbic structures which determines the overall level of hypothalamic stimulation to the autonomic nervous system.

7. The Autonomic Nervous System

7.1 Overview of the Autonomic Nervous System

The autonomic nervous system is responsible for modulating the activity of smooth muscles, cardiac muscles and glands. The term “modulate” is used here because most of these systems are capable of independent activity, which is adjusted, rather than controlled, by ANS input. This situation is illustrated by comparing the activity of heart and skeletal muscles. Skeletal muscle fibers, innervated by somatic motor neurons, do not contract unless “told” to do so by acetylcholine release at the neuromuscular junction. Cardiac smooth muscle fibers, on the other hand, are capable of independent contraction even when completely isolated in a dish and the heart as a whole will continue to beat (at an increased rate due to removal of parasympathetic inhibition) even when all neural inputs have been severed. In a similar fashion, the activity of the visceral targets of ANS innervations (smooth muscles of the vascular, digestive and urogenital systems, as well as secretory and metabolic functions the liver,

pancreas and stomach) is modulated by the balance of sympathetic and parasympathetic input.

The autonomic nervous system is composed of the sympathetic and parasympathetic divisions. This division is both anatomical (based on the origin of the preganglionic motor neurons and the locations of the ganglia) as well as chemical (based on the neurotransmitter used by the postganglionic neurons to effect a response). The sympathetic nervous system (SNS), sometimes called the thoracolumbar division because its preganglionic cell bodies are located within the lateral horn of the thoracic and lumbar regions of the spinal cord, typically releases norepinephrine from its post-ganglionic axons. Two notable exceptions, of particular import to the topic of this thesis, are cholinergic sympathetic innervations of sweat glands and some skeletal muscle blood vessels. Additionally, the SNS directly innervates the adrenal glands, thus controlling systemic release of epinephrine through the release of acetylcholine from pre-ganglionic neurons directly onto adrenal medullary cells. The SNS is most active during stressful situations and drives the mobilization of energy stores and depression of digestive functions. The parasympathetic nervous system (PNS), also called the craniosacral division, uses acetylcholine as its terminal neurotransmitter. Most of the PNS preganglionic fibers (on the order of 90%) are contained within the vagus nerves, while those that innervate head structures and the urogenital system arise in other cranial nerve nuclei (III, VII and IX) and the sacral spinal cord. The PNS is mainly involved in conserving energy and increasing digestive functions. Most organs (with the exceptions of sweat glands, arrector pili muscles and much of the vasculature, which are exclusively innervated by SNS neurons) receive dual innervations from both branches of the ANS.

The ANS is frequently described as the involuntary division of the nervous system responsible for generating the “fight or flight” or “rest and digest” responses.

While these two categories of response are distinct, and controlled by distinct divisions of the ANS (the SNS and PNS, respectively), the reality is that the physiologic state of the body is determined by the activity of both branches and is more of a balance than a situational either/or. As with nearly every aspect of physiological homeostasis, the boundaries are not always clearly delineated and any particular stimulation can have more than one effect or be employed for different purposes depending on the situation.

7.2 Illness and the ANS: Contributions to the Sickness Response

Because the ANS directly modulates many visceral functions, it also plays a role in the sickness response. While many of the processes involved in the ANS contribution to the sickness response are due to SNS activity, the PNS also likely has some critical functions and has indirect effects through decreased activity levels. As previously mentioned, the sickness responses that are the main focus of this thesis are the changes in body temperature, activity, food consumption and body weight. Increased body temperature during the febrile response relies on increased sympathetic tone to the BAT, driving non-shivering thermogenesis. In addition to hypothalamic endocrine regulation of the digestive system and glucose and lipid metabolism, the ANS also innervates the liver, pancreas, adipose tissue and portions of the digestive tract. All of these factors are employed as part of hypothalamic regulation of energy homeostasis.

8. Energy Homeostasis

8.1 Energy Homeostasis: A Possible Explanation for Sickness Responses

When viewed in its entirety, the sickness response can be seen as a re-organization of the body's priorities, directing resources away from homeostatic

maintenance and toward defense and repair.[141] From this perspective injury and infections are seen as a matter of life or death and the first priority is threat elimination. This also makes sense considering that unless the direct threat is dealt with in a timely manner then the amount of energy required to correct the situation increases. Consider bacterial infection: if invading bacteria are met with a strong response at the outset of infection, the number of bacteria is small and the number of immune cells and damaged tissue is equivalently minor; if the infection is allowed to persist, the number of bacteria multiplies and chances of spread of the infectious agent to other locations and subsequent tissue damage is greater, necessitating a greater number of immune cells to eliminate the pathogen; at the extreme, if the bacteria is sufficiently pathogenic, death is the ultimate outcome of an infection uneliminated. By re-directing energy from activities such as food gathering, reproduction and social behavior to internal consumption that actively focuses on defense and repair, the organism can more quickly return to normal function. This process requires a mechanism of coordinating physiologic and behavioral programs to limit energy expenditure on processes that do not contribute to the healing process.

8.2 Energy Homeostasis: Biological Consumer Capitalism

In order to function properly, every cell in the body requires a steady supply of nutrients (e.g. nucleic and amino acids for nucleotide and peptide synthesis and fatty acids for membrane renewal) and high energy molecules (mostly in the form of glucose and fats) that must be met by food consumption. To sustain proper bodily function, vertebrates have evolved intricate mechanisms to maintain the balance of energy intake and energy expenditure (energy homeostasis) that results in a relatively stable body weight for most of an animal's lifespan. Imbalances in energy homeostasis are

detrimental to the health of the organism: lack of sufficient and appropriate food or the inability to properly digest or metabolize consumed food can lead to malnutrition and starvation; over-consumption, food sources with high fat/calorie ratios or metabolic or signaling dysfunctions can lead to obesity and health risks such as heart disease and diabetes.

As befits a function of such importance there are multiple systems for regulating energy homeostasis. Most of these regulatory mechanisms involve reciprocal signaling (i.e. some signals that are stimulatory and others that are inhibitory to the same target/behavior) and feedback inhibition (i.e. a given signal directly and/or indirectly inhibits its own production). Furthermore, the individual components that contribute to energy homeostasis are intertwined and influence the control mechanisms for other components of the system. As such, it is more appropriate to refer to how a particular component affects the balance of the whole system (much like autonomic tone) rather than how an individual aspect is regulated in isolation. Still, for the sake of simplification, it is useful to first describe the components and some of the signaling mechanisms they employ, and then how the CNS compiles information from various systems to coordinate the whole.

The main components of energy homeostasis are food consumption (energy input), internal energy stores and energy expenditure. Food intake, and the associated need to find food sources, is one of the principle driving forces of all forms of life. Because food sources are not always readily available, higher forms of life have developed systems for internal energy storage to compensate for decreased energy intake during times of deprivation. In addition, the rate at which energy is expended can be modified to match the current state of overall nutrient and energy availability (both ingested and stored). Each of these factors produces signals (both neural and

endocrine) that converge on the hypothalamus which, in turn, alters metabolism and behavior to balance energy expenditure with energy input.

While this seems like a straight-forward and relatively simple equation, in reality there are far more factors involved than simply consuming and burning fuel. For example, before an animal eats, it must first want to eat (feel the need or desire to eat), find food (which it must also find appetitive) and then consume it (an incredibly complex behavior in itself). Such "motivated behaviors" are multi-faceted, and the neural circuitry involved in their control involve limbic (emotion and reward), cortical (movement) and autonomic (e.g. increased parasympathetic tone to the salivary glands) components of the nervous system. And that is all before the food reaches the gut and the process of digestion and absorption begins, yet another intricate interplay between nervous and endocrine systems. A complete description of all of the facets of energy homeostasis is beyond the scope of this thesis, yet a description of some of the signaling and circuitry involved is relevant in the context of sickness.

8.3 Food Intake: Supplying the Body's Demands

What causes us to eat? The simple answer seems to be that we eat because we feel hungry. That there are varying degrees of hunger seems to imply that there are either successive signals that are triggered sequentially, or certain signals that grows progressively stronger as the period between meals increases. Several models have been proposed as to how food intake is regulated, but none are sufficient to account for complete control of feeding. The two most commonly supported models are the glucostatic and lipostatic models, which postulate that feeding is controlled by circulating glucose levels or signals from fat deposits (e.g. leptin), respectively. Although both of these factors are involved in regulating eating, both models are probably overly

simplistic: It is more likely that glucose levels signal to regulate glucose and signals of adiposity regulate adiposity; neither alone is wholly responsible for food intake, though both influence feeding. To better understand how food intake is regulated, a more global perspective encompassing all of the signals relevant to energy homeostasis is necessary.

8.4 Peripheral signals of food intake

As with most homeostatic systems, the regulation of food intake involves both stimulatory (orexogenic, causing an increase in feeding) and inhibitory (anorexogenic, causing a decrease in feeding) signals. Many of these signals arise from the digestive system and convey information about how recently food was eaten. Curiously, there are more known peripheral anorexogenic signals than there are orexogenic signals.

To date, ghrelin is the only hormone produced by the digestive system known to stimulate food intake. Originally identified in a search for the endogenous agonist of the growth hormone secretagogue receptor (GHSR), ghrelin was isolated from stomach homogenates and found to stimulate the release of growth hormone (GH) from the pituitary.[142] Plasma ghrelin levels are highest prior to meal-times and drop immediately after eating, suggesting that ghrelin plays a role in meal initiation in humans.[143] The postprandial drop in plasma ghrelin is due to nutrient content (as opposed to the act of eating or distension of the stomach) as filling the stomach of fasted rats with water did not result in the same decrease.[144] Ghrelin administration causes weight gain and increases food intake and adiposity. Interestingly, ghrelin administration also results in an increase in respiratory quotient (RQ) without affecting energy expenditure, suggesting a shift from fat utilization to carbohydrate metabolism.[144] Both ghrelin and GHSR are expressed in the ARC and ghrelin administration up-regulates neuropeptide-Y (NPY)

and agouti-related peptide (AgRP) expression, suggesting a CNS mechanism of appetite regulation.[142, 145-147]

Most other gut-associated hormones are anorexogenic and are stimulated by the presence of food in the small intestine. As with ghrelin, some anorexogenic hormones are also produced in the CNS and the degree to which their effects on feeding are mediated by peripheral release is not completely clear. These hormones include secretin, cholesystokinin (CCK), peptide-YY (PYY) and glucagon-like peptide 1 (GLP-1).

When food exits the stomach into the duodenum the low pH stimulates the release of secretin, the first hormone to ever be identified.[148] The primary role of secretin is to stimulate the pancreas to release bicarbonate, thereby neutralizing the chyme for optimal digestive enzyme function. Examination of secretin receptor knockout mice also suggests a role in AVP-independent water retention and promoting water retention by promoting aquaporin expression in the kidney.[149] Secretin is also produced with the CNS and acts at both the PVN and ARC to inhibit feeding.[150]

The presence of nutrients (particularly fats) in the duodenum stimulates the release of CCK. CCK stimulates the release of pancreatic digestive enzymes.[151] CCK also inhibits feeding by stimulating vagal afferents; bilateral sub-diaphragmatic vagotomy blocks the anorexic effects of CCK administration.[152] Like secretin, CCK is also produced within the CNS where it functions as a neurotransmitter. Injection of CCK into the hypothalamus inhibits feeding, likely by stimulating VMN anorexogenic neurons.[153, 154]

After passing through the jejunum, the presence of food in the ileum causes the release of PYY and GLP-1, both of which stimulate digestion by slowing gastric emptying. Similar to CCK, both PYY and GLP-1 inhibit food intake, at least to some degree, through their actions on the vagus nerve.[155] The effects of PYY on feeding have been somewhat inconsistent: early reports demonstrated that PYY administration

resulted in hyperphagia and obesity[156, 157] while more recent reports show an anorectic response.[155, 158] This is possibly due to the use of supraphysiologic doses, resulting in off-target binding to NPY receptors.

In addition to the hormonal response of the digestive tract, nutrients absorbed during digestion also contribute to regulating food intake and energy homeostasis. Elevated plasma levels of glucose, fatty acids and amino acids all inhibit feeding through a complex interplay between peripheral and central actions. For example, elevated circulating glucose stimulates pancreatic insulin secretion (causing glucose uptake by the liver and muscles), and both glucose and insulin activate pro-opiomelanocortin (POMC) neurons and inhibit agouti-related peptide (AgRP)/NPY neurons (resulting in reduced feeding and altered metabolism, as discussed below), collectively lowering circulating glucose levels. Below a certain threshold, falling glucose levels trigger pancreatic glucagon secretion (stimulating glucose release from the liver) which acts in the opposite manner on POMC and AgRP/NPY neurons (causing increased feeding), collectively raising circulating glucose levels. Similar control mechanisms exist for amino and fatty acids, each regulated by metabolism and related to signals of stored energy.

8.5 Peripheral signals of energy stores

The main reservoirs of stored energy are glucose sequestered as glycogen in the liver (and to a lesser degree in muscles and adipose tissue) and fatty acid deposits in adipose tissue. As discussed above, blood glucose levels are tightly regulated both by the insulin-glucagon pathway and by central control of food intake. In addition, both the pancreas and liver receive sympathetic innervations that can also modulate the activity of this system to liberate glucose in stressful situations. While glycogen stores are sufficient to provide a burst of energy for relatively short periods of time, the total amount

of stored glucose is insufficient to sustain the body's requirements for long and so falling glucose levels trigger neural circuits that stimulate feeding (or decrease stimulation of anorexogenic circuits) and thereby replenish circulating glucose.

Fat stored in adipocytes provides another source of energy. The rate of deposition and liberation of fat from white adipose tissue (WAT) is controlled by hormonal (e.g. insulin, TH, GH and somatostatin) and autonomic signals. Consumption of food and adipose store usage elevates circulating levels of free fatty acids (FFA) which can signal at the level of the brain to cause decreased food intake.[159] WAT also produces the hormone leptin, and circulating levels of leptin are directly proportional to the amount of WAT (thus leading to the designation of leptin as the "obesity hormone"). Adipose-derived leptin plays an important role in regulating energy homeostasis as demonstrated by marked hyperphagia and obesity in human and animal loss-of-function mutants of either leptin or the leptin receptor.[160-165]

Each of the peripheral signals of energy balance mentioned above has been shown to affect food intake, and several have been shown to influence energy expenditure. These effects are mediated at least in part by CNS elements that respond to changes in the circulating levels of a variety of factors. As detailed below, the hypothalamus functions as the central compiler of information relating to the state of peripheral energy input and storage and modulates behavioral and metabolic responses to maintain homeostasis.

9. Hypothalamic Regulation of Energy Homeostasis

9.1 Hypothalamic Regulation of Energy Homeostasis

The recognition of what is now known as the “obesity epidemic” has stimulated research into how the body regulates weight. This has led to a vast expansion of knowledge about the neural circuits involved in regulating energy homeostasis, yet there are still many unknown components. A considerable amount of research over the past century has led to the realization that the hypothalamus compiles peripheral and central signals to regulate energy intake, storage and expenditure. The picture that has emerged is one of coordinated activity between multiple different nuclei and signaling systems. As with energy homeostasis as a whole, it is inappropriate to view any component of the hypothalamic energy balance circuitry in isolation as the coordinated activity of the whole is what determines the final output or tone of the body.

The regions of the hypothalamus most prominently implicated in the regulation of energy homeostasis are the LHA, VMN, ARC, DMN and PVN. Of these, neurons within the ARC, VMN and LHA appear to be the primary input or first-order neurons (those that sense and respond to peripheral signals of energy status and set the regulatory tone) while neurons within the PVN and DMN seem to be the primary output or effector neurons (those that transmit signals out of the hypothalamus via endocrine and autonomic pathways to effect changes to the peripheral components of energy homeostasis). Within these nuclei, several signaling mechanisms have been identified, including the central melanocortin system, brain-derived neurotrophic factor (BDNF), cilliary neurotrophic factor (CNTF) and nesfatin-1. This is still an active area of research, and new aspects of energy homeostasis regulation are constantly being discovered. Rather than discuss all of the components of hypothalamic energy homeostasis, the following discussion presents evidence for three regulatory mechanisms.

9.2 The Melanocortin System

The melanocortin system (MCS) is a G_s protein-coupled receptor (GPCR) signaling pathway involved in multiple biological processes.[166] There are five known melanocortin receptors (MC1R-MC5R) which have differing affinity for melanocortins, proteolytic cleavage products of POMC (the most relevant of which are α-melanocyte stimulating hormone, α-MSH, and ACTH). In the periphery, MC1R (originally called the MSH receptor) signaling is involved in coat color determination and skin pigmentation, while MC2R (the ACTH receptor) signaling is required for GC release from the adrenal glands.[167] Studies involving the lethal yellow (A^y) mouse, a dominant-negative mutation that is characterized in heterozygotes by yellow coat-color and obesity due to ectopic *Agouti* expression, led to the discovery of antagonistic signaling in the MCS.[168, 169] This functional antagonism is critical for the proper function of the central melanocortin system (cMCS), one of the most important components of the CNS energy homeostasis control.

9.3 The Central Melanocortin System

The cMCS is composed of neurons that express MCR and neurons that express MCR ligands. Within the CNS, MC3R and MC4R (which is largely brain-specific) are expressed in regions associated with energy homeostasis.[170-172] Expression of MC3R is more restricted, found predominantly in the thalamus and hypothalamus (particularly the ARC and VMN), while MC4R is widely distributed throughout the brain.[172, 173] Studies of genetic ablation of MC3R and MC4R demonstrated that both are involved in regulating body weight and energy expenditure. Interestingly, these two receptors appear to regulate slightly different, non-redundant aspects of energy homeostasis, possibly reflecting the difference in distribution.[174] Genetic deletion of MC3R results in an increase in adiposity without weight gain and alterations energy utilization (decreased

activity and increased RQ).[175] MC4R KO mice display characteristics very similar to the A^y phenotype, including hyperphagia, obesity (including adiposity and weight gain), hyperinsulinemia and increased longitudinal growth.[176] Human mutations of MC4R also result in a phenotype hyperphagia, obesity, hyperinsulinemia, and increased linear growth, demonstrating conservation of melanocortin homeostatic mechanisms.[177-179] Together these indicate that signaling through the brain melanocortin receptors are involved in components of energy homeostasis including body weight regulation and metabolism.

As alluded to above, the activity of the cMCS is modulated by reciprocal signals. Both MC3R and MC4R bind and are activated by α-MSH and ACTH.[170, 171] Neurons that produce POMC (the precursor for α-MSH and ACTH) are found in the ARC and in the brainstem and α-MSH projections are found in locations where MCRs are expressed (this discussion will focus on the ARC component only, although the brainstem component also plays a role in the sickness response.[180, 181] The ARC also contains are a population of neurons that co-express the proteins AgRP and NPY.[182, 183] AgRP was originally identified by similarity to the *Agouti* protein and found to act as a competitive antagonist (binding of AgRP prevents binding of α-MSH) at MC3 and 4R.[184, 185] Similar to POMC, NPY/AgRP immunoreactive fibers innervate locations where MCRs are expressed including the PVN, VMH, DMH and LHA.[180, 181, 186] These two populations of neurons, POMC and AgRP/NPY, constitute the signaling component of the cMCS. Much of the research on the cMCS revolves around obesity, but examinations of the effects of POMC-NPY/AgRP function show that food intake and body weight regulation are only one aspect of cMCS function.

The antagonistic nature of the cMCS is demonstrated by the opposite effects produced by the relevant neuropeptides. Stimulation of the brain MCRs (α-MSH and ACTH) signals positive energy balance (more energy is available than necessary),

leading to anorexia and increased energy expenditure; antagonism signals negative energy balance and results in increased feeding and decreased energy expenditure. Central injection of α-MSH (and related MC3/4R agonists) a decrease in feeding, increased energy expenditure (via increased sympathetic tone) and promotes a catabolic state (by stimulating the HPT and HPA axes).[187] These effects can be blocked by co-infusion of MCR antagonists.[181] Genetic mutations of POMC are characterized by obesity, adrenal insufficiency and alterations in pigmentation in both mice and humans.[188-190] Conversely, infusion of either AgRP or NPY causes an increase in feeding that is blocked by co-infusion of MCR agonists.[181] NPY also causes decreased energy expenditure (as measured by reduced BAT thermogenesis) and increased lipid storage (as measured by WAT lipoprotein lipase).[191] The fact that the cMCS modulates not only food intake and body weight, but also energy expenditure and metabolism demonstrates that it is one critical component of energy homeostasis regulation.

9.4 The Role of the Central Melanocortin System in Disease

Because of their location in the ARC, POMC and AgRP/NPY are ideally positioned for exposure to circulating inflammatory cytokines. Inflammatory stimuli cause decreased expression of AgRP/NPY mRNA and increased POMC expression, showing that the activity of the cMCS is regulated as though there were a positive energy balance. Our lab has shown that POMC neurons express IL-1β receptors, and increase their firing rate in response to IL-1β.[10, 88] This status also conforms to the observed sickness responses: decreased food intake and increased energy expenditure for thermogenesis coupled with catabolic tone leading to loss of body weight. This offers a possible explanation for many sickness responses, but it is only one example of the mechanisms the hypothalamus employs to regulated energy homeostasis. Two further

examples of components potentially involved in this process are brain-derived neurotrophic factor (BDNF) and the special role of hypothalamic tanycytes.

9.5 BDNF Signaling Regulates Energy Homeostasis Downstream of the cMCS

BDNF is also involved in regulating energy homeostasis.[192] Loss of function mutations (in either BDNF or its receptor, tropomyosin receptor kinase B, trkB) result in severe hyperphagia and obesity in both rodents and humans.[193-195] Conversely, central administration of BDNF decreases food intake and weight gain in rats.[196] BDNF is produced in multiple hypothalamic nuclei, including the PVN and VMH where selective deletion increases food intake, obesity and decreases energy expenditure.[193, 197] Interestingly, the functions of BDNF relevant to food intake and energy expenditure appear to derive from different populations of neurons. Selective deletion of BDNF from the VMH and DMH causes hyperphagia and obesity, but does not affect energy expenditure. In the PVN it appears that BDNF neurons in the anterior PVN regulate feeding behavior while those in the medial and posterior PVN regulate energy expenditure through BAT thermogenesis. Expression of BDNF in the brain is decreased by fasting and increased by leptin and CCK, effects that appear to be mediated by melanocortin signaling.[196, 198]

9.6 Tanycytes regulate hypothalamic input and output

Tanycytes are specialized ependymal cells that extend long processes from their cell bodies lining the 3V through the ARC to the portal vasculature at the base of the median eminence. One of the first functions attributed to tanycytes was modulation of the HPG axis. By ensheathing the neurosecretory terminals of GnRH neurons with their filopodia-like processes (called “end-feet”) they selectively restrict GnRH secretion into

the portal vasculature.[71, 199] It is possible that tanycytes regulate the activity of other releasing hormones in a similar fashion. Although generally considered to only be present around the ARC, there are cells that are morphologically similar in other CVO regions, and a small number in other structures such as the PVN.[73] In each of these regions, tanycytes serve to regulate diffusion both from the circulation and the CSF into the CVOs, and from the CVOs to surrounding brain regions.[70, 73]

In addition to the previously mentioned functions, multiple lines of evidence suggest that tanycytes participate in signaling related to energy homeostasis. Tanycytes are capable of sensing glucose and respond by releasing adenosine triphosphate (ATP).[200] Activation of ATP receptors alters feeding responses, and so it is possible that tanycyte signaling is one mechanism of communicating circulating glucose information to neurons.[201] Tanycytes are also capable of endocytosis and transcytosis of hormones, including leptin and nerve growth factor (NGF), from the blood or CSF to their target neurons.[202-204] Ependymal cells, including tanycytes, are the main source of cilliary neutrotrophic factor (CNTF), an IL-6 family cytokine that activates POMC neurons resulting in anorexia and decreased fat mass.[205-207] Tanycytes also play a complex, multifaceted role in regulating metabolism through their effects on both TRH neuron function and TRH activity. Tanycytes are the predominant CNS cell type to produce type II deiodinase, the enzyme that converts T4 to the more potent T3, which inhibits the production of TRH.[79, 80, 208] At the neurosecretory end of the TRH neuron, ME tanycytic expression of proglutamyl peptidase II, which inactivates TRH, potentially provides a mechanism for further decreasing TRH signaling at the point of release.[209] Finally, as demonstrated in chapter 3 of this thesis, tanycytes directly respond to IL-1β and thus might play a role in the sickness response.

The preceding discussions of the cMCS, BDNF and tanycytes demonstrate various examples of how components of the hypothalamus contribute to energy

homeostasis and how this process might impact the sickness responses measured in the following chapters of this thesis. The following discussion focuses on the particular feature of body temperature regulation and the febrile response.

10. The Febrile Response

10.1 Body Temperature Regulation

Regulation of body temperature is critical for survival. At temperatures above or below the normal biological range, enzymes function at sub-optimal levels and changes in membrane fluidity alter the functions of cells. Although the actual temperature of the body might vary at different points (e.g. the temperature of the surface of the skin is almost always different from the temperature within the mouth), the essential core-body temperature (CBT) is highly regulated. Heat is generated through nearly every biological process, from the basic metabolic functions of cells to heat produced from muscular contraction. In order to maintain an optimal body temperature, animals need mechanisms to alter the actual temperature back to the desired set-point.

The ultimate regulator of body temperature is the blood; excess heat is absorbed by the fluids of the body and released to the environment when blood travels near the surface of the skin. In order to maintain a constant body temperature, the temperature of the blood is tightly controlled. When body temperature elevates above the biological set-point, blood flow to skin capillaries is increased, thereby increasing the rate at which heat can be lost via classical heat transfer mechanisms of convection, conduction and radiation. In addition, sweating leads to increased heat loss through evaporative cooling. Conversely, when the temperature of the external environment decreases significantly,

blood flow to the skin is restricted and behaviors such as hunching and shivering manifest to produce heat to maintain CBT.

Although each action of each cell within an organism is a source of minute amounts of heat (mostly due to enzymatic inefficiency), the primary mechanisms for generating thermal energy are metabolism (both the catabolic breakdown of food consumed to produce components for biologic molecules and the anabolic breakdown of biologic molecules for energy) and mechanical work (mostly the contraction of muscles). A third source of heat, the uncoupling protein-1 (UCP-1) dependent direct conversion of chemical energy into heat via uncoupling of proton flux from ATP generation in mitochondria, is present in the brown adipose tissue (BAT) of most mammals.[210] While the heat generated by the first two categories (metabolism and mechanical work) is a fundamental by-product of normal biological function, they can be intentionally increased to compensate for heat lost to the environment when body temperature decreases. The third source (BAT mitochondrial uncoupling) is largely absent except in situations where increases in temperature are required to maintain thermal homeostasis.[211] Together, these processes are coordinated to increase the body temperature when environmental temperature is too low or when the organism requires a higher temperature to inhibit the success of an infectious agent.

10.2 Hypothalamic Regulation of CBT

Recent research has increased our understanding of the thermoregulatory circuitry in the central nervous system.[212] From the most simplistic perspective, CBT is maintained at a near-constant set-point through a balance of heat production and heat loss. Temperature at various points in the body is monitored by thermosensitive neurons, including those that increase their firing rates at elevated temperatures (warm-

sensitive) and at decreased temperatures (cold-sensitive). Although there is still some debate as to the exact molecular identity of the relevant “temperature receptors,” thermosensitive afferent neurons express transient receptor potential (TRP) channels whose conductance is affected by temperature. These neurons are found in multiple locations throughout the body and monitor the current temperature of various compartments. For example, warm (generally glycinergic inhibitory neurons) and cold (glutamatergic excitatory neurons) sensitive neurons in the skin transmit information about the environmental temperature while similar neurons in the abdominal cavity and within the brain itself transmit information about the actual CBT.

All of these signals ultimately converge on the pre-optic area of the hypothalamus (POA) and modulated the activity of neurons within the medial pre-optic nucleus (MnPOA). These neurons, in turn, modulate the activity of effector neurons within the DMN and PVN. The balance between stimulatory and inhibitory inputs to these effector neurons ultimately determines the excitatory output to midbrain and brainstem neurons that control autonomic neural activity.

10.3 The Febrile Response: Making life difficult for small forms of life

The febrile response is the most studied and understood aspect of the sickness response. Considerable effort has been expended to understand both why and how mammals generate fever. From the perspective of a sick animal that needs to conserve energy for healing, the febrile response is difficult to justify: in order to increase body temperature by 1°C animals need to increase metabolic output by more than 10%.[213, 214] This seems like an extraordinary burden unless the benefits offset the cost. In fact, multiple lines of evidence suggest that fever is beneficial in several ways. First, elevated body temperature enhances hematopoiesis, neutrophil production and T cell

activation.[215, 216] Each of these mechanisms increases the ability of the immune system to fight infection and aid in the healing process. Additionally, increased temperatures decrease the proliferative potential of both bacteria and viruses.[217] One possible mechanism for this is that at elevated temperatures iron and zinc, both essential for replication, are sequestered in organs such as the liver and spleen.[218]

On the other hand, in addition to being unpleasant, elevated body temperatures can also be detrimental. Excessive fever can lead to brain damage, organ failure and even death.[219] Furthermore, some research suggests that decreasing body temperature can be beneficial in certain circumstances such as cardiac arrest and other hypoxic challenges.[220, 221] For these reasons, understanding how the body generates fever and developing therapies to specifically target fever disruption could have significant medical benefit.

10.3 Mechanisms of Fever Generation

The study of fever goes back to at least the 5th century BC, when Hippocrates documented the periodic pattern of malarial fever.[222] However, it wasn't until the middle of the last century that the first studies on the biochemistry of fever began to shed light on the mechanisms that the body uses to elevate CBT.[223, 224] At that time it was recognized that "pyrogens" (interestingly the particular pyrogen used was a vaccine against the gram-negative bacteria that causes typhoid fever) caused increases in rectal temperature in alert cats and rabbits.[225] Subsequent research was focused on identifying the "endogenous pyrogens," hypothesized molecules that the body produces in order to elevate body temperature. Evidence that such a signal was produced within the CNS and exerted a local effect on body temperature and behavior first came from research showing that cerebrospinal fluid (CSF) from a shivering monkey (caused by

placing the monkey in a cold chamber) caused shivering and increased body temperature when injected into the ventricles of a warm recipient monkey.[226] Later studies identified multiple chemicals produced within the brain that caused increased CBT, including amines and prostaglandins (PG).[227] These studies initiated a nearly 40 year effort to identify the how these molecules produce fever.

Recently, the exact mechanism of fever generation has been worked out in detail. Exogenous pyrogens, such as LPS, cause mononuclear phagocytes to release pro-inflammatory cytokines including IL1-β, IL-6 and TNF-α into the general circulation.[36] Studies using genetic knockout (KO) mice lacking enzymes necessary for production of PGE2 show that endotoxin- and cytokine-induced fever depends on PGE2 production.[228-230] These enzymes are expressed by endothelial cells and microglia within the brain and their expression is up-regulated by inflammatory stimuli.[126, 231-233] Cytokine-induced PGE2 exerts its influence by binding to the E-prostaglandin receptor 3 (EP3) on MnPOA thermoregulatory neurons, resulting in activation of the thermoregulatory circuit described above.[234]

While this level of detail is extraordinary, and far superior to that of the mechanistic understanding of many other sickness responses, there are still some unanswered questions. Particularly, although both endothelium and microglia have been shown to produce PGE2, it is still not completely clear which is the relevant source. Several studies that have attempted to address this issue concluded that endothelial signaling alone is responsible for the febrile response, yet technical aspects of experimental design leave room for uncertainty.[235-238] Specifically, studies that rely on the use of transgenic animals where tissue specificity is determined by the Tie2 or Slco1c1 promoter cannot be interpreted as definitive evidence of endothelial action alone; neither strain is specific to endothelial cells, as shown in chapter 3 of this thesis. In addition, although the role of PGE2 in fever is unquestionable, other sickness

responses are not altered by interfering with PGE2 signaling. This presents the possibility of divergence of sickness responses at the level of secondary signaling.

It should also be noted that the previously described control mechanisms do not cover every aspect of the febrile response. For example, some of the heat generated to elevate body temperature likely derives from other processes (including changes to cellular metabolism driven by alterations in TH and GH release) that are also altered as part of the greater sickness response. Finally, there appears to be a complex relationship between the degree of infection/inflammation and ambient temperature that causes a switch between warm-seeking (thermogenesis) and cold-seeking (thermodissipation) behaviors that is not completely understood.[239, 240]

10.4 Behavioral Components of Thermoregulation

Feeling cold can have two meanings: the sensation that something is cold, caused by activation of cold-sensitive receptors on peripheral afferent neurons that relay information about the temperature of an object or environment; or the perception that an organism itself "feels cold," related to the central integration of sensations about the state of the body. The latter has emotional and motivational components that the former does not, and engenders behaviors (like hunching/huddling, shivering and seeking out warmer environments) intended to conserve heat. Fever triggers similar perceptions of feeling cold, and causes the same behaviors, as the body presumably uses common control mechanisms to elevate its temperature. This is one possible explanation for the decrease in movement observed in experimental models of disease.

Hypothesis and Specific Aims

The overarching goal of our research is to determine the central nervous system mechanisms of physiologic and behavioral changes in the biological response to disease. The objective of the research presented in this thesis was to *determine how inflammatory signaling within the central nervous system triggers the sickness response.*

Specific Aims:

1. Determine if CNS-specific IL-1 production is necessary for the sickness response.

2. Determine which CNS cell type(s) are responsible for triggering the sickness response.

Chapter 2:

Manuscript #1

Investigating the role of central nervous system inflammation in sickness behaviors

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Chapter 2 is an original manuscript as prepared for submission to the Journal of Neuroinflammation.

Abstract:

When an animal is sick or injured, a program of behavioral responses is initiated that promotes healing by redirecting resources to combat infection and stimulate regenerative mechanisms. These behaviors include changes in body temperature, food intake, metabolism and activity that are initiated at the level of the brain and are triggered by neural responses to inflammatory signals produced by elements of the immune system. While the direct effects of pro-inflammatory cytokine signaling are well-established, the precise cascade of events that lead to these responses is not known. One intriguing event that occurs early in the response to inflammatory stimuli is the production of pro-inflammatory cytokines within restricted areas of the brain that are involved in homeostatic control. Some evidence suggests that microglia, the resident immune cells of the central nervous system (CNS), produce cytokines as a mechanism of CNS amplification of peripheral signaling. We investigated the behavioral and physiologic responses to inflammatory stimuli in mice that lack the potent pro-

inflammatory cytokines interleukin- (IL-) 1α and β (IL-1α/β KO). No differences were found between control and IL-1α/β KO animals. We also investigated the contribution of microglia to these behaviors by examining the response of mice in which only microglia were capable of responding to IL-1 signaling. The response of these mice was not affected by restoration of microglial responsiveness.

Introduction:

Animals are continually exposed to threats to their well-being, and in order to survive have evolved defense mechanisms to deal with infection and injury. Some of these mechanisms, such as the febrile response, are shared by species that diverged up to 600 million years ago, suggesting that the benefits they convey are vital to survival.[241] The most immediate of these mechanisms are the domain of the immune system.[16, 17] Immune cells attack invading microorganisms and initiate the healing process. One of the essential features of this process is the production of molecules that signal the presence of infection or tissue damage, including both chemokines and cytokines.[242] These molecules act both locally (paracrine signaling) and systematically (endocrine signaling) to recruit other elements of the immune system to the areas where they are needed. This recruitment, and the defensive and repair mechanisms they initiate, involve an amplification of the initial signal such that the circulating levels of pro-

inflammatory cytokines and other molecules spread beyond the boundaries of the immune system and trigger systemic responses that inhibit infection and promote healing.

In addition to stimulating the immune system to promote healing, pro-inflammatory cytokines trigger neural responses that alter physiologic and behavioral profiles to aid in the healing process.[243] These responses include elevation of body temperature (fever), decreases in food consumption (anorexia), decreases in motivated behaviors (lethargy and anhedonia) and changes to metabolism and energy expenditure that result in the loss of body weight (collectively sickness behaviors).[1] While these changes are energy intensive and can have negative health impacts in the long term, they are also beneficial as evidenced by their evolutionary conservation. Although sickness behaviors have been recognized for millennia, and studied intensively in the last century, the exact mechanisms that lead from cytokine production to behavioral changes are still not completely understood.[8, 222, 224, 226, 227]

The potent pro-inflammatory cytokine interleukin-1 (IL-1) is an essential mediator of the immune response.[244] There are two forms of IL-1, IL-1α and IL-1β, both of which are agonists of the type 1 IL receptor (IL1R1).[44] Inflammatory stimuli cause the production of both forms of IL-1 not only in the periphery, but also in specific structures within the central nervous system (CNS).[55, 56, 123] While the peripheral functions of IL-1 have been studied extensively, very little is known about CNS-specific IL-1 production. A recent study of tissue-specific responses to the bacterial endotoxin lipopolysaccharide (LPS) showed that locally produced IL-1 is essential for recruiting neutrophils into the CNS but not other tissues.[245] These results demonstrate unique functions of IL-1 in the CNS and present the possibility that CNS-derived IL-1 could play a role in sickness behaviors.

The goal of the present research was two-fold: to determine whether IL-1 is necessary for sickness behaviors; and whether a microglial response to IL-1 is sufficient to trigger sickness behaviors. We hypothesized that elimination of IL-1 would either eliminate or reduce the duration of sickness behaviors. We first confirmed that IL-1 is produced within the CNS exclusively by microglia using fluorescent immunohistochemistry (IHC). To test whether IL-1 is necessary for sickness behaviors, we examined the behavioral response of mice lacking both forms of IL-1 (IL-1α/β KO) to peripheral LPS. No differences were detected between wild-type (WT) and IL-1 α/β KO mice. To determine if a microglial response to IL-1 is sufficient to trigger sickness behaviors, we generated chimeric mice in which IL-1 signaling was restored exclusively in microglia. Intracerebroventricular (ICV) administration of IL-1β triggered sickness behaviors in WT, but not chimeric, animals suggesting that restoring microglial responsiveness is insufficient to restore sickness behaviors. Our results do not support the hypothesis that IL-1 is essential for sickness behaviors.

Methods:

Animals:

All animals were housed in the Biomedical Research Building at Oregon Health and Science University (OHSU). Animals were housed at 22°C in a room with a 12-12 light-dark cycle in plastic cages with paper pellet bedding and free access to food and water. All animal husbandry and surgical procedures were conducted in accordance with the OHSU institutional animal care and use committee (IACUC).

Interleukin-1 (IL-1) α/β double knockout mice were obtained from the lab of Dr. Isamu Sugawara at the University of Tokyo.[246] A colony was established from three breeding pairs of double-heterozygous (IL-1$\alpha^{+/-}$;IL-1$\beta^{+/-}$) animals that were bred to generate the double-homozygous knockout (IL-1$\alpha^{-/-}$;IL-1$\beta^{-/-}$) genotype. For behavioral

experiments (in order to generate sufficient numbers of knockout animals) double-knockout animals were used for breeding experimental animals and C57/Bl6J (the background strain of the double-knockout line; Jackson Laboratory strain#000664) animals were used as controls. To insure as much genetic diversity as possible, offspring of all founder animals were used to construct the breeding pool and, as much as possible, animals were only mated with the offspring of different parents.

For microglia injection experiments, pan-cellular GFP (ACTb-GFP; Jackson Laboratory strain#002391) mice were used to harvest microglia that are wild-type for MyD88 but that express GFP for post-hoc localization. Whole-body MyD88 knockout (MyD88KO; Jackson Laboratory strain#009088) were used as recipient animals.

Surgical Procedures:

Emitter Implantation:

For behavioral experiments, mice were deeply anesthetized under isofluorane and a G2 E-mitter (Starr life sciences) was implanted in the peritoneal cavity through a small incision positioned 1cm below the sternum, which was then closed using surgical staples. After surgery animals were individually housed in cages placed on an ER4000 energizer/receiver platform (Starr life sciences) and allowed to recover for one week prior to experimentation.

Microglia Transplant Procedures:

Pipette Preparation:

Thick-walled pipettes were pulled on the day of injection using a Narishige vertical pipette puller with the magnet set to zero (gravity only) and the heating element set to 6.5. After pulling, the pipettes were broken to a length of 6.5 mm using a dissecting microscope. The pipettes were then sharpened to an angle of 45 degrees using a wetted spinning pipette grinder. After sharpening each pipette was inspected

under magnification to check for the presence of glass dust inside the needle; any pipettes found to contain visible debris were discarded.

Injector setup:

The injector setup consisted of an automated syringe pump, a 10 µl Hamilton syringe, a 16-gauge blunt needle and sufficient polyethylene tubing to connect the needle to the pipette when it is mounted in the electrode holder on the stereotaxic frame. Prior to performing injections pipettes were calibrated so that the progress of injection could be monitored visually. To do this, a pipette was fitted into the end of the tubing, mounted into the electrode holder and the entire line (including the needle, tubing and pipette) was filled with sterile saline. The needle was then attached to the Hamilton syringe mounted on the syringe pump and 1µl increments were marked off along the length of the pipette by aspirating and ejecting air using the syringe pump. Prior to loading cells into the pipette, a 2 µl air bubble was drawn into the pipette to act as a buffer between the saline in the line and the cell suspension.

Microglia Injection:

Microglia were harvested from neonatal pan-eGFP mice and cultured as previously described.[247, 248] After two weeks in culture, microglia were separated from mixed glia cultures and re-suspended at a concentration of ~50,000 cells/µl in culture medium.

Recipient mice were deeply anesthetized under isofluorane and placed on a Kopf model 1900 digital stereotaxic alignment system. The skull was immobilized with a combination of bite bar, nose cone and ear bars. A small elliptical piece of scalp was removed extending roughly from lambda to bregma and periosteum was removed with a sterile cotton swab. The skull was leveled laterally using a model 1905 alignment indicator and rostrocaudally using model 1915 centering scope. The stereotax was then zeroed at bregma with z=0 set to the point that the tip of the glass pipette (secured in the

electrode holder) contacted bregma. A 0.5 mm diameter hole was then drilled in the skull at (x,y)=(0,-1.7) The pipette was loaded with at least 1.5 µl of cells to insure that a total volume of 1.0 µl (50,000 cells) could be injected without the risk of injecting an air bubble. The pipette holder was then replaced on the stereotaxic frame and the tip of the pipette was lowered to (x,y,z)=(-0.25,-1.7,-5.25). Cells were then injected at a rate of 0.5 µl/min. During injection, progress was visually monitored to ensure that the pipette had not clogged during lowering. The pipette was left in place for 2 minutes and then raised 0.5 mm and left in place for an additional 2 minutes. This procedure was repeated until the pipette is raised a total of 2 mm over 10 minutes. The pipette was then completely withdrawn from the skull. This procedure was then repeated for the other side of the drill hole [(x,y,z)=(0.25, -1.0, -5.25)]. Between injections, the pipette was flushed with sterile saline to insure that the pipette was not clogged. Sham-injected control mice were prepared using the exact same methods, but with injections of culture media only in place of cell suspension.

Following microglia injection, animals were implanted with stainless steel cannula. Stereotaxic coordinates were zeroed at the point where the tip of the cannula, held in model 1966 cannula holder, just made contact with bregma. Cannula were inserted to a depth of 2.25 mm through a 0.5 mm hole drilled at (x,y)=(1.0 mm, -0.5 mm) and cemented to the skull with dental acrylic.

Once the acrylic had dried, while animals were still under isofluorane, they received intraperotineal emitter implantation as described above. After surgery animals were individually housed and allowed to recover for one week prior to experimentation. A separate cohort of animals was injected with microglia at the same time as those used for behavioral experiments, but not implanted with cannula or emitters. These animals were sacrificed at earlier time-points to examine the distribution of transplanted microglia.

Behavioral Experiments:

For three days prior to all behavioral studies, animals were handled just before lights out (6 pm) to acclimate them to handling stress. Core body temperature (°C, CBT) and gross movement counts (voluntary motor activity, VLA) were recorded by a Dell PC (running Vital View software, connected to the ER4000 Energizer/Receiver platforms) at five minute intervals for the entire time from surgery through the end of experimentation. Food and body weight were measured every twelve hours throughout the duration of the recovery. On the night of experimentation, food was manually weighed every two hours and body weight was measured every 12 hours. To reduce total animal usage and provide internal controls, a crossover experimental design was used in which each animal received both treatments, with at least three days separating experiments.

LPS Administration:

On the night of experimentation each animal was intraperitoneally (IP) injected with either 0.9% sterile saline containing 0.5% bovine serum albumin (vehicle) or lipopolysaccharide (LPS) immediately before lights out. Two concentrations of LPS in vehicle were used in separate experiments: the low-dose treatment was 50 µg/kg BW and the high-dose was 500 µg/kg BW.

Intracerebroventricular (ICV) IL-1β administration:

On the night of experimentation, animals were restrained and ICV injected with either 1µl of artificial cerebrospinal fluid (aCSF, vehicle) or 1µl of 10 ng/µl IL-1β in aCSF (10 ng total IL-1β).

Tissue Preparation

Following experimentation, animals were deeply anesthetized with a mixture of ketamine, xylazine and acepromazine, flushed with 0.01M phosphate-buffered saline (PBS; pH=7.4) to remove blood and then perfused with ice cold 4% paraformaldehyde (PFA) in 0.01M PBS. Brains were dissected free from the skull, post-fixed overnight in

4% PFA while shaking at 4°C, cryoprotected by immersion in 30% sucrose in 0.01M PBS overnight at 4°C, then frozen on dry ice and stored at -80°C. Cryopreserved frozen brains were sectioned at 30 μm on a sliding microtome (Leica Biosystems, Buffalo Grove, Illinois; SM2000R) equipped with a freezing stage (Physitemp Instruments, Clifton, New Jersey; BFS-5MP). Abscesses were dissected free and stored in 4% paraformaldehyde until they were paraffin embedded, sectioned and stained by the OHSU histology core.

Immunohistochemistry (IHC):

IHC was performed as previously described with minor modifications.[249] Briefly, free-floating brain sections washed with 0.05% PBS three times for five minutes then pretreated by quenching in 1% glycine (Sigma-Aldrich, Cat#G8898) and de-fixing for 15 minutes in 0.05% sodium borohydride (Sigma-Aldrich, Cat#71321), with three five minute PBS washes between pretreatments. Non-specific binding was blocked with 5% normal goat serum (NGS; Sigma-Aldrich) and 1% hydrogen peroxide in PBS containing 0.3% triton x-100 (Tx; Sigma-Aldrich, Cat#X100) for 30 minutes at room temperature. Sections were incubated with primary antibodies overnight while shaking at 4°C in PBS containing 1% bovine serum albumin (Sigma-Aldrich, Cat#A2153) and 0.3% Tx. The following day sections were washed three times for five minutes in PBS containing 1% NGS and 0.3% Tx and then incubated with secondary antibodies diluted in the same solution for two hours at room temperature while shaking in the dark. Finally, sections were washed three times for five minutes PBS and then mounted on gelatin coated glass slides and coverslipped using Aqua Poly/Mount (Polysciences, Warrington, Pennsylvania; Cat#18606)

The following primary antibodies were used for immunolocalization: rabbit anti-IL-1β (Abcam, Cambridge, Massachusetts; Cat#ab9722; 1:1000), rat anti-PECAM (BD Biosciences, San Jose, California; Cat#550274; 1:100), mouse anti-GFAP (EMD

Millipore, Billerica, Massachusetts; Cat. # MAB360; 1:10,000), rat anti-mouse Cd11b (eBioscience, San Diego, California; Cat#14-0112; 1:1000) and chicken anti-GFP (Abcam Cat#ab13970; 1:1000). Antibody specificity was verified by omission of primary antibody. Primary antibodies were visualized with the following secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon; Cat#A-11008), Alexa Fluor 555 goat anti-rat IgG (Molecular Probes Cat#A-21434), Alexa Fluor 555 goat anti-mouse IgG (Molecular Probes Cat#A-21422), Alexa Fluor 488 goat anti-chicken IgY (Molecular Probes Cat#A-11039). All secondary antibodies were used at a concentration of 1:500. All images were captured under epifluorescent illumination on a Leica DM4 microscope (Leica Microsystems) equipped with a DFC340X digital camera (Leica Microsystems) driven by LASv3 software.

Statistical analysis:

Raw Vital View data was exported to an Excel file along with manually recorded food and body weights for primary calculations and graphing. For 24-hour profiles, movement counts were summed and body temperature readings were averaged over one hour blocks to give total movement per hour and hourly average CBT for each animal. For statistical analysis, total movement counts were summed over the first six hours of the dark period (6 pm-12 am) and CBT was averaged over the period between 8 pm and 11 pm (to exclude stress-induced temperature increase and include the period when body temperatures were most elevated). Food intake and body weight measurements were subtracted from pre-treatment values to generate total change over various blocks of time. For graphing, group means (treatment and genotype) and standard error of the mean (SEM) were calculated from individual animal values for each measured variable. All data were analyzed by 2-way ANOVA using Prism version 6 (Graphpad).

Results:

IP LPS causes central nervous system inflammation:

Previous reports have demonstrated that peripheral administration of LPS causes expression of interleukin-1β (IL-1β) in the circumventricular organs (CVOs) of the central nervous system (CNS).[55] This expression has tentatively been attributed to microglia, although direct evidence is sparse.[56, 57] To verify these results, and concretely identify the cell-type responsible for CNS IL-1β production, we treated wild-type (C57Bl6/J, WT) animals with LPS and examined the expression of IL-1β via fluorescent immunohistochemistry (IHC). In agreement with previous results, we found that LPS administration resulted in IL-1β expression specifically within CVOs in a time-dependent manner (Fig.1). Immunoreactive (IR) IL-1β in and around the arcuate nucleus/median eminence (ARC/ME), organum vasculosum lamina terminalis (OVLT) and sub-fornical organ (SFO) was evident at four hours post-treatment and increased by eight hours post-treatment (Fig.1 A-E). The stellate morphology of IL-1β expressing cells was suggestive of glia. To determine if IL-1β expression is restricted to a particular type of glia we used dual-label, fluorescent IHC in brain sections from animals sacrificed eight hours after LPS administration (Fig.1 G-O). We examined co-labeling of GFAP (glial fibrillary acidic protein, a marker of astrocytes) and found that astrocytes do not express IL-1β (open arrowheads in Fig.1 G-I). Examination of co-labeling of Cd11b and Cd45 (cluster of differentiation antigen-11b, not shown, and -45, both markers of microglia; arrowheads in Fig.1 J-L) confirmed that IL-1β expression is restricted to microglia. Anti-IL-1β antibody specificity was confirmed by examining brain sections from IL-1α/β double knockout mice (IL-1α/β KO) eight hours after LPS administration. Although a

comparable number of Cd45+ microglia were present in the ARC/ME, no specific labeling of IL-1β was observed in brain sections from IL-1α/β KO mice (Fig.1 M-O).

The physiological and behavioral responses to LPS are normal in IL-1α/β KO animals:

To determine if IL-1 is necessary for the physiologic and behavioral responses to LPS we treated WT and IL-1α/β KO (KO) animals with a low dose (50 μg/kg BW) of LPS and monitored movement, core-body temperature, food intake and body weight for three days following treatment. Although low-dose LPS did not result in elevated core body temperature (CBT; Fig. 2 A), LPS-treated animals of both genotypes exhibited a dramatic reduction in voluntary locomotor activity (VLA; Fig. 2 B). Because the variable nature of the telemetric data can complicate visual interpretation, we analyzed the total movement in the first six hours after treatment and the average CBT for three hours after treatment. There was no effect of either treatment or genotype for the average CBT (Fig. 2 C). For total VLA, 2-factor ANOVA showed a significant effect of treatment, but no effect of genotype (Fig. 2 D).

Regardless of genotype, LPS treatment resulted in decreased food intake and loss of body weight relative to vehicle-treated animals (Fig. 3). At this dose, LPS caused a nearly 50% reduction in food consumed during the first 24 hours following treatment, but no change for the subsequent two days (Fig. 3 A). Similarly, LPS treatment resulted in the loss of roughly 6% of initial body weight at 24 hours post-treatment, but no detectable difference at later time-points (Fig. 3 B). These results indicate that short-term sickness behaviors are not altered in the absence of IL-1

Because we hypothesized that CNS IL-1 production was involved in maintaining or prolonging sickness behaviors, we tested the effects of a larger dose of LPS (500 μg/kg BW) as a model of more severe infection. Similar to the results from low-dose LPS

treatment, 24-hour telemetry profiles show high-dose LPS treatment did not have a clear effect on CBT but did cause a dramatic decrease in activity in both WT and KO animals (Fig 4 A and B). While there was no effect of treatment on the average CBT, LPS-treated KO mice had significantly higher CBT than LPS-treated WT mice (Fig. 4C). Similar to the results from low-dose treatment, high-dose LPS caused a reduction in activity of nearly 75% for both genotypes (Fig. 4 D).

The effect of high-dose LPS on food intake and body weight was more dramatic and long-lasting than that of the low-dose (Fig. 5). Food intake for the first 24 hours after treatment was decreased by nearly 75% and by roughly 20% over the second 24 hours for both genotypes. By the third day after treatment the food intake of LPS-treated animals had normalized to that of their vehicle-treated counterparts (Fig. 5 A). Animals of both genotypes lost significant body weight following high-dose LPS treatment, and this effect persisted for two days (Fig. 5 B). At 24 hours post-treatment, LPS-treated animals had lost more than 10% of their initial body weight and regained only 2% by the 48 hour time-point. By the 72 hour time-point, LPS-treated animals had regained most of their lost body weight and were no longer significantly different from their vehicle treated counterparts. Together, these results show that genetic deletion of IL-1α/β does not affect either the severity or duration of LPS-induced sickness responses.

IL-1α/β double knockout animals develop immune related health problems:

Following the second round of LPS experiments, it was noticed that some of the double knockout animals had large masses developing on their whisker pads. In order to comply with IACUC protocols for the humane treatment of animals, these mice were euthanized. It soon became obvious that this was a problem with the entire colony as no animals reached the age of three months before developing whisker pad abscesses

(Fig. 6 A). Histological examination by Dr. Chris Corless revealed that the mass was mostly composed of infiltrating neutrophils (Fig. 6 B). Although WT animals were introduced to generate genetic diversity, each time that homozygous double-knockout mice were generated the problem re-occurred. For this reason, the colony was terminated with no further experimentation.

Microglia transplant does not restore responsiveness to central inflammation:

As the resident immune cells of the brain, microglia likely play a role in the CNS response to inflammatory stimuli. Having demonstrated that microglia are in fact the only cells in the brain that produce detectable IL-1β in response to peripheral LPS, we investigated whether they play a role in the behavioral response to central inflammation. These responses are dependent upon expression of MyD88, an essential adapter protein for LPS and IL-1β receptor signaling; MyD88 KO animals show none of the stereotypical responses to intracerebroventricular (ICV) administration of IL-1β, a commonly used model of CNS inflammation.[39, 48, 123]

To determine if expression of MyD88 in microglia alone is sufficient to restore these effects, we transplanted MyD88 WT, eGFP-expressing microglia directly into the hypothalamus of MyD88 KO mice to generate chimeric animals. Behavioral experiments on chimeric and sham transplanted MyD88 KO animals showed that microglia transplant did not restore behavioral responses to IL-1β (Fig. 7). Unlike IL-1β-treated WT animals, IL-1β-treated chimeric and MyD88 KO mice consumed normal amounts of food (Fig. 7 A). There was also no difference between sham and chimeric dark-phase CBT and VLA. (Fig. 7 B and C).

Examination of brain sections from chimeric animals used in the behavioral experiments (seven days after microglia transplant) revealed that very few eGFP+

microglia could be found near the injection site (Fig. 8). Compared to brain sections from a separate cohort sacrificed 3 days after transplant (Fig. 8 A and B), there was a dramatic decrease in the number of detectable microglia in the hypothalamus seven days after transplant (Fig. 8 C). In fact, only a few microglia could be found anywhere in the brain. Some microglia were found to have migrated to distant locations including the damaged area around where the cannula had been placed and within the choroid plexus (Fig 8 B and C). These data indicate that transplant of WT microglia to Myd88KO mice is insufficient to restore IL-1β-induced sickness behaviors.

Discussion:

The production of pro-inflammatory cytokines is a critical step in the process of systemic inflammation that leads to sickness behaviors. The principle pro-inflammatory cytokines involved in this process are tumor necrosis factor (TNF) and the interleukins IL-1 and IL-6, of which IL-1 appears to be particularly important.[250, 251] Because IL-1α is not secreted, IL-1β is thought to be more relevant in the context of systemic signaling. However, new evidence suggests that IL-1α induction has essential, tissue-specific functions, particularly within the CNS.[245, 252] Because both IL-1 molecules signal through the same receptor, it is possible that elimination of either alone would be insufficient to reveal any effect on the behavioral response to disease. For this reason, we examined sickness behaviors in IL-1α/β double KO animals.

The results from the current experiments fail to support the hypothesis that IL-1 is required for sickness behaviors in the context of germline genetic deletion. While this is a negative result in the sense that no effect of experimental manipulation was detected, it does not conclusively rule out an essential role of IL-1 in the absence of genetic disruption. It is quite possible that developmental compensation accounts for the lack of

effect of deletion of the genes coding for IL-1. As previously mentioned, inflammatory stimuli induce the production and release of a plethora of cytokines, many of which have redundant or overlapping functions. For example, TNF signaling is increased in IL-1β single KO and in IL1R1 KO mice, which compensates for the lack of IL-1 signaling in these mice.[41, 42] Interfering with TNF signaling, using either anti-TNF antibodies or TNF-binding protein, revealed that genetic disruption of IL-1 signaling did in fact have consequences in the disease models studied. This issue could be addressed by the use of conditional, rather than germline, genetic engineering strategies.

Conditional knockout models might also help avoid the deleterious health effects we observed in the IL-1α/β KO line. Despite repeated back-crossing to the background strain (C57/Bl6J; WT), we were unable to eliminate this effect. That all double-knockout mice in our colony developed whisker-pad abscesses, while none were observed on any WT or heterozygous littermates, strongly suggests that this phenotype was a direct result of genetic ablation of IL-1 signaling. On the other hand, it is possible that in selecting the first mice to generate the double-knockout colony we inadvertently selected for some other recessive trait that is genetically linked to either IL-1α or β. This conclusion is supported by the fact that this phenotype has not been reported in previous experiments using IL-1α/β KO mice. Regardless of the cause, conditional deletion strategies could shed light on the effects of immune-related signaling in specific biological compartments. Before such a strategy could be employed, however, it would first be necessary to determine the sites and/or cells where IL-1 signaling is essential for the manifestation of sickness responses.

To test whether microglia are such an essential component in the signaling-to-behavior cascade, we examined the effects of central IL-1β in chimeric animals. Transplantation of WT, IL-1β-responsive microglia into MyD88 KO mice did not restore the behavioral effects of IL-1β administration. As with the previous experiments, this

negative result is not necessarily conclusive. Our assumption was that transplanted microglia would remain in the general vicinity that they were injected, and thus serve as reservoirs of signaling-competent cells. In fact, it was very difficult to find any transplanted microglia as little as one week after injection. When we were able to locate transplanted microglia, most were found far from the injection site, occasionally in areas damaged by the surgical procedures. This result is not surprising in light of the fact that microglia are known to be highly mobile cells, migrating to areas of injury and actively clearing cellular debris.[253]

The lack of sickness behaviors in chimeric animals could be explained by the migratory tendencies of microglia. Inflammatory insults cause the production of pro-inflammatory cytokines only within very restricted regions of the brain that have a less restrictive blood-brain barrier.[68, 69] The difference in vascular permeability of the CVOs could mean that they are the only regions of the brain directly exposed to circulating cytokines. We chose the arcuate nucleus of the hypothalamus (ARC) as the target of injection because the ARC is one such region which also contains neurons of the central melanocortin system that modulate many sickness behaviors. The proximity of disease-induced IL-1 production and IL-1 responsive neural elements suggests regional specificity of action: in order for microglia to trigger sickness behaviors they need to be in the right location to both receive the signal and stimulate the response.

Future experiments should be dedicated to determining which inflammatory signals are essential for triggering sickness behaviors and where those signals act to stimulate the neural circuitry that drives sickness responses. Newly published animal models, including the conditional IL1R1 mouse and the IL1R1-restore mouse could be very useful to this process.[254, 255] Understanding where and how the immune system communicates with the CNS could help alleviate some of the negative consequences of the body's response to disease.

Acknowledgements:

We would like to thank Dr. Isamu Sugawara for providing the IL-1α/β knockout mice and Dr. Chris Corless for assistance with histological examination.

Figure 1: LPS induces expression of IL-1β in circumventricular organs (**A-C**) Representative comparison of IL-1β immunoreactivity (IR) in the region of the arcuate nucleus and median eminence (ARC/ME) from a vehicle-treated animal (Veh, A) and two time-points after lipopolysaccharide (LPS) treatment. LPS causes IL-1β production in the ARC/ME within 4 hours (B) that increases by 8 hours post-treatment (C). (**D-F**) Representative images of two circumventricular organs (CVO)—the organum vasculosum lamina terminalis (OVLT, D) and sub-fornical organ (SFO, E)—and the paraventricular nucleus of the hypothalamus (PVN, F; a periventricular hypothalamic structure that is not a CVO) 8 hours after LPS-treatment shows that IL-1β expression is restricted to CVOs. (**G-I**) IL-1β IR (G; green in I) does not co-localize with the astrocyte marker glial-fibrillary acidic protein (GFAP, H; red in I). Open arrowheads point to specific IR that does not overlap. (**J-L**) IL-1β IR (J; green in L) co-localizes with the microglia marker cluster of differentiation 45 (CD45, K; red in L). Arrowheads point to overlapping specific IR. (**M-O**) Specific IL-1β IR (M; green in O) is absent in LPS-treated IL-1α/β KO animals despite similar patterns of microglia (CD45, N; red in O). Scale bars = 100μm

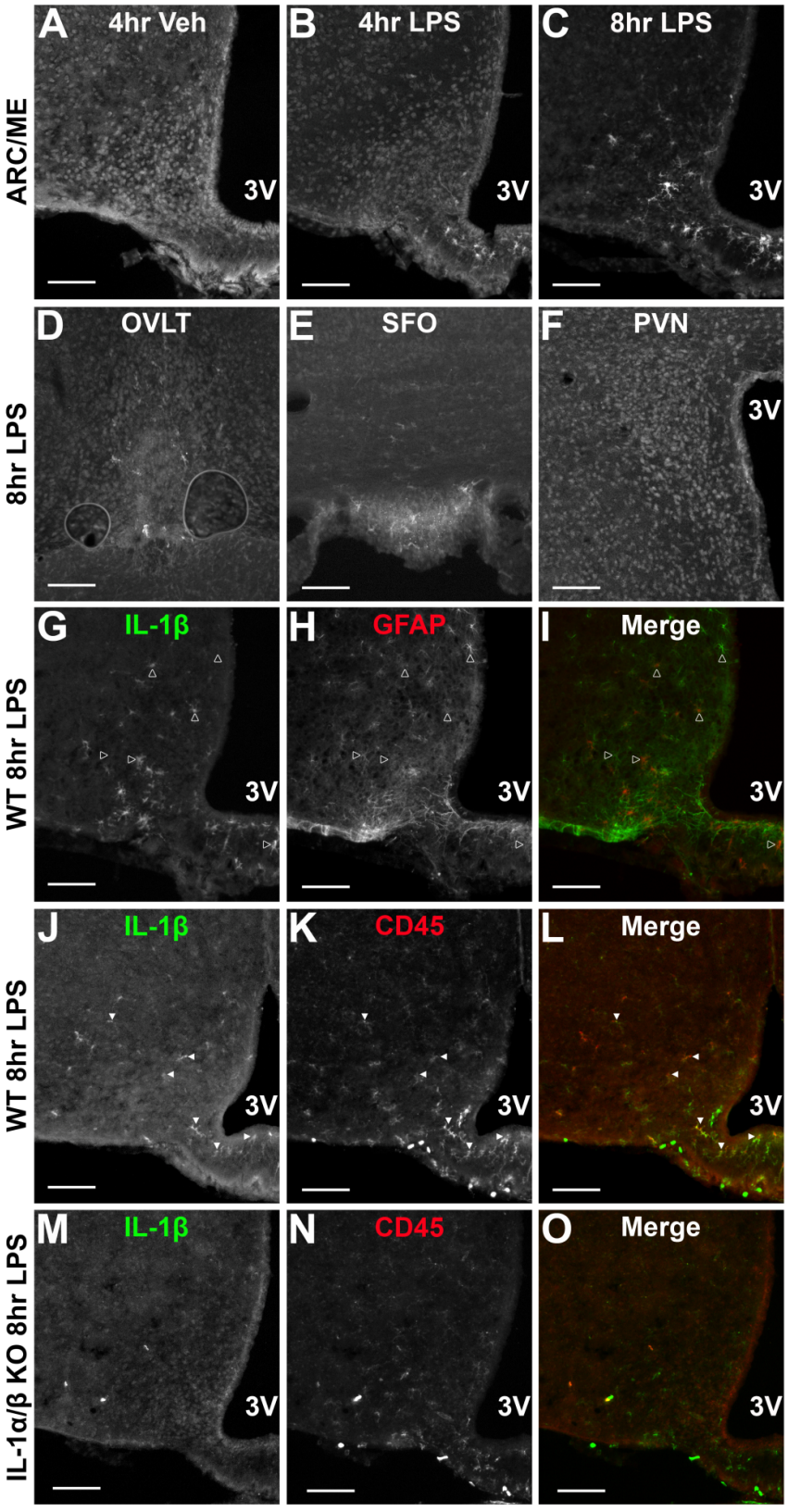

Figure 1: LPS induces expression of IL-1β in circumventricular organs

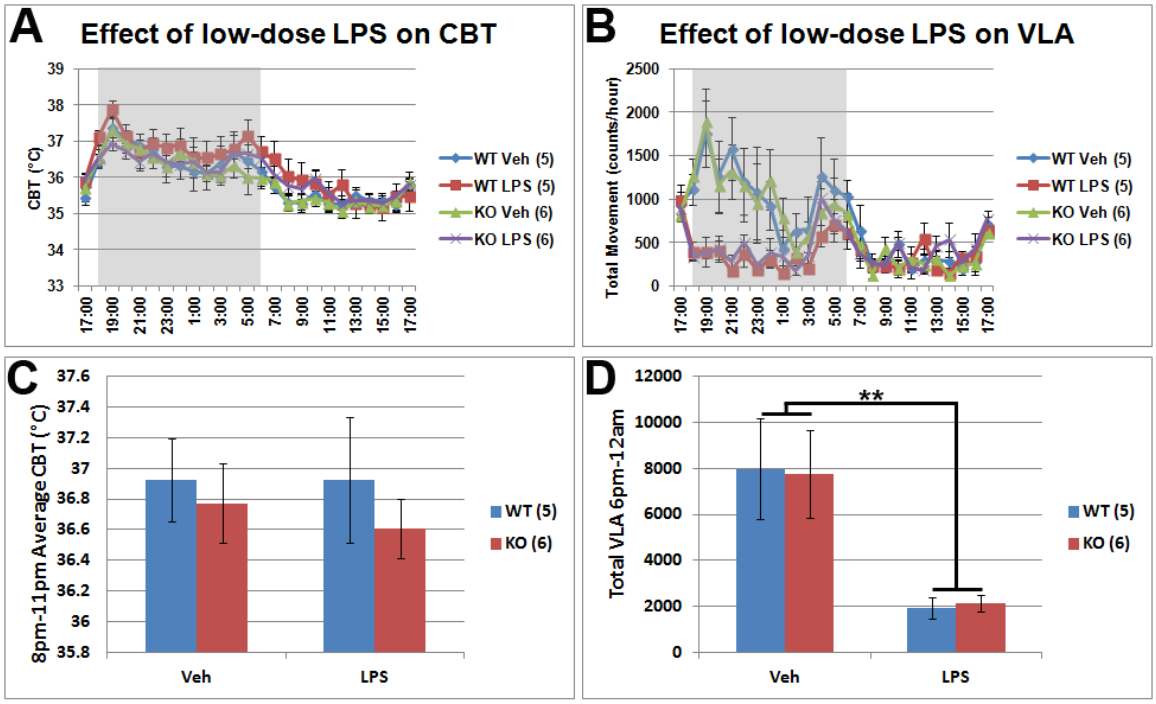

Figure 2: Low-dose (50 µg/kg) LPS decreases voluntary locomotor activity (VLA)

(**A-B**) 24-hour telemetry profiles showing the effects of LPS on body temperature and movement. Gray boxes show the dark period when mice are most awake and active. (**C**) Low-dose LPS does not cause an increase in core-body temperature (CBT) compared to vehicle (Veh) treatment in either wild-type (WT) or IL-1α/β KO (KO) animals (36.9±0.27°C and 36.9±0.41°C vs. 36.8±0.26°C and 36.6±0.19°C for WT and KO, Veh vs. LPS, respectively; p=0.64). (**D**) Low-dose LPS causes a dramatic decrease in total VLA counts compared to Veh treatment in both WT and KO animals (7969±2184 and 7767±1899 vs. 1939±469 and 2149±350 for WT and KO, Veh vs. LPS, respectively; p=0.0011). All values shown are mean±SEM for the group sizes shown in parentheses. **, p<0.01

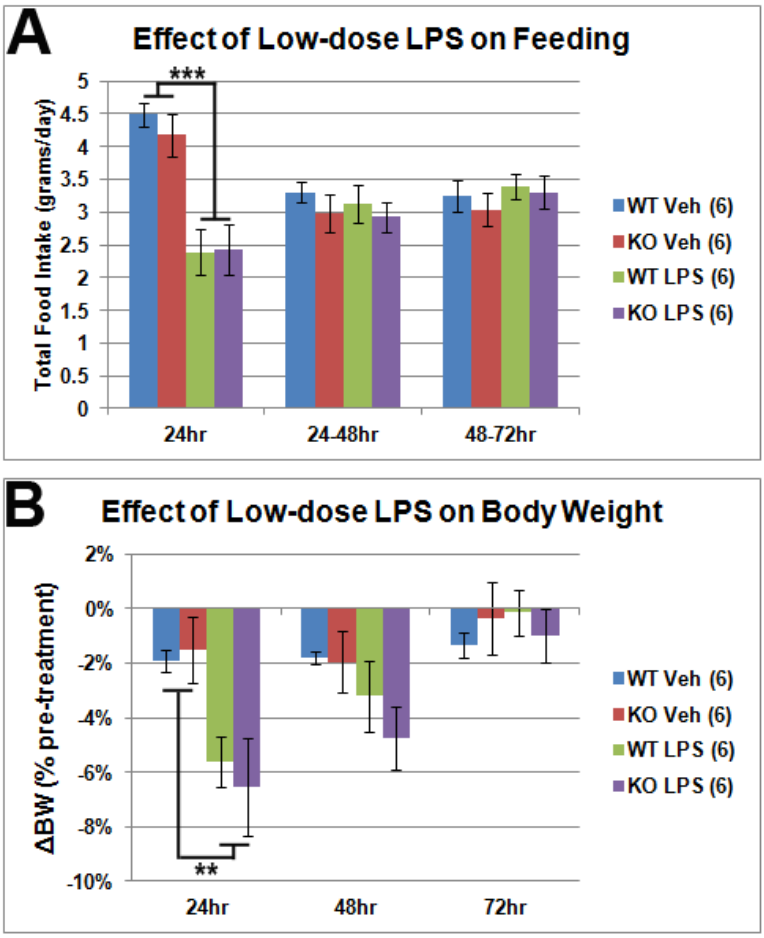

Figure 3: Low-dose LPS causes decreased food intake and loss of body weight

(**A**) Low-dose LPS caused a significant reduction in food intake for both wild-type (WT) and IL-1α/β KO (KO) mice during the first 24 hours after treatment (4.49±0.19 and 4.18±0.33g vs. 2.39±0.35 and 2.43±0.39g for WT and KO, Veh vs. LPS, respectively; p=0.0002). (**B**) Low-dose LPS also caused significant loss of body weight (ΔBW) during the 24 hours following treatment (-1.92%±0.39% and -1.52%±1.2% vs. -5.61%±0.9% and -6.55%±1.8% for WT and KO, Veh vs. LPS, respectively; p<0.0001). The ΔBW at 48 hours after treatment was not significantly different between LPS and Veh groups (p=0.076). All values are mean±SEM for group sizes in parentheses. ** p<0.01, *** p<0.001

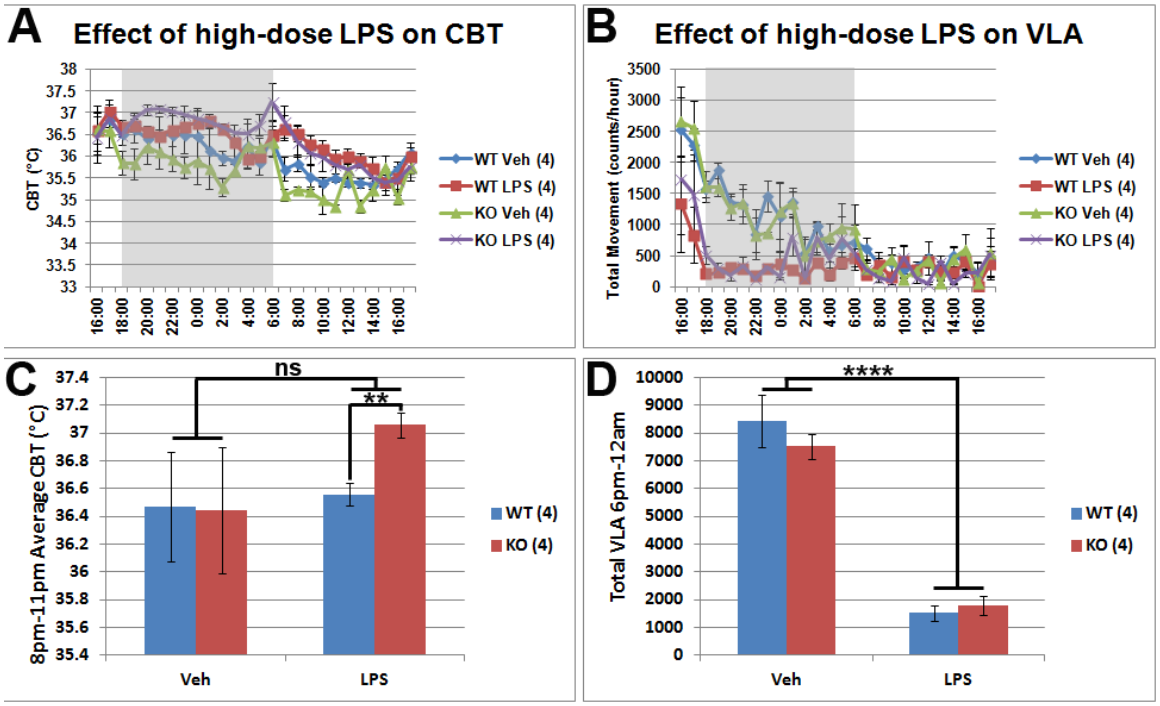

Figure 4: High-dose (500 µg/kg) LPS decreases voluntary locomotor activity (VLA)

(**A-B**) 24-hour telemetry profiles showing the effects of LPS on body temperature and movement. Gray boxes show the dark period when mice are most awake and active. (**C**) High-dose LPS does not cause a significant increase in core-body temperature (CBT) compared to vehicle (Veh) treatment in either wild-type (WT) or IL-1α/β KO (KO) animals (36.47±0.39°C and 36.45±0.46°C vs. 36.56±0.08°C and 37.06±0.09°C for WT and KO, Veh vs. LPS, respectively; p=0.296). There was a difference between the CBT of LPS-treated WT and KO mice (p=0.006). (**D**) High-dose LPS causes a dramatic decrease in total VLA counts compared to Veh treatment in both WT and KO animals (8435±939 and 7513±444 vs. 1514±266 and 1785±349 for WT and KO, Veh vs. LPS, respectively; p=0.0011). All values shown are mean±SEM for the group sizes shown in parentheses. Ns, not significant, **, p<0.01, ****, p<0.0001

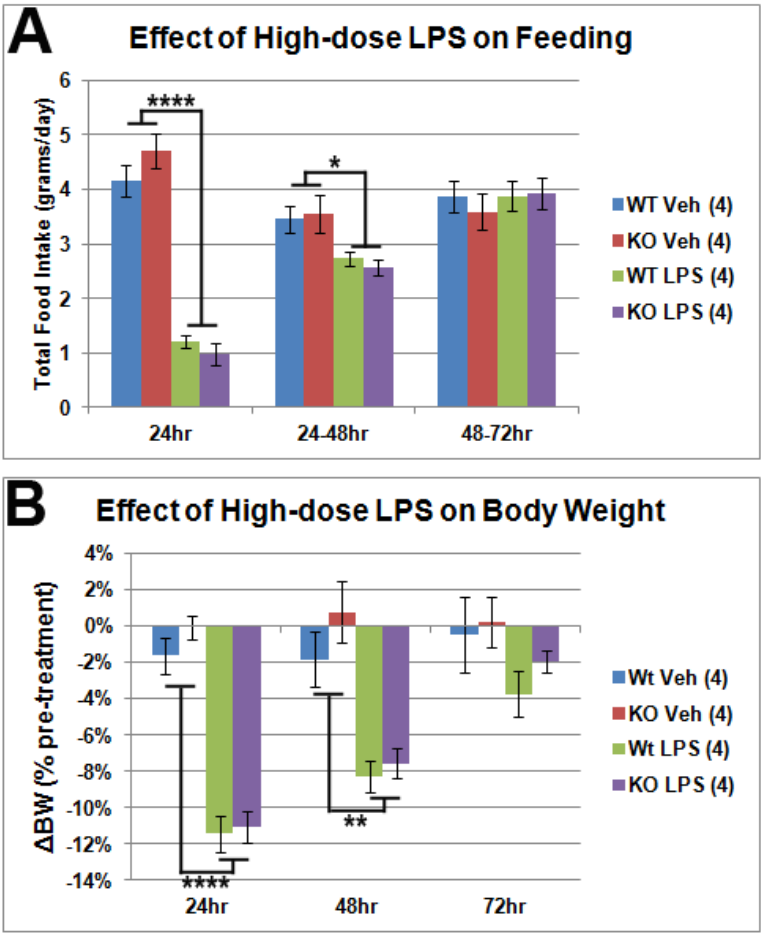

Figure 5: High-dose LPS causes long-lasting changes in food intake and body weight

(**A**) High-dose LPS caused significant reduction in food intake for both wild-type (WT) and IL-1α/β KO (KO) mice during the first (4.15±0.29g and 4.71±0.33g vs. 1.21±0.1g and 0.97±0.2g for WT and KO, Veh vs. LPS, respectively; p=0.0002) and second (3.45±0.25g and 3.56±0.34g vs. 2.73±0.13g and 2.58±0.15g for WT and KO, Veh vs. LPS, respectively; p=0.014) days after treatment. (**B**) High-dose LPS also caused significant loss of body weight (ΔBW) at 24 hours (-1.65%±1% and -0.09±0.68% vs. -11.45%±1.01% and -11.09%±0.85% for WT and KO, Veh vs. LPS, respectively; p<0.0001) and 48 hours (-1.85%±1.49% and 0.74±1.7% vs. -8.28%±0.85% and -7.56%±0.82% for WT and KO, Veh vs. LPS, respectively; p=0.002) after treatment. All values are mean±SEM. * p<0.05, ** p<0.01, **** p<0.0001

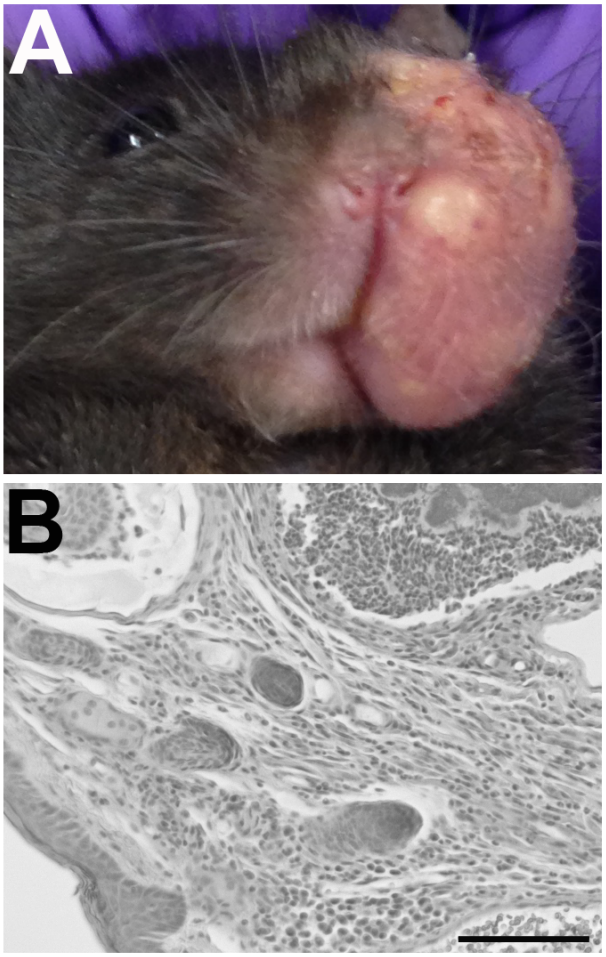

Figure 6: IL-1α/β KO mice develop whisker pad abscesses

(**A**) Example of the gross presentation of the health problems associated with the IL-1α/β KO line. (**B**) Representative image of H&E staining in a section from a fixed abscess showing neutrophil invasion and accumulation in the whisker pad. Scale bar = 100μm.

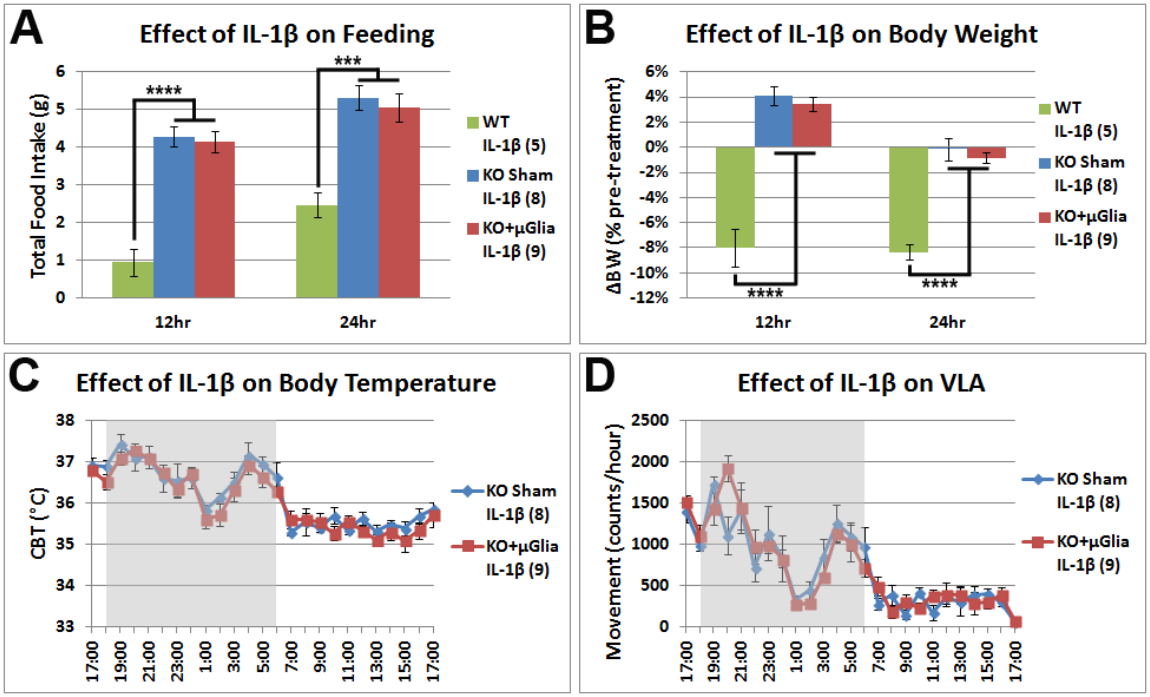

Figure 7: Microglia chimeric animals do exhibit IL-1β-induced sickness behaviors

(**A**) Intracerebroventricular (ICV) administration of 100ng IL-1β caused significant decreases in wild-type (WT IL-1β) total food intake relative to both MyD88 KO (KO IL-1β) and chimeric (KO+μGlia IL-1β) over 12 (0.94±0.35g vs. 4.28±0.27g and 4.15±0.29g for WT vs. KO and KO+μGlia, respectively; $p<0.0001$) and 24 (2.46±0.33g vs. 5.31±0.33g and 5.06±0.37g for WT vs. KO and KO+μGlia, respectively; $p=0.0001$) hours post-treatment. There was no difference between KO and KO+μGlia at either 12 ($p=0.75$) or 24 ($p=0.62$) hours post-treatment. (**B**) ICV IL-1β caused significant loss of body weight in WT relative to both KO and KO+μGlia at 12 (-8.02%±1.52% vs. 4.12±0.74% and 3.43%±0.54% for WT vs. KO and KO+μGlia, respectively; $p<0.0001$) and 24 (-8.35%±0.64% vs. -0.17±0.86% and 0.84%±0.41% for WT vs. KO and KO+μGlia, respectively; $p<0.0001$) hours post-treatment. There was no difference between KO and KO+μGlia at either 12 ($p=0.46$) or 24 ($p=0.53$) hours post-treatment. (**C and D**) 24-hour profiles show that ICV IL-1β had no effect on either core-body temperature (CBT) or voluntary locomotor activity (VLA) in KO and KO+μGlia mice. Gray boxes show the dark period when mice are most awake and active. All values shown are mean±SEM for the group sizes shown in parentheses. All values are mean±SEM. *** $p<0.001$, **** $p<0.0001$

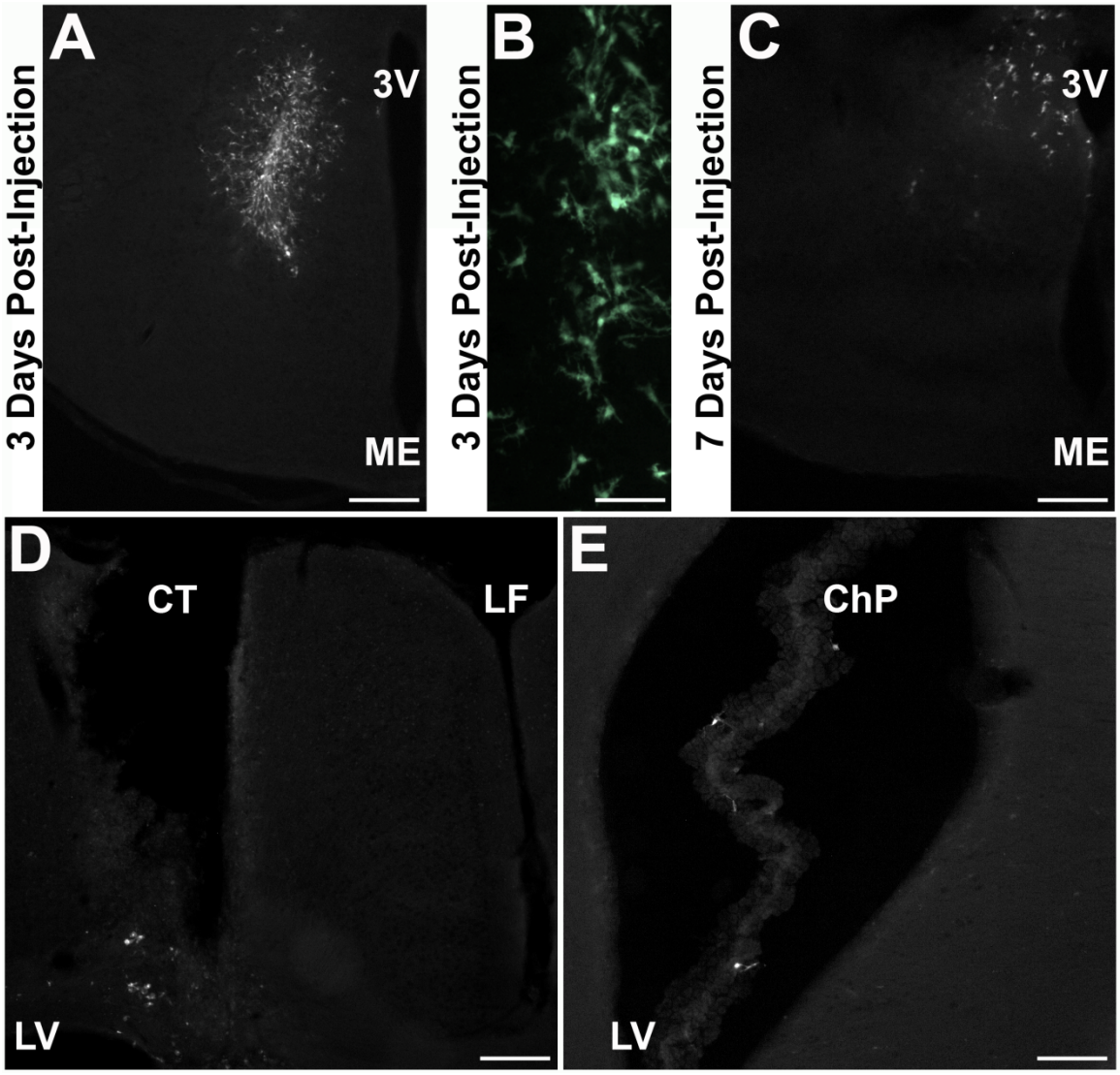

Figure 8: Transplanted microglia migrate away from injection site

Anti-eGFP immunohistochemistry (IHC) localization of transplanted microglia. (**A**) Three days after microglia transplant, a large number of microglia were still present near the site of injection. (**B**) Higher magnification image showing that transplanted microglia display characteristic morphology. (**C-E**) Seven days after microglia transplant, on the day following behavioral experimentation, very few microglia remained near the injection site. (**C**)This image is not representative, but shows a section with one of the highest number of microglia still present in the hypothalamus. Some microglia were found considerable distances from the site of injection, including to the area around the cannula tract (CT; **D**) and in the choroid plexus (ChP; **E**) within the lateral ventricle (LV). 3V, third ventricle; ME, median eminence; LF, longitudinal fissure. Scale bars in A and C = 200μm, B = 50μm, D and E = 100μm

Chapter 3:

Manuscript #2

Interleukin-1β signaling in fenestrated capillaries is sufficient to trigger sickness responses in mice.

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Chapter 3 is a modified version of an original manuscript as submitted to the journal PLOS Biology on April 21, 2017.

Abstract:

The behavioral and physiological symptoms of sickness, including fever, anorexia, depression and weight loss can be both beneficial and detrimental. These sickness responses are triggered by pro-inflammatory cytokines acting on cells within the brain. We examined where the potent pro-inflammatory cytokine interleukin-1β (IL-1β) acts in vivo via fluorescent immunohistochemistry using an experimental model that mimics central nervous system cytokine production. We found that endothelial cells, microglia, ependymal cells and astrocytes exhibit nuclear translocation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) following intracerebroventricular administration of IL-1β. Using multiple transgenic mouse lines expressing Cre recombinase under the control of cell-specific promoters, we eliminated IL-1β signaling in endothelial cells, microglia or both microglia and endothelial cells. These experiments show that IL-1β-induced sickness responses depend on intact IL-1β signaling in blood vessels and suggest that the fenestrated capillaries of circumventricular organs act as a signaling relay between the immune and nervous systems.

Introduction:

The biological response to illness and injury is an evolutionarily conserved phenomenon that promotes survival. Many disparate conditions result in the same combination of physiologic and behavioral symptoms including changes to metabolic status, fever, behavioral depression and anorexia (collectively, the sickness response).[8] As each of these symptoms is regulated at least in part by elements of the central nervous system (CNS), it is clear that there is a regulated, coordinated CNS response to disease. While there is growing understanding of the specific neural circuits that are involved in regulating individual attributes such as appetite[256] and body temperature[212], less is known about the specific cell types and signaling pathways that initiate the sickness response.

Outside of the CNS, production of cytokines is the predominant mechanism for coordinating the response to a wide variety of infectious pathogens, and also plays a key role in the response to traumatic injury, organ failure, and neurodegenerative diseases.[49-52] Pro-inflammatory cytokines are released into the general circulation by peripheral immune cells to signal the presence of disease, stimulate inflammation and promote healing.[242] Cytokines, particularly interleukin-1β (IL-1β), also act at the level of the brain to trigger changes in neuronal activity that results in sickness responses. Intracerebroventricular (ICV) administration of IL-1β causes fever, anorexia, lethargy and weight loss without raising circulating levels of IL-1β, and sickness responses triggered by peripheral inflammation are blocked by ICV administration of the endogenous IL-1 receptor antagonist (Il1ra).[123, 257, 258] Although neuronal activity is ultimately responsible for controlling physiologic and behavioral set-points, disruption of IL-1β signaling in

neurons, astrocytes and oligodendrocytes does not affect the sickness response.[39] This suggests that another cell type is responsible for modulating neuronal activity in response to inflammation.

Vascular endothelial cells, which comprise the wall of blood vessels and form an integral component of the blood-brain barrier (BBB) that prevents IL-1β from passively diffusing into the brain, represent one example of cellular signaling intermediaries between peripheral inflammation and the CNS.[53, 58] As both the conduit that carries inflammatory signals to the brain and the barricade that impedes free diffusion into the CNS, endothelial cells play multiple roles in regulating the sickness response to peripheral disease. In response to peripheral IL-1β, endothelial cells produce prostaglandins (PGs), lipid soluble signaling molecules that are not restricted by the BBB.[231, 259] This is particularly relevant to the febrile response, as interfering with IL-1β signaling or PG synthesis in endothelial cells reduces fever without altering other sickness responses.[236, 260] This implies that other mechanisms are also involved in regulating the sickness response.

In addition to causing increased circulating cytokine levels, peripheral diseases that result in sickness responses also cause the production of pro-inflammatory cytokines, including IL-1β, within the CNS. Disease models of bacterial and viral infection, cancer and organ failure all cause increased IL-1β within the brain, presenting an additional common pathway for generating sickness responses.[123, 261-263] IL-1β is produced by microglia and perivascular macrophages in and around the cicumventricular organs (CVOs) and choroid plexus (ChP), leading to site-specific amplification of peripheral inflammatory signals and elevated IL-1β in the cerebrospinal fluid (CSF) which can act via volume transmission on deeper, BBB-isolated brain regions.[55-57] ICV injections of small amounts of IL-1β achieve CSF concentrations similar to that caused by peripheral insults, providing a model for studying how sickness

responses are triggered by this shared pathway.[264, 265] Despite considerable effort, the precise cellular targets of CNS-produced IL-1β necessary for the sickness response remain unidentified.

We examined the specific cellular components of the CNS that are required for the sickness response in mice using a model that mimics CNS production of IL-1β in response to peripheral inflammation. We hypothesized that blood vessels are critical cellular components in this phase of the CNS inflammation cascade, and that disruption of IL-1β signaling in endothelial cells would alter the sickness response. Furthermore, because microglia act to amplify inflammatory signaling within the CNS, we hypothesized that eliminating microglial IL-1β signaling would decrease the severity or duration of sickness responses. Using immunohistochemistry (IHC) following ICV administration of IL-1β, we identified which cell types demonstrate the earliest observable response. We then utilized Cre recombinase-expressing, transgenic mice to systematically disrupt IL-1β signaling in specific cell populations. Our results demonstrate that the physiological and behavioral responses to IL-1β are not derived from cerebral vasculature in general, but instead depend upon a relatively small number of fenestrated capillaries located primarily in circumventricular structures.

Results:

Vascular heterogeneity in the Brain

The distribution of blood vessels in the mouse brain is not uniform. For immunolocalization experiments we focused on five regions: the organum vasculosum lamina terminalis (OVLT), the subfornical organ (SFO), the choroid plexus (ChP), the paraventricular nucleus (PVN) and the arcuate nucleus/median eminence (ARC/ME; Fig. 9 A-E). Cluster of differentiation 31 (Cd31) immunoreactivity (IR) demonstrates the

relatively high vascularity in each of these regions of interest. For greater anatomical context, low-magnification, digital photo-montages of Cd31 IR in whole coronal brain sections are shown in Figure 10.

In the three circumventricular organs (CVOs)—the OVLT, SFO and ME—and in the ChP, the increased vascular density is due to loops of fenestrated capillaries which contribute to a less restrictive blood-brain barrier (BBB) in these structures.[67] Because of this, the CVOs have long been considered likely sites of entry for circulating cytokines to access the CNS and cause sickness responses.[266] All four structures that contain fenestrated capillaries have also been directly implicated in the neural response to inflammation.[74-76, 90, 91] The PVN contains non-fenestrated, BBB-isolated vessels at a density ~3-5 times greater than most of the brain.[267] The PVN also contains the cell bodies of neurons that control the hypothalamic-pituitary-adrenal axis, modulate the autonomic nervous system and regulate the muscle catabolism that occurs during disease-associated wasting.[123, 268, 269]

Centrally administered IL-1β activates diverse cells throughout the brain

IL-1β exerts its influence predominantly through binding to a receptor complex located in the cell membrane that includes the IL-1 receptor 1 (Il1r1) and the IL-1 receptor accessory protein (Il1rap).[270] Binding of IL-1β initiates a sequence of events which requires the adaptor protein Myd88 for downstream signaling and IL-1β-induced sickness responses.[39, 47, 271, 272] Canonical IL-1β signaling culminates in activation and nuclear translocation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), resulting in changes in gene transcription.[44]

To determine the sites of IL-1β initiated signaling, we examined NF-κB IR in brain sections from animals sacrificed at various times after intracerebroventricular (ICV)

administration of either artificial cerebrospinal fluid (aCSF, vehicle) or 10ng IL-1β in aCSF (Fig. 11). Sections from vehicle-treated animals showed only diffuse, cytoplasmic fluorescence, most evident in blood vessels and ependyma at all times examined (Fig. 11A). No fluorescence above background was detected in sections incubated without primary antibody (Fig. 12A). Sections from IL-1β-treated mice displayed punctate, nuclear NF-κB IR and a marked decrease in cytoplasmic labeling, most notable as a decrease in clearly-defined periventricular vascular patterns. This change in NF-κB IR is both rapid and transient, with nuclear labeling evident at 15 minutes post-treatment, peaking by 30 minutes, persisting for at least two hours and returning to baseline, cytoplasmic labeling by 4 hours post-treatment (Fig. 12C and Fig. 11B-D). IL-1β-induced nuclear NF-κB IR was observed scattered throughout the brain with the highest density in regions immediately adjacent to the ventricles including the five regions of interest (Fig. 11E-L). Brain sections from IL-1β-treated *Myd88*KO animals do not exhibit nuclear NF-κB IR at any time examined, indicating that the nuclear translocation of NF-κB as detected by IHC is dependent on expression of functional Myd88 (Fig. 12E). Dual-label immunohistochemistry (IHC) revealed nuclear NF-κB IR in vascular endothelium (Cd31+), microglia (cluster of differentiation molecule 11b, Cd11b+), ependymal cells, including some tanycytes (vimentin+), and astrocytes (glial fibrillary acidic protein, GFAP+; Fig. 11M-P). Notably, nuclear NF-κB IR was not observed in neurons, including those within the PVN (Fig. 13).

Recombinase reporter delineates strain-specific genetic recombination

For this series of experiments we used three transgenic mouse strains that express Cre recombinase under the control of specific promoters. To assess the

contribution of blood vessels to the IL-1β induced sickness response, we utilized a strain of mice expressing Cre recombinase under the control of the promoter for the angiopoietin-1 receptor, *Tek* (also known as Tie2; *Tek*-Cre).[273] To determine sites of *Tek*-driven Cre expression, we crossed the *Tek*-Cre and Rosa26-flox-stopTdTomato (TdT) strains to generate mice that express fluorescent TdT only in cells that express *Tek*-Cre (*Tek*-TdT).[274] Examination of reporter expression and various cell-specific markers in brain sections from *Tek*-TdT animals revealed genetic recombination in all vascular endothelium (Cd31 IR) and all microglia (immunoreactive for ionized calcium-binding adapter molecule-1, Iba1 IR; Fig. 14 A and B). This result is in agreement with previously published descriptions of *Tek*-Cre expression in endothelium and myeloid cells.[275, 276] Reporter expression was absent from neurons, astrocytes and oligodendrocytes (data not shown). This pattern of expression was consistent in all brain regions examined (Fig. 15 A-D).

As the resident immune cells of the brain, microglia are likely candidates for playing a role in the brain response to immune signaling. We investigated the function of microglia in the sickness response using a line of mice that express tamoxifen-inducible Cre and yellow fluorescent protein (YFP) under the control of the endogenous promoter for the fractalkine receptor, *Cx3cr1* (*Cx3cr1* -CreERT2).[277] This allows for temporal control of Cre activity and an endogenous marker of cells that express the transgene. We verified recombination by crossing the *Cx3cr1* -CreERT2 and TdT lines (*Cx3cr1*-TdT) and found TdT expression exclusively in all microglia in brain sections from tamoxifen-treated animals (Fig 14 C and D). This result demonstrates that *Cx3cr1* -CreERT2 animals can be used to manipulate microglia without affecting blood vessels.

To examine the role of endothelium independent of microglia, we used a line of mice that express tamoxifen-inducible Cre under the control of the promoter for the thyroid-hormone transporter, solute carrier organic anion transporter family member 1C1

(*Slco1c1*-CreERT2).[236] In brain sections from tamoxifen-treated Slco1c1$^{\Delta TdTomato}$ animals, TdTomato expression was evident in multiple cell types including cuboidal cells of the ChP, a subset of β1 tanycytes[278], some hippocampal neurons and an unidentified cell type with morphology consistent with GFAP-negative astrocytes (Fig. 14 F and Fig. 15). Importantly, TdT expression was observed in all vascular endothelium within the tissue of the brain itself (parenchymal endothelium; Fig. 14 E and Fig. 15E-H). Reporter expression was absent from microglia, oligodendrocytes and GFAP+ astrocytes (Fig. 14F and data not shown). This suggests that the *Slco1c1*-CreERT2 line can be used to drive recombination in parenchymal endothelium without affecting microglia.

While comparing reporter expression with Cd31 IR in *Slco1c1*- and *Tek*-TdT animals we noted a major difference between the two lines in their vascular expression pattern: *Tek* -Cre causes recombination in all endothelium including fenestrated capillaries (* in Fig. 16A-D), while *Slco1c1*-CreERT2 causes recombination in all parenchymal endothelium but not in fenestrated capillaries (* in Fig. 16E-H). This is especially obvious in the OVLT, SFO and ME, where the fenestrated capillaries of *Slco1c1*-TdT clearly do not express TdT. Although the high level of reporter expression in ChP cuboidal cells makes it difficult to determine whether recombination occurs in the underlying fenestrated capillaries, when viewed in cross-section there does not appear to be expression in the fenestrated capillary within the tube of ensheathing cuboidal cells (* in Fig. 16G). This comparison also highlights the fact that reporter expression occurs outside of endothelium in both lines (open arrowheads in Fig. 16).

NF-κB IR confirms Cre-dependent IL-1β signaling disruption

To disrupt IL-1β signaling in specific cell populations, we utilized the conditional *Myd88* strain (*Myd88*$^{fl/fl}$) to generate promoter-specific *Myd88* knockout mice (Δ *Myd88*).

By crossing the *Myd88*$^{fl/fl}$ and the three Cre strains we produced mice that lack Myd88-dependent IL-1β signaling in all endothelium and microglia (*Tek*Δ*Myd88*), in all microglia alone (*Cx3cr1*Δ*Myd88*) and in parenchymal endothelium but not microglia (*Slco1c1*Δ*Myd88*). We also crossed the *Cx3cr1*Δ*Myd88* and *Slco1c1*Δ*Myd88* lines to generate mice that lack IL-1β signaling in microglia and parenchymal endothelium but not in fenestrated capillaries (*Cx3/Slc*Δ*Myd88*).

To confirm *Myd88* deletion and IL-1β signaling disruption, we examined NF-κB IR in brain sections from Cre- control (*Myd88*$^{fl/fl}$) and Δ *Myd88* littermates 30 minutes after ICV IL-1β treatment. In *Myd88*$^{fl/fl}$ animals, IL-1β treatment caused nuclear localization of NF-κB IR in endothelial cells within the PVN (filled arrowheads in Fig. 5A). In contrast, a vascular pattern of cytoplasmic NF-κB IR is evident in the PVN of IL-1β treated *Tek*Δ*Myd88* and *Slco1c1*Δ*Myd88* animals (filled arrowheads in Fig. 17C and E). Similarly, while many microglia in the ME of control animals had nuclear NF-κB IR (filled arrowheads in Fig. 17B), none were found in either *Tek*Δ*Myd88* or *Cx3cr1*Δ*Myd88* animals (Fig. 17D and F). The presence of nuclear NF-κB IR in ependymal cells and within the parenchyma of the brain confirms that disruption of Myd88-dependent IL-1β signaling is not global, but restricted to the sites of Cre expression (open arrowheads in Fig. 17C-F).

Of the three Cre lines used in this series of experiments, the *Cx3cr1*-CreERT2 line is unique in that it is a knock-in at the endogenous locus rather than a random insertion. This has the advantage of maintaining potential regulatory elements, and thus ensuring transgene expression in a biologically relevant distribution, but is a distinct disadvantage for this experiment as both *Cx3cr1* and *Myd88* are located on chromosome 9. To generate *Cx3cr1*Δ*Myd88* mice it was necessary to breed compound heterozygous mice (*Cx3cr1*-Cre$^{+/-}$ *Myd88*$^{fl/WT}$) until spontaneous genetic recombination was detected. While breeding was ongoing, a line of mice carrying a conditional allele of

interleukin 1 receptor 1 (*Il1r1*$^{fl/fl}$) became available, allowing disruption of IL-1β signaling in microglia independent of Myd88 (*Cx3cr1*Δ*Il1r1*).[255] NF-κB IR in brain sections from IL-1β treated control (*Cx3cr1*$^{+/WT}$), *Cx3cr1*Δ*Myd88* and *Cx3cr1*Δ*Il1r1* confirms that both *Cx3cr1*-CreERT2 knockout lines eliminated the microglial response to IL-1β (Fig. 18).

Once they were available, we crossed the *Cx3cr1*Δ*Myd88* and *Slco1c1*Δ*Myd88* lines to generate a compound knockout where MyD88 is deleted in both microglia and all brain vessels except fenestrated capillaries (*Cx3/Slc*Δ*Myd88*). NF-κB IR in brain sections from tamoxifen-treated *Cx3cr1*$^{+/WT}$ and *Cx3/Slc*Δ*Myd88* 30 minutes after ICV IL-1β treatment demonstrates that the compound knockout combines the effects of the individual knockouts (Fig. 19). In brain sections from *Cx3cr1*$^{+/WT}$ animals, nuclear NF-κB IR was present in parenchymal endothelium, microglia and β1 tanycytes (filled arrowheads and * in Fig. 19A and B). In the PVN of *Cx3/Slc*Δ*Myd88*animals there was a clear vascular pattern of cytoplasmic NF-κB IR, while in the ARC/ME both microglia and β1 tanycytes lacked nuclear labeling (Fig. 19C and D). These results show that the *Cx3/Slc*Δ*Myd88* line can be used to eliminate IL-1β signaling in both parenchymal endothelium and microglia.

Disruption of IL-1β signaling in endothelium and microglia eliminates the sickness response

We examined the effects of ICV IL-1β on *Myd88*$^{fl/fl}$ (fl/fl) and *Tek*Δ*Myd88* (KO) littermates by monitoring core-body temperature (CBT), voluntary locomotor activity (VLA), food intake (FI) and body weight (BW) for 24 hours after treatment. In agreement with previous reports, IL-1β treatment of *Myd88*$^{fl/fl}$ animals resulted in stereotypical patterns of significantly increased core-body temperature (ΔCBT) and decreased VLA and FI compared to vehicle-treated (Veh) animals (Fig. 20). These changes lasted for

several hours, with fever peaking four to five hours after treatment. In contrast, IL-1β-treated *Tek*Δ*Myd88* were indistinguishable from their vehicle-treated counterparts.

To quantify these differences we analyzed the average ΔCBT at the peak of fever (10pm-12am), total VLA counts and FI for the first six hours after treatment (6pm-12am) and change in body weight (ΔBW) 12 hours after treatment (6pm-6am; Fig.21A). In each case, two-way ANOVA revealed a significant effect of treatment and genotype (ΔCBT: treatment and genotype $p<0.0001$; VLA: treatment $p=0.0003$, genotype $p=0.036$; FI: treatment $p<0.0001$, genotype $p=0.0003$; ΔBW: treatment $p=0.014$, genotype $p=0.0044$), and for all but VLA there was a significant interaction of treatment by genotype (ΔCBT: $p<0.0001$; VLA: $p=0.07$; FI: $p<0.0001$; ΔBW: $p=0.0075$). Post-hoc analysis revealed that all differences were due to the effects of IL-1β on *Myd88*$^{fl/fl}$ animals, as only this group was significantly different from all other groups for ΔCBT (mean±SEM: IL-1β-*Myd88*$^{fl/fl}$=1.99±0.19°C vs. IL-1β-*Tek*Δ*Myd88*=0.59±0.17°C, Veh-*Myd88*$^{fl/fl}$=0.86±0.24°C, Veh-*Tek*Δ*Myd88*=0.60±0.31°C; $p<0.0001$ for all), VLA (mean±SEM: IL-1β-*Myd88*$^{fl/fl}$=3792±733 vs. IL-1β-*Tek*Δ*Myd88*=6616±583, Veh-*Myd88*$^{fl/fl}$=8124±687, Veh-*Tek*Δ*Myd88*=8352±614; $p=0.033$, 0.0011, 0.0007 respectively), FI (mean±SEM: IL-1β-*Myd88*$^{fl/fl}$=0.65±0.15g vs. IL-1β-*Tek*Δ*Myd88*=2.17±0.11g, Veh-*Myd88*$^{fl/fl}$=2.48±0.18g, Veh-*Tek*Δ*Myd88*=2.3±0.14g; $p<0.0001$ for all), and ΔBW (mean±SEM: IL-1β-*Myd88*$^{fl/fl}$= -0.49±0.34g vs. IL-1β-*Tek*Δ*Myd88*=1.05±0.18g, Veh-*Myd88*$^{fl/fl}$=0.92±0.17g, Veh-*Tek*Δ*Myd88*=0.98±0.17g; $p=0.0014$, 0.0032, 0.0022 respectively). IL-1β-treated *Tek*Δ*Myd88* animals were not different from vehicle treated animals of either genotype for any parameter measured, demonstrating that IL-1β-induced sickness responses are dependent upon Myd88 expression in endothelium and/or microglia.

Disruption of IL-1β signaling exclusively in microglia does not affect the sickness response

Because *Tek*-Cre is expressed in both endothelium and microglia it is impossible to determine the individual contribution of either cell type alone using the *Tek*Δ*Myd88* line. We examined the role of microglia independent of endothelium using the microglia-specific *Cx3cr1*-CreERT2. In both *Cx3cr1*Δ*Il1r1* and *Cx3cr1*Δ*Myd88*, the response to ICV IL-1β in experimental animals (KO) and their control littermates (fl/fl) was the same. IL-1β treatment of *Cx3cr1*Δ*Il1r1* and *Il1r1*$^{fl/fl}$ mice caused stereotypical fever and reduction in VLA and FI for several hours after treatment (Fig. 22 A). Two-way ANOVA revealed a highly significant effect of treatment ($p<0.0001$ for all; Fig. 21B), no effect of genotype and no interaction of treatment by genotype for ΔCBT (0.48±0.12°C and 0.33±0.2°C vs. 1.56±0.14°C and 1.89±0.25°C; mean±SEM for *Il1r1*$^{fl/fl}$ and *Cx3cr1*Δ*Il1r1* Veh vs. IL-1β, respectively), VLA (4903±732 and 6611±508 vs. 2435±249 and 2276±359; mean±SEM for *Il1r1*$^{fl/fl}$ and *Cx3cr1*Δ*Il1r1* Veh vs. IL-1β, respectively) and FI (2.13±0.28g and 1.89±0.32g vs. 0.88±0.18g and 0.38±0.1g; mean±SEM for *Il1r1*$^{fl/fl}$ and *Cx3cr1*Δ*Il1r1* Veh vs. IL-1β, respectively). Analysis of body weight data revealed a highly significant effect of treatment ($p<0.0001$), a significant effect of genotype ($p=0.025$), and no interaction of treatment by genotype for ΔBW (1.08±0.14g and 0.99±0.14g vs. 0.35±0.1g and -0.28±0.11g; mean±SEM for *Il1r1*$^{fl/fl}$ and *Cx3cr1*Δ*Il1r1* Veh vs. IL-1β, respectively). The effect of genotype on ΔBW reflects the fact that IL-1β-treated *Il1r1*$^{fl/fl}$ animals did not lose as much weight as *Cx3cr1*Δ*Il1r1* animals. Analysis of the effects of ICV IL-1β on *Cx3cr1*Δ*Myd88* animals revealed a significant effect of treatment, no effect of genotype and no interaction of treatment by genotype for both 12 hour (6pm-6am) FI (4.83±0.56g and 5.3±0.23g vs. 2.57±0.53g and 3.25±0.71g; mean±SEM for *Myd88*$^{fl/fl}$ and *Cx3cr1*Δ*Myd88* Veh vs. IL-1β, respectively; $p=0.0037$) and ΔBW (0.5±0.2g and

0.84±0.12g vs. -0.86±0.25g and -0.7±0.66g; mean±SEM for *Myd88*$^{fl/fl}$ and *Cx3cr1*Δ*Myd88* Veh vs. IL-1β, respectively; p=0.0045; Fig. 23). Together these results clearly demonstrate that disruption of IL-1β signaling in microglia alone does not alter the sickness response to ICV IL-1β.

Deletion of MyD88 from brain parenchymal endothelium does not alter the sickness response

Having eliminated microglia as the sole causative agent of the IL-1β induced sickness response, we queried the contribution of blood vessels independent of microglia using *Slco1c1*-CreERT2. ICV IL-1β caused increased ΔCBT and decreased VLA and FI for both groups relative to their vehicle treated counterparts for several hours after treatment (Fig. 22 D-F). Similar to the *Cx3cr1*-CreERT2 results, we found no differences between *Slco1c1*Δ*Myd88* and *Myd88*$^{fl/fl}$ animals (Fig. 21C). The effect of treatment was highly significant (p=0.0008 for ΔCBT and p<0.0001 for VLA, FI and ΔBW) and there was no effect of genotype and no interaction of treatment by genotype for ΔCBT (0.34±0.10°C and 0.45±0.27°C vs. 1.38±0.32°C and 1.22±0.07°C; mean±SEM for *Myd88*$^{fl/fl}$ and *Slco1c1*Δ*Myd88* Veh vs. IL-1β, respectively), VLA (8191±1146 and 8388±893 vs. 2282±1042 and 2235±348; mean±SEM for *Myd88*$^{fl/fl}$ and *Slco1c1*Δ*Myd88* Veh vs. IL-1β, respectively), FI (4.02±0.34g and 3.52±0.38g vs. 0.65±0.26g and 0.54±0.13g; mean±SEM for *Myd88*$^{fl/fl}$ and *Slco1c1*Δ*Myd88* Veh vs. IL-1β, respectively) and ΔBW (1.3±0.12g and 0.88±0.26g vs. -0.77±0.42g and -0.34±0.14g; mean±SEM for *Myd88*$^{fl/fl}$ and *Slco1c1*Δ*Myd88* Veh vs. IL-1β, respectively). These results demonstrate that elimination of Myd88-dependent signaling in brain parenchymal endothelium, but not in microglia or fenestrated capillaries, is insufficient to alter the sickness response to IL-1β.

Deletion of MyD88 from brain parenchymal endothelium and microglia does not alter the sickness response

Taken together, the previous results suggest two possible conclusions: 1) Myd88 expression in either blood vessels or microglia alone is sufficient to maintain the IL-1β-induced sickness response, and it is only when Myd88-dependent IL-1β signaling is disrupted in both cell types simultaneously that the sickness response is eliminated; or 2) Myd88 expression in CNS vessels that express *Tek*-Cre but not *Slco1c1*-CreERT2—the fenestrated capillaries of CVOs—is sufficient to maintain the IL-1β-induced sickness response. To address this issue, we used the compound knockout *Cx3/Slc*Δ*Myd88* to determine whether elimination of IL-1β signaling in both parenchymal endothelium and microglia is sufficient to alter the sickness response.

In response to IL-1β, both *Myd88*$^{fl/fl}$ and *Cx3/Slc*Δ*Myd88* mice had increased ΔCBT and decreased VLA and FI for several hours after treatment (Fig. 22 G-I). As with the individual knockouts (*Cx3cr1*Δ*Il1r1*, *Cx3cr1*Δ*Myd88* and *Slco1c1*Δ*Myd88*) and control animals, IL-1β treatment of *Cx3/Slc*Δ*Myd88* resulted in the full set of sickness responses (Fig. 21D). There was a significant effect of treatment, no effect of genotype and no interaction of treatment by genotype for ΔCBT (-0.09± 0.17°C and 0.35±0.16°C vs. 1.09±0.35°C and 1.15±0.2°C; mean±SEM, p=0.0028 for *Myd88*$^{fl/fl}$ and *Cx3/Slc*Δ*Myd88* Veh vs. IL-1β, respectively), VLA (8328±1183 and 8219±521 vs. 4111±1084 and 4311±2078; mean±SEM, p=0.016 for *Myd88*$^{fl/fl}$ and *Cx3/Slc*Δ*Myd88* Veh vs. IL-1β, respectively), FI (2.65±0.17g and 2.37±0.15g vs. 0.61±0.27g and 0.48±0.23g; mean±SEM, p<0.0001 for *Myd88*$^{fl/fl}$ and *Cx3/Slc*Δ*Myd88* Veh vs. IL-1β, respectively) and ΔBW (1.28±0.31g and 0.86±0.21 vs. -0.66±0.25g and -0.66±0.51; mean±SEM, p=0.0009 for *Myd88*$^{fl/fl}$ and *Cx3/Slc*Δ*Myd88* Veh vs. IL-1β, respectively). These results show that

disruption of Myd88-dependent signaling in parenchymal endothelium and microglia, but not in fenestrated capillaries, is insufficient to eliminate the sickness response.

Discussion:

One of the enduring questions in the field of inflammation research is the precise mechanism and central anatomical location where cytokines produced within the CNS act to generate sickness responses. To address this question we first examined the pattern of NF-κB IR after central IL-1β. In agreement with previous studies we found that IL-1β treatment results in a change from a diffuse, cytoplasmic distribution to a punctate, nuclear pattern, indicating nuclear translocation of NF-κB in a time- and region-dependent manner.[57, 279] The fact that this change is absent in IL-1β-treated *Myd88*KO animals, which do not exhibit sickness responses when given IL-1β, demonstrates that nuclear translocation is dependent on Myd88.[39] It also validates NF-κB IHC as a tool for both visualizing the sites of IL-1β action and for verifying Cre-mediated signaling disruption.

We found that vascular endothelium, choroid plexus, ependyma, astrocytes and microglia all demonstrate nuclear translocation of NF-κB following ICV IL-1β administration. Although the concentration of NF-κB+ nuclei was highest around CVOs, we observed nuclear translocation throughout the brain. Such a broad response complicates the task of determining which components are critical for the physiologic and behavioral sickness responses. For example, microglia undergo activation in response to inflammatory stimuli, altering their transcription profiles and appearance.[280-282] Components of the BBB, including microvascular endothelial cells and ependymal cells, also undergo cytoarchitectural modifications in inflammatory settings, potentially altering the diffusion barriers between the blood, CSF and brain.[283, 284] Both of these

lead to profound changes within the CNS, but this does not prove that they are essential for generating sickness responses.

In order to determine which IL-1β responsive cells are necessary for the resultant fever, lethargy, anorexia and loss of body weight, we systematically eliminated Myd88-dependent signaling from identified targets. Previously we found that deletion of *Myd88* in neurons and astrocytes (utilizing *Nes*-Cre mice) did not affect the behavioral response to IL-1β.[39] This finding was surprising considering that a behavioral response to a stimulus requires neuronal involvement. Indeed, we and others presented evidence that ICV IL-1β induces activation of restricted populations of neurons involved in regulating appetite, body temperature, metabolic homeostasis and hedonistic behaviors.[10, 57, 88, 279] An early study of IL-1β in rats found that neurons activated in the PVN and elsewhere do not express Il1r1, indicating an indirect mechanism of stimulation.[285] The current result that neurons do not display nuclear localization of NF-κB following ICV IL-1β is further evidence that alterations in neuronal activity are not exclusively due to direct action of IL-1β, but instead require an intermediate signal from a different cell type.

Prostaglandin-E2 (PGE2) is one example of an intermediate signaling molecule that plays a role in the sickness response. Long recognized as an important endogenous pyrogen, PGE2 might also play a role in generating other sickness responses.[227] PGE2 is produced in the brain by endothelial cells and microglia in response to inflammatory stimuli.[126, 232, 233] Wilhelms et. al. show that deletion of PGE2 synthesizing enzymes using the *Slco1c1*-CreERT2 line reduces the febrile response to peripheral LPS and IL-1β without affecting changes in locomotor activity.[260] In a similar study, Ridder et. al. report that deletion of *Tak1*, a component of the IL-1β signaling cascade, using the *Slco1c1*-CreERT2 line reduced the febrile and lethargic responses to intravenous IL-1β without affecting anorexia, weight loss or corticosterone production.[236] The loss of IL-1β-induced PGE2 production by endothelium and/or microglia could explain our result

that the febrile response is eliminated only in the *Tek*Δ*Myd88* line; only *Tek*-Cre causes recombination in microglia and all endothelium, including fenestrated capillaries, thus affecting all sources of PGE2.

Alternatively, it is possible that a specific cellular or regional source of PGE2 is responsible for fever. PGE2 causes fever by directly activating neurons in the thermoregulatory median preoptic nucleus (MnPOA), a structure adjacent to the OVLT.[234] Previous reports demonstrated inflammatory stimulus-induced expression of the prostaglandin-synthesizing enzyme cyclooxygenase-2 (COX-2) in the OVLT, one of the CVOs where we observed the highest density of IL-1β-induced nuclear NF-κB.[231, 232] This raises the possibility that PGE2 produced specifically within the OVLT acts on the nearby MnPOA neurons to generate fever, while other cellular/regional sources might contribute to different aspects of the sickness response. For example, PGE2 can activate neurons in the PVN that control the hypothalamic-pituitary-adrenal (HPA) axis, which in turn drives muscle catabolism, a hallmark of sickness-induced wasting.[12, 123, 126]

In contrast, other studies demonstrate that while genetic disruption of PGE2 production eliminates the febrile response to IL-1β, it does so without affecting inflammation-induced depression of locomotor activity.[230] Similarly, Fritz et. al. found that *Slco1c1*Δ*Myd88* mice did not demonstrate IL-1β-induced place aversion, but had a normal anorexia response, further supporting our conclusion that anorexia is not dependent on IL-1β signaling in parenchymal endothelium.[286] This same group showed that LPS- and IL-1β-induced conditioned place avoidance requires MyD88-dependent PGE2 production in endothelial cells but not microglia. This result is in agreement with the current finding that genetic disruption of IL-1β signaling in microglia did not affect any of the sickness responses that we measured. Collectively, these results demonstrate that sickness responses can be disrupted individually, rather than collectively, and

implicate a subset of vascular endothelium as a critical relay site in the initiation of sickness responses to IL-1β.

The fenestrated capillaries found in CVOs and ChP represent one such category of specialized vasculature. Our results using *Tek*Δ*Myd88* and *Slco1c1*Δ*Myd88* show that fenestrated capillaries are capable of transducing inflammatory signals into sickness responses. Crosses with the Rosa 26-flox-stop TdTomato reporter strain revealed that although there were some differences in reporter expression between the two strains—notably *Tek*- but not *Slco1c1*-Cre expression in microglia and fenestrated capillaries—each drives recombination in all parenchymal brain endothelium. Since both strains eliminate parenchymal endothelial IL-1β signaling, any observed differences between the two strains are due to the non-overlapping areas of Cre expression; in this case, *Tek*-driven *Myd88* deletion in microglia and/or fenestrated capillaries is responsible for the absence of sickness response in *Tek*Δ*Myd88* animals. Considering that *Cx3cr1*-mediated disruption of microglial IL-1β signaling—even when combined with *Slco1c1*-mediated vascular disruption—failed to affect the sickness response, the most logical conclusion is that the differences between the two vascular Cre strains is due to Myd88 expression in fenestrated capillaries alone.

It is possible that Myd88 expression in any endothelial cells, as opposed to specifically in fenestrated capillaries, is sufficient to maintain the sickness response. IL-1β-induced production of diffusible signals such as PGE2 could allow for widespread neuronal activation from any responsive population of endothelium. On the other hand, multiple lines of evidence implicating CVOs in the sickness response make fenestrated capillary-specific signaling a more credible possibility. First, Takahashi et. al. found that electrolytic lesion of the SFO reduced the febrile response to intravenous LPS, demonstrating that the SFO is critical in the transduction of to circulating signals into physiologic responses.[76] Subsequent studies showing that production of inflammatory

cytokines, including IL-1β, is restricted to CVOs demonstrate that these structures are uniquely capable of producing this key inflammatory amplification step.[55, 56, 123] Finally, a recent study examining the CNS effects of peripheral LPS administration produced results remarkably similar to our own. Nakano et. al. showed that IP LPS caused nuclear localization of signal transducer and activator of transcription 3 (Stat3) specifically within the CVOs.[287] Taken together, these studies show that the CVOs are an exclusive niche where peripheral immune signals interact with elements of the CNS to generate the sickness response.

The unique anatomy and physiology of fenestrated capillaries suggests that they are a targetable signaling node for treatments designed to prevent or reverse sickness responses. Here we demonstrate that disruption of IL-1β signaling in microglia or parenchymal endothelium does not affect the IL-1β induced sickness responses, and it is only when signaling is disrupted in all endothelium, including fenestrated capillaries, that these responses are eliminated. Future studies will be dedicated to determining how these CNS vessels transduce the IL-1β signal into a neuronal response. Unfortunately there is currently no genetic model that would allow for disruption of IL-1β signaling exclusively in fenestrated capillaries; final confirmation of this pathway will require development of this research tool. This would also allow for identification of the secondary signaling molecule(s) that directly stimulate neuronal circuits responsible for generating the sickness response.

Acknowledgements:

We would like to thank Dr. Marcus Schwaninger, Dr. Wen-Biao Gan and Dr. Randy D. Blakely for providing mice used in this series of experiments.

Methods:

Animals:

For this series of experiments, the following animal strains were used: C57BL/6J (Jackson Laboratory, Bar Harbor, Maine; stock #000664), *Myd88* knockout (*Myd88*KO; Jackson Laboratory stock #009088), *Tek*-Cre (Jackson Laboratory stock # 004128), conditional MyD88 (MyD88$^{fl/fl}$; Jackson Laboratory stock #008888), Rosa26-stop-flox TdTomato (Jackson Laboratory stock #007908), *Slco1c1*-CreERT2 (provided by Dr. Marcus Schwaninger)[236], *Cx3cr1*-CreERT2 (provided by Dr. Wen-Biao Gan)[277], and conditional IL-1 receptor (*Il1r1* $^{fl/fl}$ provided by Dr. Randy D. Blakely).[255] Each of the Cre strains was backcrossed onto the *Myd88*$^{fl/fl}$ background for at least three generations with the Cre allele carried by the paternal line (father was Cre +/-; *Myd88*$^{fl/fl}$ mother was Cre-/-; *Myd88*$^{fl/fl}$). All experiments were conducted using Cre +/-; *Myd88*$^{fl/fl}$ as the experimental group and Cre-/-; *Myd88*$^{fl/fl}$ littermates as the control group. *Cx3cr1*-CreERT2 animals were also bred to the conditional *Il1r1* strain using the same breeding and control strategies.

All animals were housed at Oregon Health and Science University (OHSU) in plastic cages with paper pellet bedding and environmental enrichments on a 12:12 h light:dark cycle in a room maintained at 22°C. For transgenic strains carrying one of the tamoxifen inducible Cre transgenes, both experimental and control groups were given two treatments of 10mg tamoxifen (Sigma-Aldrich, St. Louis, Missouri; Cat#T5648) by oral gavage (dissolved in sesame oil at a concentration of 100mg/ml) with 48 hours between treatments. Animals were treated with tamoxifen one week prior to cannulation and emitter implantation. All animal care, handling and experimentation were conducted in accordance with OHSU institutional animal care and use committee (IACUC) guidelines.

Experimental Design

In order to reduce overall animal use and to provide internal controls, a crossover experimental design was employed whereby each animal received both vehicle and IL-1β with at least three days between treatments. Group sizes were determined empirically based on previous research that demonstrated that the effects of treatment are large enough (generally greater than 40%) that statistical difference is detected using 5-6 animals per group. Most experiments were first conducted in pilot experiments using three animals per group. These experiments were then replicated, when possible, using a larger number of animals. All data presented are from single experiments, and therefore the results presented reflect biological replicates (individual animals receiving the same treatment within a single experiment). Data from all animals used in a given experiment were included in all analysis; no animals were excluded.

Surgical Procedures:

During all surgical procedures mice were kept deeply anesthetized with isofluorane. Mice were placed on a stereotactic alignment system (Kopf Instruments, Tujunga, California; model 1900) and a scalp incision was made to expose the skull. Stereotactic coordinates were zeroed at the point where the tip of the cannula touched bregma. Cannula were emplaced in the lateral ventricle to a depth of -2.25mm through a 0.5mm diameter hole drilled at (x,y)=(1.0mm, -0.5mm) and secured with dental acrylic (Yates & Bird, Chicago, Illinois; Cat#44118). For behavioral experiments each animal was also implanted with a transponder (G2 E-mitter; Starr Life Sciences, Oakmont, Pennsylvania; Cat #870-0010-01) in the peritoneal cavity to monitor core body temperature and movement. Following surgery, animals were individually housed in cages on telemetry platforms (ER4000 Energizer/Receiver; Starr Life Sciences) and allowed to recover for at least one week prior to experimentation. During recovery,

animals were monitored for fever and movement and were required to reach pre-operative body weight in order to be included.

Time-Course of IL-1β Induced Inflammation:

Male C57Bl/6J mice with permanent cannula implanted into the lateral ventricle were treated with either 1µl artificial cerebrospinal fluid (aCSF; vehicle) or 1µl 10ng/µl recombinant mouse interleukin 1-beta (IL-1β; 10ng total in aCSF; R&D Systems, Minneapolis, Minnesota; Cat#401-ML-005) and sacrificed at 15 minutes, 30 minutes, 90 minutes, 2 hours, 4 hours or 8 hours post-treatment. For co-localization experiments, the 30 minute time point was selected as this represented the time of maximal NF-κB nuclear localization and allowed for consistent, large-scale experiments. At the time of sacrifice, each animal was deeply anesthetized with a mixture of ketamine, acepromazine and xylazine, flushed with 0.01M phosphate-buffered saline (PBS; pH=7.4) to remove blood and then perfused with ice cold 4% paraformaldehyde (PFA) in 0.01M PBS. Brains were dissected free from the skull, post-fixed overnight in 4% PFA while shaking at 4°C, cryoprotected by immersion in 30% sucrose in 0.01M PBS overnight at 4°C, then frozen on dry ice and stored at -80°C.

Behavioral Experiments:

For three days prior to experimentation, animals were handled, restrained and cannula caps were removed to acclimate the animals to handling stress. On the evening of experimentation each animal was restrained and injected with either 1µl aCSF or 1µl 10ng/µl IL-1β immediately before lights out (6pm). Core body temperature and movement counts were automatically recorded every five minutes by the E-mitter system. Food was manually weighed every two hours throughout the dark phase and at 12 and 24 hours post-treatment. Body weight was recorded at the time of treatment and at 12 and 24 hours post-treatment. In order to reduce overall animal use and to provide internal controls, a crossover experimental design was employed whereby each animal

received both vehicle and IL-1β with at least three days between treatments. Following experimentation, each animal was sacrificed and tissue harvested as described in the previous section.

Immunohistochemistry (IHC):

Cryopreserved frozen brains were sectioned at 30μm on a sliding microtome (Leica Biosystems, Buffalo Grove, Illinois; SM2000R) equipped with a freezing stage (Physitemp Instruments, Clifton, New Jersey; BFS-5MP). Brain sections were first washed with three changes of PBS then pretreated by quenching with 1% glycine (Sigma-Aldrich, Cat#G8898) in PBS and de-fixing in 0.05% sodium borohydride (Sigma-Aldrich, Cat#71321) in PBS, with three PBS washes between pretreatments. Sections were then blocked in 5% normal goat serum (NGS; Sigma-Aldrich) and 1% hydrogen peroxide in PBS containing 0.3% triton x-100 (Tx; Sigma-Aldrich, Cat#X100) for 30 minutes at room temperature. Sections were incubated with primary antibodies overnight shaking at 4°C in PBS containing 1% bovine serum albumin (Sigma-Aldrich, Cat#A2153) and 0.3% Tx. The following day sections were washed with three changes of PBS containing 1% NGS and 0.3% Tx and then incubated with secondary antibodies for two hours at room temperature while shaking in the dark. Sections were washed with three changes of PBS and then mounted on gelatin coated glass slides and coverslipped using Aqua Poly/Mount (Polysciences, Warrington, Pennsylvania; Cat#18606)

The following primary antibodies were used for immunolocalization: rabbit anti-NF-κB (Cell Signaling, Danvers, Massachusetts; Cat#8242; 1:1000), rat anti-Cd31 (BD Biosciences, San Jose, California; Cat#550274; 1:100), rabbit anti-Iba1 (Wako Pure Chemicals, Osaka, Japan; Cat#019-19741; 1:500), chicken anti-vimentin (EMD Millipore, Billerica, Massachusetts; Cat#AB5733; 1:10,000), mouse anti-NeuN (EMD Millipore Cat#MAB377; 1:1000), mouse anti-GFAP (EMD Millipore cat. # MAB360; 1:10,000), rat anti-mouse CD11b (eBioscience, San Diego, California; Cat#14-0112; 1:1000) and

chicken anti-GFP (Abcam, Cambridge, Massachusetts; Cat#ab13970; 1:1000). Antibody specificity was verified by omission of primary antibody. Primary antibodies were visualized with the following secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon; Cat#A-11008), Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes Cat#A-21428), Alexa Fluor 555 goat anti-rat IgG (Molecular Probes Cat#A-21434), Alexa Fluor 555 goat anti-chicken IgY (Molecular Probes Cat#A-21437). All secondary antibodies were used at a concentration of 1:500. Observation of TdTomato expression was through native fluorescence only, with no IR amplification. All images were captured under epifluorescent illumination on a Leica DM4 microscope (Leica Microsystems) equipped with a DFC340X digital camera (Leica Microsystems) driven by LASv3 software.

Statistical Analysis:

Movement counts (voluntary locomotor activity, VLA) and core body temperature (CBT) measurements for individual animals were recorded by the E-mitter system at five minute intervals. Change in core body temperature (ΔCBT) was calculated by subtracting the baseline CBT (average of recorded CBT for four hours prior to treatment) from the measured CBT for each animal at each time point. For 24-hour profiles, VLA was summed over each hour to give total counts per hour and ΔCBT values were averaged to give average hourly ΔCBT. For group-wise comparisons of the effects of IL-1β treatment, VLA counts were summed for six hour blocks (6pm-12am) for each animal for total movement analysis, while ΔCBT values were averaged over the two hour blocks of peak fever (10pm-12am). Food intake was calculated from bi-hourly manual food-weight measurements and changes in body weight were calculated by subtracting the body weight at the time of treatment from body weight measurements taken 12 hours after treatment. Statistical analysis was carried out using Prism 6.0 (GraphPad Software, San Diego, California). All data were analyzed by 2-way ANOVA for treatment and

genotype with Tukey's multiple comparisons for each group (treatment and genotype) relative to all other groups.

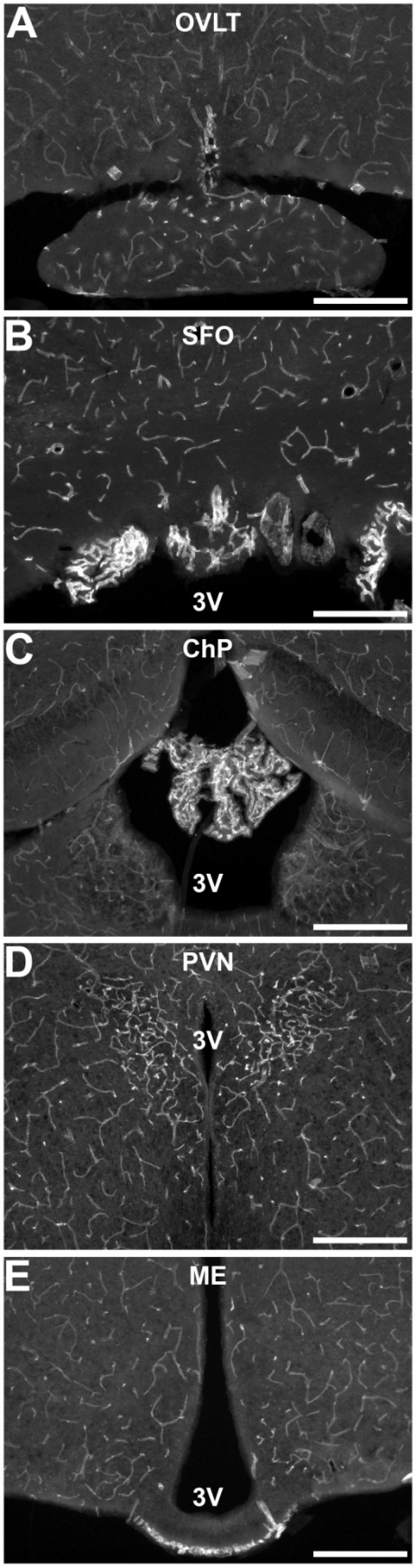

Figure 9: Cd31 immunoreactivity (IR) shows brain vascular heterogeneity

Increased vascular density demonstrated by Cd31 IR in representative epifluorescent images of the organum vasculosum lamina terminalis (OVLT, A), subfornical organ (SFO, B), choroid plexus (ChP, C), paraventricular nucleus (PVN, D) and arcuate nucleus/median eminence (ARC/ME, E). 3V, third ventricle. Scale bars = 500 μm.

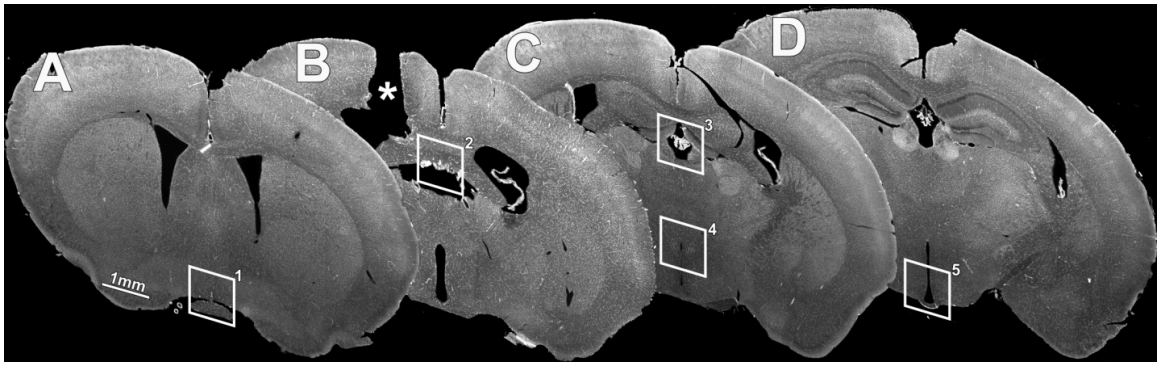

Figure 10: Vascular heterogeneity in the mouse brain

(A-D) Cd31 IR in epifluorescent digital montages of representative brain sections from animals used for various experiments at bregma (A), bregma -0.5 (B; * shows the location where a cannula was placed to give access to the lateral ventricle), -1.0 (C) and -1.5mm (D) according to the mouse brain atlas by Paxinos and Franklin (2012 Paxinos). Boxes indicate the five regions of interest shown in Figure 1. Increased vascular density is evident in the organum vasculosum lamina terminalis (OVLT, 1), subfornical organ (SFO, 2), choroid plexus (ChP, 3), paraventricular nucleus (PVN, 4) and arcuate nucleus/median eminence (ARC/ME, 5). Scale bar=1mm.

Figure 11: Intracerebroventricular (ICV) administration of IL-1β induces NF-κB nuclear localization in a time- and region-dependent manner. The effect of ICV IL-1β is both rapid and transient as demonstrated by NF-κB immunoreactivity (IR) in representative confocal images of the paraventricular nucleus (PVN). IR in vehicle- (aCSF, n=6) treated animals shows cytoplasmic labeling of blood vessels (A). IL-1β treatment causes NF-κB nuclear localization that peaks by 30 minutes (B, n=8), persists for at least two hours (C, n=4) and returns to near baseline, with cytoplasmic vascular patterns reappearing, by four hours after treatment (D, n=4). This effect was most prominent in and around the PVN, organum vasculosum lamina terminalis (OVLT; E and F), subfornical organ (SFO; G and H), arcuate nucleus/median eminence (ARC/ME; I and J) and choroid plexus (ChP; K and L). High-magnification confocal images of co-labeling with cell-specific markers shows that vascular endothelial cells (M), microglia (N), astrocytes (O) and tanycytes (P) directly respond to IL-1β. Scale bars: A-L = 50 μm, M-P = 10 μm.

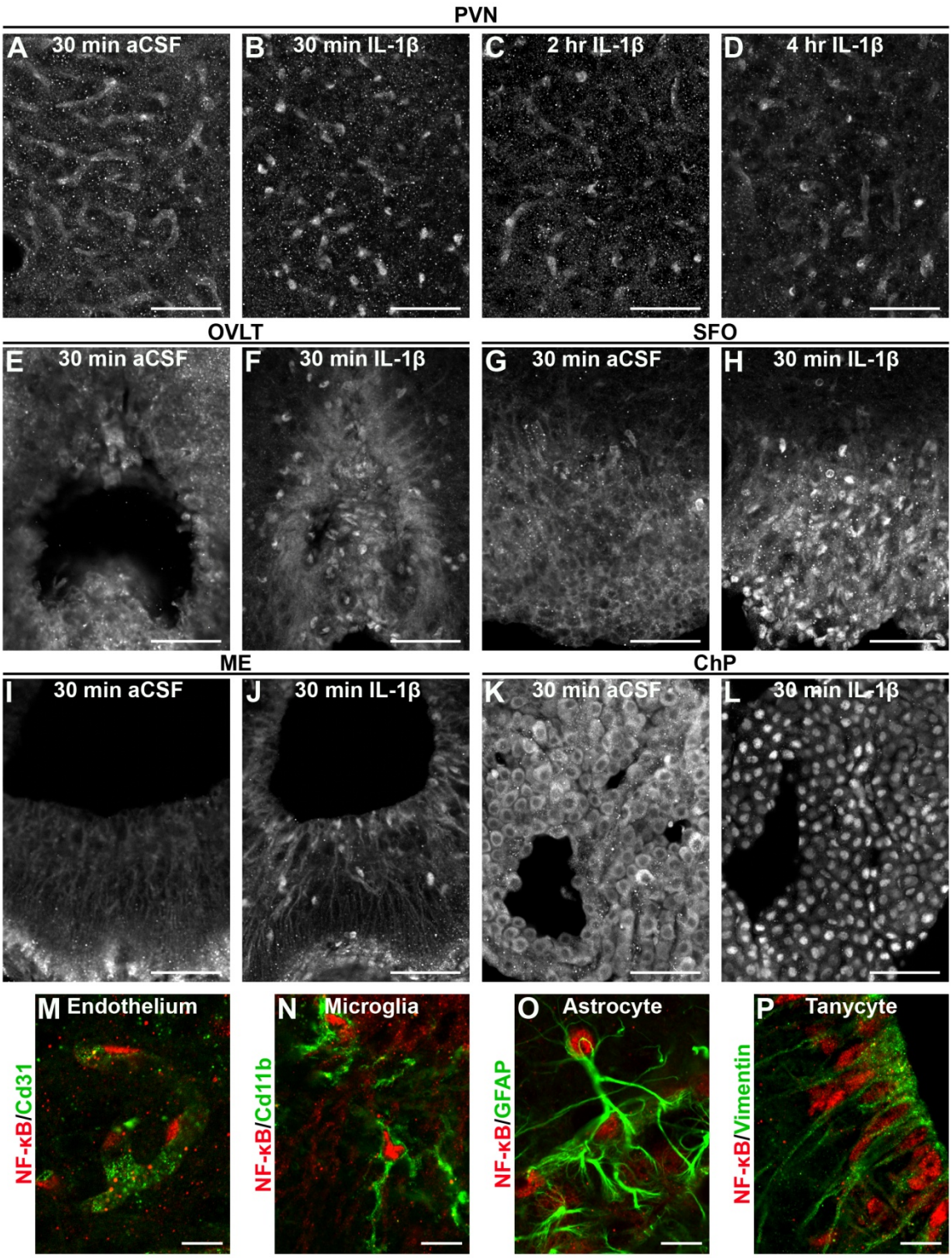

Figure 11: Intracerebroventricular (ICV) administration of IL-1β induces NF-κB nuclear localization in a time- and region-dependent manner

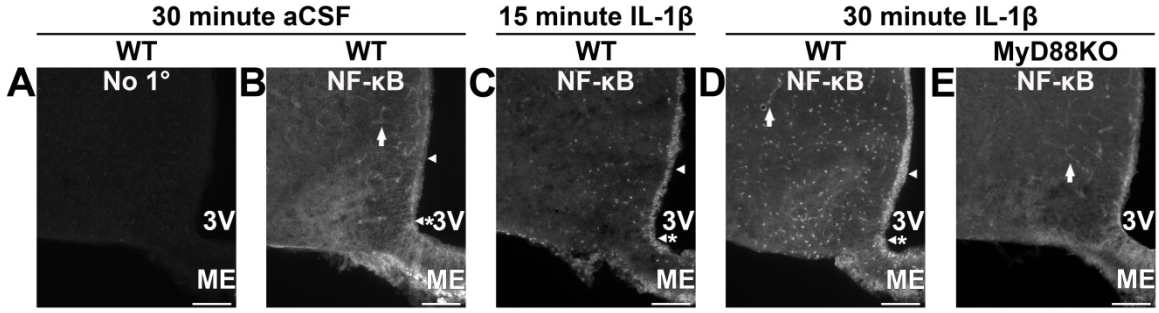

Figure 12: IL-1β-induced nuclear localization of NF-κB requires Myd88

Representative epifluorescent images of the effects of central IL-1β. Omission of primary antibody (A) demonstrates that the vascular pattern of cytoplasmic immunoreactivity (IR, arrows) observed in vehicle (aCSF) treated animals (B, n=3) is specific to the NF-κB antibody. At the level of the arcuate nucleus/median eminence (ARC/ME), nuclear NF-κB IR is evident by 15 minutes (C, n=4) and peaks around 30 minutes (D, n=8) after ICV IL-1β treatment. Ependymal cells lining the third ventricle (cuboidal nuclei, arrowheads), tanycytes (columnar nuclei, *) and endothelial cells (arrow in D) demonstrate nuclear NF-κB IR. As with vehicle treated animals, NF-κB IR remained cytoplasmic in IL-1β treated *Myd88*KO (E, arrows, n=3) animals at all times examined. 3V = third ventricle, ME = median eminence. Scale bars = 100 μm.

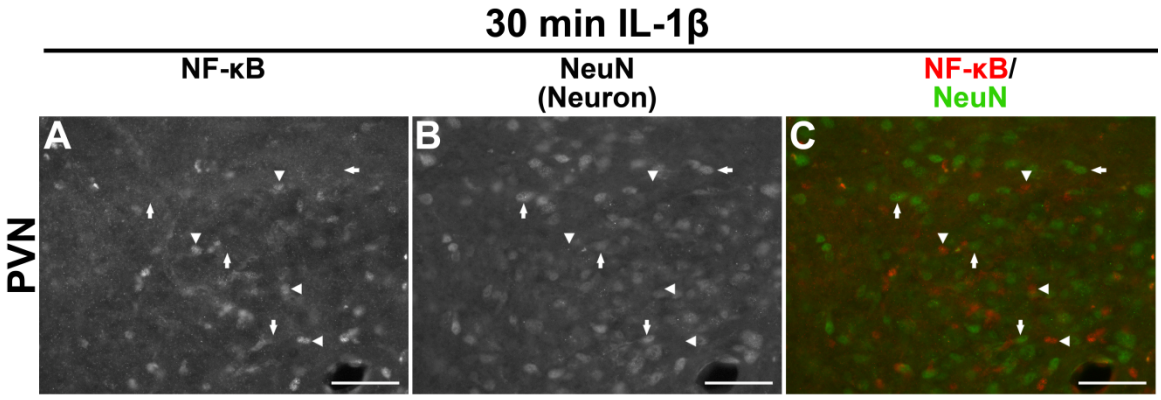

Figure 13: Neurons in the PVN do not exhibit IL-1β-induced nuclear NF-κB

Representative epifluorescent images show that IL-1β causes nuclear localization of NF-κB (green) within the paraventricular nucleus (PVN; A, arrowheads). Despite a high density of neuronal nuclei (NeuN, red; B, arrows), there was no evidence of co-localization (C, n=3). Scale bars = 50 μm.

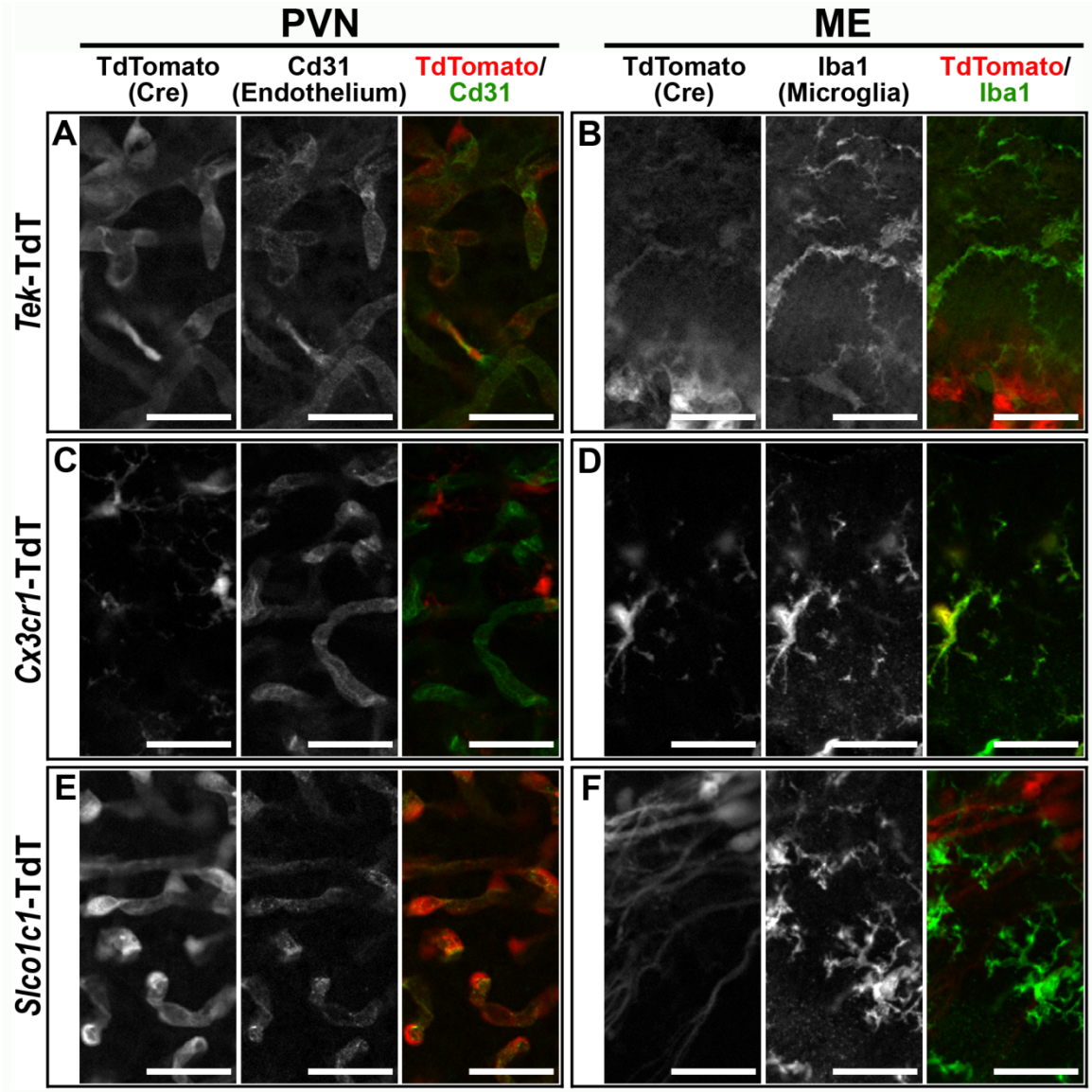

Figure 14: Recombinase reporter shows strain-specific Cre expression

Representative confocal images demonstrate that *Tek*-Cre is expressed in Cd31+ vascular endothelial cells (A) and Iba1+ microglia (B, n=4). *Cx3cr1*-CreERT2 is only expressed in microglia in both the PVN and ME (C and D, n=6). *Slco1c1*-CreERT2 is expressed in blood vessels (E) but not microglia (F, n=4). TdTomato+ cells with long processes in F are β1 tanycytes. Scale bars = 25 μm.

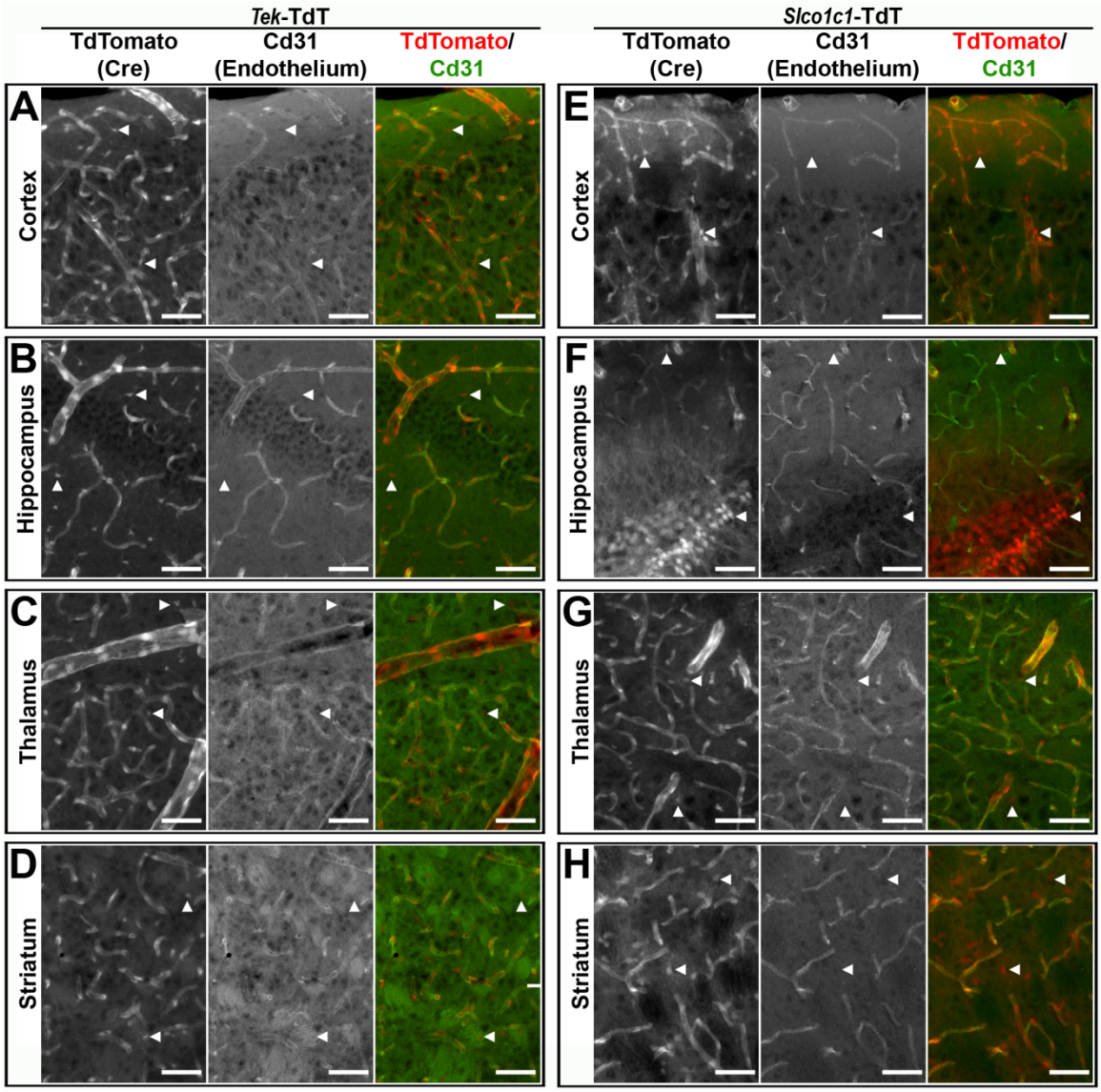

Figure 15: Both *Tek*-Cre and *Slco1c1*-CreERT2 drive recombination in parenchymal endothelium

Representative epifluorescent images demonstrate that TdTomato (TdT) expression was present in all parenchymal endothelium (Cd31+) in both *Tek*-TdT (A-D, n=4) and *Slco1c1*-TdT (E-H, n=4) animals in all brain regions examined. Expression outside of blood vessels (arrowheads) was also present in both lines in all regions. Scale bars = 50 μm.

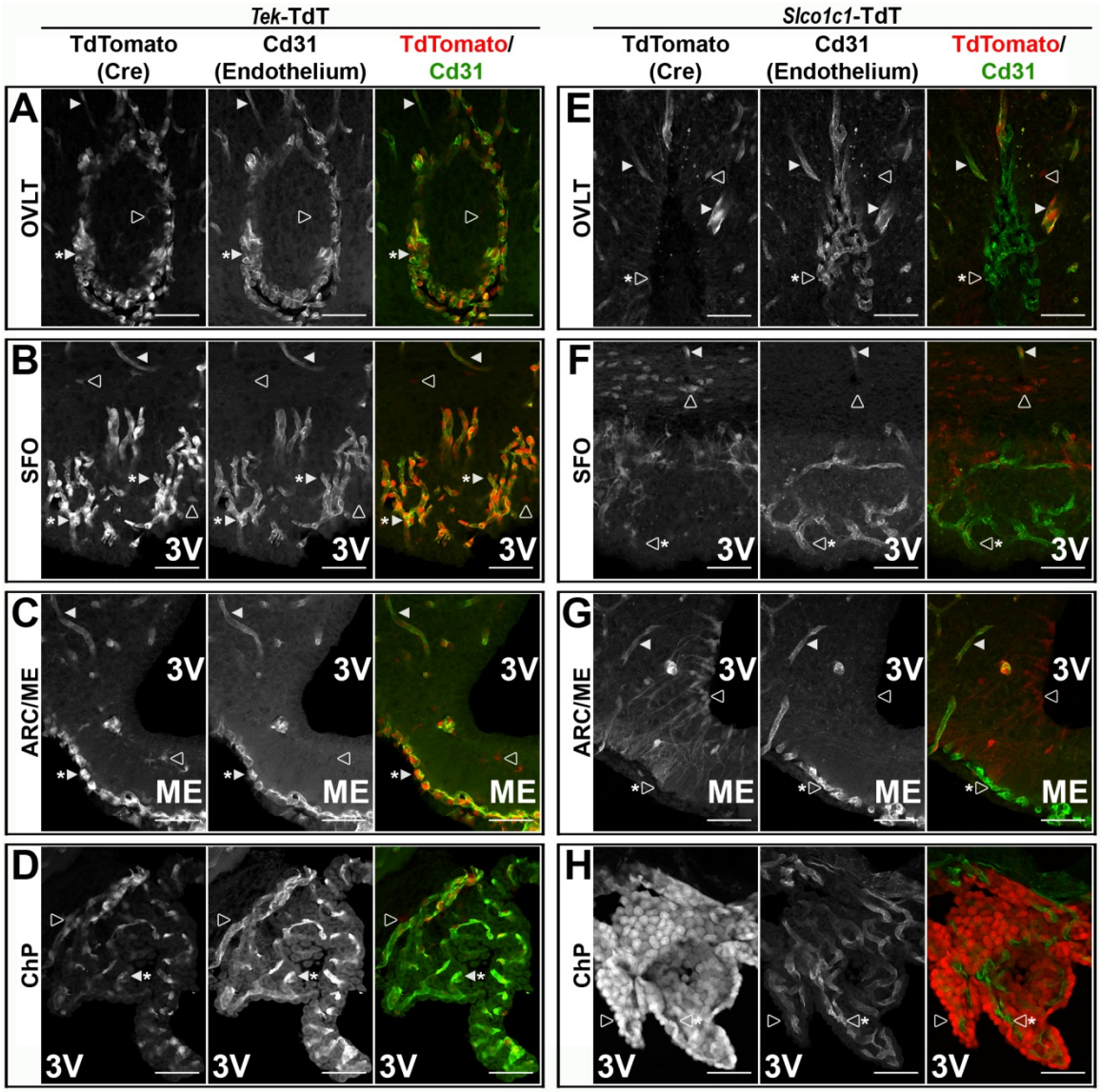

Figure 16: *Tek*-Cre, but not *Slco1c1*-CreERT2, is expressed in fenestrated capillaries of circumventricular organs

Representative confocal images of co-labeling of TdTomato (TdT, red) and Cd31 (green) in *Tek*-TdT animals (A-C, n=4) confirms co-expression in parenchymal endothelium (filled arrowheads) and fenestrated capillaries (filled arrowheads with *) in the organum vasculosum lamina terminalis (OVLT, A), subfonical organ (SFO, B), arcuate nucleus/median eminence (ARC/ME, C) and choroid plexus (ChP, D). Reporter expression is also seen in microglia in each of these regions (open arrowheads in A-D). Co-labeling of TdT (red) and Cd31 (green) in *Slco1c1*-TdT animals (E-H, n=4) shows Cre activity in all parenchymal endothelium (filled arrowheads), but not fenestrated capillaries (open arrowheads with *). Reporter expression is also seen in other cells that do not express Cd31 (open arrowheads in E-H). Scale bars = 50 μm.

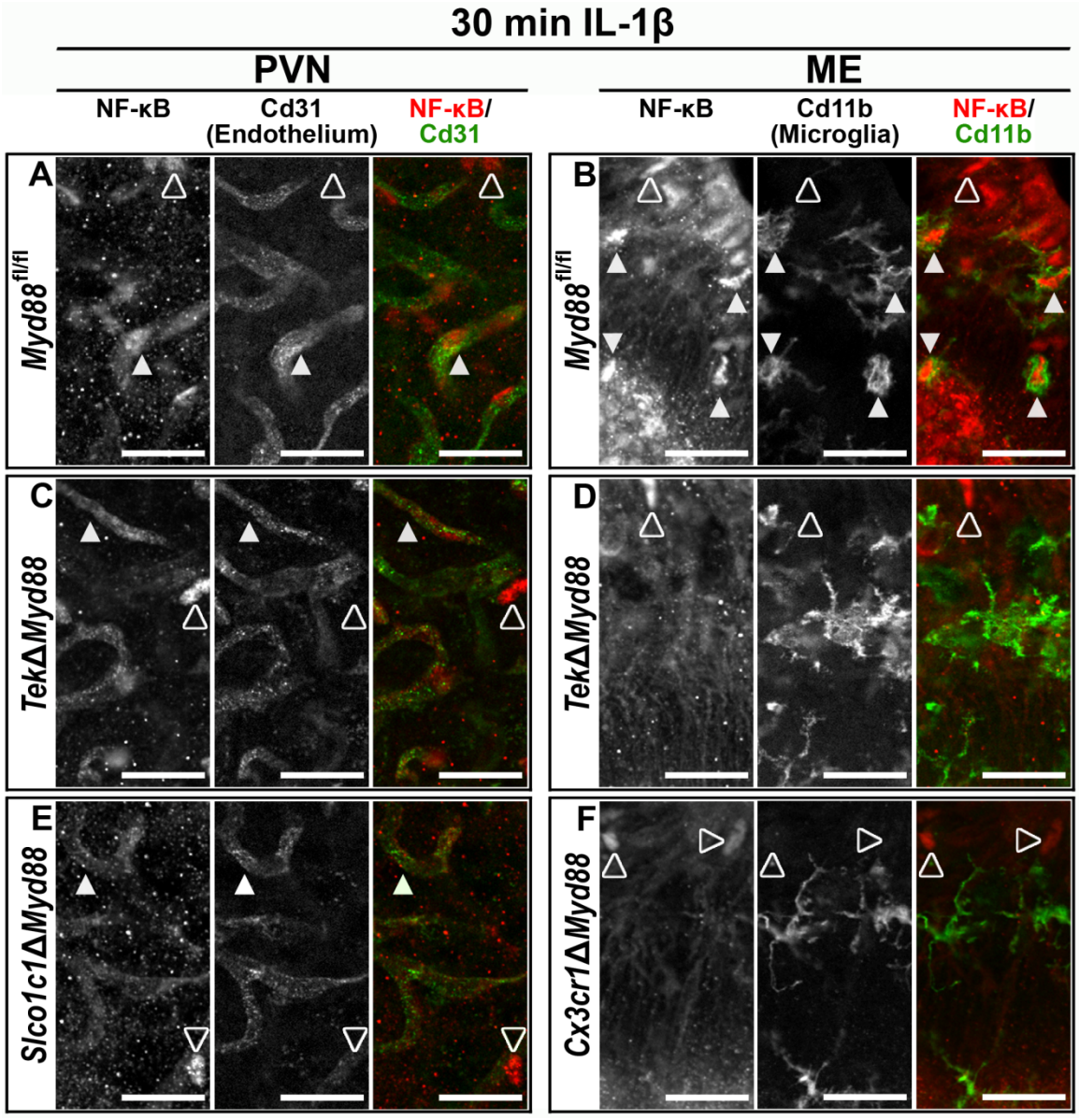

Figure 17: NF-κB immunoreactivity (IR) confirms strain-specific IL-1β signaling disruption

Representative confocal images show that in control animals (*Myd88*$^{fl/fl}$) IL-1β causes nuclear localization of NF-κB (red) in endothelial cells (Cd31+, green; filled arrowheads in A) and microglia (Cd11b+, green; filled arrowheads in B, n=3). Nuclear IR was also present in cells that did not express co-labeled proteins (open arrowheads in A-F). *Tek*Δ*Myd88* disrupts IL-1β signaling in endothelium and microglia as NF-κB remains cytoplasmic in blood vessels (filled arrows in C) and nuclear IR was absent from microglia (D, n=3). The vascular pattern of cytoplasmic NF-κB IR shows that *Slco1c1*Δ*Myd88* disrupts signaling in parenchymal endothelium (filled arrowheads in E, n=3). The absence of nuclear NF-κB in microglia of *Cx3cr1*Δ*Myd88* demonstrates microglia signaling disruption (F, n=3). Scale bars = 25 μm.

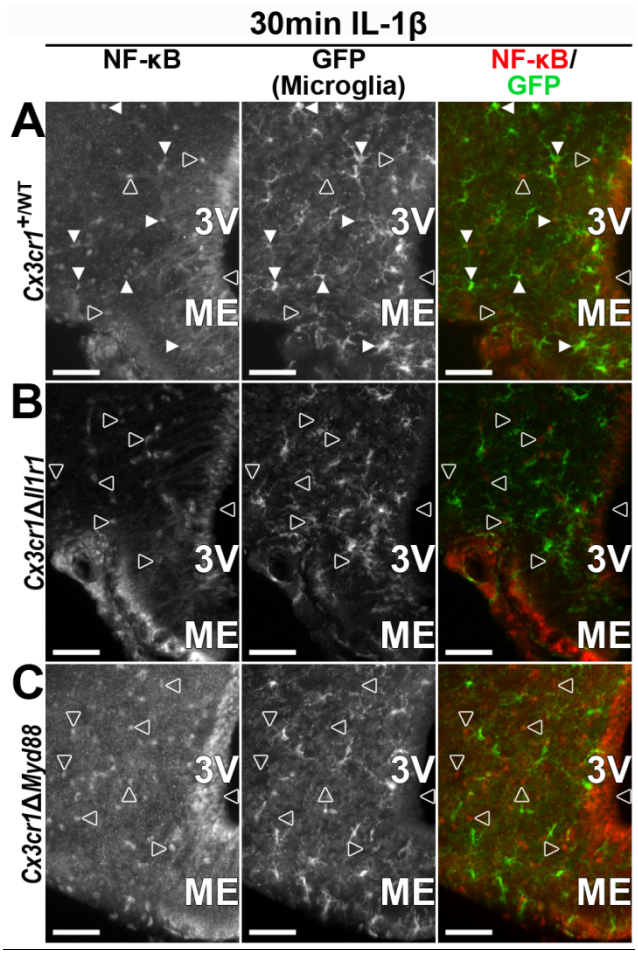

Figure 18: *Cx3cr1*-CreERT2 causes genetic recombination exclusively in microglia

Representative epifluorescent images of NF-κB immuoreactivity (IR) 30 minutes after ICV IL-1β demonstrates *Cx3cr1*-CreERT2 mediated disruption of signaling in microglia when either the interleukin-1 receptor (IL1R1) or MyD88 is deleted. The *Cx3cr1*-CreERT2 transgene contains a sequence coding for YFP that allows for visualization of microglia using an anti-GFP antibody. In Cre+ animals that do not have floxed IL1R1 or MyD88 (*Cx3cr1*$^{+/WT}$, n=5), nuclear NF-κB is found in GFP+ microglia in the ARC/ME (co-expression denoted by filled arrowheads in A) and in cells that do not express GFP, including ependymal cells lining the third ventricle (3V; open arrowheads). In Cre+ animals that are homozygous for floxed alleles of IL1R1 (*Cx3cr1*Δ*Il1r1*, B, n=4) or MyD88 (*Cx3cr1*Δ*Myd88*, C, n=3) nuclear NF-κB IR is absent from GFP+ microglia. Nuclear NF-κB IR in cells that do not express GFP (open arrowheads in B and C) demonstrates that IL-1β signaling is not disrupted globally. Scale bars = 50 μm.

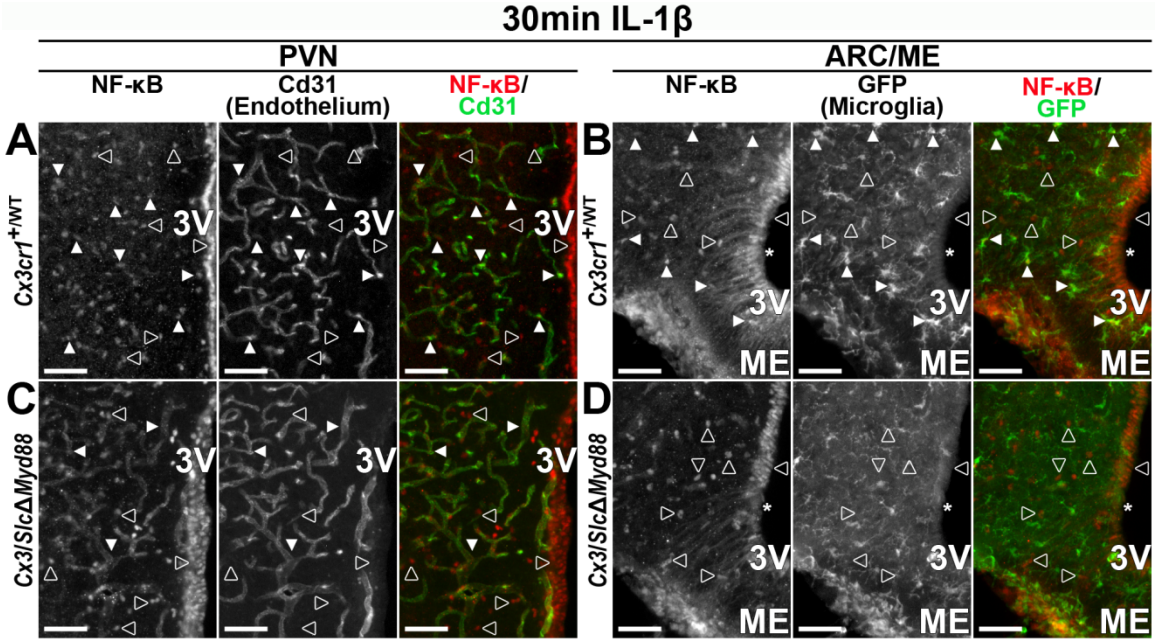

Figure 19: Combined *Cx3*/*Slc*-CreERT2 eliminates IL-1β signaling in parenchymal endothelium and microglia

A and B) Representative epifluorescent images of the paraventricular nucleus (PVN) and arcuate nucleus/median eminence region (ARC/ME) showing IL-1β-induced nuclear NF-κB immunoreactivity (IR) in parenchymal endothelium (co-expression with Cd31 denoted by filled arrowheads in A) and GFP+ microglia (co-expression denoted by filled arrowheads in B) in *Cx3cr1*-CreERT2+ animals that do not have floxed MyD88 (*Cx3cr1*$^{+/WT}$, n=5). Nuclear NF-κB IR is also found in cells that do not express either marker (open arrowheads in A and B), including ependymal cells lining the third ventricle (3V) and β1-tanycytes (* in B). (C and D) Representative images from an IL-1β-treated combined Cre animal (*Cx3/SlcΔMyd88*, n=3) showing cytoplasmic NF-κB IR in PVN parenchymal endothelium (filled arrowheads in C) and an absence of nuclear NF-κB IR in GFP+ microglia (B). Similar to control animals, nuclear NF-κB IR is found in cells that do not express either marker (open arrowheads in C and D), but with an apparent decrease in β1-tanycytes (* in D) where *Slco1c1*-CreERT2 is expressed (see Figure 4G). Scale bars = 50 μm.

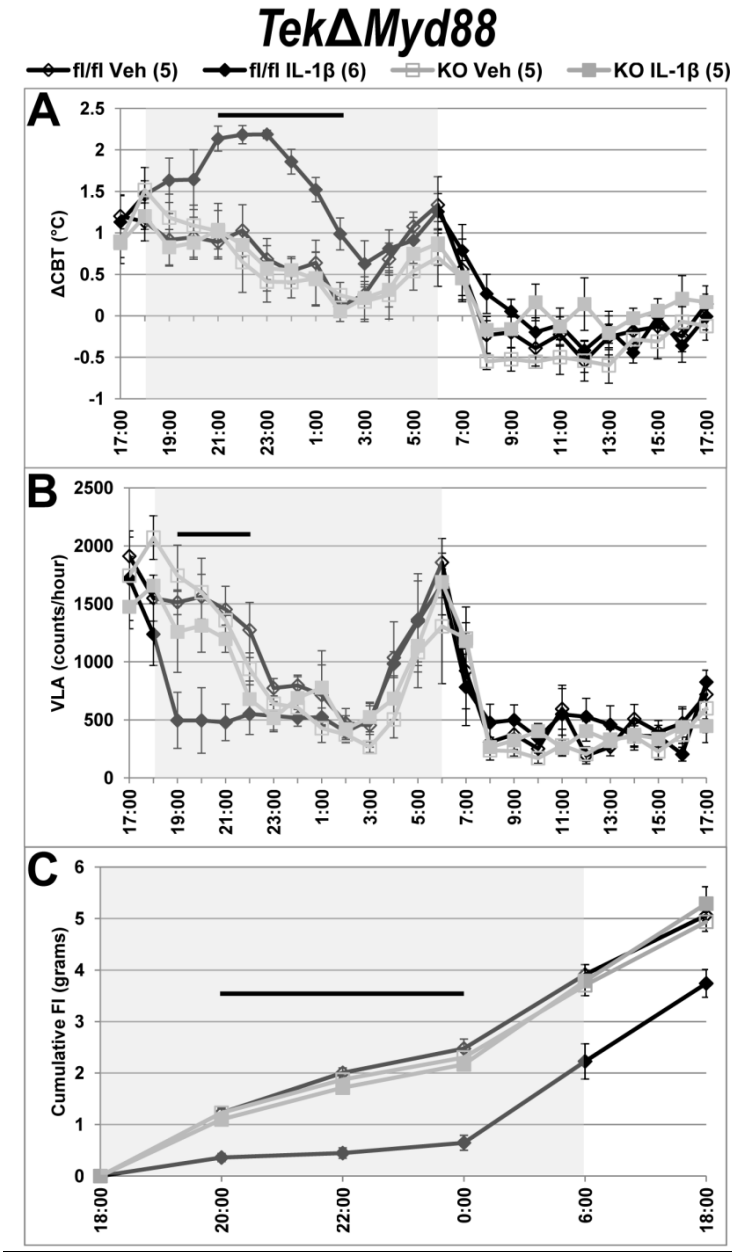

Figure 20: *Tek*Δ*Myd88*-mediated disruption of IL-1β signaling in all endothelium and microglia eliminates sickness response

24-hour profiles of telemetric and feeding data shows the stereotypical IL-1β-induced elevation in core body temperature (ΔCBT, A) and decrease in voluntary locomotor activity (VLA, B) and food intake (FI, C) in *Myd88*$^{fl/fl}$ (fl/fl) but not *Tek*Δ*Myd88* (KO) mice. IL-1β-treated control ΔCBT, VLA and FI were significantly different from vehicle- (Veh) treated values for several hours following treatment ($p<0.05$ for times below black bar above traces in A-C). IL-1β-treated KO animals were not different from Veh-treated animals at any time. Gray boxes show dark-phase, when mice are most active. All values shown are mean ± SEM for group sizes listed in the legend above A.

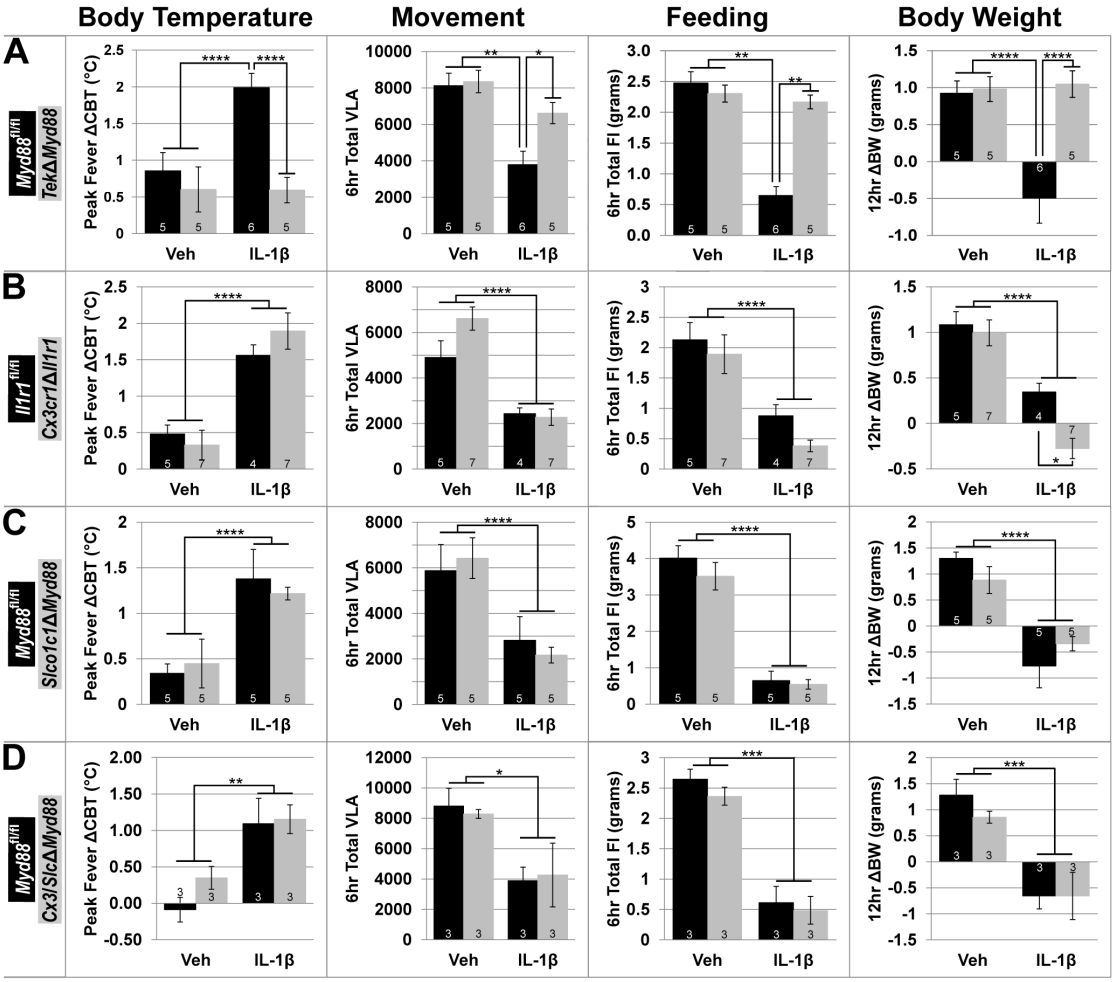

Figure 21: The IL-1β-induced sickness response is only eliminated when signaling is disrupted in all endothelium and microglia

*Tek*Δ*Myd88*-mediated disruption of IL-1β signaling in all endothelium and microglia (KO) eliminates the increase in core body temperature (ΔCBT) and decrease in voluntary locomotor activity (VLA), food intake (FI) and body weight (ΔBW) associated with IL-1β treatment of *Myd88*$^{fl/fl}$ mice (fl/fl, A). Targeted disruption of IL-1β signaling in microglia alone (*Cx3cr1*Δ*Il1r1*, B), parenchymal endothelium (*Slco1c1*Δ*Myd88*, C) or both parenchymal endothelium and microglia (*Cx3/Slc*Δ*Myd88*, D) was insufficient to alter the sickness response. IL-1β treatment is the only factor that influences ΔCBT, VLA, FI and ΔBW for all genotypes except *Tek*Δ*Myd88*. All values shown are mean ± SEM for group sizes listed in graph bars. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

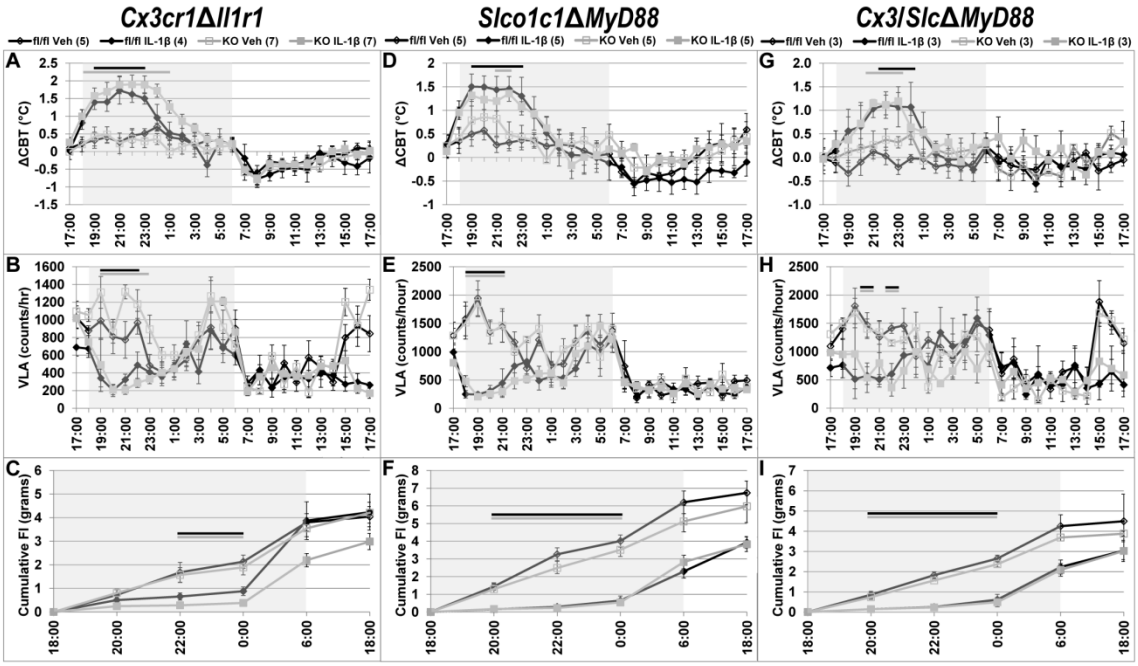

Figure 22: *Cx3cr1ΔIl1r1*-, *Slco1c1ΔMyd88*-, and *Cx3/SlcΔMyd88*-mediated disruption of IL-1β signaling in microglia, parenchymal endothelium or both together does not affect the sickness response

24-hour profiles of telemetric and feeding data shows the stereotypical IL-1β-induced elevation in core body temperature (ΔCBT) and decrease in voluntary locomotor activity (VLA) and food intake (FI) in both control (fl/fl) and strain-specific knockout animals (KO). *Cx3cr1ΔIl1r1* (A-C), *Slco1c1ΔMyd88* (D-F) and *Cx3/SlcΔMyd88* (G-I) mice all exhibit IL-1β-induced sickness responses. Regardless of genotype ΔCBT, VLA and FI were significantly different from their vehicle treated counterparts for several hours following treatment ($p<0.05$ for times below black (fl/fl) and gray (KO) bars above traces in A-C). Gray boxes show dark-phase, when mice are most active. All values shown are mean ± SEM for group sizes listed in the legend above A, D and G.

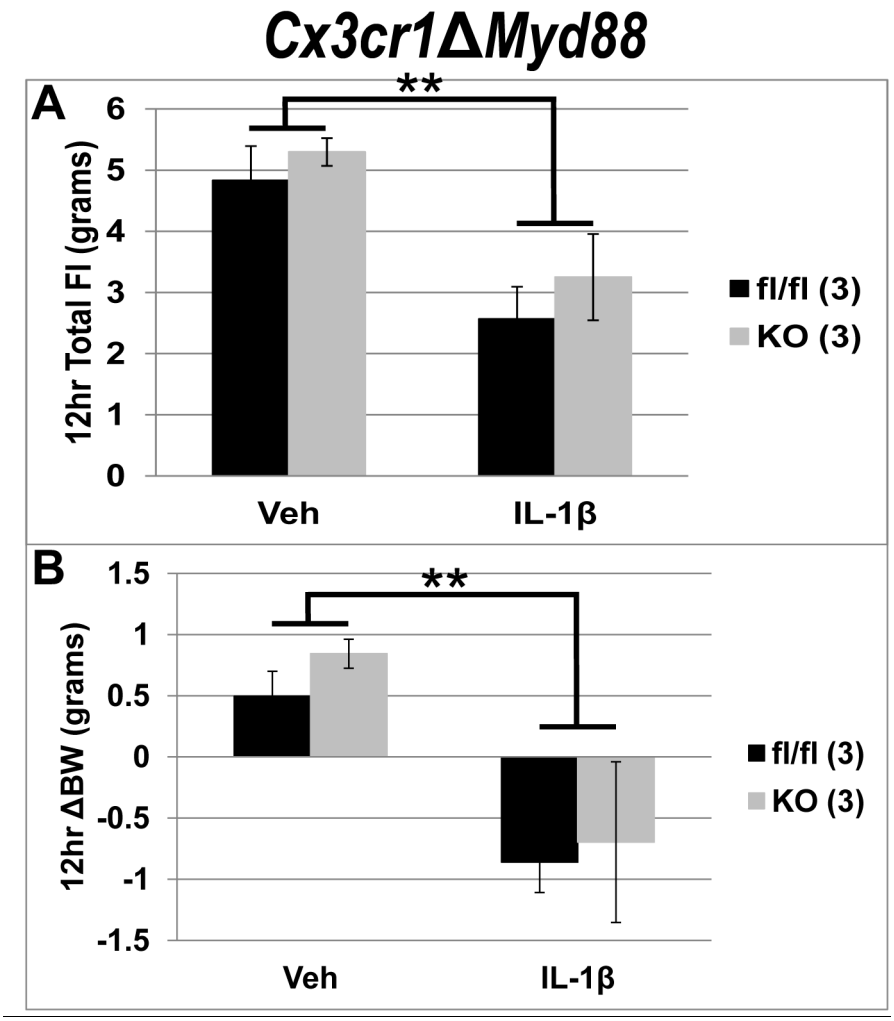

Figure 23: *Cx3cr1ΔMyd88*-mediated disruption of IL-1β signaling exclusively in microglia does not affect sickness responses

Both *Cx3cr1ΔMyd88* and their Cre-, *Myd88*$^{fl/fl}$ littermates (fl/fl) exhibit IL-1β-induced sickness responses. Regardless of genotype IL-1β treatment caused a significant reduction in both overnight (6pm-6am) total food intake (FI; A) and change in body weight (ΔBW; B). All values shown are mean ± SEM for group sizes shown in legend. **, $p<0.01$.

Chapter 4:

Summary and Conclusions

The last thirty years has seen a dramatic increase in our understanding of how disease affects the body and how the brain coordinates the biological responses that promote survival. It is now generally accepted that inflammatory cytokine signaling is a critical component of this process and that the physiologic and behavioral alterations induced in response to disease are the direct result of cytokine-initiated changes in neural activity. How these immune signals are communicated to the CNS to exert their influence on brain structures that subsequently alter specific aspects of homeostasis and patterns of behavior is still an active area of research.

The goal of the research presented in this thesis was to identify whether 1) CNS-specific production of inflammatory cytokines is essential for the manifestation of sickness responses and 2) which specific cell type(s) are required to translate immune system messages into neuronal-mediated responses. Based on a significant body of previous data we hypothesized that CNS production of inflammatory cytokines is an important step in initiating and/or prolonging sickness responses. From the results of our initial experiments we further hypothesized that cytokine signaling in microglia and endothelial cells was responsible for triggering these responses. Although we were unable to concretely delineate the role that a particular cytokine (IL-1β) plays in the sickness response, we demonstrated that cytokine signaling confined to the brain (as a model of CNS-specific cytokine production) engages a highly restricted and specialized population of blood vessels (fenestrated capillaries) to trigger the entire sickness response.

These results conform to the expanding understanding that the sickness response is a reflection of a sequential process of signaling between the brain and the body. The first steps in this process, those of identifying a problem and raising the biological alarm, are initiated by components of the peripheral immune system. An

integral aspect of these early events is the production of inflammatory cytokines—released initially into the surrounding tissues and subsequently amplified to raise cytokine levels in systemic circulation—that recruit not only other elements of the immune system but myriad systems throughout the body in the struggle for survival. How these signals translate into alterations in neuronal activity, and subsequently to changes in physiology and behaviors, was the principle question we sought to answer.

One of the most intriguing aspects in the sequence of events initiated by the immune system is the production of inflammatory cytokines, particularly IL-1β, within the brain. Although this phenomenon has been known about for nearly 30 years, its exact cause and significance to the sickness response remains elusive. Because CNS IL-1β production precedes inflammation-induced activation of neurons, and because interfering with IL-1β signaling specifically within the CNS reduces sickness responses, we suspect that IL-1β produced within the brain plays an important role.

Some previous research suggests that microglia are responsible for producing these cytokines. Our first step in investigating the consequences of CNS IL-1β production was to confirm these results. We tested four different antibodies before finding one that worked in our hands (verifying the specificity by examining IR in IL-1α/β KO negative controls), and confirmed that only microglia make detectable IL-1β. IL-1α/β KO mice did not show IL-1β IR when given LPS, despite showing sickness responses, demonstrating that IL-1β is not absolutely required for sickness behaviors. Because the IL-1α/β KO is a germline deletion and cytokine cross-talk/redundancy/compensation has been demonstrated by previous research, we cannot comment on what role IL-1β plays in the absence of genetic manipulation.

The use of conditional genetic models could provide interesting avenues of inquiry to specifically address the role of CNS cytokine production. Conditional alleles of

IL-1 are not available, but the recently developed Il1r1$^{fl/fl}$ and Il1r1-restore mice could address the question from a different angle. If IL-1 production within the brain is necessary for sickness responses, then eliminating (or restoring) IL-1 responsiveness should indicate this independent of the *Il1* genes. For example, low doses of IP IL-1β have been reported to cause sickness responses and brain IL-1 (via vagal transmission) without elevating circulating levels.[99] Our results from the second part of this thesis suggest that IL-1 signaling in fenestrated capillaries is necessary for sickness responses caused by central IL-1. It should be possible to determine the consequences of CNS IL-1 production by using Tie2-Cre (or better yet, the fenestrated capillary-specific Cre that is in development) to delete/restore *Il1r1*. While such mice would still maintain whatever response(s) depends solely on vagal transmission, any differences in the initiation or duration of the behavioral response to peripheral LPS would indicate a specific function of CNS-derived IL-1.

The fact that IL-1α/β KO animals develop immune-related health issues indicates that germline deletion is not without consequences. It would be interesting to investigate why IL-1α/β KO mice develop whisker pad abscesses, and whether other, unobserved complications also arise. Assuming that this issue is not due to linked transmission of an unknown mutation, determining what causes leukocyte invasion in IL-1α/β KO mice (or conversely what prevents such problems in IL-1α/β WT) could lead to interesting discoveries. The specificity of the lesions—we only ever observed them on a single whisker pad, potentially providing internal control by the contralateral pad—should aid in determining if they are directly due to absence of IL-1 signaling or to the up-regulation of compensatory cytokine factors.

Having demonstrated that microglia exhibit a clear response to inflammatory stimuli, we next asked if a microglial response to IL-1β contributed to physiologic and behavioral changes induced by inflammation. Our results did not support the hypothesis that microglia alone could trigger a sickness response; Myd88 KO animals with WT microglia did not respond to ICV IL-1β. Whether this is because microglia alone are insufficient to drive a behavioral response is still unknown due to several caveats. As discussed in chapter 2, it is possible that the migratory nature of microglia explains the absence of a response. It is also possible that the culture process changed something about the injected microglia, or that we simply did not inject enough to elicit a response. Examination of injected microglia shortly after transplant, showed that they exhibit morphology consistent with resident microglia, but this brings up a curious observation: microglia found near CVOs are morphologically and transcriptionally distinct from those found in much of the rest of the CNS. CVO-proximal microglia display characteristics commonly associated with an activated state (thickened, shorter processes) and express markers (including Cd45) that are found at lower levels, if at all, in other regions. This raises specific questions that are worthy of follow-up. Why, if microglia freely move about the brain, does there appear to be static differences? Are there different populations of microglia, or do microglia that wander into proximity with CVOs alter their phenotype? Such questions warrant further investigation.

Our next goal was to identify which cell type(s) respond to IL-1β to trigger the sickness response. The problem with a marker of activation such as cFos is that it only indicates which cells have increased activity without identifying what exactly caused said activation. To avoid this issue, we sought to first verify a previously reported tool (NF-κB nuclear localization as a marker of IL-1β-initiated signaling) using our variation on standard methods of fluorescent IHC. This allowed us to use multiple labels

simultaneously to definitively identify which cells demonstrated a response to a single stimulus (ICV IL-1β administration). We tested four antibodies before identifying one that showed clear nuclear localization in IL-1β-treated mice that was absent in vehicle-treated mice. As a means of validating that nuclear NF-κB was a direct response to IL-1β we tested the response in animals that are incapable of canonical IL-1β signaling (Myd88 KO); at no time was there any detectable change in NF-κB IR, indicating that this tool, in our hands, would serve as a method for determining cellular candidates for IL-1β-initiated signaling that could contribute to the sickness response.

While these controls (IL-1β vs. aCSF and Myd88 KO vs. WT) confirm that the change in IR requires IL-1β treatment and Myd88, they do not absolutely demonstrate a direct action of IL-1. It is possible that some secondary signal that also mediates NF-κB activation is responsible for some of the observed changes in IR. Similarly, it is still possible that Myd88 KO animals are capable of mounting a response to IL-1β through an alternative signaling pathway (such as the neuron-specific Il1racpb pathway) that does not involve activation of NF-κB, but the relevance of such pathways to the sickness response is unlikely given that Myd88 KO animals do not demonstrate sickness responses to IL-1β.[45, 237] Furthermore as neuronal Myd88 KO animals respond to IL-1β indistinguishably from control animals, it is unlikely that a direct effect in neurons is responsible for measured changes in physiology and behavior.

In addition to identifying IL-1β responsive cell types, we also used this tool to verify Cre-mediated signaling disruption in each of the promoter-specific Myd88 KO lines by examining differences in IR compared to control animals. This is, at best, an imperfect proxy of what would be the appropriate control: verification that Myd88 is not expressed in specific cells due to recombination. Despite testing six different antibodies we were unable to find one that could distinguish between Myd88 KO and WT animals.

This is an important point of diligence that is commonly neglected: In order to properly support the conclusions drawn from the use of Cre animals, it should be required that some method be employed to test whether genetic recombination occurred in the predicted pattern. Many times this is not adequately pursued; most studies using previously published Cre models do not examine Cre expression, and initial reports often rely on reporter strains alone to determine the sites of Cre expression and take that as an indicator of genetic recombination. In such studies it is important to keep in mind that Cre or reporter expression is not equivalent to genetic recombination at the target locus. Epigenetic modifications (among other factors) can influence the accessibility of particular regions of DNA that can influence how readily they are recombined. The Rosa locus is frequently used as a reporter insertion site specifically because it is so readily accessible and transcriptionally active and therefore conveys sensitivity in determining sites of Cre expression. A recent comparison of various Cre lines and detection strategies highlights this issue, but more needs to be done on the part of researchers to either properly verify Cre expression and conditional target deletion or emphasize caveats in order to ensure that data can be properly interpreted.[288]

Our examination of reporter expression in the various Cre lines used in the second part of this thesis provides an excellent example of why thorough characterization needs to be done and properly reported. Most reports using the Tie2-Cre line refer to it as “endothelial-specific,” despite the fact that extra-vascular Cre expression (in myeloid cells) was documented in the initial reports. Verification can seem like an unnecessary burden—requiring additional time and money and possibly inviting skepticism—especially when the model in question has been in use for more than 15 years, but the integrity of the scientific record demands it. Accurate and complete reporting is essential to prevent the propagation of misconceptions, potentially

benefitting future researchers, and is certainly preferable to having the validity of results questioned in hindsight.

The results from our analysis of reporter expression patterns and signaling disruption studies imply that minor differences in expression patterns can have dramatic consequences. Fenestrated capillaries in the CNS are only found in CVOs, a highly restricted distribution, yet they seem to be the sole element necessary for the full complement of sickness responses elicited by central IL-1β. Elimination of Myd88-dependent signaling from neurons/astrocytes/oligodendrocytes (via Nes-Cre), microglia (Cx3cr1-CreERT2), parenchymal endothelium/tanycytes/GFAP- astrocytes/hippocampal neurons (Slco1c1-CreERT2) or a combination of Cx3/Slc-CreERT2 all failed to eliminate the sickness response to ICV IL-1β, while elimination in all endothelium/microglia (Tie2-Cre) completely eliminated all responses. We interpret these results to mean that fenestrated capillaries represent a unique signaling niche for transducing inflammatory signals. This is a rather surprising result considering the wide-spread response to IL-1β as detected by NF-κB nuclear localization. On the other hand, because of the fact that CVOs have an altered BBB structure, they have long been considered as likely sites of action of many different circulating signals including hormones, nutrients and cytokines.

As mentioned above, it is important to recognize the caveats of the model. It is possible that there is some other population of cells that undergo recombination in the Tie2-Cre line that we did not detect. We attempted to fully characterize the expression pattern in all three Cre lines, but it is possible that some vital compartment escaped our notice. It should also be noted that the experimental design that we chose might favor signaling in CVOs relative to other brain regions. The ependymal cells that line the ventricles prevent passage of molecules in the CSF into the brain, but this barrier is also less restrictive in the CVOs. Additionally, just as circulating signals are prevented from

entering the brain by the presence of the BBB, so are molecules in the CNS restricted free contact with blood vessels by the BBB elements that reside on the brain side of the barrier. These possibilities are somewhat less compelling given the dramatic difference in vascular NF-κB IR induced by IL-1β and the extent to which nuclear NF-κB is observed throughout the brain. How the results of our current experiments apply to other models of disease, and therefore to the CNS response to illness in general, needs to be assessed. A definitive answer to these questions awaits the advent of the fenestrated capillary-specific Cre model currently under development.

As with most aspects of biology, the exact elements and sequence of events related to inflammation-induced sickness responses involved are complex and rarely linear. Biologists frequently attempt to categorize cause and effect into particular domains (e.g. glucose homeostasis is the domain of the liver and pancreas) in an effort understand how biological processes work. Such simplification helps in communicating scientific results to a broad audience, but risks obscuring the underlying reality that no aspect of biology is truly independent from the whole. In the given example, the reality is that it is impossible to segregate glucose homeostasis from energy homeostasis as a whole; the body's ability to maintain essential plasma glucose (outside of relatively short windows of time) requires food intake and the engagement of other endocrine and neural systems.

By examining multiple responses in a given experiment (as opposed to only monitoring fever or individual measures of behavior) we gain a better understanding of the global CNS-coordinated response to disease. Many previous experiments have indicated that individual responses might be segregable (e.g. genetic manipulations of PGE2 synthesis alter the febrile response without affecting anorexia or weight loss), implying that specific, detrimental symptoms could be targeted for treatment without

affecting potentially beneficial processes. This is the goal of much of the research on obesity and cachexia: If the wasting associated with chronic disease could be prevented without compromising the healing process (or, conversely, if adiposity could be decreased independent of other homeostatic mechanisms), many disease outcomes would likely be improved. Whether such targeted therapies are possible requires a much better understanding of the mechanisms involved in translating peripheral immune signals into changes in neural activity and which specific CNS circuits are involved in modulating particular symptoms. We can only gain such understanding by observing multiple aspects of the sickness response in a given experimental model of disease.

As new details emerge about the signaling pathways and circuitry involved in modulating physiologic and behavioral parameters in the context of disease we are gaining a better understanding of how immune-neural interaction works. The global picture that is slowly coming into focus is daunting in its complexity, but careful experimentation with emphasis on synthesizing broad perspectives, will ultimately result in complete understanding. We hope that the small contribution to this understanding provided by our current results will ultimately benefit the greater scientific community.

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